STUDIES ON PROTEIN METABOLISM DURING THE DEVELOPMENT OF *BOMBYX MORI*

Thesis submitted to the University of Calicut for the Degree of Doctor of Philosophy under the faculty of Science

By

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2007

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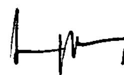
CERTIFICATE

This is to certify that this thesis is an authentic record of work carried out by Miss. Tanuja David from February 2002 to September 2007 under my supervision and guidance, in partial fulfillment of the requirements of the Degree of Doctor of Philosophy under the Faculty of Science, University of Calicut. No part of this thesis has been presented before for any other degree. I certify also that Miss. Tanuja David has passed the Ph.D. Preliminary Qualifying Examination held in 2003.



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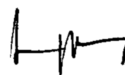
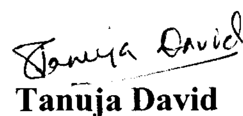


Dr. K.V. Lazar

DECLARATION

I, Tanuja David do hereby declare that this thesis entitled "**Studies on protein metabolism during the development of *Bombyx mori*"** submitted by me to the University of Calicut for the award of degree of Doctor of Philosophy under the Faculty of Science is the result of the research work carried out by me under the guidance of Dr. K.V. Lazar, Reader, Department of Zoology, University of Calicut. I further declare that the results presented in this thesis have not been submitted previously for any degree.



Tanuja David

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ACKNOWLEDGEMENTS

With immense pleasure and high regard, I express my gratitude to Dr. K.V. Lazar, Reader, Department of Zoology, University of Calicut, for guidance.

I am grateful to Dr. U.V.K. Mohamed, Professor and former Head of the Department of Zoology, University of Calicut, for his support and constant encouragements during the course of this work.

I wish to express my thanks to Dr. K.P. Janardanan, Professor and Head of the Department of Zoology, University of Calicut, for providing necessary facilities.

I wish to express my thanks to Dr. M. Gokuldas Professor, Department of zoology, University of Calicut for his support and encouragements during the course of this work.

I wish to express my thanks to Dr. Anandan, Scientist, CIFT, Cochin, for helping me to carry out HPLC analysis.

I would like to acknowledge SERIFED, Kozhikode, for providing sufficient experimental animal for completing my work.

I express my gratitude to my colleagues in the Molecular Biology Laboratory, Department of Zoology, University of Calicut, for their help and co-operation in completing this work

I express my gratitude to Mrs. Dr. Mangalalakshmi and Mrs A. N. Smitha senior colleagues for encouraging me during the course of this work.

I am indebted to my parents for their support and encouragements in completing this work.

I am thankful to the University of Calicut for awarding University Research Fellowship during the tenure of the present work.

Finally, I express my thanks to BINA PHOTOSTAT, Chenekkal, Calicut University, for DTP of this work.

TANUJA DAVID

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FOREWORD

Biochemical studies of insect development have attracted the interest of many investigators. This is mainly because of the short life span and distinct morphological types during the stages of development. The stages in the development of the holometabolous insects are particularly striking that the morphological and biochemical features of its larva, pupa and adult are not comparable. In contrast to the larval and adult development the pupal development, which is interposed between the larva and adult takes place in a closed system. Further the larva like silkworm inhabiting in a humid environment has to cope with water stress when they change to a different environment such as that of a pupa and adult. The various biochemical processes dealing with water conservation and maintenance of internal environment in a closed system like pupa would elucidate the biochemical homeostasis undergoing in organisms during development and hence the present study.

Homeostatic mechanisms maintain stable conditions with respect to any given physiological parameter. In insects fat bodies are in intimate contact with circulating haemolymph which is consistent with movements of molecules between the two compartments. Fat bodies respond to physiological and biochemical needs in many ways, including very high rates

of protein biosynthesis and detoxification of nitrogenous waste products. Many of the responses to physiological needs occur on a relatively large scale, and they can substantial impact on insect biology.

In the present study biochemical studies were conducted during the pupal and adult developmental stages of the silkworm, *B. mori* maintained in the laboratory. The pupal stages of *B. mori* represent a transitory phase and adult stages represent their final phase of development. During the pupal period of the development of the insect it undergoes various morphological and physiological changes. These changes must accompany a change in the biochemical composition of the animal in order to maintain a biochemical homeostasis of its internal environment. The major objectives of the present study were to evaluate the nitrogenous constituents of the insect such as proteins, ammonia, urea, uric acid, creatinine, total and individual free amino acids and their metabolism during the development to the paupal and adult stages. The changing profiles of the enzymes arginase, urease, alanine aminotransferse and aspartate aminotransferase were also evaluated during the development to the paupal and adult stages. The experimental results are interpreted in the context of the physiological modification of the internal environment of the insect.

REVIEW OF LITERATURE

Introduction

Insects are the most successful class of organisms (according to their number and distribution) in the animal kingdom. They inhabit practically all ecological niches of the world; and numerous morphological, anatomical, nutritional and physiological specialisations are found in relation to their mode of life. The important adaptive features of insects are the restriction in body-size, specialisation in the integumentary structures and in the metamorphic pattern. The biochemical economy in the formation of integumentary structures lies in its lability during the life cycle, which provides an opportunity to recycle the precursors of the cuticular structures. In insects, the metamorphosis has achieved to a high degree of perfection, especially in advanced orders, by the evolution of holometabolous forms, which are the dominant group among them. The morphological changes during metamorphosis enables the animal to adapt to different ecological habitats. These changes are invariably followed by corresponding changes in the biochemical make up of the body metabolic pool, though a change in the basic metabolic pattern is not expected. This point to a high degree of physiological radiation of the organic molecules/biochemical mechanisms in individual species during different phases of life-cycle.

Metamorphosis in insects

Insect metamorphosis is characterized by the remodeling of existing tissues and involves the destruction of larval/pupal tissues and their replacement by an entirely different population of cells. Insects grow by molting-shedding their cuticle and growing new cuticle as their size increases. In holometabolous insects the larva, pupa and adults possess striking morphological features. During the larval-pupal and pupal-adult development changes in the pattern of protein metabolism and nitrogen excretion is seen in order to maintain biochemical homeostasis.

The role of the excretory system in maintaining homeostasis

Dynamic changes in salt, water, acid-base and nitrogen amounts occur from time to time in all organisms as a result of food ingested, environmental conditions, and metabolism. Regulatory mechanisms that respond rapidly to these changes are necessary to preserve the integrity of cells and tissues. For example, herbivore ingest relatively large amounts of potassium and little sodium with their plant–based diet, while blood–feeders such as some hemipterans and mosquitoes ingest relatively large amounts of sodium (mostly as sodium chloride) and little potassium with their food. Nitrogen metabolites from proteins, amino acids, and purines must be disposed off by all cells. Maintenance of the constancy of the internal environment of cells,

tissues and organisms is the process of homeostasis, and the excretory system plays a major role (Nation and Thomas, 1965).

Acid-Base homeostasis

The excretory system is important in maintaining the acid-base balance of body fluids and tissues (Harrison, 2001). Acidosis or alkalosis may be experienced by an insect, depending on various foods, the presence of certain types of chemical compounds in plants eaten, the type of proteins metabolized (whether proteins yield a high proportion of acidic, basic, or neutral amino acids), and metabolic conditions such as exercise (e.g., flight) that produce acids in the tissues (Harrison and Kennedy, 1994). The western lubber grasshopper *Taeniopoda eques* exhibits flexibility in shifting between excretion of excess acid or excess base equivalents (Harrison and Kennedy, 1994), depending on need. This flexibility and regulatory ability in a highly polyphagous insect seems adaptive, and many other insects may show similar ability (Harrison and Kennedy, 1994).

Acid–base regulation has been most thoroughly studied in the desert locust *S. gregria* (Philips *et al.*, 1994; Harrison and Kennedy, 1994). Secretion of H^+ and formation of ammonium ions (NH_4^+) in the ileum is a principal mechanism for excreting excess acid equivalents (Harrison, 1994; Harrison and Kennedy, 1994; Phillips *et al.*, 1994). The ileum is a major site of ammoniagenesis, the formation of ammonia from precursors, in locusts in

which hindgut cells specifically metabolize amino acids and glucose for energy (Peach and Phillips, 1991). The excess nitrogen from amino groups is incorporated into the formation of ammonia. The excretion of total ammonia nitrogen serves several functions in locusts (Harrison and Phillips, 1992; Phillips *et al.*, 1994; Harrison, 1995), including,

1. Ammonium urate allows the insects to conserve Na^+, an ion that is not high in the food of locusts.

2. Conversion of ammonia (NH_3) to ammonium (NH_4^+) in the ileal cells is equivalent to removal of protons (H^+), and excretion of ammonia is more than sufficient to explain the recovery of haemolymph pH after a load of HCl is injected into the haemocoel.

3. Excretion of ammonia by locusts conserves water (because of precipitation of not-very-soluble ammonium urate salt).

4. Increases nitrogen excretion by 25% more than excretion of only sodium or potassium urate.

Nitrogen homeostasis

Harrison (1995) has addressed the fact that nitrogen, although known to be a growth–limiting nutrient for some insects, is nevertheless excreted in several forms by insects. Excess protein nitrogen is excreted as uric acid (a purine), or purines related to uric acid, as ammonia or ammonium salts, and in

several other (usually minor) forms. Nitrogen from nucleic acids is also excreted as uric acid (or as related purine metabolites or metabolites of uric acid). The complete sequence of enzymes in the ureotelic pathway for synthesis of urea has not been found in insects, but arginase, a primary enzyme in the pathway, is active in fat body of the abdomen and thorax throughout the life cycle of *Aedes aegypti* mosquitoes. Although uric acid is the primary excretory product, small amounts of urea are excreted (Dungern and Briegel, 2001). Bursell (1967) and Cochran (1975) provided thorough reviews of early literature on nitrogen excretory products in insects.

Electrolyte homeostasis

Beyenbach (1995) reviewed mechanisms for maintaining electrolyte homeostasis with special emphasis on the blood-feeding mosquito *A. aegypti* as a model insect. Adult female mosquitoes need a blood meal in order to mature each batch of eggs, but with the blood large salt (NaCl) must be excreted. Sodium excretion is an active process and occurs in malpighian tubule cells of the adult mosquito in response to stimulation from the mosquito natriuretic peptide (MNP) released from the corpora cardiaca (Wheelock *et al.*, 1988; Beyenbach, 1995). Larval *A. aegypti* live in fresh water, and in response to an increase in salinity they secrete 5-hydroxy-tryptamine (serotonin) into the hemolymph, leading to an increase in cAMP formation in the malpighian tubules (Clark and Bradley, 1993). The blood-

feeding hemipteran *R. prolixus* also secretes Na^+ (and K^+) into the lumen of malpighian tubules. Hematophagous behaviour may have driven the evolution of Na^+ secretion by malpighian tubule cells, thus enabling blood-feeders to regulate ion homeostasis after a large, salty meal, Ianowski and O'Donnell (2001) suggest a stoichiometry of Na^+ : K^+ : 2Cl co-transport across the basolateral membrane of tubule cells.

Protein metabolism

Proteins provide the chief structural elements of the muscles, glands and other tissues. They comprise some 2.2 per cent of the fresh substance of the adult bee when newly hatched, 3.2 per cent in the foraging bee with fully developed flight muscles. In addition, a certain amount of protein is stored in the fat body and much is deaminated or converted into carbohydrate or fat and used for energy production. During growth, and particularly at metamorphosis, there is extensive synthesis of protein. The available evidence suggests that this new protein is always the product of synthesis from free amino acids. The individual amino acids and the total amino acid concentration in the tissues, go through widely different cycles during the growth and development of different insect species (Chen, 1958).

The general processes of intermediary metabolism in insects are closely similar to those of other animals. Thus amino acids undergo extensive transamination. This provides for the synthesis of the 'non-essential' amino

acids from existing amino acids and sugars (Kilby and Neville, 1957) and it provides for the oxidative removal of amino groups from aspartate, glutamate, alanine, and other amino acids with the production of α-ketoacids (α-ketoglutarate). These can enter the tricarboxylic acid cycle (citric acid cycle) which operates in insects as one of the chief sources of energy (Bheemeswar, 1959). A 'balanced amino acid pool' is necessary for protein synthesis, and transamination is the chief mechanisms for the regulation of this pool. Transaminase activity in *Hyalophora* pupae follows a U-shaped curve which suggests a close relation between protein synthesis and transamination. The muscles are largely responsible, but in *Periplaneta* the fat body is an important site, maintaining the high amino acid level in the haemolymph. Amino acid glutamine is probably concerned in the biosynthesis of glucosamine and therefore a chitin (Hackman, 1974).

The major biochemical process underlying insect morphogenesis is protein synthesis. So the number and patterns of tissue specific proteins vary at different stages of development and become increasingly more complex with advances in development. The structural or enzymatic protein is immediately responsible for the developmental stages.

At the initiation of the larval development, growth is the predominant phenomenon. In *Drosophila* the increase in total protein content parallels closely to that in both wet and dry weight during the first 72 hours of

development. An important aspect of protein metabolism during larval development is the synthesis of haemolymph proteins. In general the protein concentration in haemolymph increases rapidly during the later half of larval development, falls at metamorphosis and decline to its lowest level in early adult life. An accumulation of haemolymph protein in *Calliphora* larvae was reported by Munn *et al.* (1967). Ruegg (1968) demonstrated that under *in vitro* conditions the specific rate of protein synthesis in larval fat body of *Drosophila* declines rapidly between 65 hours and pupation.

Protein metabolism during metamorphosis of holometabolous insects has been the subject of numerous studies (Chen, 1971). The majority of the earlier studies address the question to what extend the drastic morphogenetic alterations involved in the transformation of larva to adult is reflected in the pattern of protein metabolism. It is now generally accepted that nearly all protein in the insect haemolymph are synthesized in the fat body (Wyatt, 1980). Thus the increase in haemolymph protein concentration is accompanied by a fall in the synthetic capacity of the fat body.

Fat body of insects is analogous to the liver and adipose tissue of mammals in their functional aspects but its functional diversity cannot be equated to any other metazoan cell type (Wyatt, 1980). The insect fat body consists of loosely aggregated or compact masses of cells enclosed in a membranous sheath that are freely suspended in the haemocoel. It is

structurally organized to provide maximal exposure to the haemolymph. The fat body is well suited for both absorbing and releasing metabolites since it is the principal metabolic storage tissue in an organisms having open, diffusion type circulatory system.

The insect fat body is a major organ of multiple metabolic processes. Metabolism involves, among other things, the utilization of substance absorbed from the gut, their assimilation into substances in the body or their oxidation to provide energy. Fat body is composed of two or three cell types. The predominant metabolic storage cells are the adipocytes. In a well nourished insect, the cytoplasm of these cells is packed with droplets of fats, glycogen and proteins showing that the tissue serves as an important storage depot for reserve materials. The second common cell type is urocyte that sequesters uric acid for storage-excretion.

The fat body appears to be most conspicuous in the larvae of holometabolous insects. The fat body undergoes growth and development along with other insect tissue and its functions change in accordance with the developmental stage of the insect. Shigematsu (1958) was the first person who confirmed that insect haemolymph proteins are synthesized by the fat body. Protein synthesis shows variation with the age of the insect. Price (1966) observed that highest level in four-day-old larvae of *Calliphora erythrocephala* and the rate of synthesis falls as the larvae grows old. The fat

body of holometabolous insect larvae synthesizes major haemolymph proteins during the feeding stage and during prepupal stage, the fat body cells incorporate these proteins (Roberts and Brock, 1981; Levenbook, 1985).

In general, the rate of protein synthesis in the fat body is high in early growing larvae and declines rapidly with the advance of larval life. Following protein production, the fat body changes to a storage organ for several selected haemolymph proteins for use during adult development (Tojo *et al.*, 1980).

Haemolymph is the circulating body fluid in insects that fills body cavity or haemocoel. The haemolymph serves as an excellent barometer in determining the biochemical status of the developing insect. The transfer of metabolites by haemolymph is particularly evident in Endopterygota, at the beginning of metamorphosis (Wyatt, 1961). The chemical composition of haemolymph is highly variable among the diverse species examined at different developmental stages of the same species (Florkin and Jeuniaux, 1974). The volume of haemolymph varies widely as developmental stages proceed. In *Schistocerca gregaria* it appears that the increase in haemolymph volume occurring prior to ecdysis is derived from cellular and gut water, since the total percentage of dry weight remains constant (Lee, 1961). The variability of haemolymph volume is an important adaptation in insects to deal with the changes in its external environment (Florkin, 1966). It is shown

that adipokinetic hormone induces a hypertrehalosemic response and is involved in the homeostasis hemolymph trehalose concentration in the 5th instar larvae of *Bombyx mori* during feeding (Oda *et al.*, 2000). 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 fluid of other invertebrates (Florkin and Jeuniaux, 1974). The protein components of haemolymph comprises a structurally and functionally heterogeneous array of macromolecules that include storage proteins, vitellogenins, lipophorins, immunoproteins, clotting proteins, tanning proteins, lysozymes, enzymes etc. (Wyatt and Pan, 1978; Miller and Silhacek, 1982; Riddiford and Law, 1983). Haemolymph proteins may be used directly as a source of material for the synthesis of protein by developing adult tissues. This has been confirmed in *Phormia regina* using radioactive labelled proteins and in the fifth instar larvae of *Locusta migratoria* (Tobe and Loughton, 1969). Protein levels may decline prior to larval-pupal ecdysis, rise in the pupae followed by another decline during adult development (Wyatt and Pan, 1978).

The origin of the protein storage granules in mitochondria is reported in *Drosophila* (Von Gaudeker, 1963), *Philosamia* (Walker, 1966) and *Rhodnius* (Wigglesworth, 1967). During the feeding stage, the fat body of holometabolous insect larvae synthesizes storage proteins that are secreted to haemolymph (Marinotti and de Bianchi, 1986). During pupal life, the accumulated storage proteins are hydrolyzed and their amino acids utilized for

the synthesis of adult proteins (Munn and Greville, 1969; Levenbook and Bauer, 1984). Since the last review on insect storage proteins appeared (Levenbook, 1985), important advances have been made with respect to classification, understanding of control of synthesis, uptake by the fat body and the function of these abundant larval haemolymph proteins. The bulk of information is available for Diptera and Lepidoptera, and additional reports represent Orthoptera, Hymenoptera and Dictyoptera (Evans and Wigglesworth, 1990). There is an account of storage proteins in vespid wasps related to the characterization, developmental pattern and occurrence in adults (Hunt *et al.*, 2003).

A detailed study of the origin and development of protein containing bodies of *Calpodes* fat body was undertaken by (Collins, 1969). The best studied storage-protein class is arylphorins. This class includes the prototype storage proteins, calliphorin isolated and characterized from *Calliphora erythrocephala* (Munn *et al.*, 1969). Arylphorins have been identified in Lepidoptera and Hymenoptera. Synthesis and accumulation of storage proteins arylphorins and female specific protein were studied during the final 2 larval instars of the tobacco hornworm, *Manduca sexta* (Webb and Riddiford, 1988). Arylphorin was present in both stages, but its synthesis ceased during the moult, during starvation, and at the wandering stage, and then resumed about 24 hours after the onset of feeding. Three storage proteins have been identified in *Corcyra cephalonica* with molecular weights of

86KDa, 84KDa and 82KDa. Fat body of last instar larvae show a low uptake of all the three storage proteins. The selective uptake of storage proteins into the fat body cells at the end of the larval development after cessation of feeding is well documented in lepidopteran insects (Miller and Silhacek, 1982; Tojo *et al.*, 1982; Caglayan and Gilbert, 1987). The fat body from the last instar larvae of *Corcyra cephalonica* was able to incorporate only small amounts of radio labelled storage protein under *in vitro* and *in vivo* conditions (Ismail and Dutta–Gupta, 1990; Kiran Kumar *et al.*, 1997). The occurrence of tyrosine-rich storage proteins was investigated in two weevils. Three monomers (two major, 44 and 31 KDa, and one minor, 51KDa) were purified from pupae of *Sitophilus oryzae*, and one major monomer (65 KDa) from *Rhynchophorus palmarum*. These occur as insoluble granules in the pupal fat body.

Two electrophoretically and immunologically distinct storage hexamers (Hex 1 and Hex 2) have been identified in *Camponotus festinatus* workers. The molecular weights of the native molecules were estimated to be 460,000 (Hex 1) and 580,000 (Hex 2) by pore limiting gradient electrophoresis. Both proteins are composed of a single type of apoprotein of approximately 73 KDa (Hex 1) and 80 KDa (Hex 2). *Camponotus festinatus* storage hexamers bear some homologies in their N-terminal sequence with the arylphorins of Diptera and Lepidoptera, as well as with crab haemocyanin. In the pupa of *Apis cerana indica*, a number of small and large darkly stained

rounded albuminoid globules appear in the fat cells which increase in the mid pupal stage and decrease in the late pupal stages. This decrease suggests that the fat cells store the energy reserves during the larval stages and get depleted during metamorphosis (Venkatesh *et al.*, 1995). The ultrastructure of fat body in workers of *Camponotus festinatus* confirms that they store large quantities of protein, lipid and carbohydrate under some conditions. Rounded electron-dense granules, which are abundant in workers maintained in groups isolated from the parent colony, probably contain an arylphorin like protein. The relationship between storage of nutrient reserves and the presence of larvae suggests that the stores may function in regulating seasonal brood production (Rosell and Wheeler, 1995).

