EFFECTS OF ANTIOXIDANTS ON *BOMBYX MORI* AGEING

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By

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CERTIFICATE

This is to certify that the thesis entitled **"**Effects of antioxidants on *Bombyx mori* ageing**"** is an authentic record of research work carried out by Ms. Liji, C. under my supervision and guidance, in partial fulfilment 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 Ms. Liji, C. has passed the Ph.D. preliminary qualifying examination held in 2004.

Dr. K. V. Lazar

DECLARATION

I, Liji, C. do hereby declare that this thesis entitled **"**Effects of antioxidants on *Bombyx mori* ageing**"** submitted by me to the University of Calicut for the award of the degree of Doctor of Philosophy under the Faculty of Science, is the bonafide record of research work carried out by me in the Laboratory of Insect Physiology and Biochemistry, Department of zoology, University of Calicut, under the guidance of Dr. K. V. Lazar. I further declare that no part of this thesis has been submitted previously for any other degree.

University of Calicut, **Liji, C.**

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INTRODUCTION

Ageing is a universal process whose manifestations are familiar and unambiguous and the old age in human as well as other animals can be recognized readily after minimal assessment. Despite this an accepted definition for ageing and a detailed understanding of the biological mechanisms underpinning ageing are elusive. Ageing has been defined as the progressive loss of function accompanied by decreasing fertility and increasing mortality and disability. In addition ageing has dramatic effects on the response to pharmacological, surgical and rehabilitative interventions. Altered response to therapeutic interventions might be considered in any future definitions of ageing (Lean and Counter, 2004).

Mortality rate increases exponentially in old age. Consequently, old age is considered to be the major risk factor for many diseases. Oxidative modification of DNA, proteins, lipids and small cellular molecules by reactive oxygen species (ROS) along with impaired antioxidant mechanism play some role in a wide range of common diseases and age-related degenerative conditions. These include cardio vascular diseases, inflammatory conditions and neuro degenerative diseases such as Alzheimer's disease and Parkinsonism, mutational disorders, chronic stress included perturbed homeostasis, immunodepression, inflammation, diabetes mellitus, peptic

ulcer, cancer and other diseased conditions. Oxidant damage by ROS is also associated to photo ageing radiation toxicity, cataract formation and muscular degeneration (Maxwell, 1995).

ROS include free radicals and non- radical species. The free radicals carry an unpaired electron and are unstable and reactive. They include super oxide, nitric oxide and the most reactive and toxic hydroxyl radical. Non-radical oxidants include hydrogen peroxide, single oxygen and ozone, which form free radicals in tissues through various chemical reactions. Once free radicals are initiated, they can propagate by involving in chain reactions with other less reactive types, the resulting chain reaction compounds generally survive longer in the body and thus increase the potential for cellular damage. Naturally free radicals occur as a result of aerobic oxidation in cells. Their formation also occur when oxygen in the blood stream combine with any of a diverse group of chemicals including those commonly found in polluted air, in primary and secondary cigarette smoke and such other chemical toxins. Additionally free radical production and damage are accelerated by the normal sunlight radiations and increased aerobic activities.

To protect molecules against toxic free radicals and other ROS, cells have developed antioxidant defence system that include the enzymes super oxide dismutase (SOD), which dismutates superoxide and glutathione peroxidase, which destroy toxic peroxides and small molecules including

glutathione. External sources of antioxidant vitamins like vitamin C, vitamin E and phytochemicals from plant rich diets provide important protection against oxidant damage (Borek, 2001).

A free radical has three stages - initiation, propagation and finally termination. They are terminated or neutralized by nutrient antioxidants, enzymatic mechanisms or by recombination with each other. The aim is to attain a delicate balance between free radical activity and optimum antioxidant activity. When a specific antioxidant meets a free radical in the blood stream at its appropriate activity site, they naturally combines and the free radical is converted into harmless water and oxygen. As a result antioxidant induced cellular damage lessens and health improves. However, the natural antioxidant mechanism can be inefficient and hence dietary intake of anti oxidant compounds become important. In addition to this there is an inverse relationship between dietary intake of antioxidant rich foods and incidence of a number of diseases. Therefore search into the determination of antioxidant capacity of different compounds become important.

Experimental ageing studies have been done in various groups of animals such as insects, nematodes, rodents *etc*. Theoretically, the ageing process pertains to single individuals; therefore studies concerned with the effect of ageing on physiological function should ideally be conduct on the same individual during its entire lifespan. But in practice such measurements,

especially at the cellular and molecular level can rarely be obtained without killing the organism. Most commonly age associated patterns are obtained from the populations by sampling different members of a cohort as it ages. Such cross – sectional sampling therefore often gives highly variable and confounding results (Donato *et al*., 1979). A convenient way to overcome this difficulty is to conduct studies prior to the period of 10 – 20% mortality in the population (Sohal, 1985). Accepting the universality of ageing process in multicellular animals, it is easier to overcome such difficulties by using insects as experimental animals. In insects the fruit fly *Drosophila melanogaster* (Sohal, 1985; Nicolsi *et al*., 1973) and the housefly *Musca domestica* (Rockstein and Miquei, 1973; Sohal *et al*., 1985) have been extensively used for ageing studies. Since larvae are the representatives of juvenile form they exhibit maximum protection against ageing activities (Pant and Srivastava, 1979; Reddy *et al*., 1994). Despite of the absence of glutathione peroxidase (GPx) in insects, the catalase mediates the main mechanism for H_2O_2 breakdown (Sohal, 1993). So insects are most useful in analyzing the effect of H_2O_2.