Transaminases

Aminotransferase activity has been demonstrated in a few insects. Enzyme activities are found in different tissues and the transamination reactions involving alanine, glutamate, aspartate and the corresponding keto acids appear most active (Chen and Bachmann, 1964). Regulation of glutamine metabolism studied in the last larval stadium of silkworm shows that ammonia is re-assimilated into amino acid through glutamine synthetase/glutamate synthase pathway for silk synthesis in *Bombyx mori* (Hirayama and Nakamura, 2002). Compared to mammals there is very little

information on the structure and function of aminotransferases, which are known to play a key role in the intermediary metabolism of amino acids.

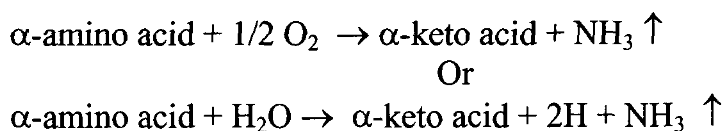
The most important physiological functions of aminotransferases, mainly alanine aminotransferase (ALAT) and aspartate aminotransferase (AAT), are the maintenance of the amino acid pool at a proper level for protein synthesis (Meister, 1965), the supply of metabolites for energy metabolism (Sacktor, 1974) and the catalysis of interactions between protein and carbohydrates metabolism (Katunuma *et al.*, 1968). The ALAT activity in *Drosophila nigromelanica* increases rapidly during larval growth, declines to a minimum at the middle of the pupal development, and rises again at emergence and during the early adult life (Schneider and Chen, 1981). As both growth and differentiation are closely related to protein synthesis, the elevated activity in growing larva and young flies appears logical. The same profile has been reported for AAT activity during development of house fly, *Musca domestica* (McAllen and Chefurka, 1961). Wadhwa *et al.* (1986) suggested that ALAT and AAT activities were higher in the feeding insects than the non-feeding insects. In the army worm, *Spodoptera mauritia*, an increase in the level of enzyme activity only in the feeding stages and the activity declines with the cessation of feeding until pupation (Lazar and Mohamed, 1988). However, the larva maintains a relatively higher level of enzyme activity during larval-pupal transformation. The high levels of aminotransferases observed in the feeding stages of larva are in tune with its

anabolic phase of development. The protein synthesis requires a balanced amino acid pool and transamination is one of the chief mechanisms, which functions as a regulator for this (Reddy *et al.*, 1991). The ALAT activity forms a general index of amino acid break down and AAT marks towards the mobilization of amino acids into gluconeogenesis (Adibi, 1968; Davidson and Longslow, 1975).

Ammonia

Ammonia is the chief by-product of protein and amino acid metabolism. Ammonia is readily soluble in water and is diffusible and can thus be lost relatively readily through the skin and gills of aquatic organisms. The maximal ammonia concentration in the blood of vertebrates is about 0.01 mg/100 ml (Florkin, 1949).

Ammonia is formed by deamination reactions of amino acids. Oxidative deamination transforms amino acids into keto acids that can be further oxidized in the TCA cycle.



$$\alpha\text{-amino acid} + 1/2\ O_2 \rightarrow \alpha\text{-keto acid} + NH_3 \uparrow$$

Or

$$\alpha\text{-amino acid} + H_2O \rightarrow \alpha\text{-keto acid} + 2H + NH_3 \uparrow$$

The α-keto acid enters the TCA cycle or the glycolytic pathway or the β-oxidation pathway and is broken down to CO_2 and water.

Ammonia in those animals in which it is not a major waste product, may be utilized in acid–base balance. Non oxidative deamination reactions also are known that are catalyzed by specific dehydrases. Generally, these reactions lead to the formation of ammonia and either acetate or pyruvate. In mammals, non-essential amino acids usually go to form pyruvate. Such amino acids are glucogenic because pyruvate can be used to synthesize carbohydrate in the form of glycogen. Essential amino acids generally form acetate upon deamination. These amino acids are ketogenic because the acetate, in the form of acetyl-CoA, is used in fatty acid synthesis.

In mammals, for example, nearly all the excreted ammonia comes from glutamic acid of the blood and is liberated by glutaminase in the kidney. The amount of glutamine amide nitrogen removed from the blood is increased, and urine ammonia nitrogen is increased during acidosis, while both quantities are decreased during alkalosis (Rector *et al.*, 1954).

Glutamine as a source of ammonia and the role of NH_3 excretion in acid–base regulation has not been much investigated. The sculpin *Myoxocephalus* excretes most of its nitrogenous waste as ammonia through the gills, only 14 percent of this nitrogenous waste comes from blood NH_3 but the gills have active glutaminase and glutamic acid dehydrogenase, hence the excreted NH_3 is formed in the gills from blood glutamine (Goldstein and Forster, 1960). In the earthworm *Lumbricus*, food leads to increased acid

production, which is neutralized by increase in excreted ammonia (Needham, 1957). Ammonia is also formed from urea by the action of urease, an enzyme purified from plants, certain molds, and bacteria and reported from several animals.

Free ammonia occurs naturally in the haemolymph of insects (Buck, 1953; Florkin and Jeuniaux, 1974). But the origin and physiological significance of this important compound is not fully elucidated in insects. Different views exist as to whether it is a true metabolites or it is produced as a result of microbial metabolism (Wigglesworth, 1972; Mullins, 1974). The pathway of ammonia assimilation into amino acids and used for silk protein synthesis in the silkworm, *Bombyx mori* has been demonstrated (Shinbo *et al.*, 1997). The physiological adaptations of these organisms to cape up the toxicity were not investigated in detail. Recycling of urea into ammonia by the action of mulberry leaf urease in the midgut lumen for nitrogen source has been demonstrated in the silkworm larvae, *Bombyx mori* (Hirayama *et al.*, 1999).

Ammonia Excretion

Ammonia is a product of protein and amino acid metabolism. Free ammonia cannot be stored in tissues or cells because it is a very strong base influencing pH, and in its free form it is very toxic to all cells. It must be rapidly excreted or transformed into a less toxic compound. If water is

readily available for dilution, ammonia can be excreted as the free base or as an ammonium salt. Animals that excrete ammonia as their primary nitrogenous waste product are described as ammoneotelic.

Ammonia is a major excretory product for larval stages of some Diptera that live in very wet environments. Larvae of *Calliphora erythrocephala*, the common blowfly; *Wohlfahrtia vigil*, a sarcophagid fly (Brown, 1936), *Phormia regina*, a blowfly; and *Lucilia cuprina*, the sheep ked (Hitchcock and Haub, 1941) excrete ammonia into wet surroundings that dilute it to non toxic levels. *Lucilia sericata*, another blowfly, excretes up to 15-folds more ammonia than uric acid (Brown, 1938). Although uric acid is synthesized, most of it is stored in the tissues (Storage excretion).

Staddon (1955, 1959) found that most of the nitrogen excreted by the aquatic larva of the neuropteran *Sialis lutaria* and the Odonate *Aeshna cyanea* is ammonia.

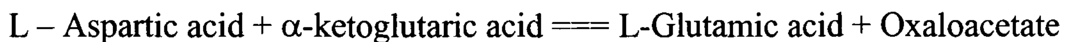
Some terrestrial insects excrete the majority of their excretory nitrogen as ammonia or ammonium salts. The American cockroach *P. americana* excretes ammonia as a major excretory product (Mullins and Cochran, 1972), but the precise mechanism of its excretion has not been elucidated. Ammonia and ammonium nitrogen can account for from 10 to 46% of the total nitrogen excreted by the desert locust, *S. gregaria* according to Harrison (1995). The locust sectum secreter significant quantitier of endogenously produced

ammonia peferentially into the lumen as NH_4^+ rather than NH_3. Thus, the results suggest that the ammonia crosses the apical membrane via an amiloride-inhibitable Na^+-NH_4^+ exchange mechanism (Thomson *et al.*, 1998).

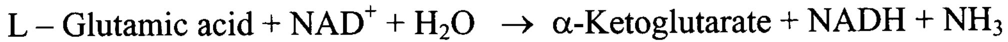
Most animals, including insects, synthesize ammonia into less toxic compounds such as urea (mammals) or uric acid (birds, reptiles, insects, Dalmatian dog). Enzymes involved in amino acid metabolism and ammonia production include amino transaminases (or transferases), glutamic acid and alanine dehydrogenases, L-and D-amino acid oxidases, adenosine deaminase, and monoamine oxidase. All of these enzymes have been detected in a number of insects (Cochran, 1975).

The amino transaminases are widely distributed in insect tissues and enable the amino group of one amino acid to be transferred to a ketoacid, thereby forming a new amino acid.

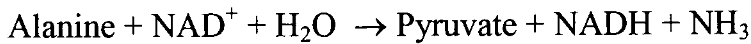
For example,


L – Aspartic acid + α-ketoglutaric acid === L-Glutamic acid + Oxaloacetate

Although ammonia is not directly released in transaminase reactions, the reactions provide a way to interconvert non essential amino acids and to make amino acids available to enzymes that deaminate with release of ammonia, such as glutamic dehydrogenase and alanine dehydrogenase in the following reactions:



$$L - Glutamic\ acid + NAD^+ + H_2O \rightarrow \alpha\text{-Ketoglutarate} + NADH + NH_3$$



$$Alanine + NAD^+ + H_2O \rightarrow Pyruvate + NADH + NH_3$$

The ammonia formed in these reactions is rapidly excreted or converted into less toxic compounds. The α-ketoglutaric acid and pyruvate formed can be metabolized through the Krebs cycle as an energy source (Cochran, 1975).

Ammonia production may also come from turnover and replacement of an insect's own nucleic acids, as well as from the metabolism of nucleic acids ingested with food.

Urea is a significant nitrogenous excretory product of insects

The synthesis of urea in vertebrate is mediated primarily through the classical ornithine cycle. Though the case is well known for vertebrates, insect biochemists have tended to take a rather restricted view of urea production and excretion. The occurrence of urea in insect excreta is well known (Gilmour, 1961; Razet, 1966; Bursell, 1967; Corrigan, 1970; Schoffeniels and Gilles, 1970; Wigglesworth 1972; Lazer and Mohamed, 1979). The usual finding is that urea comprises a small percentage of the total nitrogen in excreta, sometimes being present in only trace amounts. Wigglesworth (1972) was of the opinion that it is always present in the urine of insects. However, the results of certain other workers do not necessarily

support that generalization (Brown, 1938a; Nation and Patton, 1961; McNally *et al.*, 1965), but when one considers trace amounts and levels of detectability the question is more difficult to resolve.

Certain species excrete relatively large amounts of urea (Powning, 1953; Berridge, 1965). This finding is intriguing and both authors concluded that urea is of metabolic origin. In the nymphs of *Dysdercus fasciatus*, the gut is discontinuous, there by preventing direct voiding of undigested urea with the faeces (Berridge, 1965). In addition there is ample evidence for the occurrence of urea in the blood and internal tissues of numerous insect species (Ludwing, 1954; Ludwing and Cullen, 1956; Ramsay, 1958; Hayashi, 1961; Mitlin *et al.*, 1964a; Wang and Patton, 1969; Lazar and Mohamed, 1979), which presumably also argues for the metabolic origin of urea. Contrarily Wigglesworth (1931) stated that urea excreted by the blood feeding bug *Rhodnius* is derived largely from ingested blood.

Bursell (1967) concluded that food is not the source of urea in most species of insects. Rather it is apparently produced and excreted by insects (Gilmour, 1965). In support of this concept, Ramsay (1958) found that urea and certain other low molecular weight materials are present in the primary filterate of *Carausius* and diffuse into the malpighian tubule with this fluid.

Many insect species are capable of producing and excreting certain degradation products of uric acid. The occurrence of uricase and

allantoninase in insect tissue is well documented (Razet, 1966; Bursell, 1967). However, further degradation of the purine structure beyond allantoic acid by insect tissues has only rarely been reported (Gilmour, 1965; Razet 1966; Kilby, 1965; Bursell, 1967; Wang and Patton, 1969). The synthesis of urea by the classical ornithine cycle is envisaged in insects. The synthesis of urea can be either via degradation of purines through uric acid (uricolytic pathway) or through the urea cycle. The production of urea by the former pathway is not demonstrated in insects. However urea cycle was partially operative in insects. Pant and Kumar (1978) reported the presence of appreciable activity of the urea cycle enzymes in the tissue of *Sacrophaga ruficornis*, a carnivorous dipteran insect.

Powless *et al.* (1972) found an active ornithine transcarbymalase resulting in the incorporation of bicarbonate into citrulline in the bug *Nezara*. They concluded that urea produced by *Nezara* is the result of arginase acting on excess arginine in the diet. The adult silkworm *Bombyx mori* showed a high arginase activity and the activity in male moths is five times than in females (Osanai and Aigaki, 1984; Osanai and Yonezawa, 1985). The high arginase activity in males are localized almost entirely (96% of the total activity in the whole body) in the vesicula seminalis in the male reproductive system (Aigaki and Osanai, 1985).

Importance of arginase enzyme in the production of urea in insects

The presence of arginase has been demonstrated in insect fat body and muscle (Reddy and Campbell, 1966a, b). Arginase has been reported from many insect species, especially in extracts derived from fat body and flight muscles (Garcia *et al.*, 1956; Kilby and Neville, 1957; Szarkowska and Porembska, 1959; Porembska and Mochnacka, 1964; Reddy and Campbell, 1966a, b, Powless *et al.*, 1972). The insect tissues form a part of the vertebrate ornithine cycle, the most important among them being the enzyme arginase which catalyses the conversion of arginine to ornithine and urea (Pant, 1988).

Razet (1966) stated that urea production alone is sufficient justification for wide spread presence of arginase in insects. A perusal of the literature indicates the arginase reaction to be the major mechanism of urea production. Reddy and Campbell (1966a) have demonstrated the occurrence of arginase, ornithine transaminase and pyrroline-carboxylate reductase in the fat body and flight muscle of *Hyalophora.* Inokuchi *et al.* (1969) concluded that a pathway from citrulline to proline via arginine and ornithine which exists in *Bombyx* will result in the production of urea.

Urease

Urease activity was detected in the haemolymph of the silkworm, *Bombyx mori* from the beginning of spinning to the pharate adult stage, if the

larvae were reared on mulberry leaves through out the 5th instar (the last larval instar), selective transport of the mulberry leaf urease from the midgut into the larval haemolymph of the silkworm, *Bombyx mori* has been suggested (Hirayama *et al.*, 2000; Sugimura *et al.*, 2001). The exact origin of urease enzyme is not fully elucidated in insects.

Uric acid

One of the most studied nitrogenous compounds in insects is uric acid. Consequently its presence in the fat body has been investigated in a number of insects (Nolfi, 1970; Maddrell, 1971; Wigglesworth, 1972; Cochran, 1973). The general finding of these investigations is that uric acid is extensively stored in the fat body of insects at certain stages of its life cycle. Thus, the internal urate storage has been demonstrated in cockroaches (Srivastava and Gupta, 1961; Roth and Dateo, 1965; Cochran, 1973; 1976; 1979a, b; Mullins and Cochran, 1975a, b; Cochran *et al.*, 1979) *Chrysopa carnea* (Spiegler, 1962), *Dysdercus fasciatus* (Berridge, 1965), *Glossina morsitans* (Brown *et al.*, 1973), *Hyalophora cecropia* (Jungries and Tojo, 1973), *Meodiprion sertifier* (Fogal and Kwain, 1974), *Pieris brassicae* (Lafont and Pennetier, 1975) and *Manduca sexta* (Buckner and Caldwell, 1980; Williams Boyce and Jungreis, 1980).

The storage of uric acid was interpreted as an efficient mechanism of excretion in insects (Maddrell, 1971). This is based on the concept that the

effective removal of uric acid from the metabolic pool can be attained by storing it in a particular tissue in an almost inert form. But its mobilisation at certain periods of life cycle poses many problems with regard to the concept of storage excretion. It was found out that urate storage in cockroaches was associated with ammoniotelic excretion (Cochran, 1975).

High concentration of uric acid in the haemolymph of insect which distinguishes them from all other invertebrates (Florkin and Morgulis, 1949) indicates uricotelic nature of excretion and is correlated with terrestrial habitat. The occurrence of uric acid in the haemolymph has been demonstrated in a number of insects (Buck, 1953; Wyatt, 1961; Jeuniaux, 1971). Terrestrial animals are adapted to synthesize and excrete uric acid as a means to conserve water.

Uric acid synthesis and excretion

Uric acid is synthesized in insects from protein nitrogen as well as from nucleic acid nitrogen. The major portion is synthesized from protein nitrogen simply because insects ingest relatively much more protein nitrogen (or amino acid nitrogen) in their diet than nucleic acid nitrogen (Cochran, 1975).

The fat body is the primary site for uric acid synthesis. Barrett and Friend (1970) found that glycine contributes a carboxyl carbon to position-4 and an α-carbon to position–5 during synthesis of uric acid in *R. prolixus*.

Although the origin of the nitrogen atoms in uric acid from *Rhodnius* was not determined, it seems reasonably certain that they are derived from NH_3 resulting from metabolism of proteins, amino acids, and nucleic acids (Barrett and Friend, 1970). The final steps in the synthesis of uric acid involve the conversion of hypoxanthine and xanthine to uric acid. The enzyme that catalyzes the two-step conversion is xanthine dehydrogenase (Irzykiewicz, 1955). Evolution of xanthine dehydrogenase in insects, instead of xanthine oxidase, may have occurred as a potential way to recover some of the costs of urate synthesis (Cochran, 1975).

Uric acid and related uricotelic compounds are admirably adaptive excretory products for animals that live with water stress and that need to conserve water. Uric acid is the least soluble of the compounds (6 mg/100 ml of water) followed by allantoin (60 mg/100 ml), hypoxanthine (70 mg/100 ml), and xanthine (260 mg/100 ml) (Bursell, 1967). Data presented by Harrison (1995) shows that ammonium urate is much less soluble than either sodium or potassium urate, and it has the advantage that the NH_4^+ rids the body of additional nitrogen and acid equivalents.

Storage excretion of uric acid, or deposition in various parts of the body, is common in cockroaches and some other insects. Male cockroaches deposit uric acid in accessory glands associated with their reproductive tract (Cochran, 1973; Mullins and Keil, 1980).

Animals that excrete most or all of their excretory nitrogen as uric acid are described as uricotelic. Bursell (1970) proposed the definition of uricotelism to include excretion of allantoin and allantoic acid because both are derived from further metabolism of uric acid. Cochran (1975) concurred with the broader definition, and extended it further to include excretion of the uric acid precursors hypoxanthine, xanthine, and guanine excreted by some insects (Morita, 1958; Mitchell *et al.*, 1959; Nation and Patton, 1961; Nation, 1963; Mitlin and Vickers, 1964; Nation and Thomas, 1965).

Creatinine

The excretions of creatine and creatinine have been studied in a few insects. The importance in its investigation lies in that fact that the earlier studies on the subject have cast some doubt on its metabolic origin in insects as food is the most likely source (Bursell, 1967; Cochran, 1975). Bursell (1967) considered these materials to be the product of digestion rather than metabolism. However the findings of Wigglesworth (1931), Mitlin *et al.*, (1964a) and McNally *et al.*, (1965) do not necessarily support the above view. Investigations on the creatine and creatinine in the excreta and haemolymph of the larva of *Spodoptera mauritia* revealed that the materials occupy a significant position among its nitrogenous constituents (Lazar and Mohamed, 1991).

MATERIALS AND METHODS

Biology of the Experimental Animal

The silk moth, *Bombyx mori* is a member of the family *Bombycidae*, in the order Lepidoptera, class Insecta and phylum Arthropoda.

The life cycle of *B. mori* demonstrates the most advanced form of metamorphosis. In holometabolous, the serial progressions of four distinct stages of development complete one generation of the species; egg, larva, pupa and adult.

The number of generations per year or season depends on the voltinism of the silkworm strain and variables including temperature. Voltinism is when some members of a species enter hibernation- like period of diapause, while others do not. Under natural conditions, silkworm strains producing one generation per year are univoltine. Those that are producing two generations per year are bivoltine, and multivoltine are those strains which produce many generations per year. The life cycle of *B. mori* has the following stages:

The egg of *B. mori* is a very small hard structure. The egg shell provides a protective covering for embryonic development. Fertile ova darken to a blue gray within 24 hrs.

The larva is the vegetative stage where growth takes place. The larva of *B. mori* is an elongated caterpillar commonly called a silkworm. Larvae are monophagous and feed only on mulberry plants. The newly hatched larvae in about four weeks time, undergo moulting four times and start spinning a silk cocoon of one continuous fiber for pupation. Pupation completes in one or two days.

The silk cocoon serves as a protection for the pupa. Cocoons are shades of white, cream and yellow depending on silkworm genetics. After a final molt inside the cocoon, the larva develops into the brown, chitin covered structure called the pupa. Metamorphic changes of the pupa result in an emerging moth.

The pupal moult occurs within the cocoon spun by the final instar larva. Pupae can be seen only by cutting open the cocoon. Pupae are soft and white soon after the moult but become hard and brown with the tanning of the pupal cuticle.

The pupa is a non-motile and non-feeding stage. The larval organs are destroyed and adult organs are differentiated during this stage. It is divided into three regions- head, thorax and abdomen. The head is small and situated on the ventral side of the thorax. It has a pair of compound eyes, a pair of large antennae and vestigeal mouth parts. The thorax is large and prominent. Two pairs of wings and three pairs of legs are immovably pressed against the

body on the ventral side and enclosed in a chitinous case. The abdomen is composed of eleven segments but only nine are visible dorsally. Seven pairs of spiracles are represented in the first seven segments of the abdomen and of these the last pair is non-functional.

The sex-markings are more clearly visible than in the larva. The female pupa is larger with a broader abdomen while the male is thinly built with narrower abdomen. The female pupa can be distinguished from the male by the vertical line in the centre of the eighth abdominal segment on the ventral side. In the male there is a small round spot on the ninth segment.

The adult stage completes the life cycle of *B. mori*. The moth emerges after about 11-12 days of life span as a pupa. The moth inside the cocoon secretes an alkaline fluid from its mouth to soften the cocoon layer before emerging from the cocoon. The moth usually comes out of the cocoon early in the morning. Adult moths have heavy, rounded, furry bodies and cannot feed because of undeveloped mouth parts. The forewing has a hooked tip, characteristic in this family, however it is flightless. Wings and body are usually white, but may vary to shades of light brown.

It is the reproduce tive stage where adults mate and female lays about 400-700 eggs. Hatching occurs only in the early morning because the light influences hatching.

The moths have lost their flight due to several centuries of domestication. It does not feed during its short life span of three to six days. The size of the moth is about 4cm x 2cm. The entire body and the wings are covered with epidermal scales. The body is divided into head, thorax and abdomen.

The head is small and hypognathous. The paired compound eyes are prominent on the sides of the head. On the inner side of the eye arise a pair of bipectinate antennae. The coiled probosis is non-functional.

Of the three thoracic segments, the mesothorax is the most prominent. The cuticle of the thoracic segments is hardened to form sclerites. The mesothoracic and metathoracic segments each bear a pair of wings. The forewings overlap the hind wings. The pale coloured wings are covered with scales.

The abdominal segments are also covered with scales. Eight segments are visible in the abdomen in the male and only seven in the female. The male and female moths can be identified from the size of the abdomen, the size of the antennae and the external genitalia.

In males, the caudal end has a pair of hooks known as harpes, and in females, the caudal end has sensory hairs- which is protruded and retracted to expel the pheromone. Abdomen is long, narrow with eight visible segments. Antennae are large in males and small in females.

Collection and rearing of silkworm

The bivoltine silkworm hybrid, Elite-CSR 2x4 was used for the present study. The rearing of the silkworm was undertaken with collection of last instar larvae from farmers of SERIFED, Kozhikode, Kerala. The rearing house and all the rearing appliances were disinfected in advance with bleaching powder to free the rearing environment and the surrounding from pathogens. The last instar larvae were cultured in plastic trays with matured mulberry leaves. The plastic trays also disinfected in advance. The worms were fed twice a day. As the animal required a humid atmosphere (78 to 82% relative humidity) for normal growth, they were kept away from wind and intense light. The duration of fifth instar was about six days. During the end of fifth instar the larvae stopped feeding and began to spin cocoons. The silkworm pupates inside the cocoon in another two days. The transparent or very delicate cocoon with pupa inside is called as pre-pupa.

The pre-pupal stages were separated from the colony. The pre-pupae continued to spin cocoon and after two days the pre-pupae were transformed into pupa with hard cocoons. The biochemical estimations were conducted throughout the pupal and adult period.

Biochemical assays

The insects were anaesthetized with diethyl ether and their weights noted. The whole pupa/adult was homogenized in respective buffer using an

all-glass homogenizer. For estimations, pooled samples, extracted from appropriate number of insects were used. The samples were immediately processed before melanization and clotting takes place. Satisfactory results were obtained when this was done within 2-3 min. The estimations comprise protein, total free amino acids (FAA), glucose, aspartate aminotransferace, alanine aminotransferase, arginase, urease, ammonia, urea, uric acid and creatinine. The changes in the concentration of the materials per unit fresh weight of the insect and total insect were estimated throughout the pupal and adult period at an interval of 24 hrs. The physiological significance of the variations in each material was discussed and attempts have been made to elucidate the homeostatic mechanisms with which the animal undergoes in its developmental phases.

Total proteins

A definite volume of tissue homogenate in phosphate buffer (0.1M, pH 7) was used and the proteins precipitated by adding equal volumes of 2/3 N sulphuric acid and 10% sodium tungstate. The suspension was centrifuged at 2000 rpm. The precipitate was dissolved in a known volume of 1 N sodium hydroxide by boiling for 5 min. in a boiling water bath. The solution was taken and estimated for protein according to the phenol-biuret method of Lowry *et al.* (1951). The blue colour developed was read in a ultra violet spectrophotometer at 540 nm spectrum. Bovin serum albumin (Fraction V,

sigma) was used as a standard. A calibration graph was prepared using standard Bovin serum albumin and was used to evaluate the spectrophotometer readings.

Ammonia

A known quantity of whole insect homogenate was deproteinized using barium hydroxide zinc sulphate isotonic solution. The supernatant was used to estimate ammonia according to Miller and Rice (1963). The ammonia in the aliquots of the sample were estimated based on the reaction of ammonia with hypochlorite and phenol in the presence of nitroprusside (Berthelot reaction) forming the blue indophenol. The colour developed was read in a spectrophotometer at 640 nm. Standard ammonium sulphate processed in the same way was used for calibration.

Urease

The assay of urease was done in whole insect homogenate. A known quantity of insect homogenate in 0.1 M phosphate buffer, pH 7.4 was used for the enzyme assay. A known volume of the homogenate10% urea was incubated in the water bath at 37°C for half an hour. The reaction was stopped with zinc sulphate and barium hydroxide deproteinizing the solution and the resultant supernatant was estimated for ammonia according to Miller and Rice (1963). Heat denatured insect homogenate was used as control.

Urea

A definite quantity of tissue homogenate in phosphate buffer (0.1 M, pH 7) was used and extracted with a known volume of zinc sulphate-barium hydroxide isotonic solution. The solution was centrifuged and aliquots of the supernatant were analysed using Fearon reaction, modified by Beale and Croft (1961), using diacetyl monoxime. The pink chromogen formed was estimated colorimetrically using 535 nm spectrum, in an ultraviolet spectrophotometer. A calibration graph was prepared using the standard urea and was used to evaluate the spectrophotometer readings.

Arginase assay

A definite volume of tissue homogenate prepared in CTB 0.1% in (0.2 M, pH 9.5) sodium glycine buffer solution was used as enzyme extract. Heat denatured enzyme extract was used as control. The enzyme extract of known quantity with substrate arginine was allowed to incubate for 1 hour at 37°C water bath. After 1 hour the enzymatic reaction was stopped with known volume of perchloric acid. The whole medium was centrifuged and the filterate were analysed for enzyme arginase according to Reddy and Campbell (1969a). The pink chromogen formed was estimated colorimetrically using 535 nm spectrum. The denatured enzyme estimation was done using same method mentioned above. Arginase activity was expressed as one micro mole urea formed per minute at 37°C under the assay condition.

Uric acid

A definite quantity of tissue homogenate in phosphate buffer (0.1 M, pH 7) was used and extracted with equal volumes of 2/3 sulphuric acid and 10% sodium tungstate. The solution was centrifuged and aliquots of the supernatant were analysed for uric acid according to Brown (1945). The blue colour developed due to the formation of phosphotungstic-uric acid complex was measured in an ultraviolet spectrophotometer using 680 nm spectrum. The standard for uric acid treated in the same way was used for calibration.

Creatinine

A definite volume of tissue homogenate in phosphate buffer (0.1 M pH 7), was deproteinized with equal volumes of 2/3 N sulphuric acid and 10% sodium tungstate. The solution was centrifuged and aliquots of the supernatant obtained were analysed for creatinine based on Jaffe reaction (Mc Fate *et al.,* 1954). The yellow orange colour developed was read in a ultra violet spectrophotometer using 520 nm spectrum. The standard was prepared and used for calibration.

Total free amino acids

The definite volume of tissue homogenate in phosphate buffer (0.1 M, pH 7) was deproteinized with 80% ethanol. The precipitate was centrifuged off and supernatant was estimated for total free amino acid according to Lee

and Takahashi (1966). The colour intensity developed was read in an ultraviolet spectrophotometer at 540 nm spectrum. An equal mixture of all standard amino acids prepared in 0.1% solution of ethanol was used. A calibration graph was prepared using standard amino acid mixture and was used to evaluate the spectrophotometer readings.

Individual free amino acid estimation using HPLC

The individual amino acids were determined using HPLC according to Ishida *et al.,* (1981). A known quantity of them were carefully washed with 6 N HCI and transferred into the test tubes. The volume was made up and the tubes were heat –sealed after filling pure nitrogen gas. Hydrolysis was carried out in a hot air oven at 110°C for 24 hours. After hydrolysis, the contents were removed and filtered in to round bottom flasks through Whatman filter paper No.42. The flasks were flash-evaporated to remove traces of HCI and the process was repeated for three times with distilled water. The residue was made up to 1 ml with 0.05 M HCI. The samples thus prepared were filtered again through a membrane filter of 0.45 μm and 20μl was injected into Shimadzu HPLC-LC 10 AS having a column packed with a strongly acidication exchange resin i.e., styrene divinyl benzene copolymer with sufinic group. The column is of sodium type [ISC-07/s 1504 Na-Shimadzu]. The mobile phase consists of two buffers. Buffer A (sodium citrate, absolute alcohol, perchloric acid, pH 3.2) and buffer B (sodium citrate,

boric acid, 4 N NaOH, pH 10). The oven temperature was maintained at 60°C, the amino acids were eluted from the column by step wise elution i.e., acidic amino acids first, followed by neutral and then finally basic amino acids. The eluted amino acids were detected by using a fluorescence detector after post column derivatization with O-phthalaldehyde. In the case of proline and hydroxyproline, imino groups was converted to amino group with sodium hypochlorite.

Aspartate aminotransferase (AAT) and alanine aminotransferase (ALAT)

A definite quantity of whole insect homogenate in 0.1 M phosphate buffer, pH 7.4 was used for the enzyme assay. The substrate mixture containing alpha oxoglutaric acid and L-aspartic acid fro AAT and substrate mixture containing alpha oxoglutaric acid and L-alanine for ALAT were used. The substrate mixture and insect extract of known volume were incubated in the water bath at 37°C for one hour. The activity of AAT and ALAT was estimated according to Reitman and Frankel (1957) using pyruvic acid as standard. The colour developed due to the formation of keto acid was read after 10 minutes at 520 nm against a reagent blank in an ultra violet spectrophotometer. One unit enzyme activity corresponds to the formation of one mole of keto acid per min. at 37°C under the experimental conditions.

Heat denatured enzymes was used as control, and same procedure was carried out.

Glucose

A definite quantity of whole insect homogenate was used for the glucose estimation. Homogenate of the extract was deproteinized using barium hydroxide-zinc sulphate isotonic solution. The resultant aliquot of the supernatant was used for glucose estimation according to Morgan (1975). The colour developed was read at 540 nm in a spectrophotometer. The standard was prepared using glucose and calibration graph was prepared using spectrophotometer readings.

RESULT AND DISCUSSION

Total Proteins

The content of total protein of the insect during the developmental stages of pupa and adult are presented in table 1 and figure 1.

Table 1. Total Protein Content of the insect during the development of the pupal and adult stages of B. mori

Day of development	mg/g, mean ± SD	mg/insect, mean ± SD
Pupal days		
Day 1	46.32 ± 7.225	75.50 ± 11.778
Day 2	49.57 ± 6.146	64.44 ± 7.990
Day 3	45.22 ± 4.838	62.17 ± 6.652
Day 4	46.93± 8.165	65.70 ± 11.431
Day 5	41.48 ± 5.475	51.24 ± 6.763
Day 6	43.87 ± 3.904	43.87 ± 3.904
Day 7	43.37 ± 5.204	52.91 ±6.349
Day 8	41.27 ± 3.673	55.31 ± 4.922
Day 9	45.64± 4.883	55.23 ± 5.909
Day 10	34.22 ± 3.969	45.85 ± 5.318
Day 11	31.32 ± 4.196	32.89 ± 4.407
Adult days		
Day 1	49.99 ± 6.398	17.92 ± 2.293
Day 2	55.47± 8.930	26.23 ± 4.233
Day 3	50.33 ± 7.046	16.04 ± 2.245
Day 4	43.65 ± 5.412	13.00 ± 1.612
Day 5	29.13 ± 4.952	13.28 ± 2.257

Values are the means of five determinations with standard deviations.

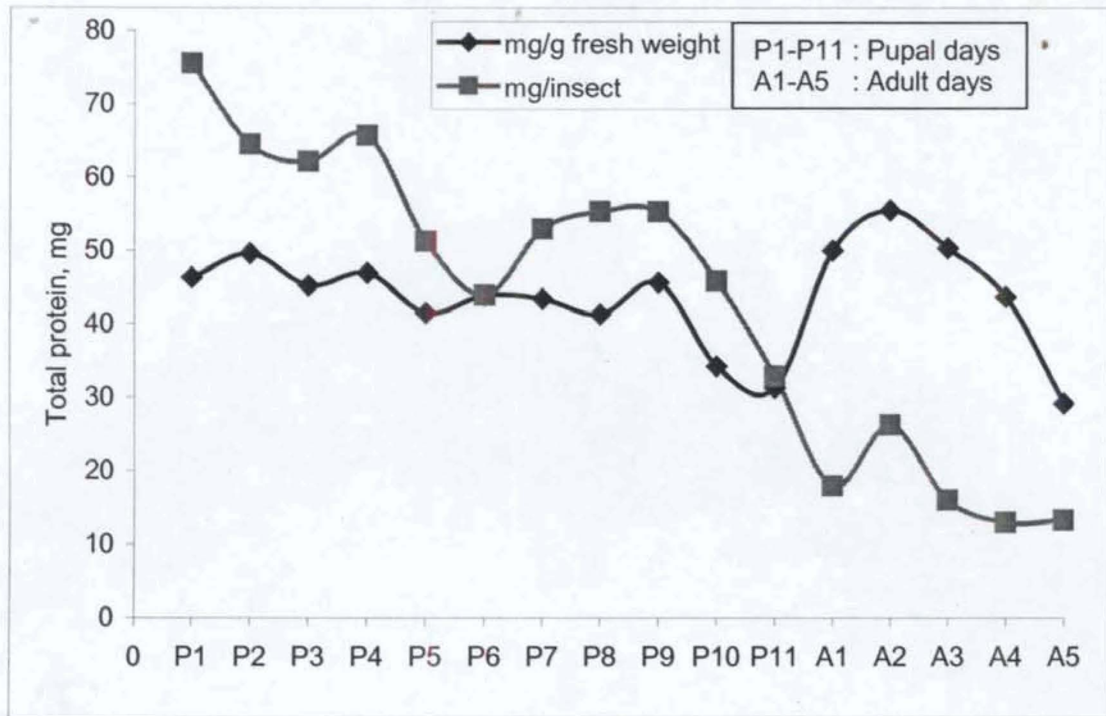


Fig. 1. Total protein in the pupal and adult development days of *B. mori*

The amount of total protein per unit weight of the insect remained more or less same from Day 1 to Day 11 of pupal stage. But towards the first day of adult, the amount of total protein increased recording the peak value on Day 2, followed by a decline up to day 5 adult. The pattern of changes in the levels of protein per insect was more or less similar to that observed per unit weight.

Proteins are the most studied nitrogenous compounds in the insect fat body. These macromolecules are involved in the regulation of all biochemical events in the organism (Harper *et al*., 1993). The various aspects of the haemolymph proteins in insects have been studied by many investigators (see review, Buck, 1953; Wyatt, 1961; Chen, 1966, 1978; Jeuniaux, 1971; Price, 1973; Wyatt and Pan, 1978). Accumulation of proteins

in haemolymph and fat body during the final instar insect development has been established by Chen (1985). It has been established that the proteins are synthesized in the fat body and released into the haemolymph, which are subsequently sequestered into the fat body and stored there depending upon the physiological condition of the animal. The protein concentration of insect haemolymph is generally higher than that of the internal fluids of other invertebrates and is almost similar to that of the blood of man (Florkin and Jeuniaux, 1974). The present finding that the pupal and adult stages of *B. mori* contains 29.13 to 55.47 mg protein per ml and 13.001 to 75.50 mg protein in per insect is in good agreement with the above view and comes within the range of values reported (Buck, 1953; Engle and Woods, 1960; Lazar, 1983; Mangalalaxmy, 2002).

The variation in the protein content observed in the developmental stages of *B. mori* was very stricking that there was a 5.7 fold decrease in protein content per insect.

The storage of proteins is of universal occurrence in insects. The fat body is the major source of the haemolymph proteins, the other sources being midgut, pericardial cells and haemocytes (Coles, 1966; Price and Bosman, 1966). Therefore, the synthesis of proteins in the fat body must be maximum in the feeding phases in order to cope up with the requirements of the haemolymph and of the growth of pupa. Lazar, 1983 has suggested that the raw materials required for synthesis and storage of proteins after the cessation of feeding activity may have received from the haemolymph where these

materials are obtained either from the food materials absorbed or from the degeneration of the larval tissues. The selective uptake and storage of haemolymph proteins by the fat body of the larva have been demonstrated in many insects (Loughton and West, 1965; Locke and Collins,1968; Chippendale and Kilby,1969; Tobe and Loughton, 1969a, b; Chippendale, 1970; Collins and Downe, 1970; Patel,1971; Price, 1973; Collins, 1974, 1975; Wyatt, 1975; Wyatt and Pan, 1978).

Insects are known to its capacity to regulate the osmotic pressure of the haemolymph inspite of the variation in its volume (Florkin, 1966). The fat body cells being intact, can store materials without causing any imbalance to the osmoregulatory mechanism. During this process the fat body cells enlarge in size, as they do not multilply during the instar (Price, 1973). Thus, it can be visualized that the storage of proteins in the fat body is an adaptation of the animal to withstand the effects of desiccation and to maintain the imbalance arising out of the water deprivation in the pupal and adult stages. A survey of the haemolymph proteins of insects (Wyatt and Pan, 1976) revealed that many low molecular weight proteins are present in it. Therefore, the various ingredients of the haemolymph may contribute to the maintenance of its osmotic pressure.

Another important property of plasma proteins is its buffering capacity (Engle and Woods, 1960; Foster, 1960; Putnam, 1965). For eg., in the parasitic larva of Diptera, *G. intestinalis*, the serum proteins account for about 45% of the total buffering (Levenbook,1950b). The unaccounted materials

which affect the rest of the osmotic pressure is more in Lepidoptera. It is possible that the haemolymph proteins beyond its capacity to act as enzymes may also take part in the maintenance of osmotic pressure.

Ammonia

The concentration of ammonia in the insect during the developmental stages of pupa and adult are presented in the table 2 and figure 2.

Table 2. **Ammonia content of the insect during the pupal and adult stages**

Day of development	**μ mole/g, mean ± SD**	**μ mole /insect, mean ± SD**
Pupal days		
Day 1	1.72 ± 0.251	2.67 ± 0.389
Day2	0.99± 0.107	1.55 ± 0.168
Day 3	0.99 ± 0.097	1.37 ± 0.134
Day 4	1.07 ± 0.173	1.33 ± 0.215
Day 5	0.24 ± 0.028	0.29 ± 0.033
Day 6	0.30 ± 0.037	0.36 ± 0.045
Day 7	0.32 ± 0.054	0.40 ± 0.068
Day 8	0.25 ± 0.038	0.29 ± 0.044
Day 9	0.36 ± 0.051	0.41 ± 0.058
Day 10	0.81 ± 0.094	0.87 ± 0.101
Day 11	1.29 ± 0.140	1.47 ± 0.160
Adult days		
Day 1	0.53 ± 0.064	0.19 ± 0.023
Day 2	0.63 ± 0.108	0.21 ± 0.036
Day 3	0.54 ± 0.086	0.20 ± 0.032
Day 4	0.41 ± 0.062	0.18 ± 0.027
Day 5	0.42 ± 0.049	0.19 ± 0.022

Values are the means of five determinations with standard deviations.

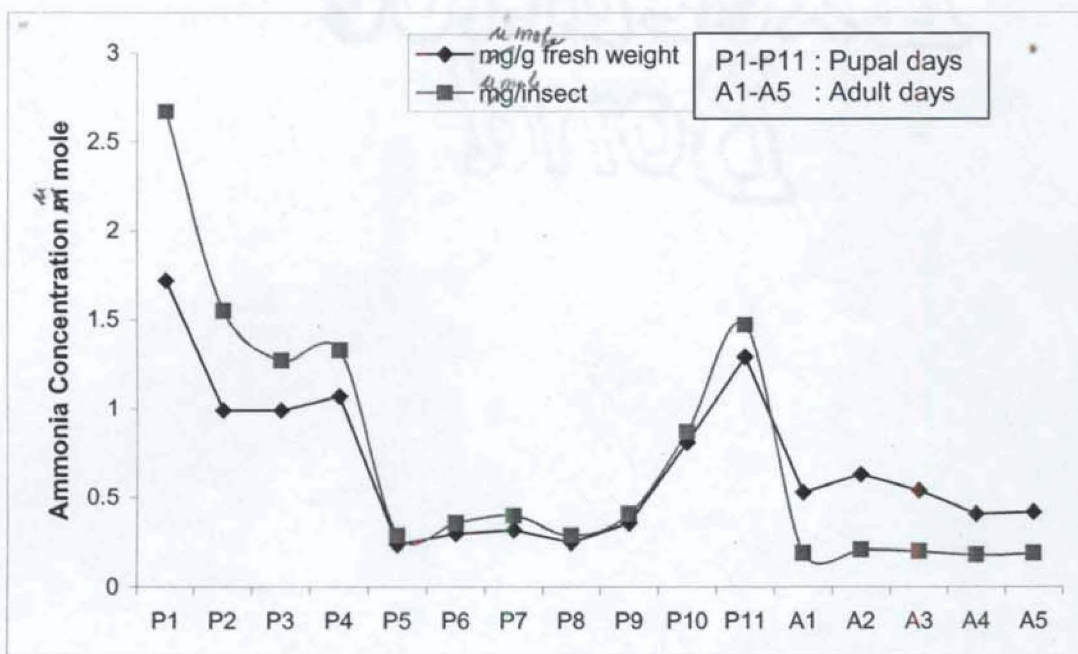


Fig. 2. Ammonia content in the pupal and adult development days of *B. mori*

The level of ammonia on the basis of unit weight of insect was initially high on Day 1 of pupal stage, but declined during the middle pupal stages, followed by an increase up to Day 11. In the day 1 adult the concentration of ammonia showed a sharp decline which remained more or less same throughout the adult stages.