The reducing amino acids like tryptophan and tyrosine are potent inhibitors of lipid peroxidation and oxidative stress. In addition to this tryptophan is an important precursor of tryptophan hydroxylase enzyme and directly involved in the biogenesis of the neurotransmitter serotonin, the

decreased level of which increases neurodegenerative changes in central nervous system (Mosmann and Behl, 2002).

In the present study, a reducing amino acid tryptophan was administered through the feed material of final instar larvae of silkworm, *Bombyx mori*, in order to study its antioxidant effects on the growth and longevity of the larvae. The specific objectives of the study are to evaluate the physiological and biochemical changes during the development of the final instar larvae of silkworm, on the administration of antioxidant amino acid, tryptophan. The profiles of pro-oxidant H_2O_2 and antioxidant enzyme catalase during the development of the larvae was evaluated. The turnover of total protein and glucose in the larvae was also evaluated. The results are evaluated in the context of reduced oxidative stress and enhanced growth of the larva on the administration of tryptophan.

REVIEW OF LITERATURE

Ageing

The actual time of death or total longevity of an organism is determined either by the innate rate of physiological decline or by the rate of ageing and also by the severity of the extrinsic challenges destabilizing its homeostatic balance. Ageing is a highly complex phenomenon whose proper understanding involves the elucidation of many processes besides those involved in debilitation during the post reproductive phase (Sohal, 1985). According to the view of Rockstein and Miquei (1973), the lifespan of all metazoan animals can be subdivided in to four major stages; *viz* successful fertilization and embryonic development, growth, maturation and senescence. They defined senescence as the sum total of all those changes in structure and function which are deleterious and degradative in nature. It ultimately results failure of the individual to survive and thereby in the death.

Ageing is accompanied by many biochemical changes at cellular and subcellular levels. A salient feature of the ageing process in all populations is that the probability of death increases as a function of age. Statistical analysis of this relationship, indicates the age-specific death rate increases exponentially with the age. Ageing shows a broad phylogenetic distribution

but is not universal as some species exhibit no age associated increase in mortality or decline in fertility (Kirkwood and Austad, 2000). There exist no current reliable methods for the measurement of the rate of ageing and hence many aspects of the ageing process are outside of a clear conceptual framework. So it is difficult to explain when the developmental period stops and the senescent phase begins.

Theories regarding the mechanism of ageing

The most acceptable opinion among gerontologists is that, ageing is a mufti- casual phenomenon. Medvedev in 1990 cited that, about three hundred different hypotheses have been proposed to explain the mechanism of ageing. Since all the components in the body undergo some kind of alteration with age it is vital to separate the cause from the effect.

Theories of ageing fall almost exclusively within the domain of either the programmatic or stochastic schools of thought (Lints, 1971). According to the former view, ageing is a continuation of the process of differentiation and is under the direct control of genes specifically coding for a programmed impairment of certain functions. The later emphasize the uncorrectable intrinsic and extrinsic damages of living system. Another view, expressed by Cutler (1976) and Sacher (1978) envisioned that ageing is caused by the byproduct of cellular metabolism and the rate of ageing is governed by the efficiency of the mechanism, encoded by longevity determinant genes.

Free radical theory

Harman (1956) proposed that oxygen species with one unpaired electron (Free radical) induces deleterious reactions in the cells and the net effect of which constitutes the ageing process. Before Harman (1956), Gerschman *et al*. (1954) recognized that the mechanism of radiation and oxygen toxicity involve in the generation of free radicals.

Free radicals are produced within the cells during autoxidation of a variety of substances, action of some oxidative enzymes and most importantly by mitochondria during respiration (Chance *et al.*, 1979). Most of the oxygen consumed by the cells is reduced tetravalently to water due to the activity of cytochrome oxidase in mitochondria and a small proportion of oxygen is reduced univalently. Univalent reduction of oxygen yields intermediate substances such as superoxide anion radical ($O_2^{\cdot}$), Hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$). $O_2^{\cdot}$ is rapidly converted to H_2O_2 by the enzyme super oxide dismutase (SOD).

Reactions of free radicals with organic molecules can assume a cyclic propagation sequence where each reaction results, either by addition or subtraction of an atom, in the formation of a product and another free radical that can carry on the chain. Hence the termination of the chain reaction in biological systems usually depends upon the presence of an antioxidant molecule (Pryor, 1976; 1978).

In two different studies, Finkel and Holbrook (2000) and Longo (1999) proved that the over expression of antioxidant enzymes extend longevity and most long lived model organisms were shown to be resistant to oxidative stress. Thus they were overwhelmed the possible doubts about the widely accepted nature of free radical theory.

Somatic mutation theory

Since radiations accelerate the rate of mutations it was postulated that natural ageing may also be due to mutations occurring randomly in the somatic cells (Curtis, 1963). He has reported the evidence from regenerating liver to support this theory. The mutation load of cells would increase with age that lead to disfunctioning and altered expression of an ever-increasing number of genes. The consequences would be a decrease in the functional capacity of somatic cells. Studies of Clark and Rubin (1961) on the wasp, *Hapro bracon*, indicated that ageing may be due to accrual of genetic damage but it is occurred during irradiation.

The daur larva is an alternative larval stage of *Caenorhabditis elegans*, which allows animals to survive through periods of low food availability. Well-fed worms live for about three weeks, but daur larvae can live for at least two months without affecting post – daur lifespan. Mutations in the genes daf-2 and age-1 that produces a daur constitutive (Daf-C) phenotype

and in the gene clk- 1, which are believed to slow down the metabolism are found to be markedly increasing the adult lifespan (James *et al*., 2000).