The pattern of change in the levels of ammonia per insect was more or less the same as observed on the basis of unit weight of the insect. There was a 55% increase in enzyme activity on Day 1 pupa than the activity recorded on Day 1 pupa in per unit weight of the insect.

Urease activity

The urease activity in the insect during the developmental stages of pupa and adult are presented in table 3 and figure 3.

Table 3. Activity of urease in the insect during the pupal and adult stages

Day of development	Units/g, mean ± SD	Units/insect, mean ± SD
Pupal days		
Day 1	0.056 ± 0.007	0.087 ± 0.010
Day2	0.050 ± 0.004	0.078 ± 0.007
Day 3	0.045 ± 0.007	0.063 ± 0.010
Day 4	0.041 ± 0.005	0.051 ± 0.007
Day 5	0.031 ± 0.004	0.039 ± 0.005
Day 6	0.019 ± 0.002	0.023 ± 0.002
Day 7	0.027 ± 0.004	0.034 ± 0.005
Day 8	0.025 ± 0.002	0.032 ± 0.002
Day 9	0.022 ± 0.003	0.025± 0.004
Day 10	0.059 ± 0.007	0.064 ± 0.007
Day 11	0.063 ± 0.006	0.072 ± 0.006
Adult days		
Day 1	0.034 ± 0.0049	0.012 ± 0.001
Day 2	0.040 ± 0.0068	0.013 ± 0.001
Day 3	0.035 ± 0.0059	0.012 ± 0.002
Day 4	0.028 ± 0.0044	0.011 ± 0.001
Day 5	0.021 ± 0.0026	0.009 ± 0.001

Values are the means of five determinations with standard deviations.
One unit of urease activity was defined as 1 micro mole NH_3 liberated per min. at 37°C under the assay conditions.

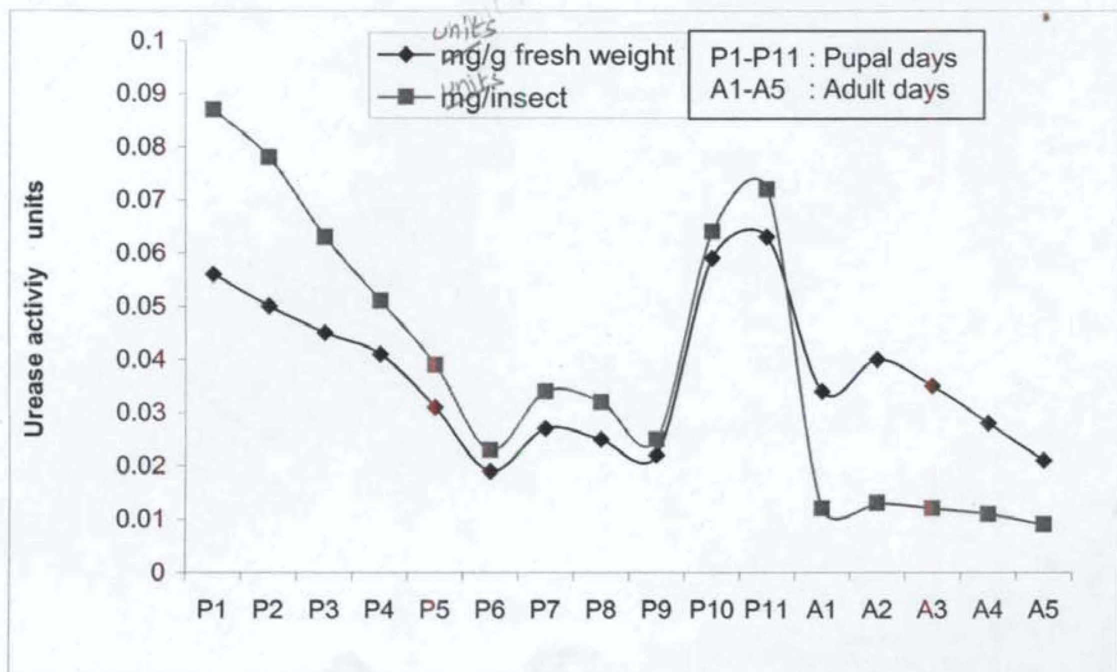


Fig. 3. Urease activity the pupal and adult development days of *B. mori*

The urease activity estimated per unit weight of insect exhibited high on Day 1 of pupa then gradually decreasing. On Day 11, the activity rose gradually and showing peak value. The enzyme activity was low on Day 1 of adult stage. There was 45 – 50% decrease in enzyme activity when the adult eclosion took place. The enzyme activity slowly declined through out the adult phases recording minimum value on Day 5.

On the basis of per insect the pattern of change observed was same as that in concentration per unit volume. The first day pupa showed almost 55% increase in enzyme activity than the one based on per unit volume. There was almost 83% decrease in enzyme activity towards the first day of adult when compared to last pupal day.

Ammonia and Urease activity

As a consequence of the general concept of uricotelism in insects the presence and significance of ammonia was considered to be universally present in insects. A few studies on concentrations of ammonia in insects revealed varying ammonia levels (Lennox, 1941; Levenbook, 1950a; Staddon, 1955; Wang and Patton, 1959; Prusch, 1971). The previous findings of Lennox (1941) showed high levels of ammonia in 14.286 μ mole /NH_3/ml in the blowfly, *L. sericata*. Staddon (1955) demonstrated that toxic symptoms developed in aquatic larvae of *S. lutaria* when the ammonia concentration reached 5 μ mole /ml, but death occurred only at 14.286 μ mole/ml concentrations. The larva and adult of *A. domesticus* contains 4.165 and 4.305 μ mole of ammonia/ml in the haemolymph inspite of its terrestrial habitat. They however, concluded that this ammonia is the product of deamination. The present finding suggests that the developmental stages of pupa contains 1.72 μ mole NH_3/g and 2.67 μmole NH_3 per insect is comparable with that observed in *A. domesticus* (Wang and Patton, 1969) and in the larva of *S. mauritia* (Lazar, 1983). Invertebrates are more tolerant to ammonia toxicity than vertebrates (Campbell, 1973). It is observed that high titre of ammonia in the pupa of *B. mori* and toxicity due to such high concentrations of ammonia in the animal may be nullified by the presence of a high titre of amino acids, uric acid and other organic acids. In the present

study it is observed that there is a direct correlation between the concentration of ammonia and free amino acid in the tissues while the concentration pattern of uric acid is inversely proportional to that of ammonia. The concentration pattern of uric acid also denotes the utilization of ammonia for its synthesis. In the chicken, *Gallus domesticus,* (Karasawa and Tasaki, 1976) have demonstrated that the ability to synthesize uric acid was significantly increased by the infusion of ammonia.

The changes in the concentration of ammonia observed during the development of the animal may be interpreted in the context of the maintenance of homeostatic equilibrium of its internal environment. The significance of ammonia in the context of homeostatic mechanisms has been already mentioned elsewhere. The concomitant fall observed during the post maturation phases is significant with regard to the maintenance of the ammonia toxicity.

The present study demonstrated activity of urease in the pupal and adult stages of *B. mori*. The studies conducted by Hirayama *et al.* (1999, 2000) and Sugimura *et al.* (2001) on the silkworm larvae shows that there is only selective uptake of mulberry plant urease into the midgut of larva. They have suggested that urea is converted into ammonia by the action of mulberry leaf urease in the midgut lumen and used as a nitrogen source. Singer (2003) has suggested that ammonia is a regulatory molecule and an important signal

communicating between amino acid catabolism following an increase in protein intake and the sequence of events leading to a change in excretory function. Birds and terrestrial insects are uricotelic and a high protein intake increase tubular urate secretion by the kidney (birds) or malpighiain tubule (insects). Ureogenic fish, given NH_4Cl, increase gill and renal clearance of urea and gill clearance of ammonia. Renal mass increase in mammals, birds and reptiles given a high protein intake. Thus, animals in general respond to an increase in protein intake with a change in excretory function such as to increase the clearance of the major nitrogenous end products of protein metabolism. Scaraffia *et al.* (2005) investigated mechanisms by which *Aedes aegypti* mosquitoes are able to metabolize ammonia when female mosquitoes were given solutions containing NH_4Cl or to a blood meal, haemolymph glutamine and proline concentrations increased markedly, indicating that ammonium/ammonia can be removed from the body through the synthesis of these two amino acids.

Urea

The urea levels in the insect during the developmental stages of pupa and adult are presented in table 4 and figure 4.

Table 4. Urea Content of the insect during the development of pupal and adult stages

Day of development	μ mole /g mean ± SD	μ mole / insect mean ± SD
Pupal days		
Day 1	1.34 ± 0.241	2.40 ± 0.432
Day 2	1.78 ± 0.288	2.76 ± 0.447
Day 3	2.17 ± 0.314	2.97 ± 0.430
Day 4	2.54± 0.322	3.15 ± 0.400
Day 5	1.92 ± 0.228	2.40 ± 0.285
Day 6	2.21 ± 0.355	2.72 ± 0.437
Day 7	2.51± 0.356	3.07 ± 0.435
Day 8	2.68 ± 0.292	3.44 ± 0.374
Day 9	2.94 ± 0.285	3.35 ± 0.324
Day 10	3.61 ± 0.321	3.90 ± 0.347
Day 11	3.36 ± 0.473	3.84 ± 0.541
Adult days		
Day 1	1.35 ± 0.156	0.51 ± 0.059
Day 2	2.51 ± 0.404	0.85 ±0.136
Day 3	2.43 ± 0.369	0.96 ± 0.145
Day 4	2.37 ± 0.258	1.03 ± 0.112
Day 5	2.43 ± 0.345	1.10 ± 0.156

Values are the means of five determinations with standard deviations.

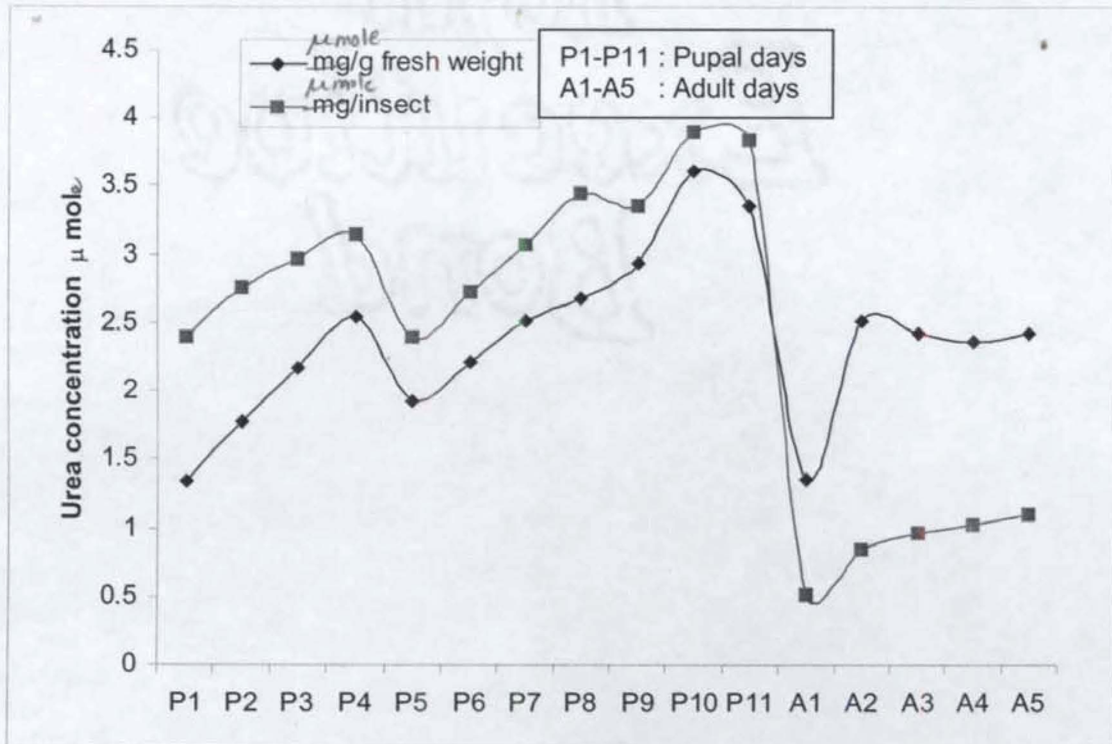


Fig. 4. Urease content in the pupal and adult development days of *B. mori*

The amount of urea per unit weight of insect gradually increased from Day 1 of pupa recording the peak value on Day 10, followed by a conspicuous decline on first day of adult. There was almost 60% reduction of urea on Day 1 of adult stage when compared to last pupal day. There was a gradual increase in the content of urea the following days of adult stage. The level of urea almost doubled in day 2 adult and remained more or less same throughout the adult stage.

On the basis of total insect the content of urea showed a similar pattern of change as that observed for unit weight of the insect. There was almost

80% reduction of urea in the Day 1 of adult stage, comparative to last pupal day.

Arginase activity

Arginase activity in the insect during the developmental stages of pupa and adult are presented in table 5 and figure 5.

Table 5. Activity of Arginase in the insect during the pupal and adult stages

Day of development	units/g, mean ± SD	units/insect, mean ± SD
Pupal days		
Day 1	0.0110 ± 0.00128	0.0156 ± 0.00182
Day2	0.0132 ± 0.00163	0.0136 ± 0.0016
Day 3	0.0207 ± 0.00312	0.0210 ± 0.00317
Day 4	0.0219 ± 0.00310	0.0231 ± 0.00328
Day 5	0.0208 ± 0.00355	0.0224 ± 0.00383
Day 6	0.0232 ± 0.00276	0.0314 ± 0.00373
Day 7	0.0173 ± 0.00185	0.0192 ± 0.00265
Day 8	0.0163 ± 0.00293	0.0183 ± 0.00329
Day 9	0.0121 ± 0.00119	0.0139 ± 0.00137
Day 10	0.0060 ± 0.00053	0.0069 ± 0.00061
Day 11	0.00096 ± 0.00011	0.00096 ± 0.00011
Adult days		
Day 1	0.0514 ± 0.00647	0.0257 ± 0.00323
Day 2	0.0336 ± 0.00359	0.0205 ± 0.00219
Day 3	0.0138 ± 0.00132	0.0083 ± 0.00079
Day 4	0.0027 ± 0.00040	0.0021 ± 0.00031
Day 5	0.0056 ± 0.00060	0.0039 ± 0.00042

Values are the means of five determinations with standard deviations.
One unit of arginase activity was defined as 1 micro mole urea liberated per min. at 37°C under the assay conditions.

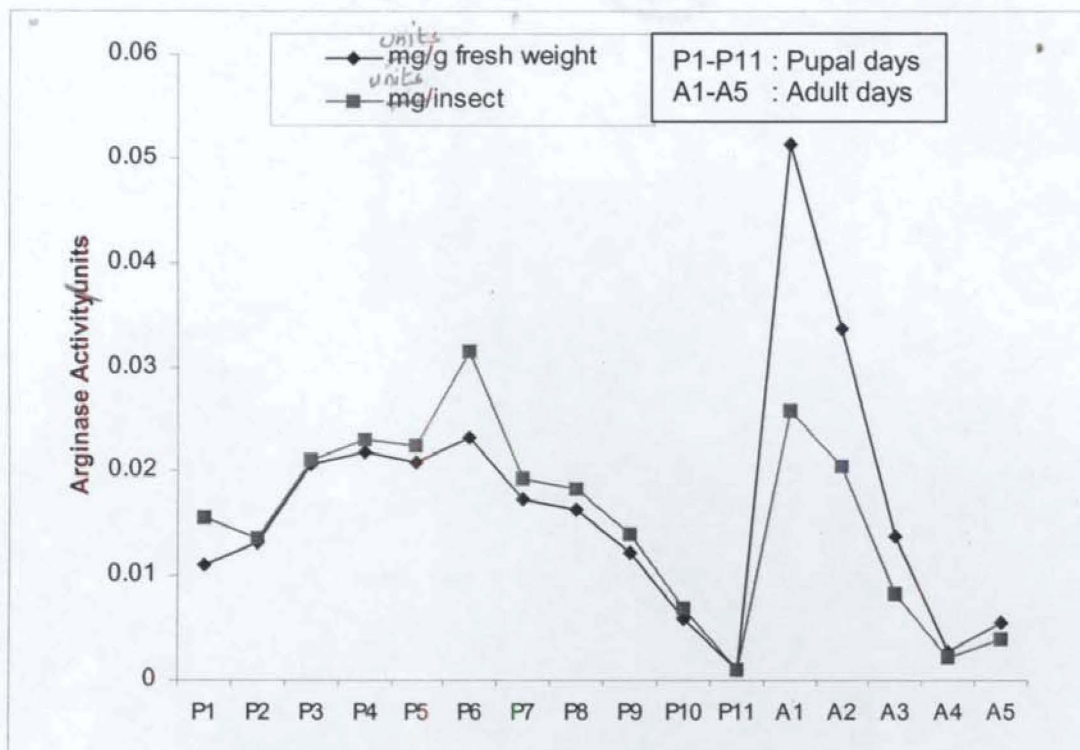


Fig. 5. Arnginase enzyme activity in the pupal and adult developmental days of B. mori

Arginase activity estimated per unit weight of the insect was low at the beginning of pupal stage, but gradually increased in the middle phases of pupa followed by a decline recording the least value on Day 11. The enzyme activity decreased to about 24 fold on final pupal day when compared to day 6 pupa. The enzyme activity was maximum on day 1 of adult stage showing the peak value and thereafter dropped towards final stages of adult. There was 54 fold increases in enzyme activity on day 1 of adult stage when compared to last pupal day.

A similar pattern of variations was observed in the activity of arginase estimated per insect. The maximum activity was recorded on Day 6 and minimum on Day 11 of pupal stage.

Urea and arginase activity

Urea is a protein–unfolding agent that can accumulate to locally high concentrations in tissues of many organisms (David *et al.*, 1999). The accumulation of urea during periods of water stress, has been demonstrated in semiterrestrial and terrestrial animals (Balinsky *et al.*, 1961, 1967b; Gordon *et al.*, 1961; Scheer and Markal, 1962; Tercafs and Schoffeniels, 1962; Janssens, 1964; Janssens and Cohen, 1968; De Jorge and Peterson, 1970; Horne, 1971, 1973, 1977a, b; Jungreis, 1971; Campbell *et al.*, 1972; Tramell and Campbell, 1972). Accumulation of urea during water stress is probably due to increased availability of the urea cycle intermediates. The present finding suggest that the tissues of the pupa contain as much as 3.61 μ mole/g of urea is comparable to some of the reported values: 0.514 to 16.925 μ mole/ml in the blood of insects (Leifert, 1935; Babers, 1938; Donato, 1938; Wang and Patton, 1969; Lazar, 1983). The results also demonstrate that the urea levels in the insect declined sharply from Day 10 of pupal stage up to adult eclosion. It has been shown that urea levels in the haemolymph tend to decline toward

larval–pupal transformation of *B. mori* (Sumida *et al.* (1990, 1995) and *S. mauritia* (Lazar and Mohamed 1989).

The primary function of urea synthesis is detoxification of ammonia, but it also includes the maintenance of osmotic pressure (Campbell, 1973). Urea is a low molecular weight molecule and therefore it can impart a crystalloid osmotic pressure to the medium. Urea also functions as a molecule in the control of blood volume in terrestrial and semiterrestrial animals (Janssens, 1964; Campbell and Bishop, 1970; Goldstein, 1970; Lazar and Mohamed, 1989). When in the blood, urea reduces the vapour pressure and thus reduces the evaporative water loss. The accumulation of urea during periods of water shortage has been demonstrated in many animals (De Jorge and Peterson, 1970; Horne, 1971, 1977 a, b; Campbell *et al.,* 1972; Tramell and Campbell, 1972; Newman and Thomas, 1975). Since the ureogenesis in a number of cases is connected with ormoregulatory aspects, Florkin (1966) has suggested that it can be considered as a physiological radiation of the arginine biosynthesis. In the case of the pupa of *B. mori* it is reasonable to suggest that the urea synthesis is more directed towards its function in ormoregulation and arginine biosynthesis rather than nitrogen elimination. During dormancy the metabolism in general is low (Campbell, 1973; Cohen, 1976) in order to conserve the energy resources of the animal. Thus the accumulation of urea in the pupal stages of *B. mori* suggests that it maintains a hyperosmotic

environment inside in order to survive periods of water shortage. Similar results have been observed by Baldwin (1969) in marine elasmobranch, where there was a urea build up in the tissues in order to conserve water.

Arginase is one of the chief enzymes for the production of urea in insects. Arginase activity appears to be present throughout the life cycle of several species of insects (Garcia *et al.*, 1956; Kilby and Neville, 1957; Szarkowska and Porembska, 1959; Porembska and Mochnacka, 1964; Kameyama and Miura, 1968; Reddy and Campbell, 1966a, b; Powless *et al.*, 1972). The present study demonstrates the presence of arginase activity throughout the pupal and adult development of *B. mori*. Conspicuous changes in the levels of arginase were also observed during pupal and adult development. Reddy and Campbell (1966a) observed that there is an increase in enzyme activity with adult emergence. High level of arginase activity in male moths have been detected in adult silkworm, *B. mori* (Osanai and Aigaki, 1984; Osanai and Yonezawa, 1985). Pant (1988) has suggested that the insect tissues exhibit a part of the vertebrate ornithine cycle, the most important among them being the enzyme arginase which catalyses the conversion of arginine to ornithine and urea.

Uric acid

The concentration of uric acid in the insect during the developmental stages of the pupa and adult are presented in table 6 and figure 6.

Table 6. Uric acid Content in the insect during the pupal and adult stages

Day of development	μ mole/g, mean ± SD	μ mole/insect, mean ± SD
Pupal days		
Day 1	8.232 ± 1.168	12.801 ± 1.817
Day2	11.783 ± 1.248	18.299 ± 1.939
Day 3	11.971 ± 1.807	16.600 ± 2.506
Day 4	12.490 ± 2.135	15.462 ± 2.644
Day 5	13.248 ± 1.404	16.560 ± 1.755
Day 6	13.455 ± 1.654	16.510 ± 2.030
Day 7	12.476 ± 1.434	15.570 ± 1.790
Day 8	13.015 ± 1.848	16.659 ± 2.365
Day 9	14.302 ± 1.372	16.304 ± 1.565
Day 10	16.339 ± 2.467	17.662 ± 2.666
Day 11	15.682 ± 1.819	17.893 ± 2.075
Adult days		
Day 1	9.683 ± 1.016	3.471 ± 0.364
Day 2	10.255 ± .892	3.473 ± 0.302
Day 3	11.109 ± 1.466	6.013 ± 0.793
Day 4	13.055 ± 1.227	6.067 ± 0.570
Day 5	13.355 ± 1.415	6.090 ± 0.645

Values are the means of five determinations with standard deviations.

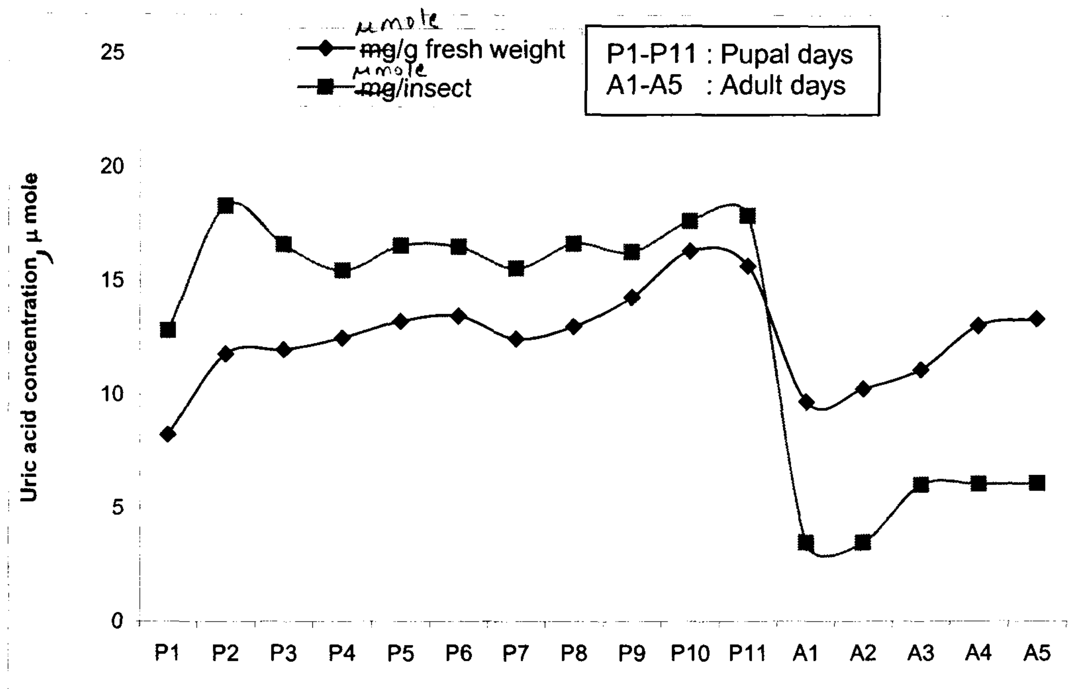


Fig. 6. Uric acid content in the pupal and adult developmental days of *B. mori*

Based on unit weight the amount of uric acid was least at the beginning of the pupa, but gradually increased. The content of uric acid reduced to 40% on Day 1 of adult, when compared to final stages of pupa. This was followed by a slight increase on Day 5 of adult.

The content of uric acid estimated per insect showed a similar pattern of variation. There was 80% reduction in uric acid content from final stages of pupa to initial stages of adult.

Uric acid metabolism in insects

One of the characteristic features of insect haemolymph is the high concentration of uric acid (Florkin and Morgulis, 1949; Buck, 1953; Wyatt, 1961; Jeuniaux, 1971). The presence of uric acid in the fat body of insects has been investigated (Nolfi, 1970; Maddrell, 1971; Wigglesworth, 1972; Cochran, 1973).The levels of uric acid observed in the present study are comparable with the range of values reported in insects (Buck, 1953; Wyatt, 1961; Jeuniaux, 1971). The high concentration of uric acid observed in the pupa of *B. mori* indicates the capacity of the insect to hold the material without affecting the normal metabolism. The low solubility of uric acid and its inert nature explains the suitability of this compound as an end-product of metabolism and as a storage material.

Urate storage is a universal phenomenon in insects. In insects uric acid is extensively stored in the fat body which is the major storage tissue of the animal (Nair *et al.*, 1967; Jungreis and Tojo, 1973; Buckner and Caldwell, 1980; Lazar, 1983). The physiological aspects of urate storage have been reviewed by Nolfi (1970), Maddrell (1971), Wigglesworth (1972) and Cochran (1975). The general contention on urate storage is that the material is stored in a particular stage of the life cycle of the organism concerned and is utilized for various anabolic purposes during the forthcoming developmental phases, conditions of stress and eliminating it from the active metabolic pool

for the purpose of nitrogen excretion. Nolfi (1970) has classified functions of stored purines into following categories: 1) storage excretion, 2) pigmentation, 3) a general source of nitrogen and carbon and 4) nucleic acid and purine source. Evidence for all these functions has been found in insects (Cochran, 1975). The storage function of uric acid can be interpreted in terms of laws of thermodynamics governing the living organisms, i.e., the conservation of energy. In the pupal and adult stages the conservation of water is inevitable to its survival and is causatively linked with the synthesis and storage of uric acid. Uric acid is known to contribute to the osmotic pressure of the medium and involves in the acid-base balance and buffering capacity (Florkin and Morgulis, 1949; Chen, 1966). In a closed system like pupa the uric acid plays an important role in the maintenance of the homeostatic equilibrium of the internal environment. The removal of materials from the haemolymph of the insect either by sequestration or by utilizing them for the synthesis of products like uric acid neutralizes the effects caused by desiccation.

Creatinine

The concentration of creatinine in the insect during the different developmental stages of pupa and adult are presented in table 7 and figure 7.

Table 7. Creatinine content in the insect during the pupal and adult stages

Day of development	mg/g, mean ± SD	mg/insect, mean ± SD
Pupal days		
Day 1	0.190 ± 0.030	0.31 ± 0.050
Day2	0.164 ± 0.023	0.213 ± 0.030
Day 3	0.130 ± 0.013	0.166 ± 0.017
Day 4	0.119 ± 0.015	0.167 ± 0.022
Day 5	0.146 ± 0.014	0.212 ± 0.020
Day 6	0.199 ± 0.030	0.286 ± 0.043
Day 7	0.262 ± 0.042	0.353 ± 0.056
Day 8	0.306 ± 0.043	0.429 ± 0.060
Day 9	0.240 ± 0.025	0.356 ± 0.038
Day 10	0.192 ± 0.029	0.259 ± 0.039
Day 11	0.140 ± 0.021	0.157 ± 0.023
Adult days		
Day 1	0.095 ± 0.0099	0.032 ± 0.0033
Day 2	0.082 ± 0.012	0.030 ± 0.0045
Day 3	0.063 ± 0.0067	0.029 ± 0.0031
Day 4	0.061 ± 0.0074	0.026 ± 0.0031
Day 5	0.074 ± 0.012	0.020 ± 0.0032

Values are the means of five determinations with standard deviations.

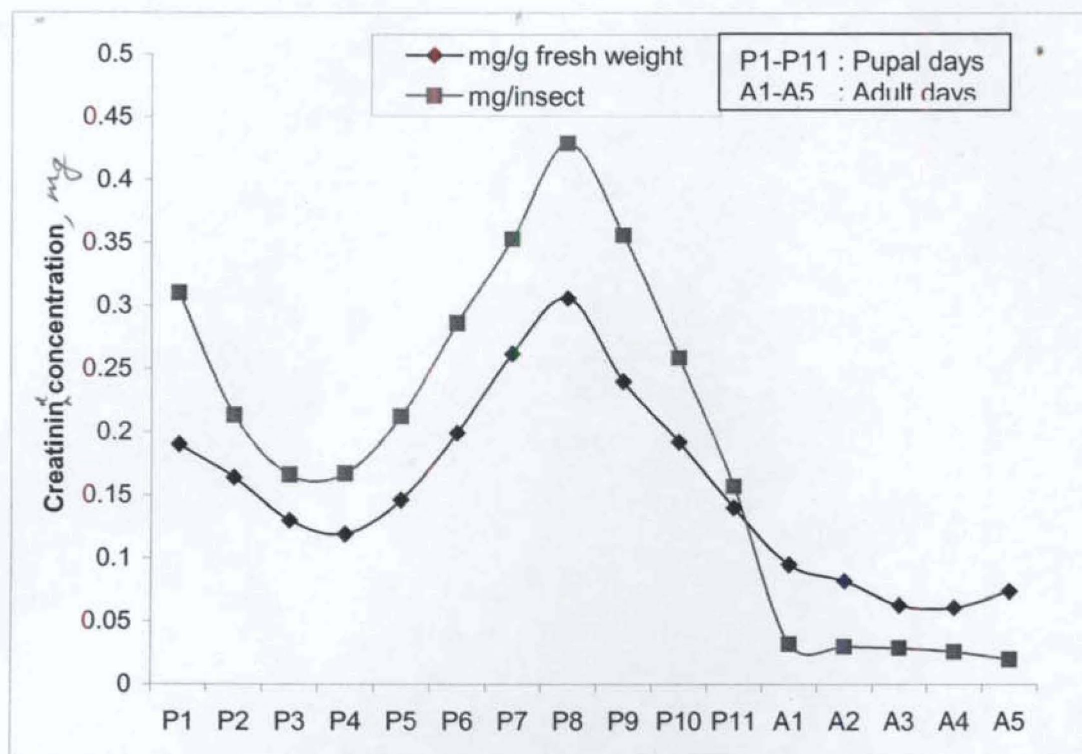


Fig. 7. Creatinin content in the pupal and adult developmental days of *B. mori*

The concentration of creatinine per unit weight was high at the beginning of the pupa, but declines slowly up to day 4 pupa followed by a gradual rise recording the peak value on Day 8 pupa. The levels of creatinine were low during the adult stages.

The presence of creatinine has been reported in the final instar larvae of *Orthaga exvinacea* and *Spodoptera mauritia* (Lazar and Mohamed, 1991; Kuzhivelil and Mohamed, 1997). El Allaf *et al*. (1984) has studied the effects of age and sex on serum urea, serum creatinine and on creatinine clearance.

Creatinine generation rate is a possible indicator of protein nutritional status (Shinzato *et al.*, 1997). The levels of creatinine in the pupal and adult stages indicate the turnover of creatinine as product of protein metabolism. In vertebrates where these compounds are found it is used as a phosphagen in muscle metabolism (Ennor and Morrison, 1958). But in pupal and adult stages no such energy requirements (as in muscle metabolism) can be envisaged. Thus, it can be suggested that these compounds can arise as a waste product of amino acid metabolism which effects the elimination of waste nitrogen.

The metabolism of phosphagens in animals indicates that arginine is the precursor for the synthesis of both phospharginine and phosphocreatine. But in insects energy metabolism during flight is related with proline metabolism (Sacktor, 1975), which in turn is incidentaly a derivative of arginine. As creatinine is a minor nitrogenous constituent, its levels in different pupal and adult stages of development reflects their metabolic interrelationships and the homeostatic mechanism operating in the animal.

Total free amino acids

The total free amino acid content in the insect during the developmental stages of pupa and adult stage are presented in table 8 and figure 8

Table 8. Total free amino acids in the insect during the pupal and adult stages

Day of development	mg/g, mean ± SD	mg/insect, mean ± SD
Pupal days		
Day 1	17.263 ± 1.847	26.844 ± 2.872
Day2	20.261 ± 2.877	27.371 ± 3.886
Day 3	22.172 ± 2.084	28.012 ± 2.633
Day 4	23.196 ± 3.061	28.716 ± 3.790
Day 5	21.232 ± 3.418	26.540 ± 4.272
Day 6	23.655 ± 3.595	29.025 ± 4.411
Day 7	22.770 ± 2.390	28.417 ± 2.983
Day 8	21.810 ± 3.075	27.917 ± 3.936
Day 9	21.525 ± 2.733	24.539 ± 3.116
Day 10	24.558 ± 3.290	26.547 ± 3.557
Day 11	26.686 ± 2.908	30.449 ± 3.318
Adult days		
Day 1	28.663 ± 4.614	10.275 ± 1.654
Day 2	26.489 ± 4.079	8.971 ± 1.381
Day 3	25.072 ± 3.083	8.462 ± 1.040
Day 4	21.231 ± 2.208	8.321 ± 0.865
Day 5	20.544 ± 3.513	9.368 ± 1.601

Values are the means of five determinations with standard deviations.

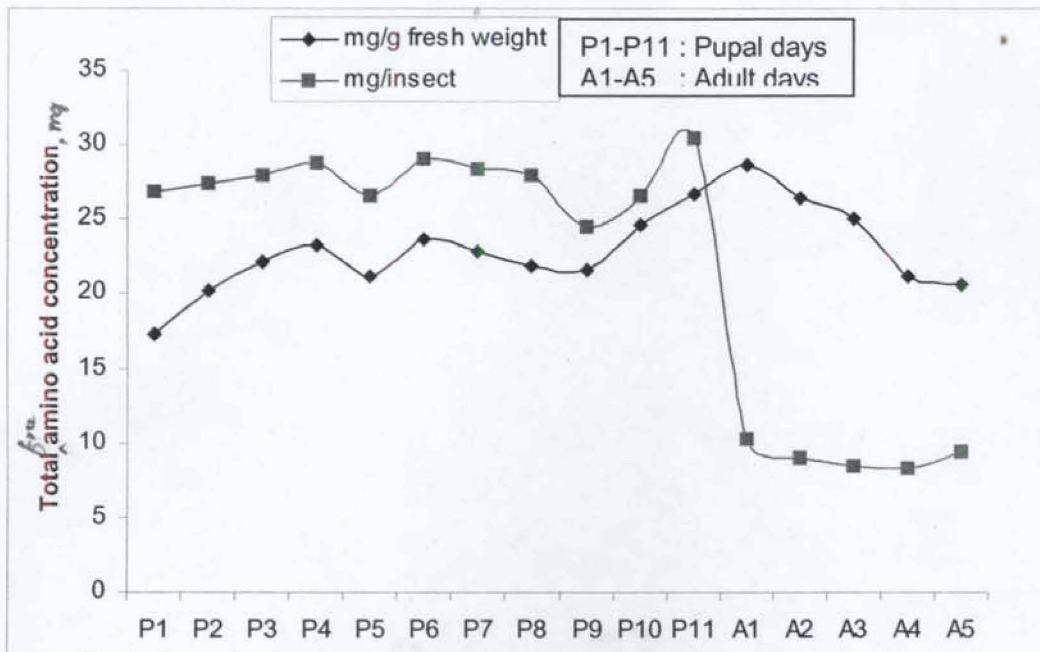


Fig. 8. Total free amino acid concentration in the pupal and adult developmental days of *B. mori*

The total free amino acid content based on unit weight of the insect remained consistent throughout the pupal development. The same pattern was observed in the adult developmental stages.

The concentration of total free amino acid per insect was more or less the same during the pupal days but with a sharp dip in the day 1 adult and remained steady thereafter.

The occurrence of a high titre of free amino acids in insects is a universal phenomenon. The titre of amino acids in insects can be used as a pointer in the evaluation of the dynamic metabolism of proteins and of the organism in total. A change in the free amino acid pool will directly influence

the protein turnover, that is, the synthesis and degradation of proteins, and thus obviously reflect the physiological state of the organism. The amount of free amino acids in the tissues varies from 17.26 mg/g to 28.66mg/g in the pupal and adult stages. It has been established that there is a dynamic equilibrium between the synthesis and degradation of proteins in the organism. As there is no intake of food in the pupal and adult stages of *B. mori* the sources of materials are restricted to the proteins in the animal. Therefore, any depletion or repletion of amino acids will indicate a shift in equilibrium between synthesis and degradation of body proteins. The changes observed in the total protein content of the insect were in agreement with the variations observed in the total free amino acid content.

One of the important functions of amino acid in insects is the regulation of osmotic pressure (Florkin and Morgulis, 1949; Buck, 1953; Wyatt, 1961, Chen, 1966; Florkin and Jeuniaux, 1974). Other physiological function of free amino acids includes its buffering capacity and the maintenance of acid–base balance.

Wigglesworth (1972) suggested that insect haemolymph contains a total concentration of amino acids 50 –100 times that which is normal for mammalian plasma. Amino acids may perform a number of functions and so may be particularly important for tissues such as the malpighian tubules that are bathed in the haemolymph. In most cells, amino acids act as intracellular

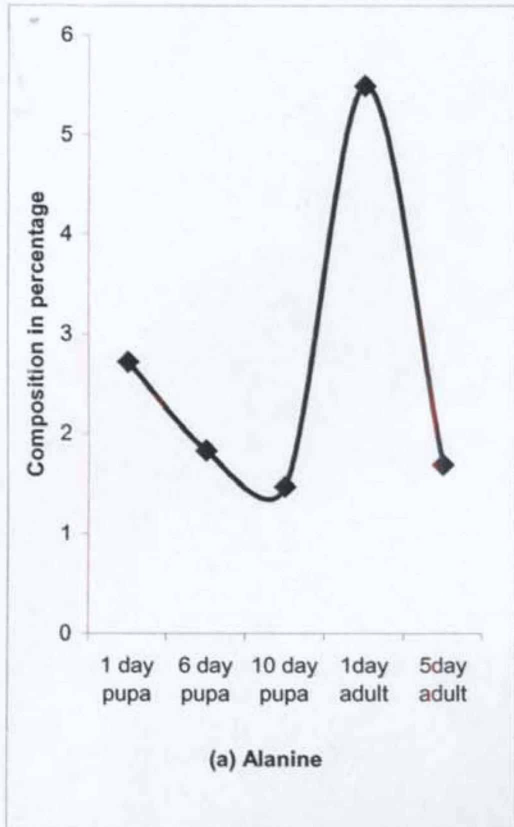
compatible osmolytes (Yancey *et al.*, 1982). Some amino acids play pivotal roles in metabolism by insect tissues. Proline and alanine are equally as important as carbohydrates in supplying energy to the flight muscles of the African fruit beetle, *Pachnoda sinuate* (Auerswald *et al.*, 1998). Fluid secreted by isolated malpighian tubules of the desert locust, *Schistocerca gregaria,* contains high concentration of proline, where it acts as a respiratory substrate to drive electrogenic chloride reabsorption across the lumen-facing membrance of the rectum (Chamberlin and Phillips, 1982). The studies conducted on the malpighian tubules of *Rhodnius prolixus* and *Drosophila melanogaster* demonstrated that amino acids can modulate the secretion of ions and water (Mathew *et al.*, 2003). Free amino acids such as glutamine and proline play important roles as compatible osmolytes in the regulation of cell volume. Isolation of tubules in amino- acid free saline may thus compromise cell volume regulation as amino acids are gradually lost from the cells. In such conditions, the slowing of transepithelial ion transport may be protective of cell function, in that further loss of intracellular osmolytes may also be slowed. Changes in cell hydration that is in cell volume, can act as important regulators of cell function and therefore changes in cell volume through the effects of hormones and amino acids can thus alter cell function (Haussinger, 1996).