Error catastrophe theory

Orgel in 1963 proposed this theory, otherwise called Orgel's hypothesis. It suggested that alteration in gene expression can occur in the absence of somatic mutations through a mechanism involving random errors in protein- synthesizing apparatus. The assumption behind this hypothesis is that random errors should occur in the synthesis of mRNA and in the reaction between amino acids and their respective activating enzymes. The two postulates of the error catastrophe theory are that positive feedback leads to an increase in error frequency and that the altered products significantly contribute to senescence. Harrison and Holiday (1967) fed five different amino acid analogues to larvae of *Drosophila melanogaster*. The lifespan of the adult flies decreased as a result of this treatment, which was interpreted to support Orgel's hypothesis.

Wilson (1973) raised an argument against this theory that the activity of many enzymes does not decline with age in insects or mammals, nor does the total amount of solute proteins within cells greatly increases with age, which would suggest that ageing is unlikely to be a result of the accumulation of aberrant protein molecules.

Theory of clonal ageing

Hayflick and Moorhead (1961) found that normal human embryonic fibroblast-WI 38 cells, when cultured *in vitro*, undergo a finite number of about 250 ± 10 replications prior to death. The total number of cell doublings remains constant even if cultures are preserved by freezing during an early passage for prolonged periods. In 1977 Hayflick has interpreted the limited proliferative capacity of normal cultured cells to a manifestation of ageing at cellular level. The presumption behind this hypothesis is that the factors which limit the proliferative capacity of cells in *in vitro* are substantially the same that are responsible for ageing *in vivo*. It is also found that the potential number of cell replications inversely related to the age of the donor. But several other factors such as inadequacy of the culturing protocol, poor correlation between maximum lifespan of the species and the potential cell doubling, long term survival capacity of the tissue transplants, poor influence of proliferative capacity towards cell death etc never support this theory.

Bell *et al.* in 1978 raised argument against this theory. According to their opinion cessation of cell proliferation in culture is a manifestation of cell differentiation rather than ageing and was suggested that ageing may not be a consequence of an intrinsic programme of death rather a product of extrinsic damage.

Gene regulation theory

The underlying assumption sustaining this school of thought is that senescence and death are an extension of the process of development and differentiation. According to the models proposed by Hahn (1970) the age related alterations such as phosphorylation, acetylation, methylation and ADP- rybosylation may occur in chromosomal proteins affecting their association with DNA. The distinct shifts in the pattern of protein synthesis during development and reduced thermolability of chromatin in old animals have been claimed to constitute supportive evidence for gene regulation theory of ageing. Experimental evidences were provided by Strehlar *et al*. (1971) and Krooth (1974).

It is known that as cell division proceeds the telomere length get shortened, unless their repeated sequences are replenished by telomerase. Bondar *et al*. (1998) proposed that cells become senescent when progressive telomere shortening during each division produces a threshold telomere length. The simplest way to explain the prevalence of telomerase in human malignancies is to assume that telomere shortening is a tumor suppressing mechanism. This interpretation was assailed by studies of a knockout mouse lacking the essential RNA sub unit of telomerase (De Lang, 1998). Cells from the mice still could undergo malignant transformations and form tumors after transformation with viral onco protein contrary to the predictions of the

telomere - clock model. However, alternative mechanism of telomere maintenance could have been activated in these cells. Telomere shortening may be one mechanism operating in conjunction with other metabolic pathways.

Evolutionary theory

This theory postulates that ageing could occur by the accumulation of deleterious mutations which are expressed later in life (Medawar, 1952). Findings of Williams in 1957 which gave much support to Medawar's postulates was that many genes which have a beneficial effect during early part of life have a pleiotropic deleterious effect on fitness of the organism in later life.

According to Patridge and Bartson (1993), the key to the evolutionary theory of ageing is that the force of natural selection decreases with age. A young organism has its entire reproductive period ahead of it, however long that period may be. An old organism has a smaller fraction of that reproductive period remaining. A mutation with a negative effect expressed in the young organism would affect the entire reproductive period, and thus there is a strong selection against that mutation. A mutation with a negative effect expressed only later in life would not affect the early part of the reproductive period and there is less selection against that mutation. Thus, with increasing age there is progressively less force of natural selection, and

therefore less selection for “fitness” of the old individuals. This situation is proposed to give rise to two genetic phenomena that causing ageing. The first one is the mutation accumulation, which explain the random accumulation of inherited mutations that have adverse effect late in life. The second one is the antagonistic pleiotropy, which is the effect of a gene that has a positive benefit early in life and a negative effect late in life. These phenomena are proposed to result in the structural and functional deterioration and increased mortality rate that characterized ageing (Tower, 1996).

Factors affecting lifespan and ageing

Clark and Rockstein (1964) has categorized the factors governing and affecting the course of ageing and lifespan into two namely the genetic factors and the environmental factors. According to them the programmed cessation of growth, failure of juvenile hormone, production and depletion of essential substances are the main genetic factors, while the extrinsic radiation, pathological insults and influences of a changing environment are included under the later. On the basis of various analyses of mortality in a variety of populations under different experimental condition, Sacher (1977) believed that the actual lifespan of an organism is determined by two major factors: the rate of ageing and the vulnerability of the organism to death from age independent pathological causes.

Role of central nervous system

Different facts show in total that the brain is an initial substratum of ageing and DNA of neural cell is primarily susceptible for this process. Age dependent changes in the epiphysis - a part of central nervous system - bring a significant contribution to organismal ageing. It has been suspected for a long time that gradual loss of nerve cell play a role in ageing. A 33% reduction in cell number was reported by Rockstein (1950) in the brain of old worker honeybees. Age associated structural changes in the brain have been examined in *Drosophila melanogaster* (Herman *et al.,* 1971) and in *Musca domestica* (Sohal and Sharma, 1972). Their results suggested that neurons in old flies exhibited a reduction in cytoplasmic volume and accumulation of age pigment lipofuscin. Comparison of the brains from young and old *Musca* indicated no reduction in the number of neurons in old flies but several degenerative alterations such as the loss of ribosome, focal cytoplasmic degeneration, cellular autophagy and most prominently the accumulation of lipofuscin were observed in the nerve cells. Accumulation of myeloid residual bodies is the most striking feature of ageing in the nerve cells of the brain of the scorpion fly, *Panorpa vulgaris* (Collatz and Collatz, 1981).

Olovnikov (2003) in a detailed study about the redusome hypothesis of ageing and the control of biological time during individual development, provided a very clear picture with enough evidences about the key role of the

nervous system and its energy metabolism in the lifespan of animals. He sited that, a selective irradiation of an animal brain does not evoke accelerated ageing in absence of a radiation disease. The irradiation of the whole fruit fly larvae did shorten the lifespan of the imago. Being administered at the larval stage, a radio sensitizer 5 - bromo - 2- deoxy uridine (Brd U) deteriorates photo sensitivity of an imago and he concluded that the initial substratum of an imago ageing was solely the DNA of nervous ganglia. Montemayer *et al.* (1990) were detected an acidic protein of approximately 40,000 Da in the nervous system of the moth, *Manduca sexta* coincident with neuronal death.

Among the target organs of hypertensive vascular disease the brain is most affected by ageing and oxidative stress. The brain contains a high concentration of polyunsaturated fatty acids in its cell membranes. These fatty acids are targets of oxygen derived free radicals which cause chain reactions of lipid peroxidation (Ohtsuki *et al*., 1995). Kinoshita *et al.* (1999) studied the lipid peroxidation and oxidative stress in spontaneously hypertensive (SHR) rats compared with those of normal Wistar Kyto rats (WKY) and suggested that thio barbituric acid reactive substances (TBARS), end product of lipid peroxidation and an indirect marker of oxidative stress are increased in the brains of spontaneously hypertensive rats (SHR). Kishi *et al*. (2004) studied the effect of ROS in the rostral ventrolateral medulla (RVLM) in the brain stem, where the vaso motor center is located, in spontaneously hypertensive

rats. Their results suggest that oxidative stress in the RVLM increase blood pressure which might be caused by an increase in sympathetic nerve activity.

Influence of endocrine system

Growth hormone (GH) and Insulin like growth factor-1(IGF-1), a mediator of GH actions, have been of particular interest because of their thymopoetic effects and declining serum concentration during ageing (Rodriguez *et al*., 1998). Effect of human growth hormone on human ageing was studied by Corpas *et al*. (1998). According to their view, increased adiposity exerts an effect, independent of age, to decrease IGF-1 levels because both ageing and obesity are associated with elevation of plasma insulin levels, resulting in enhanced feed back inhibition of GH, and hence IGF-1 secretion. They also suggested that, a decrease in physical activity may contribute to the reduction in serum IGF-1 levels with age. This is the evidence for the age- independent positive correlation of plasma IGF-1 binding protein, IGF BP-3 with IGF-1 levels. A recent study on mammalian systems (Richardson *et al*., 2004) shown that a reduction of insulin and IGF-1 levels in plasma or reduction in insulin/ IGF-1 signaling are correlated with increased longevity and retarded ageing.

Juvenile hormone (JH) plays a significant role in insect metamorphosis and reproduction. John and Muraleedharan (1993) noticed that adult emergence was inhibited in larvae of *Achaea janata* treated with juvenile

hormone analogue. It was also noticed that juvenile hormone analogue can make changes in lipophorin of the haemolymph (Gonzalex *et al.*, 1991). 1n a study about the modulatory influence of juvenile hormone analogue and 20 hydroxy ecdysone (20 HE) on lipophorin synthesis in red cotton bug *Dysdercus cingulatus*, Mohan and Muraleedharan (2005) suggested that juvenile hormone analogue inhibits lipophorin production while 20 HE stimulates it and it was found that in *Drosophila melanogaster* juvenile hormone, which result from InR signal pathway mutation is sufficient to extend lifespan and insulin like ligands, non autonomously mediate ageing through retardation of growth or activation of specific endocrine tissue.

Reddy *et al.* (1996) proved that thyroxin has a direct control on metabolism and age dependent changes through influencing ATP ase system in tasar silkworm, *Antheraea mylitta.* The secretory peptides lutenising hormone – releasing hormone, enkephalin, angiotensin and oxytocin are biochemical antioxidants in aqueous medium. These hormones scavenge free peroxy radicals, prevent the oxidation of low - density lipoprotein, and inhibit lipid peroxidation in brain membranes. They also can scavenge reactive nitrogen species derived from nitric oxide and peroxy nitrite. An analysis of the structure - activity relationship indicates that their antioxidant activity is derived from the occurrence of solvent exposed tyrosin and tryptophan residues which is consistent with the mass spectrometry results. (Moosmann and Behl, 2002).