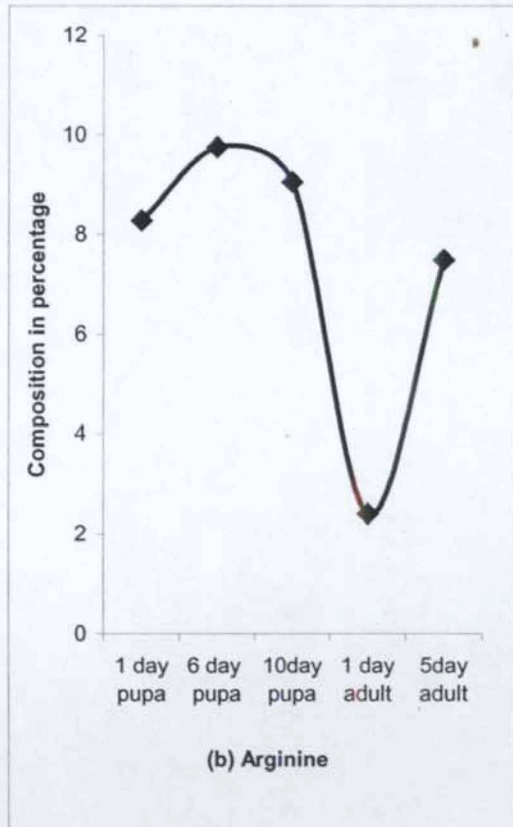
Individual free amino acids

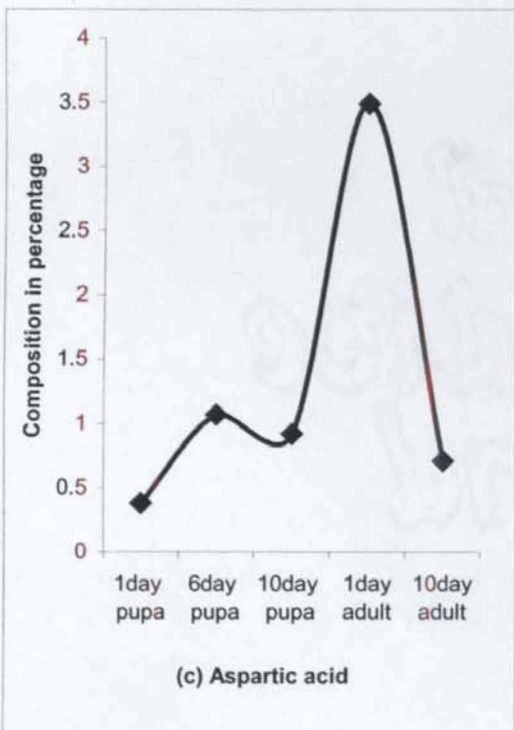
The percentages of individual free amino acids in the insect during the developmental phases of pupa and adult stages are presented in table 9 and figure 9.

Table 9. Individual free amino acid composition in Percentage

Amino acids	Percentage of individual free amino acid				
	1 day pupa	6 day pupa	10 day pupa	1 day Adult	5 day Adult
Alanine	2.72	1.83	1.47	5.49	1.69
Arginine	8.28	9.45	9.05	2.40	7.48
Aspartic acid	0.38	1.07	0.919	3.49	0.71
Glutamic acid	12.94	8.66	6.38	10.97	6.19
Glycine	2.08	6.383	2.39	2.66	1.99
Histidine	19.95	17.61	18.24	9.36	8.32
Hydroxy-proline	13.17	11.86	11.26	20.34	18.37
Isoleucine	2.34	3.44	2.29	2.18	4.65
Leucine	2.70	1.72	2.01	1.77	4.52
Lysine	6.14	2.66	15.78	0.47	6.38
Phenyl-alanine	4.11	4.32	6.69	5.55	7.25
Proline	1.73	2.74	4.02	0.79	1.07
Serine	1.98	6.36	4.17	5.51	7.31
Taurine	9.40	3.98	2.31	18.04	13.39
Threonine	2.46	6.68	2.53	3.85	3.69
Tyrosine	9.55	4.38	6.61	4.93	4.55
Valine	N.D	6.49	3.82	2.09	2.36
Total	100	100	100	100	100







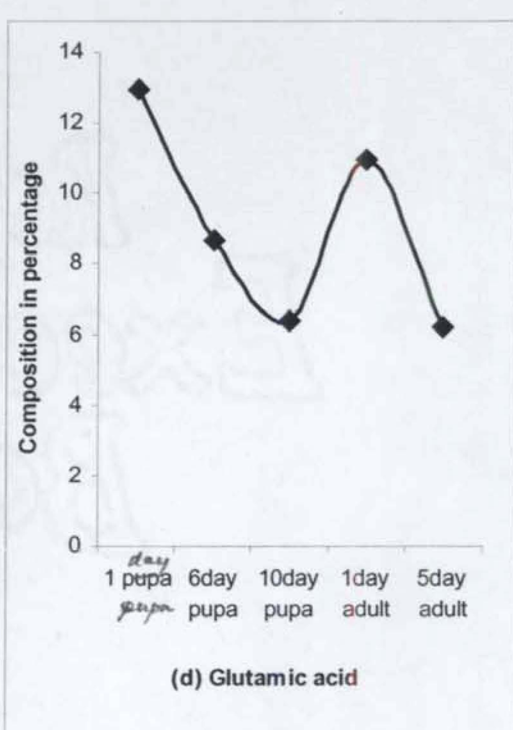
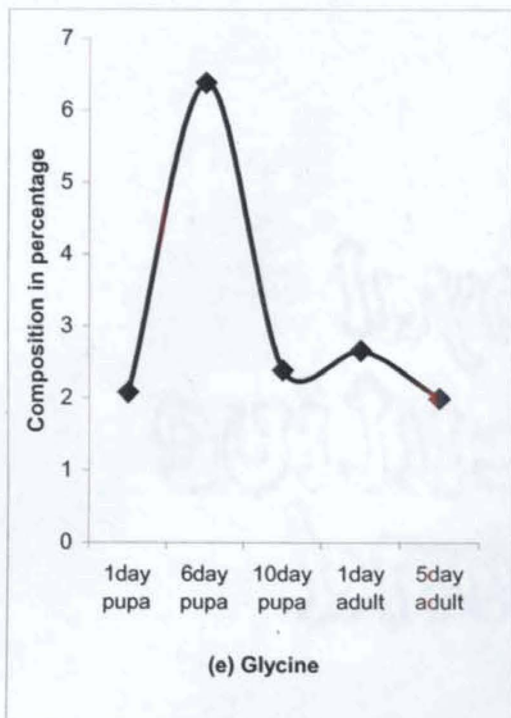
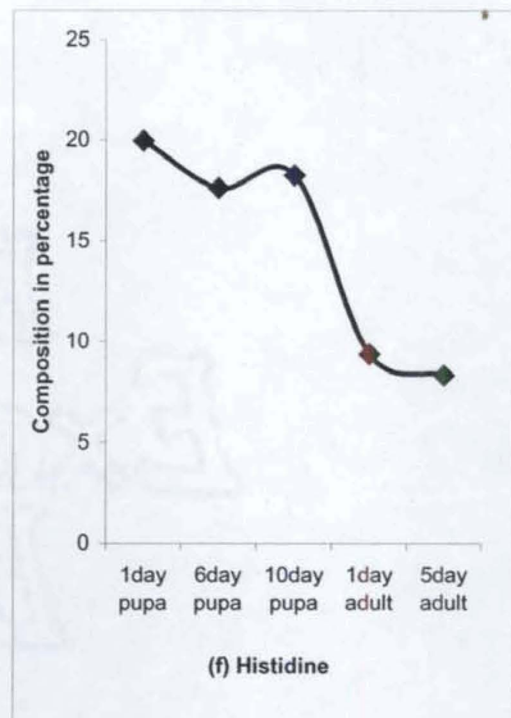
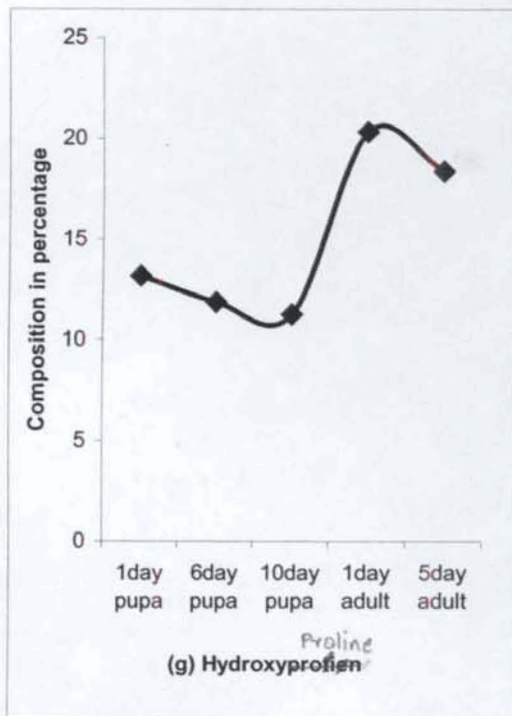


Fig. 12 (a-d). Individual free amino acid as the percentage of the total free amino acids in the insect.







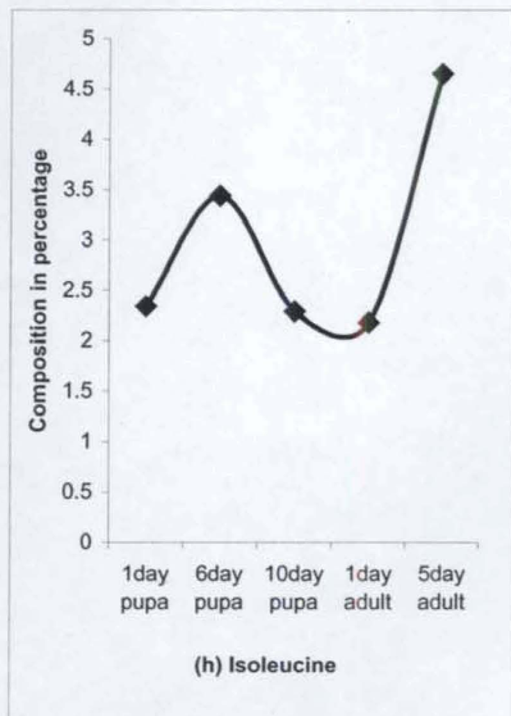
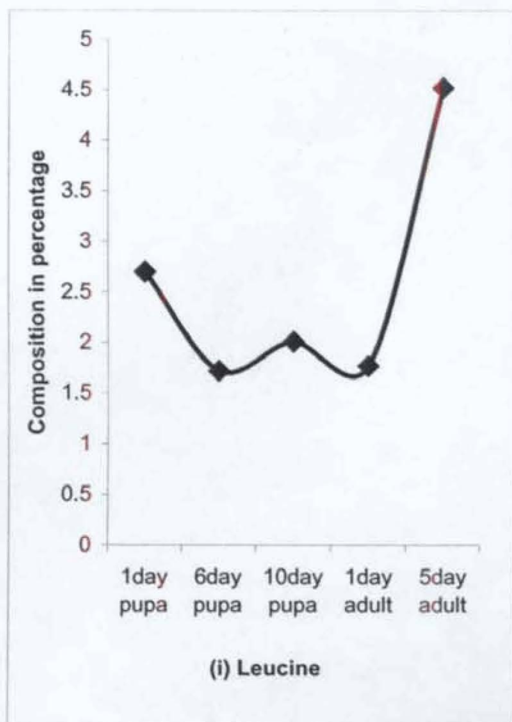
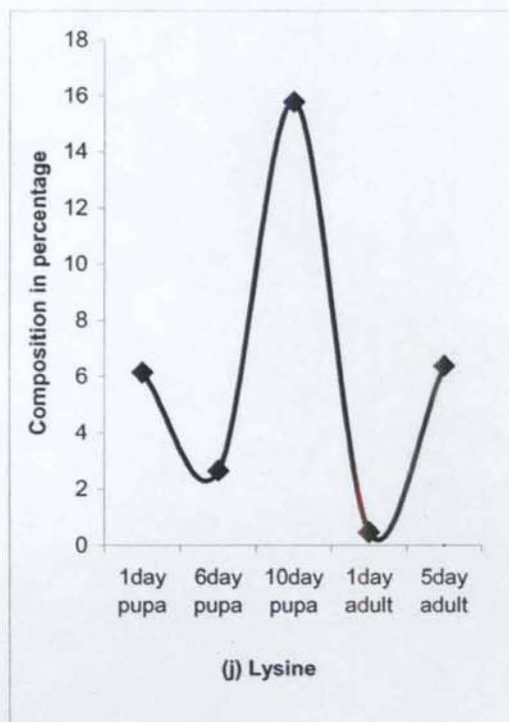
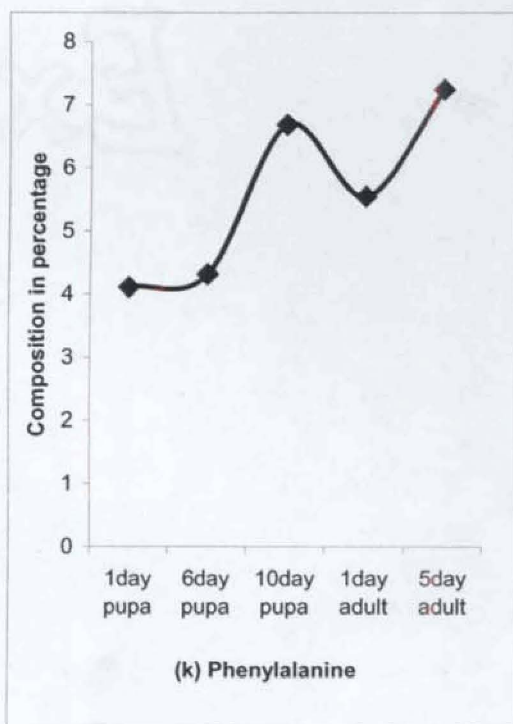


Fig. 9 12 (e-h). Individual free amino acid as the percentage of the total free amino acids in the insect.







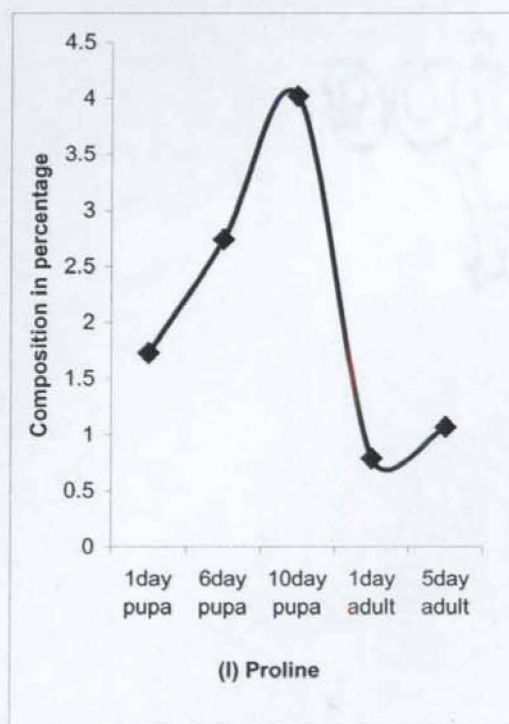
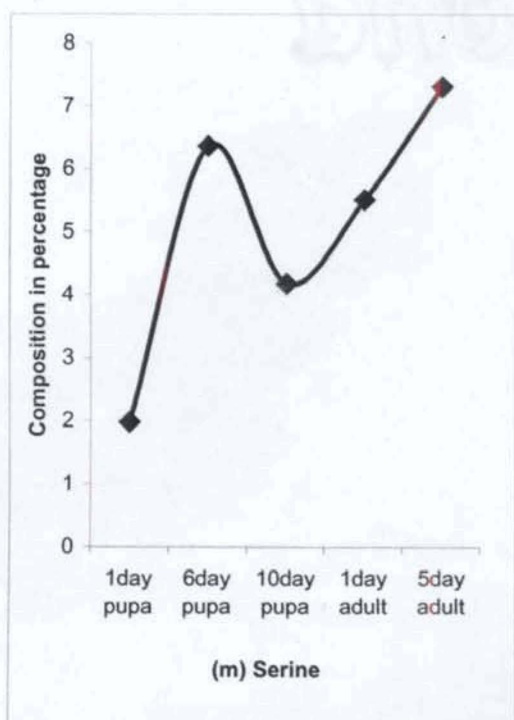
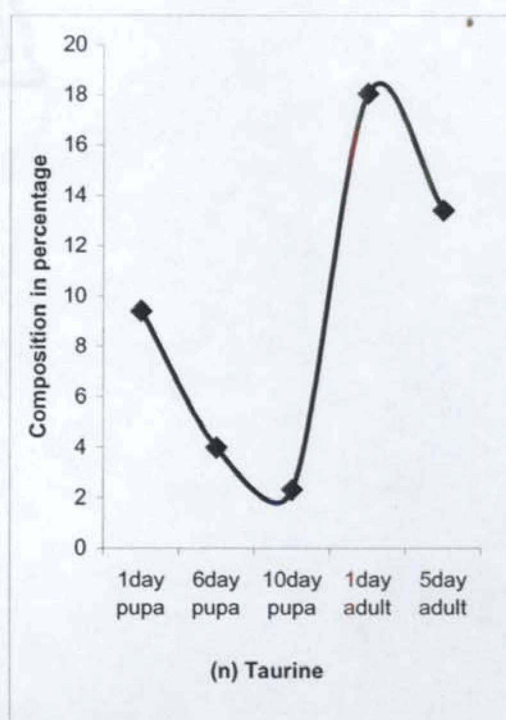
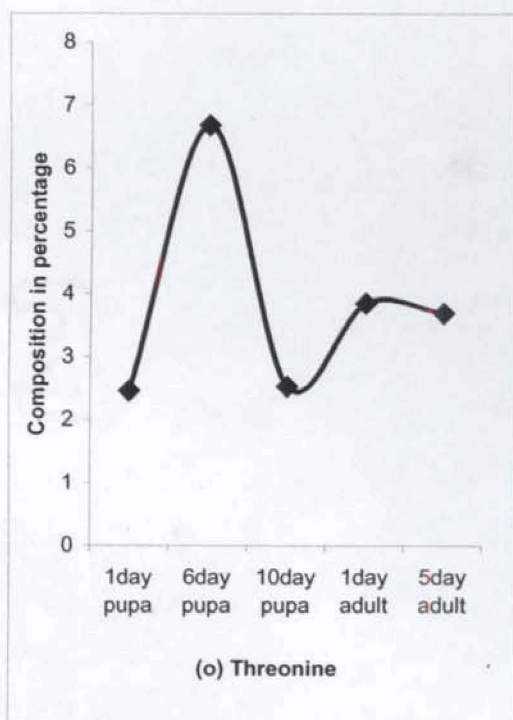


Fig. 12 (i-l). Individual free amino acid as the percentage of the total free amino acids in the insect.







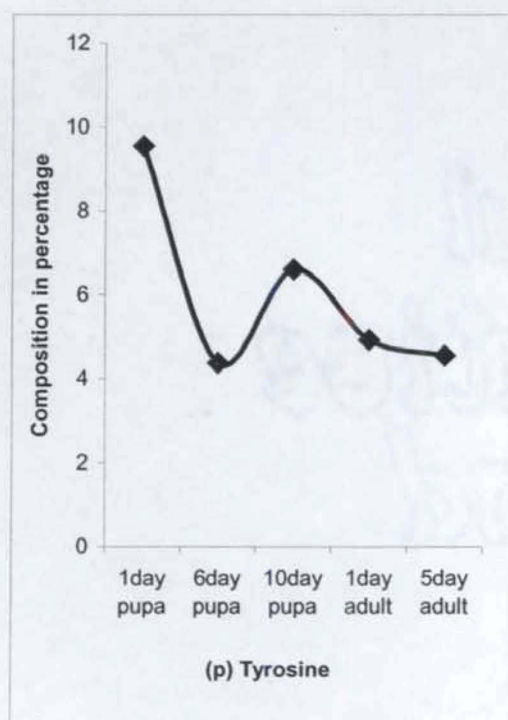


Fig. 12 (m-p). Individual free amino acid as the percentage of the total free amino acids in the insect.

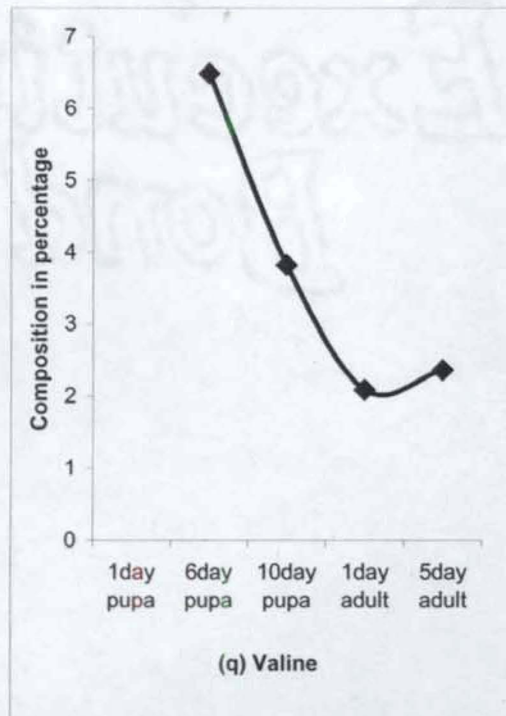


Fig. 12(q). Individual free amino acid as the percentage of the total free amino acids in the insect.