Role of mitochondria in cellular ageing

As mitochondriae are the principal site of cellular oxidation processes and energy production, they are more susceptible to age related oxidative damages. The mitochondriae are the major intracellular producers of O_2 and H_2O_2. The rate of mitochondrial O_2 and H_2O_2 generation increases with age in houseflies and the brain, heart and liver of rat (Sohal and Brunk, 1992). They argued that the rate of mitochondrial O_2 and H_2O_2 generation rather than the antioxidant level, may act as a longevity determinant. In a previous study Sohal *et al*. (1990) observed that, even though the rate of H_2O_2 generation was inversely related to the maximum lifespan potential (MLSP) of the species, under identical conditions mitochondria from animals with MLSP release relatively greater amount of H_2O_2. The comparative study of rate of mitochondrial H_2O_2 generation in houseflies of similar chronological but different physiological age by Sohal (1991) reveals that, at same chronological age, mitochondria from flies with a shorter life expectancy had a markedly high rate of H_2O_2 generation than those with a longer life expectancy.

Results of two different studies by Sohal and Sohal (1991) and Sohal and Dubey (1994) proved that oxidative damage to mitochondrial membranes, especially to the inter molecular cross- linking in the inner mitochondrial membrane can contribute towards the increased H_2O_2

generation. Partial inhibition of cytochrome oxidase activity lead to the stimulation of mitochondrial H_2O_2 production was noticed by Sohal (1993) in housefly. He also suggested that rather than NADH and cytochrome b, cytochrome c is more involved in the production of H_2O_2.

In a comparison between two age groups of *Drosophila* mitochondrial proteins, Fleming *et al*. (1986) noticed that age dependent changes in the expression of mitochondrial proteins are quantitative and not qualitative. Sohal *et al*. (1995) conducted a comparative study between five dipteran flies to elucidate the role of oxidative stress, mitochondrial super oxide anion radicals and the concentration of protein carbonyls. They noticed that the average lifespan potential of these flies was found to be inversely correlated with the rates of mitochondrial super oxide and H_2O_2 production and with the level of protein carbonyls, and to be directly related to the activity of cytochrome c oxidase. They concluded that longer lifespan potential in these insect species is associated with relatively low levels of oxidant generation and oxidative molecular damage. Yan *et al.* (1997) showed the importance of mitochondrial aconitase, an enzyme in the citric acid cycle, since it act as a specific target during aging of housefly.

Studies of Waris *et al*. (2001) on mitochondrially associated hepatitis B virus X protein (HBx), shed new light on the physiological significance of Its mitochondrial association and role in inducing oxidative stress which can

contribute to the liver pathogenesis associated with the hepatitis B virus infection. They show that, as a consequence of association with mitochondria, HBx constitutively induces activation of transcription factors.

It is now evident that ageing in higher animals may be due to accumulated mutations in the mitochondrial genome which reduce their functioning as respiratory units, and also that once a mitochondrial mutation appeared in a cell it leads to the overgrowth of the wild type mitochondria (Kadenbach and Hocker, 1990). It is also assumed with indirect evidence that antioxidants may reduce mutationally relative events such as the development of malignant disorders (Troll, 1991). Mitochondriae are also the major site of reactive oxygen species (ROS) production. The ROS produced immediately react with the mitochondrial membrane to initiate lipid peroxidation and cause formation of Conjugated Dienes (CD), the initial index of lipid peroxidation (Soumya *et al*., 2006).

Caloric restriction and ageing

Caloric restriction (CR) extends lifespan in a wide spectrum of organisms and for decades it was the only regimen known to promote longevity in mammals (Roth *et al*., 2001). But long term caloric restriction lead to the extinction of lifespan in laboratory mammals (Barrews and Kokonnen, 1981; Hagopian *et al*., 2003). Kopec (1928) reported that under nutrition prolonged the period of larval development in *Drosophila*

melanogaster but the lifespan of the underfed adults was distinctly shorter than the controls. CR can also delay the onset or reduce the incidence of many age related disease including cancer and diabetes and also reduce the levels of reactive oxygen species through a slowing in metabolism. (Roth *et al.*, 2001).

Several studies showed that caloric restriction increases the replicative lifespan in yeast by activating Sir-2, a highly conserved NAD- dependent deacetylase which act as a key regulator of lifespan. (Kaeberlein *et al*., 1999; Tissenbaum and Guarente, 2001; Lin *et al.*, 2000; 2002) Sin-2 is an NAD - dependent histone deacetylase and is required for chromatin silencing and lifespan extinction (Imai *et al*., 2000; Landry *et al*., 2000; Smith *et al*., 2000). Electron transport is required for longevity during CR (Lin *et al*., 2002). He further proved that the requirement of electron transport during CR is due to a shunting of carbon metabolism from fermentation to the mitochondrial TCA cycle. The concomitant increase in respiration is necessary and sufficient for the activation of Sir-2 mediated silencing and extension of lifespan. Lin *et al*. (2000) added some more findings like CR decreases NADH levels, and the NADH is a competitive inhibitor of Sir-2. CR increases the intracellular NAD/ NADH ratio by up- regulating respiration, thereby decreasing NADH levels. CR experimental studies in old mice by Hagopian *et al*. (2003) proved the significant increase in the activities of pyruvate carboxylase, phospho enol pyruvase, carboxy kinase, fructose-1, 6-di phosphatase and glucose-6-phosphatase when compared with controls. While branched chain aminoacid

transaminase was unchanged, increased activities of tyrosine, tryptophan, histidine, phenylalanine, alanine and aspartate transaminases, as well as of malate and glutamate dehydrogenases were also observed as an indication of increased gluconeogenesis.

The impact of CR on ageing was recently proved by Wang *et al*. (2007) in dog populations. According to them long term restriction of energy intake without malnutrition is a robust intervention that has been shown to prolong life and delay age related morbidity.

Stress resistance and longevity

Stress is known to disturb the physiological homeostasis of the organism and the ability to cope with such stressful stimuli is crucial determinant of health and diseases. Complex neuro chemical pathways have been demonstrated to regulate stress responses and interactions between the host factors that are instruments in deciding the nature and the extent of impact of such aversive inputs on the biological system. Factors like emotionality and prior stress exposure are also known to influence the stress responses, and adaptation to stress is known to occur after repeated exposure to such aversive stimuli (Selye, 1936; Chapman *et al*., 1954; Henke, 1987; Ray *et al*., 1987; Henke,1988; Whitton, 1995).

Prokaryotic and eukaryotic cells at elevated temperatures have been shown to mount a typical heat shock response resulting in the synthesis of a

new set of proteins called heat shock proteins-HSPS. They participate in antigen presentation, intracellular trafficking and apoptosis (Kiang, 1998). Nylanstend *et al*. (2000) suggested that heat shock proteins play an important role in cancer. HSPS-70 is abundantly expressed by human tumors and tumor cell lines and has been shown to be essential for the survival of cancer cells. Mammalian cells are known to respond to a stress response when subjected to unfavorable conditions like hypothermia or hyperthermia. Cells produce heat shock proteins, which protect the host from death (Welch, 1990). Studies about heat shock proteins by Wu (1995) and Rassow *et al*. (1997) proved that they represent conserved family proteins that are regulated at the transcriptional and translational level. They also function as supervisors and there by helping proteins to retain their native conformation.

Several studies support a major role for multiple stress resistance in the regulation of longevity in yeast and worms. In yeast, the down regulation of glucose signaling by ras-2, cyrl and sch 9 mutations increases longevity and stress resistance by activating stress resistance transcription factors, which induce the expression of genes encoding for several heat shock proteins (Longo and Fabrizio, 2002). Analogously, in worms, the activation of the insulin/ IGF-1 LIKE/ DAF-2 Pathway extends survival and increase thermo tolerance and antioxidant factor DAF-16 (Kenyon, 2001). Longo and Fabrizio (2002) also proved that, lifespan regulation of more complex organisms such as *Drosophila* and mice appears to depend on the activity of insulin/ IGF-1

stimulated pathways similar to those identified in yeast and worms. These pathways include serine threonine kinases (AKT/ pKB) and regulate the activity of several stress resistance proteins including SODs, catalase and HSPs. The analogous role of glucose or hormone/ growth factor signaling in stress resistance and ageing in the major genetics model systems suggest that longevity can be extended by similar mechanism in many organism. Longo (2003) suggested that the Ras pathway, which was already proved in yeast by him, might also negatively regulate longevity in higher eukaryotes.

Amstad *et al*. (2001) suggested the special ability of an anti apoptotic molecule Bcl-2 in rats, which protect the cells from undergoing apoptosis. Recently Khar *et al*. (2006) also suggested a significant role for Bcl-2 in heat induced apoptosis in BC-8 cells- a rat histiocytoma, and the loss of Bcl-2 protein following heat stress might contribute to apoptosis.

Emotional factors like stress can influence the neurobehavioral profile of the organism and precipitate an anxiety like syndrome and studies have indicated that the behavioral factors such as emotions are useful predictors of stress susceptibility and adaptation (Selye, 1936; Henke *et al*., 1991; Chrouses and Gold, 1992). Pal *et al*. (2006) showed that emotional stressors may induce free radical generation in the CNS, which might result in anxiogenic behaviour, which are attenuated by antioxidants.

Age related changes in the metabolism of insects

Insect ontogeny consists of both embryonic development and post embryonic development. One outstanding feature of the post-embryonic development is the striking change of forms occurring at successive ontogenetic periods. This is especially true for the holometabolous insects where a pupal stage is interposed between the larva and the adult. Phenomenon of metamorphosis is known to be complex. The final instar larvae of holometabolous insects undergo important physiological modifications associated with somatic growth in the feeding phase and reorganization of tissues in the non-feeding phase effecting the larval pupal transformation. Despite such a complexity there is apparently no principal difference between the development of insect and that of other animals.

The basic mechanism is the transformation of a developing organism from an immature to an adult state by the process of cell differentiation. As differentiation involves the formation of proteins, the primary importance of protein metabolism in insect development appears evident. Protein synthesis in holometabolous insects is well established by Chen (1985).

Results of various studies suggest that the total content of haemolymph proteins increases during larval development. The increase is most rapid during the time approaching pupation. In the larva of *Culex pipiens* the total protein concentration is low at earlier stages, but increases fourfold within

24h prior to puparium formation (Chen, 1959). Wyatt *et al*. (1956) reported that the blood protein of *B. mori* increased about 1- 2% in early third instar. Apparently the same is true for *Samia cynthia* where protein concentration, according to Laufar (1960a), increases rapidly from the third instar to a maximum in the spinning fifth larval instar. Similar changes have been noted for the beetle *Popillia japonica* (Ludwig, 1954).

From the morphogenetic point of view investigations of the haemolymph proteins are of particular interest because they provide us with an adequate background to judge the synthetic activity associated with the differentiation process in the developing organism. According to Florkin and Jeuniaux (1974) 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. The protein concentrations of plasma rises to a peak of over 200 mg/ml at the end of the actively feeding phase of third instars larval life, then falls slowly during the wandering period of late larval life with an accelerated decrease near pupation (Kinnear *et al*., 1968). Studies on the nature, origin and fate of major haemolymph proteins in *Calliphora* by Kinnear and Thomson (1975) proved that four quantitatively major proteins are found in the plasma of haemolymph which are immunologically, electrophoretically and structurally distinct and do not share any common subunits. Further these proteins are specific for different stages of development such as larva, pupa and adult.