Chromatography of the amino acid extracts of pupa and adult stages using HPLC showed 17 identifiable amino acids.On the basis of the percentage of individual amino acids in the total free amino acids, the content of histidine was the most predominant followed by hydroxyproline. The relative abundance of the predominant amino acids in each stage is as follows:

1 day pupa: histidine > hydroxyproline > glutamic acid > tyrosine> taurine > arginine > lysine > phenyl alanine > alanine > leucine > threonine > isoleucine > glycine > serine > proline > aspartic acid > threonine. Valine was not detected.

6 day pupa: histidine > hydroxyproline > arginine > glutamic acid > threonine > valine > glycine > serine > tyrosine > phenylalanine > taurine > isoleucine > proline > lysine > alanine > leucine > aspartic acid.

10 day pupa: histidine > lysine > hydroxyproline > arginine > phenylalanine > tyrosine > glutamic acid > serine> proline > valine > threonine > glycine > taurine > isoleucine > leucine > alanine > aspartic acid.

1 day adult: hydroxyproline > taurine > glutamic acid > histidine > phenylalanine > serine > alanine > tyrosine > threonine > aspartic acid > glycine > arginine > isoleucine > valine > leucine > proline > lysine.

5 day adult: hydroxyproline > taurine > histidine > arginine > serine > phenylalanine > lysine > glutamic acid > isoleucine > tyrosine> leucine > threonine > valine > glycine > alanine > proline > aspartic acid.

Individual free amino acids

A perusal of the literature reveals that there is no much difference in the metabolism of amino acids in different orders of insects and other animals except that there is a change in the magnitude of the metabolism of individual amino acids. Every animal has its own specific characteristic biochemical features which make them unique from the other though the basic pattern will always be the same. The characteristic features of the insect cuticle and their periodic remodelling during the life cycle demand a ready supply of amino

acids for the synthesis of various integumentary structures. Since the adult structures of holometabola are entirely different from that of its larva, an entire reorganization of tissues takes place inside their pupa. The advantages derived from the retention of a high titre of amino acids are 1) it ensures a ready supply of materials for the synthesis of integumentary structures (2) metabolism of amino acids provide energy 3) it helps the animal to maintain the acid base balance and osmotic pressure.

a) Glutamic acid

The relative proportion of glutamic acid was maximum in the first day pupal stage with 12.94 percentage of the total but declined thereafter. The proportion of glutamic acid was also high in 1st day adult but declined in the 5 day adult. Glutamic acid and glutamine are important precurssors for the synthesis of purines, pyrimidenes, NAD and for various transamination reactions, which forms connecting link between carbohydrate and protein metabolism. Further, the assimilation of ammonia is also important in the biosynthetic pathway to glutamate and glutamine pathways of glutamine metabolism in *Spodoptera frugiperda* and evidence for the presence of nitrogen assimilation system (Drews *et al.*, 2000). High concentration of uric acid observed also invariably requires large amount of glutamine.

b) Proline and hydroxyproline

The high proportion of proline and hydroxyproline indicates their importance in the overall metabolism of amino acids in the insect. The proportion of proline slowly increased from first pupal stage reaching the peak in the late pupal stage but declined sharply in the adult stages. Generally, the proportion of hydroxyproline was high compared to the amounts of other amino acids. The pattern of changes observed for hydroxyproline was inversely proportional to proline. Hydroxyproline occurs exclusively in structural proteins of animals. As proline and hydroxyproline form important amino acids in the synthesis of integumentary structures, their enhanced synthesis is expected during pupal-adult transformation.

Proline is synthesized from arginine. Thus, the metabolism of arginine results in the subsequent increase in the amount of proline which is hydroxylated to form hydroxyproline and also urea production. The proline metabolism has multiple function, that is, as an energy source, precursor for cuticular structure and the synthesis of urea (Bursell, 1963, 1966; Bursell *et al.*, 1974; Sacktor, 1975). Mosquitoes utilize proline as a temporary nitrogen sink to store ammonia arising from deamination of amino acid and as an additional mechanism for shuttling ammonia between flight muscles and fat body of mosquitoes (Goldstein *et al.*, 2003; Scaraffia and Well, 2003).

c) Arginine

The active metabolism of arginine would result in the production of urea and synthesis of proline. The proportion of arginine was high during the pupal period and with a dip in the 1st day adult. The relatively high proportion of arginine in the first, middle and late pupal stages indicate its active synthesis during the respective stages. The high levels of urea found in the pupal stages of the insect can be explained in the above context.

d) Lysine

Lysine occurs consistently in the haemolymph of insects (Chen, 1962). There was a striking variation in the proportion of lysine during the development of pupal and adult stages of *B. mori*.

e) Glycine

Estimation of glycine showed that its proportion was more or less same in the pupal and adult stages of *B. mori* except that of the middle pupal stage where its proportion was maximum. One of the important functions of glycine is synthesis of structural proteins (Lucas *et al*., 1960; Bailey and Weis-Fogh, 1961; Seifter and Gallop, 1966; Srivastava, 1971). Silkworms, being lepidopterans need ready supply of raw materials for the synthesis of structural proteins during pupal-adult transformation. The conspicuous change in the proportion of glycine in the middle pupal stage indicate the

reorganization of the integumentary structures of the insect during the pupal period of development.

f) Serine

The proportion of serine was high in the pupal and adult stages except in the 1 day pupa. Serine has been reported to be a significant fraction in lepidopteran haemolymph, but its origin and metabolism are unknown (Chefurka, 1965).

g) Taurine

Taurine is involved in the synthesis of structural proteins. The proportion of taurine was considerably high in the adult stages of the insect. Massive synthesis of taurine occurs in the pupa of insects (Boolnaryk, 1981) and high levels of taurine was observed in the haemolymph of *D. melanogaster* and *P. regina* (Chen and Hanimann, 1965; Levenbook and Dinamarca, 1966). Chen (1985) has reported that the taurine synthesized in the pupa is carried over to the adult.

h) Aspartic acid

Aspartic acid is important in the synthesis of structural proteins and it is an important precursor for the synthesis of purines and pyramidines (Lazar and Mohamed, 1988). The proportion of aspartic acid was low in the pupal and adult stages except in the 1st day adult. Aspartate plays a major role in

transamination reactions and thus forms a connecting link between carbohydrate and protein metabolism (Katunuma *et al.*, 1968).

i) Threonine

Threonine accounts to about 2.46 percentage to 6.68 percentage of the total free amino acids. The peak value of threonine was observed in the middle pupal stage. Threonine can be converted to glycine and then to serine which in turn can be catabolised liberating energy.

j) Alanine

The proportion of alanine was comparatively low during the pupal and adult stages except in the 1st day adult. Alanine is one of the important amino acid in transamination reaction and is involved in gluconeogenesis (Harris,1988; Lazar and Mohamed, 1998).

k) Valine, leucine and isoleucine

The proportion of leucine and isoleucine was low during the pupal stage and 1st day adult but with a sharp rise in the 5th day adult. The proportion of valine was high in the middle stage pupa but declined in the adult stages. Leucine, isoleucine, and valine can be transaminated to corresponding α-keto acids, which are then oxidatively decarboxylated to yield a derivative of CO-A.

l) Phenylalanine and tyrosine

The proportion of phenylalanine and tyrosine was relatively high in the pupal and adult stages. Phenylalanine and tyrosine are involved in the tanning of insect cuticle. Tyrosine exhibits a conspicuous variation during the development of insects (Lazar and Mohamed, 1988).

m) Histidine

Histidine was found to be the most predominant free amino acid during the pupal stages of *B. mori*. The proportion of histidine accounts to 17.61 to 19.95 percentages and 8.32 to 9.36 percentages of the total free amino acids of the pupal and adult stages of *B. mori* respectively. It has been reported that histidine is the most predominant free amino acid of the fat body of the larva of the army worm, *Spodoptera mauritia* (Lazar and Mohamed, 1988). The role of histidine as a nitrogen store and in the removal of ammonia from the effective metabolic pool is well known (Bursell, 1967). The high levels of histidine observed in the pupal and adult stages can be explained in the above context.

Aspartate aminotransferase (AAT) activity

The AAT activity in the insect during the developmental stages of pupa and adult are presented in table 10 and figure 10.

Table 10. Aspartate aminotransferase activity in the insect during the pupal and adult stages

Day of development	AAT units/g, mean ± SD	AAT units/insect, mean ± SD
Pupal days		
Day 1	0.181 ± 0.021	0.295 ± 0.035
Day2	0.410 ± 0.039	0.533 ± 0.051
Day 3	0.432 ± 0.069	0.612 ± 0.098
Day 4	0.459 ± 0.065	0.642 ± 0.091
Day 5	0.470 ± 0.057	0.613 ± 0.075
Day 6	0.526 ± 0.045	0.579 ± 0.050
Day 7	0.442 ± 0.066	0.523 ± 0.078
Day 8	0.345 ± 0.048	0.483 ± 0.067
Day 9	0.263 ± 0.034	0.373 ± 0.049
Day 10	0.194 ± 0.031	0.262 ± 0.042
Day 11	0.107 ± 0.015	0.113 ± 0.016
Adult days		
Day 1	0.183 ± 0.019	0.061 ± 0.006
Day 2	0.142 ± 0.021	0.041 ± 0.006
Day 3	0.123 ± 0.019	0.023 ± 0.003
Day 4	0.082 ± 0.011	0.010 ± 0.001
Day 5	0.028 ± 0.003	0.007 ± 0.008

Values are the means of five determinations with standard deviations.
One unit of aspartate amino transferase activity was defined as 1 micro mole keto acid liberated per min. at 37°C under the assay conditions.

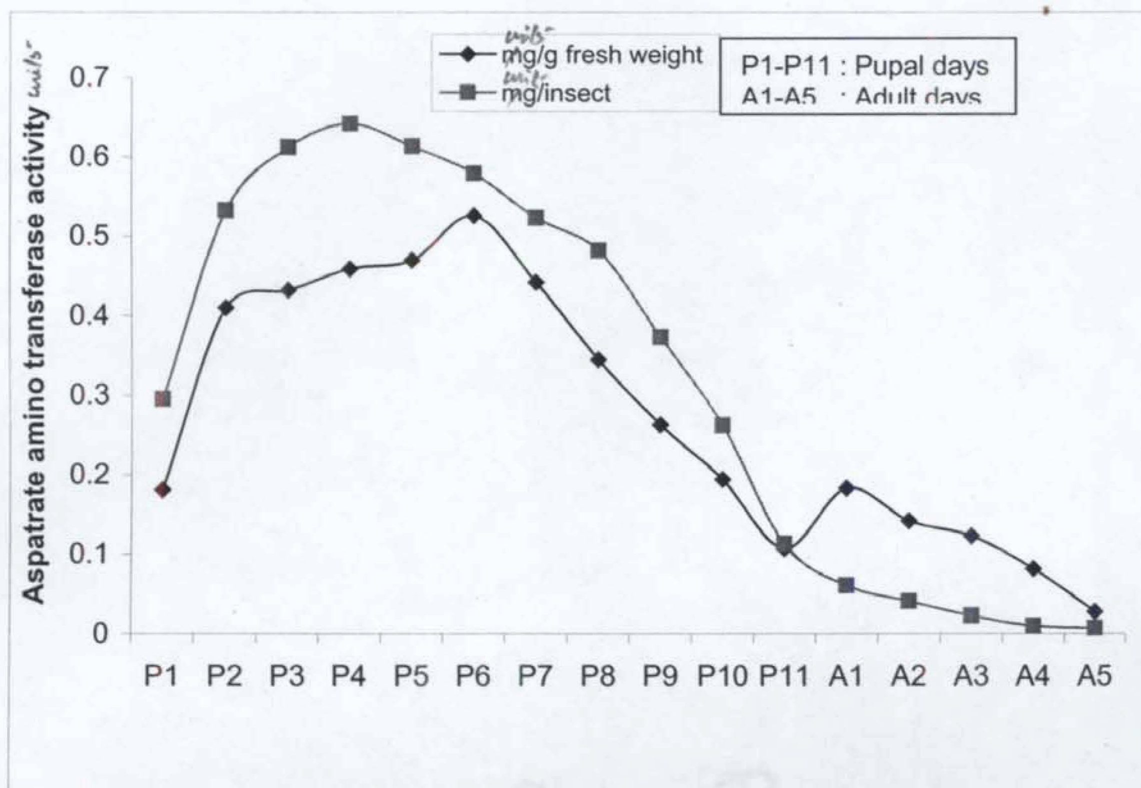


Fig. 10. Asparate amino transferase activity in the pupal and adult developmental days of *B. mori*

The AAT activity estimated per unit volume gradually increased from Day 1 of pupa, the activity was maximum towards the middle phase on Day 6. The activity slowly dropped on Day 11. There was rise of almost 70% of enzyme activity on Day 1 of adult stage, but declined towards the final stages of adult.

The AAT activity of per insect also showed similar pattern of variation in the pupal stages. The enzyme activity slowly declined throughout the developmental stages. The maximum activity recorded on Day 4 of pupal stage.

Alanine aminotransferase (ALAT) activity

The ALAT activity in the insect during the developmental stages of pupa and adult are presented in table 11 and figure 11.

Table 11. Alanine aminotransferase activity in the insect during the pupal and adult stages

Day of development	ALAT units/g, mean ± SD	ALAT units/insect, mean ± SD
Pupal days		
Day 1	0.329 ± 0.050	0.537 ± 0.082
Day2	0.367 ± 0.043	0.478 ± 0.056
Day 3	0.392 ± 0.040	0.532 ± 0.054
Day 4	0.429 ± 0.060	0.601 ± 0.084
Day 5	0.456 ± 0.062	0.570 ± 0.077
Day 6	0.502 ± 0.080	0.552 ± 0.088
Day 7	0.424 ± 0.049	0.515 ± 0.060
Day 8	0.357 ± 0.043	0.499 ± 0.060
Day 9	0.262 ± 0.036	0.392 ± 0.055
Day 10	0.195 ± 0.030	0.263 ± 0.041
Day 11	0.085 ± 0.011	0.089 ± 0.011
Adult days		
Day 1	0.113 ± 0.019	0.038 ± 0.006
Day 2	0.093 ± 0.013	0.030 ± 0.004
Day 3	0.073 ± 0.009	0.029 ± 0.003
Day 4	0.070 ± 0.007	0.021 ± 0.002
Day 5	0.055 ± 0.007	0.015 ± 0.002

Values are the means of five determinations with standard deviations.
One unit of alanine amino transferase activity was defined as 1 micro mole keto acid liberated per min. at 37°C under the assay conditions.

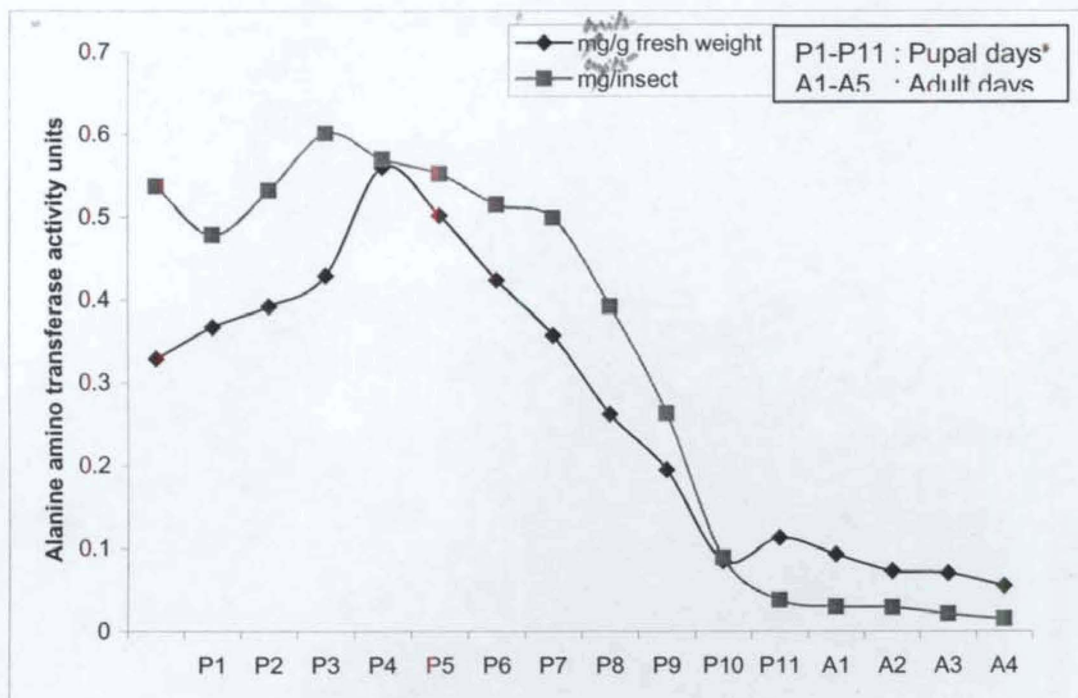


Fig. 11. Alanine amino transferase activity in the pupal and adult developmental days of *B. mori*

The ALAT activity estimated per unit volume gradually increased from Day 1 of pupal stage and reached maximum on Day 6, thereafter declining towards the last pupal day. The enzyme activity rose on Day 1 of adult, the increase was almost 30%. Thereafter the activity slowly declined towards the final adult stage.

The ALAT activity of per insect also showed similar pattern of variation in the pupal stages. The maximum activity recorded on Day 4 of pupal stage.

Glucose

The glucose content in the insect during the developmental stages of pupa and adult are presented in table 12 and figure 12.

Table 12. The content of glucose in the insect during the pupal and adult stages

Day of development	μ mole/g, mean ± SD	μ mole/insect, mean ± SD
Pupal days		
Day 1	18.354 ± 2.330	29.918 ± 3.799
Day2	30.123 ± 5.181	39.160 ± 6.735
Day 3	27.371 ± 3.194	37.492 ± 5.361
Day 4	25.840 ± 3.074	36.177 ± 4.305
Day 5	32.271 ± 4.905	38.912 ± 5.914
Day 6	40.178 ± 4.138	44.195 ± 4.552
Day 7	51.394 ± 4.882	62.700 ± 5.956
Day 8	49.325 ± 6.510	69.055 ± 9.115
Day 9	37.821 ± 4.046	45.769 ± 4.897
Day 10	20.700 ± 1.738	27.738 ± 2.329
Day 11	58.852 ± 9.475	61.794 ± 9.948
Adult days		
Day 1	32.634 ± 4.634	11.013 ± 1.563
Day 2	24.324 ± 3.137	8.218 ± 1.060
Day 3	19.456 ± 3.346	6.732 ± 1.157
Day 4	15.127 ± 2.193	5.121 ± 0.742
Day 5	14.151 ± 1.528	3.891 ± 0.420

Values are the means of five determinations with standard deviations.

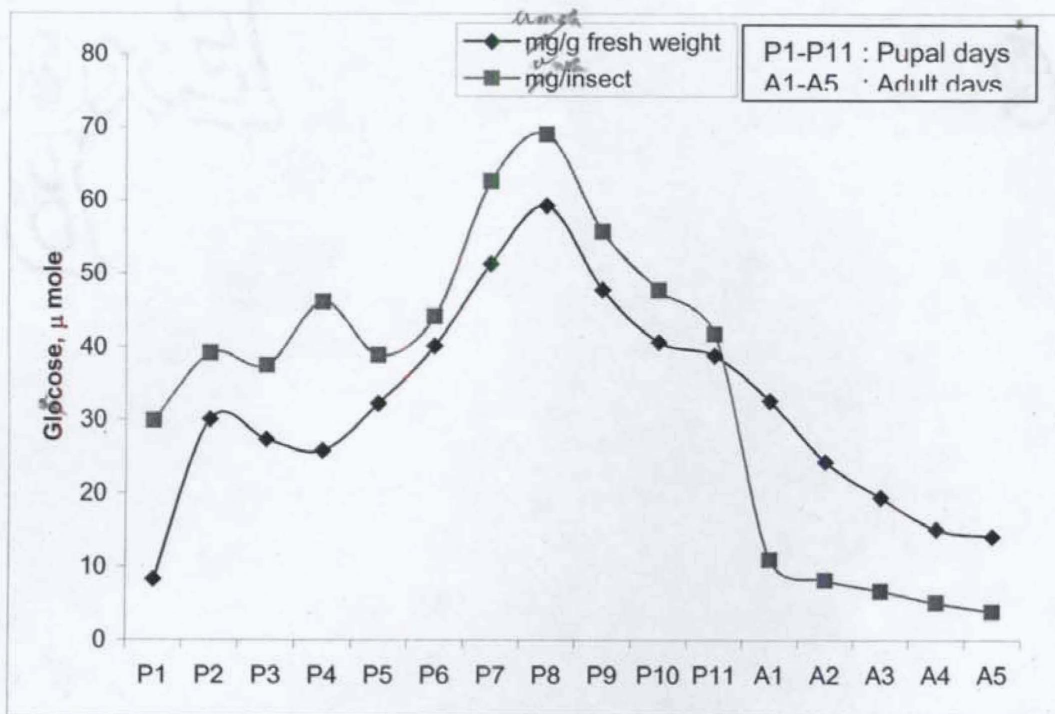


Fig. 12. The glucose content in the pupal and adult developmental days of *B. mori*

The glucose content in per unit volume in pupal stage rose from Day 1showing the peak value on Day 11 and was followed by a similar decline up to eclosion. The concentration of glucose showed a sharp dip in the first day adult which remained more or less the same during the adult stages.

The glucose content estimated per insect also followed similar pattern of variation throughout the developmental phases.