Different developmental pattern of proteins was also identified in different animals including mammals and some of these are homologous to insect proteins also. In a study Tagawa *et al*. (1994) identified a novel protein tyrosine phosphatase gene from an embryonal rat brain. Its transcription is specific to brain and is developmentally regulated, as it is expressed at high levels in embryonal and neonatal stages but scarcely in an adult. Homology search found that this gene belongs to the same family of the membrane type tyrosine phosphatase gene of *Drosophila* (DPTPIOD), whose expression is specific to the central nervous system of the embryo.

Kilby (1963) stated the importance of fat body which functions both as storage center for fat, carbohydrate and protein and as a principal site for intermediary metabolism. In a study about the relationship between the proteins of the haemolymph and fat body during development of *Pieris brassicae*, Chippendale and Kilby (1969) revealed that the fat body was in fact selectively storing two major haemolymph proteins. During early larval life no blood proteins could be detected in the tissues of *Malacosoma.* At the onset of pupation haemolymph proteins were found in fat body, midgut wall and heart muscle. The pattern of incorporation was specific for each tissue, indicating that the absorption process was selective. Loughton and West (1964) also noted that the levels of blood proteins increased in the early pupa but declined in the late pupa.

A detailed analysis of the amino acid pattern at different embryonic stages would provide us with valuable information about the biochemical processes, which accompany morphogenetic events. Insects are known to contain an unusually large amount of free amino acids whose total concentration in some species has been estimated to be more than thirty times higher than that in other groups of animals (Florking, 1959). It is found that the ten amino acids arginine, histidine, lysine, tryptophan, phenylalanine, methionine, threonine, leucine, isoleucine, valine – which are known to be necessary for the growth of mammals have been proved to be also essential for insects (Lipke and Frankel, 1956; House, 1962). Chen (1962) claimed that a number of amino acids such as arginine, cystine, glycine, proline, tryptophan, tyrosine and phenylalanine are especially concerned with either moulting, differenciation, pupation or emergence of the adult. Chen (1960) found that in *Drosophila* the quantity of free amino acids per larva increases rapidly from 48h to 72h after oviposition at 25ºC, remains rather constant for the subsequent 24h and then drops steadily near the time of pupation. Buck (1953) has suggested that the high titre of amino acids in the haemolymph is correlated with active protein degradation and synthesis, which occurs during growth, moulting and metamorphosis. The changes in the total content of free amino acids in the haemolymph and fat body of *Spodoptera mauritia* showed a bell-shaped pattern of variation during the development of the larva (Lazar and Mohamed, 1988c).

A preliminary step in the intermediary metabolism of amino acids is the deamination which is catalysed by the aminases by cleaving oxidatively two amino groups from the amino acid molecule. The resulting ketoacids may act as acceptors for the transfer of amino groups from other amino acids serve as substrates for fat and carbohydrate synthesis, or join the main channel of oxidation *via* tricarboxylic acid cycle, the operation of which has been demonstrated in various insects (Levenbook, 1985).

The most active transamination reaction always involves glutamate, aspartate and alanine with the corresponding keto acids. There is also an active synthesis of glutamine from glutamate and ammonia in the presence of ATP and Mg. Although transaminase activity has been demonstrated in various insect tissues, Kilby (1963) has an opinion that the major part of the transamination process takes place in the fat body, which has been shown to exhibit many other synthetic activities and fulfill a large variety of metabolic functions similar to the midgut gland in mollusca and crustacea and the liver in vertebrates.

The changes in free amino acid concentrations with regard to the physiological state of insect are reported by Maddrell and Gardiner (1980) and Whitton *et al.* (1995). Since a high titre of free amino acids in the haemolymph is one of the most outstanding characteristic features of insects, it is not surprising to find amino acids in the excreta of many insect species

(Lazar and Mohammed, 1988c; Sinha *et al.*, 1991). Cochran (1985) proposed at least three patterns of amino acid voiding in insects *viz.*, normal nitrogenous excretion, unabsorbed digestive waste and high level specific amino acid excretion. It has been demonstrated by Ramsay (1958) that in the stick insect amino acids from the haemolymph can enter the malpighian tubules by passive diffusion. Some of them are probably reabsorbed in the rectum, but significant quantities may be eliminated with the faeces.

In insects as in other animals, glucose has a central place in carbohydrate metabolism. In the majority of species that have been examined, however, the amount of free glucose is quite small (Howden and Kilby, 1960). The most characteristic sugar of insect haemolymph is trehalose. Hansen (1964) found that insect haemolymph often contains so much trehalose that the sugar can easily be obtained in crystalline form. Typical carbohydrate content in haemolymph appears to reflect specialized diet. For example abundant fructose is found in larval haemolymph of the horse bot fly, *Gastrophilus intestinalis* is probably attributable to the high level content of the host's food. (Leevenbook, 1985) Glycogen has been recognized in insects and the greatest glycogen deposits in insects are commonly found in the fat body. For example in *B. mori* during the fifth larval instars the glycogen content of the fat body rises from 5 to 20% of dry weight (Shigematsu and Takeshita, 1959). According to them fat body glycogen constitutes a reserve that can be built up and drawn on as required.

When insects are starved, this reserve is drawn on to maintain blood trehalose level. Insect chitin, which accounts for a major part of cuticle is a polymer of N acetyl glucosamine units and an elevated level of glucose in the tissues is necessary to supplement the same during the remodeling of integumentary structures. Kramer *et al*. (1985) stated that chitin synthesis in insects involves utilization of glucose derived from trehalose. Variations in the glucose levels during larval development of *Spodoptera mauritia* was studied by Lazar and Mohammed (1998) and found that the feeding stage of larva was characterized by low levels of glucose in the tissue, where as the same increased sharply in the non feeding stage. In insects as in the vertebrates, the regulation of carbohydrate metabolism is effected in part by hormonal mechanism. Steel (1961) gave the most satisfactory evidence for action of an insect hormone on an enzyme system. He reported that injection of aqueous extract of corpora cardiaca in to adult cockroaches was followed by elevation of haemolymph trehalose.