Alanine aminotransferase activity , aspartate aminotransferase activity and Glucose levels

The pupal to adult transformation of holometabolous insects undergo important physiological modifications during which major reorganization of

tissues and structures takes place. The AAT and ALAT activity provides a balanced amino acid pool, which is necessary for protein synthesis (Reddy *et al.*, 1991). Higher transaminase activities shows enhanced mobilization of free amino acids. The ALAT activity forms a general index of amino acid breakdown and AAT marks the mobilization of amino acids and gluconeogenesis (Adibi, 1968; Davidson and Longslow, 1975). Both amino acid oxidases and transaminases have been demonstrated in various tissues of insects (Kilby and Neville, 1957; Desai and Kilby, 1958; Price, 1961; Chen and Bachmann- Diem, 1964; Martigoni and Milstead, 1967).

In the present study, an increase in ALAT activity together with AAT activity was observed from beginning of the pupal development of *B. mori* which gradually declined towards final stages. The first day of adult insect showed an increased ALAT and AAT activity per unit weight of the insect. McAllan and Chefurka (1961) showed that the transaminase activity in the cockroach and the housefly increases during larval development and adult differentiation parallel to the increase in protein synthesis. An increase in aminotransferase activity was observed in *D. melanogaster* larva fed with carbohydrate free diets (Geer and Zacharias, 1974), in aestivating *Xenopus* laevis (Balinsky *et al.*, 1967b) and in mammals during postnatal development (Miller, 1969). This is inevitable for keeping pace with the increasing demand of keto acids for gluconeogenesis during starvation or postnatal development. However, with the cessation of feeding activity (a situation

almost similar to starvation) the activity of amino transferases may be linked to gluconeogenesis. The most important physiological functions of L-alanine amino transferase are the maintenance of the amino acid pool at a proper level for protein synthesis (Meister, 1965), the supply of metabolites for energy metabolism (Sacktor, 1974) and the catalysis of interactions between protein and carbohydrate metabolism (Katunuma *et al.*, 1968).

The level of glucose was seen increasing from Day 1 of pupal stage reaching peak value towards mid-pupal stage and declined towards final stages of pupa. There was a continuous decline in the amount of glucose towards the final stages of adult. As there is no external source of glucose for pupa, the increase in glucose levels suggests an increase in gluconeogenesis, which corresponds with the increasing level of AAT/ALAT activity. Alanine aminotransferase and aspartate aminotransferase activities in the final instar larvae of *Spodoptera mauritia* showed an increase with the larval growth, accompanied by a sharp rise in the level of glucose in the larval fat body and haemolymph indicating an elevated level of gluconeogenesis during development (Lazar and Mohamed, 1998).

SUMMARY

1. The developmental stages of pupa and adult stages of *B. mori* were marked by changes in the levels of protein metabolites and their enzymes.

2. The turnover of total proteins in the pupal and adult stages revealed that the reduction in the total protein content is not followed by a reduction per unit volume, indicating its functional role in the maintenance of the internal environment.

3. The adult insect contains about one-half of the total proteins of the pupa indicating extensive conversion of pupal proteins takes place during adult development.

4. Toxicity due to high titre of ammonia in the pupa of *B. mori* may be nullified due to the high titre of free amino acids and uric acid present during that period.

5. The changing levels of ammonia in the pupal and adult stages of *B. mori* indicate its conversion to other nitrogenous metabolites.

6. The levels of urease in the insect were in tune with the levels of ammonia in the insect indicating the hydrolysis of urea.

7. The results demonstrated the presence of urease activity in *B. mori* pupa and adult.

8. The titre of urea was about twice in the pupa when compared to the adult *B. mori*.

9. The present study suggests urea synthesis and its accumulation in *B. mori.*

10. The changing profiles of urea in the *B. mori* pupa indicate its function in osmoregulation rather than nitrogen elimination.

11. The high levels of arginase in the adult insect were not accompanied by an accumulation of urea indicating its de novo synthesis and excretion during that stage.

12. The changing levels of arginase in the insect indicate *de novo* synthesis of urea in *B. mori.*

13. The levels of uric acid were high in the pupal stages of *B. mori* demonstrating its suitability as a storage nitrogenous end-product.

14. It was suggested that storage of uric acid in the pupa of *B. mori* effectively eliminates it from the body metabolic pool.

15. The high concentration of uric acid in the pupal and adult stages of *B. mori* shows its suitability as an end-product of metabolism and as a storage material.

16. The high levels of creatinine in the pupal stages indicate high protein turn-over in the pupa.

17. The reduced level of creatinine in the adult stages of *B. mori* indicates reduced turnover of proteins during that period.

18. The high titre of amino acids and their changing profiles during the development of *B. mori* indicates its extensive metabolism during the development of the insect.

19. The levels of total free amino acids of the insect during the pupal and adult stages indicate its extensive utilization in the formation of adult structures.

20. The AAT and ALAT activities and levels of glucose in the pupal and adult development stages indicate gluconeogenesis.

BIBLIOGRAPHY

Adibi S.A. (1968). Influence of dietary deprivation on plasma concentration of free amino acids of man. *J. Appl. Physiol.*, 25, 52-57.

Aigaki T. and Osanai M. (1985). Arginase activity in the silkworm, *Bombyx mori:* development profiles, tissue distribution and physiological role. *J. Comp. Physiol.*, 155, 653-657.

Averswald L., Schneider P. and Gade G. (1998). Utilisation of substrates during tethered flight with and without lift generation in the African fruit beetle *Pachnoda sinuata* (Cetoricnae). *J. Exp. Biol.*, 201, 2333-2342.

Babers F.H. (1938). An analysis of the blood of the sixth instar southern army worm *(Prodenia eridania). J. Agricult. Research.*, 57, 697-706.

Bailey K. and Weis-Fogh T. (1961). Amino acid composition of a new rubber - like protein, reslin. *Biochem. Biophys. Acta.*, 48, 452-459.

Baldwin E. (1937). *An Introduction to Comparative Biochemistry.* Ed.I, Cambridge University Press, Cambridge.

Baldwin E. (1967). *Dynamic Aspects of Biochemistry,* V Ed., Cambridge University Press, London.

Balinsky J.B., Choritz E.L., Coe C.G.L. and Van der Schans G.S. (1967b). Amino acid metabolism and urea synthesis in naturally aestivating *Xenopus laevis. Comp. Biochem. Physiol.*, 22, 59-68.

Barrette F.M. and Friend W.G. (1970). Uric acid synthesis in *Rhodnuis prolixus. J. Insect Physiol.*, 16, 121-129.

Berridge M.J. (1965). The physiology of excretion in cotton stainer. *J. Exp. Biol.*, 43, 511-552.

Beyenbach K.W. (1995). Mechanism and regulation of electrolyte transport in malpighian tubules. *J. Insect Physiol.*, 41, 197-207.

Bheemeswar B. (1959). Some aspects of amino acid metabolism in insects. IV *Int. Congr. Biochem.*, 12, 72-87.

Bodnaryk R.P. (1981). Developmental changes in brain taurine during metamorphosis of the noctuid moth *Mamestra configurata. Insect. Biochem.* 11, 9-16.

Brown A.W.A. (1936). The excretion of ammonia and uric acid during larval life of certain muscoid flies. *J. Exp. Biol.*, 13, 131-139.

Brown A.W.A. (1938). The nitrogen metabolism of an insect (*Lucilia sericata* Meig): Uric acid, Allantoin, and Uricase. *Biochem. J.., 32, 895-902.*

Brown I.R.F.,D'costa M.A. and Rutessaria A. (1973). Uric acid metabolism in the tsetse fly, *Glossina morsitans. Comp. Biochem. Physiol.*, 46B, 741-747.

Buck J.D. (1953). Physical properties and chemical composition of insect blood. In *Insect Physiology*, pp.147-190. Ed. by Roeder K.D., Wiley, New York.

Buckner J.S. and Caldwell J.H. (1980). Uric acid levels during last larval instar of *Manduca sexta*, an abrupt transition from excretion to storage in fat body. *J. Insect Physiol.*, 26, 27-32.

Bursell E. (1963). Aspects of the metabolism of amino acids in the tsetse fly, *Glossina* (Diptera). *J. Insect Physiol.*, 9, 439-452.

Bursell E. (1966). Aspects of the flight metabolism of tsetse flies (*Glossina*). *Comp. Biochem. Physiol.*, 19, 809-818.

Bursell E. (1967). The excretion of nitrogen in insects. *Adv. Insect Physiol.*, 4, 33-67.

Bursell E. (1970). *An Introduction to Insect physiology*, Academic Press, London.

Bursell E., Billing K.C., Hargrove J.W., Mc Cabe C.T. and Slack E. (1974). Metabolism of the bloodmeal in tsetse flies (a review). *Acta Tropica*, 31, 297-320.

Caglayan S.H. and Gilbert L.J. (1987). *In vitro* synthesis, release and uptake of storage proteins by the fat body of *Manduca sexta. Comp. Biochem. Physiol.*, 876, 989-997.

Campbell J.W. (1973). Nitrogen excretion. In *Comparative Animal Physiology,* III, pp.279-316. Ed. by Prosser C.L., Saunders, Philadelphia.

Campbell J.W. and Bishop S.H. (1970). Nitrogen metabolism molluscs. In *Comparative Biochemistry of Nitrogen Metabolism*: *The invertebrates*, pp.103-206. Ed. by Campbell J.W., Academic Press, London.

Campbell J.W., Drotman R.B., McDonald J.A. and Tramell P.R. (1972). Nitrogen metabolism in terrestrial invertebrates. In *Nitrogen Metabolism and the Environment*, pp.1-54. Ed. by Campbell J.W. and Goldstein L., Academic Press, London.

Chamberlin M.E. and Phillips J.E. (1982). Regulation of haemolymph amino acid levels and active secretion of protein by malpighian tubules of locusts. *Can. J. Zool.*, 60, 2745-2752.

Chefurka W. (1965). Intermediary metabolism of nitrogenous and lipid compounds in insects. In *The Physiology of Insecta*, Vol.II, pp.669-768. Ed. by Rockstein, Academic Press, London.

Chen P.S. (1958). Studies on the protein metabolism of *Culex pipens* L.I. Metabolic changes of free amino acids during larval and pupal development. *J. Insect Physiol.*, 2, 38-51.

Chen P.S. (1962). Free amino acids in insects. In *Amino Acid Pools*, pp.115-138. Ed. by Holden J.T., Elsevier, Amsterdam.

Chen P.S. (1966). Amino acid and protein metabolism in insect development. *Adv. Insect Physiol.*, 3, 53-132.

Chen P.S. (1971). Biochemical Aspects of Insect Development. *Monogr. Dev. Biol.*, 3, 1-230.

Chen P.S. (1978). Protein synthesis in relation to cellular activation and deactivation. In *Biochemistry of Insects*, pp.145-203. Ed. by Rockstein M, Academic Press, London.

Chen P.S. (1985). Amino acid and protein metabolism. In *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, pp.177-218. Ed. by Kerkut G.A. and Gilbert L.I, Pergamon Press, Oxford.

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Chen P.S. and Bachamann-Diem C. (1964). Studies on the transamination reactions in the larval fat body of *Drosophila melanogaster. J. Insect Physiol.*, 10, 819-829.

Chen. P.S. and Haniman F. (1965). In exaustauschchromato graphische. Untersuchungen ijber die freien Aminosauren and Derivate wahrend der Entwicklung on *Drosophila melanogaster. Z. Naturforsch.*, 116, 305-314.

Chippendale G.M. (1970). Metamorphic changes in fat body proteins of the southwestern corn borer, *Diatraea grandiosella. J. Insect Physiol.*, 16, 1057-1068.

Chippendale G.M. and Kilby B.A. (1969). Relationship between the proteins of the haemolymph and fat body during development of *Pieris brassicae. J. Insect Physiol.*, 15, 905-926.

Clark T.M. and Bradley T.J. (1993). Short term changes in haemolymph properties of larvel *Aedes aegypti* in response to physiological challenges affect malpighian tubule secretion rater *in vitro. Am. Zool.*, 33, 43A.

Cochran D.G. (1973). Comparative analysis of excreta from twenty cockroach species. *Comp. Biochem. Physiol.*, 46A, 409-419.

Cochran D.G. (1975). Excretion in Insects. In *Insect Biochemistry and Function*, pp.177-281. Ed. by Candy D.J. and Kilby B.A., Chapman and Hall, London.

Cochran D.G. (1976). Excreta analysis on additional cockroach species and the house cricket. *Comp. Biochem. Physiol.*, 53A, 79-81.

Cochran D.G. (1979a). Comparative analysis of excreta and fat body from various cockroach species. *Comp. Biochem. Physiol.*, 64A, 1-4.

Cochran D.G. (1979b). Uric acid accumulation in young American cockroach nymphs. *Entomol. Exp. Appl.*, 25(2), 153-157.

Cochran D.G., Mullins D.E. and Mullins K.J. (1979). Cytological changes in the fat body of American cockroach, *Periplaneta americana*, in relation to dietary nitrogen level. *Ann. Ent. Soc. Am.*, 72, 197-205.

Cohen P.P. (1976). Evolutionary and comparative aspects of urea synthesis. In *The Urea Cycle*, pp.21-38. Ed. by Grisolia S., Baguena R. and Mayor F., John Wiley and Sons, New York.

Coles G.C. (1966). Studies on resiten biosynthesis. *J. Insect Physiol.*, 12, 679-691.

Collins J.V. (1969). The hormonal control of fat body development in *Calpodes ethlius* (Lepidoptera, Hesperiidae). *J. Insect Physiol.*, 15, 341-352.

Collins J.V. (1974). Hormonal control of protein sequestration in the fat body of *Calpodes ethlius* stall. *Can. J. Zool.*, 52, 639-642.

Collins J.V. (1975). Secretion and uptake of 14C proteins by fat body of *Calpodes ethlius* stall (Lepidoptera, Hesperiidae). *Differentiation*, 3, 143-148.

Collins J.V. and Downe A.E.R. (1970). Selective accumulation of haemolymph proteins by the fat body of *Galleria mellonella*. *J. Insect. Physiol.*, 16, 1697-1708.

Corrigan J.J. (1970). Nitrogen metabolism in insects. In *Comparative Biochemistry of Nitrogen Metabolism: The Invertebrates*, Vol. 1, pp.387-488. Ed. by Campbell, J.W., Academic Press, London.

David C.L., Pierce V.A., Aswad D.W. and Gibbs A.G. (1999). The effect of urea exposure on isoaspartyl content and protein L-isoaspartate methyl transferase activity in *Drosophila melanogaster. Comp. Biochem. Physiol.* B, 124, 423-427.

Davidson T.F. and Lonslow D.R. (1975). Change in plasma glucose and liner glycogen following the administration of gluconeogenic precursors to the starving fowl. *Comp. Bioch. Physiol.*, 52, 645-649.

De Jorge F.B. and Petersen J.A. (1970). Urea and uric acid contents in the hepatopancreas, kidney and lung of active and dormant snails,

Strophocheilus and *Thaumastus* (Pulmonata, Mollusca). *Comp. Biochem. Physiol.*, 35, 211-219.

Delaunay H. (1931). L' excretion azottee des invertebres. *Biol.Rev.*, 6, 265-301.

Desai R.M. and Kilby B.A. (1958). Some aspects of nitrogen metabolism in the fat body of the larva of *Calliphora crythrocephala. Arch. Int. Physiol. Biochem.*, 66, 248-259.

Donato B.G. (1938). Urea in blood and excreta during development of the silkworm. *Bull. Soc. Ital. Biol. Sper.*, 13, 735-736.

Drews M., Doverskog M., Ohman L., Chapman B.E., Jacobsson U., Kuchel P.W. and Haggstrom L. (2000). Pathways of glutamine metabolism in spodoptera frugiperda (sf g) insect cells: evidence for the presence of the nitrogen assimilation system, and a metabolic switch by IH/15 N NMR. *J. Biotechnol.*, 78, 23-37.

Dungera Ven P. and Brieged M. (2001). Protein catabolism in mosquitoes: Ureotely and uricotely in larval and imaginal *Aedes aegypte. J. Insect Physiol.*, 47, 131-141.

El Allaf D., Marchal C., El Allaj M., Chapella J.P., Deleixhe A. and Carlier J. (1984). Development of urea, creatinine and createnine clearance as a function of age and sex. *Arch. Int. Physiol. Biochemi.*, 91, 249-254.

Engle R.L. Jr. and Woods K.R. (1960). Comparative Biochemistry and Embryology. In *The Plasma Proteins*, Vol.II, pp.183-265. Ed. by Putnam F.W., Academic Press, London.

Ennor A.H. and Morrison J.F. (1958). Biochemistry of the phosphagens and related guanidines. *Physiol. Rev.*, 38, 631-674.

Evans P.D. and Wigglesworth V.B. (1990). *Advances in Insect Physiology*, Vol. 22, 303-304, 313-314. Academic Press.

Florkin M. (1949). *Biochemical Evolution*, pp. 157. Academic Press, New York.

Florkin M. (1966). *A Molecular Approach to Phylogeny*, Elsevier Publishing Company, Amsterdam, London and New York.

Florkin M. and Jeuniaux C. (1974). Haemolymph composition. In *The Physiology of Insecta*, pp.255-307. Ed. by Rockstein M., Academic Press, London.

Florkin M. and Morgulis S. (1949). *Biochemical Evolution*, Academic Press, London.

Florkin M. and Schoffeniels E. (1965). Euryhalinity and its concept of physiological radiation. In *Studies in Comphrensive Biochemistry*. pp. 6-40. Ed. by Munday K., Pergamon Press, London.

Fogal W.H. and Kwain M.J. (1974). Metabolism and excretion of nitrogen during metamorphosis and egg production in the saw fly, *Neodiprion sertifer. J. Insect Physiol.*, 20, 1287-1301.

Foster J.F. (1960) Plasma albumin. In *The Plasma Proteins,* Vol. I, *Isolation, Characterization* and *Function*, pp.179-239. Ed. by Putnam F.W., Academic Press, London.

Garcia I., Roche J. and Tixier M. (1956). Sur le metabolisme hydrolytique de I arginine chez les insectes et sa signification metaboliques. L.R. Seanc. Soc. Biol., 150, 632-634.

Geer B.W. and Zacharia C.E. (1974). Alteration of L-alanine amonitransferase, L-aspartate aminotranspherase and 6-hydroxyacetyle dehydrogenase activities in *Drosphila inclanogaster* larvae by nutritional manipulation. *Comp. Biochem. Physiol.*, 47 B, 53-62.

Gilmour D. (1961). *The Biochemistry of Insects*, Academic Press, London.

Gilmour D. (1965). *The metabolism of insects*. Freeman, San Francesco.

Goldstein D.A., Pennigton J.E. and Wells M.A. (2003). The role of haemolymph protein as a nitrogen sink during flood meal digestion by the mosquito *Aedes aegypte. J. Insect Physiol.*, 49, 115-121.

Goldstein L. (1970). Renal ammonia and acid excretion in infant rats. *Amer. J. Physiol.*, 218, 1394.

Goldstein L. and Forster R.P. (1960). Ammonia excretion from gills of sculpin *Myxocephalus Fed. Proc.*, 19, 359.

Gorden M.S., Schmidt-Nielsen K. and Kelly H.M. (1961). Osmotic regulation in the crab-eating frog (*Rana cancrivora*). *J. Exp. Biol.*, 659-678.

Hackman R.H. (1974). Chemistry of the insect cuticle. In The Physiology of Insecta, Ed.II, Vol.VI , pp. 215-270. Ed. by Rockstein M., Academic Press, London.

Harper H.A., Rodwell V.W. and Mayer P.A. (1993). Review of Physiological Chemistry. Lange Medical Publication, Los Altos, California. 131-182.

Harris R.A. (1988). Carbohydrate metabolism : Major Metabolic pathways and their control. In *The Text Book of Biochemistry with Clinical Correlation*, 2nd edition, pp.261-310. Ed. by Devlin T.H., John Wiley and Sons, New York.

Harrison J.F. (1994). Respiratory and ionic aspects of acid-base regulation in insects: An introduction. *Physiol. Zool.*, 67, 1-6.

Harrison J.F. (1995). Nitrogen metabolism and excretion in locusts. In *Nitrogen Metabolism and Excretion*, pp. 119-131. Ed. by Walsh P.J. and Wright R., CRC press, Boca Raton, FL.

Harrison J.F. (2001). Insect acid-base physiology. *Annv. Rev. Entomel.*, 46, 221-250.

Harrison J.F. and Phillips J.E. (1992). Recovery from acute haemolymph acidosis in unfed locusts :Role of ammonium and titrable acid excretion. *J. Exp. Biol.*, 165, 97-110.

Harrison J.G. and Kennedy M.J. (1994). *In Vivo* studies of the acid-base physiology of grasshoppers: The effect of feeding state on acid-base and nitrogen excretion. *Physiol,. Zool.*, 67, 120-141.

Haussinger D. (1996). The role of cellular hydration in the regulation of cell function. *Biochem. J.*, 313, 697-710.

Hayashi Y. (1961). Urea formation in the tissues of *Bombyx mori* larva. *J.Sericult.Sci.* Japan, 30,13-16.

Hirayama C. and Nakamura M. (2002). Regulation of glutamine metabolism during the development of *Bombyx mori larvae. Biochem. Biophys. Acta.*, 1571, 131-137.

Hirayama C., Sugimura M. and Shinbo H. (1999). Recycling of urea associated with the host plant urease in the silkworm larvae, *Bombyx mori. J. Insect Physiol.*, 45, 15-20.