Lipid peroxidation and oxidative stress

Lipid peroxidation can be defined as the oxidative deterioration of lipids containing varying numbers of carbon - carbon double bond. Lipid peroxidation is widely used as an indicator to relief oxidative stress and cell membrane damage (Halliwell and Gutteridge, 1990).

Lipid hydro peroxides are non- radical intermediates derived from unsaturated fatty acids, phospholipids, glycol lipids, cholesterol itself. Their formations occur in enzymatic or non enzymatic reactions involving activated chemical species known as reactive oxygen species (ROS). Free radicals exert their toxic effect by acting on DNA, membrane proteins and lipids. Anti cancer drugs like cyclo phosphamide are known to exert their cytotoxic effects by a free radical mediated mechanism (Dumonet *et al*., 2001) While such reactions might prove beneficial in chemotherapy, they will result in the build up of free radicals in normal animals that leads to membrane damage and inactivation or alternation of dietary oils rich with unsaturated fatty acids make target tissues like kidney more vulnerable to peroxidative damage (Venkatraman *et al*., 1998).

Fatty acids may be autoxidized either in free form or combined into glycerol lipids or glyco lipids. Thus oxidized tri acyl glycerol monomers include molecules containing different oxygenated groups, mainly hydroxyl keto and epoxy, as well as short – chain fatty acyl and short chain n-oxo fatty acyl groups as the main product. The majority of chain cleavage products formed from monohydroperoxides are molecules belonging mainly two groups, simple hydrocarbons and short chain aldehydes. Other far less characterized molecules were described and bear epoxy alcohol or ketone group, either alone or in combination, even with an aldehyde function. In contrast with unsaturated fatty acids cholesterol exists as a single molecular

species, its oxidation products are much less complicated to isolate and characterize (Smith and Johnson, 1989). Cholesterol may undergo autoxidation and photoxidation processes both give to oxysterols of various structures depending on the type of oxidation and the physical state of the substrate.

All unsaturated aldehydes may undergo further changes by autoxidation producing other volatile compounds. Thus hydroxyl aldehydes may undergo cleavage to give shorter chain aldehydes, some along with other chemical groups. Among all these substances malonaldehyde MDA is an object of a great interest despite of its complex and yet unknown origin. It is believed to be that poly unsaturated fatty acids (PUFA) of the cell membrane which undergo peroxidation, giving rise to products like conjugated dienes and malonaldehyde (Soumya *et al.*, 2006).

Oxidative stress, implicated in the pathogenesis of a wide variety of clinical disorders, refers to the cytological consequence of a mismatch between the production of free radicals and the ability of the cell to defend against them. Oxidative stress can thus, occur when the generation of free radicals increases or the capacity to scavenge free radicals and repair of oxidatively modified macromolecules decreases, or both (Sies, 1997). This imbalance leads to the accumulation of oxidatively modified molecules called reactive oxygen species (ROS) which produce significant functional

alterations in lipids, proteins and DNA molecules (Jacob and Burri, 1996). Griesmacher *et al*. (1993) observed that antioxidants which offer protection to oxidative damage, are disturbed in diabetes as reflected by greater levels of biochemical markers of free radical activity including malonaldehyde and decreased levels of vitamin E, vitamin C and uric acid.

Reactive oxygen species formations have the ability to alter reversibly or irreversibly the cellular function in humans. ROS alters the biochemistry and the physiology of the sperm and the antioxidant mechanism could protect the sperms from the damages produced by free radicals. Gallardo (2007) evaluated the antioxidant system in normal semen and made a conclusion that the measurement of the antioxidative and oxidative agents could serve to evaluate human infertility in those cases where the result of the spermato bioscopy appears normal.

Free oxygen radicals

In eukaryotic organisms ROS are mainly produced by cells come from the following four sources: a) Normal aerobic respiration in mitochondria, which generates superoxide radical ($O_2^{\cdot}$) and the ensuring toxic products, hydrogen peroxide (H_2O_2) and the highly reactive hydroxyl radical ($OH^{\cdot}$), b) Stimulated macrophages and polymorpho nuclear leucocyte, which release superoxide and nitric oxide radical ($NO^{\cdot}$), c) Peroxisomes, cell organelles that produce H_2O_2 as a by- product of degrading fatty acid and other molecules

and d) Oxidant by products that occur during the induction of cytochrome P450 enzymes. Exogenous source of ROS include the following: a) Tobacco smoke, which has a broad spectrum of oxidant - ionizing radiation, and generates free radicals in exposed tissues, notably the hydroxyl radical ($OH^{\cdot}$), b) UV light, which produces singlet oxygen (O_2) and OH, c) Ozone (O_3) and oxides of nitrogen in polluted air, d) Industrial toxins such as carbon tetrachloride, e) Drugs such as Phenol barbital, which is a known tumor promoter in liver and f) Charcoal-boiled foods, which form a variety of carcinogens, notably benzo (a) pyrene (Borek, 2001).

Mitochondrial respiration provides ROS including superoxide radical ($O_2^{\cdot}$) and hydrogen peroxide (H_2O_2). It is estimated that about 1-4% of consumed oxygen is converted to O_2^{-} and H_2O_2 at the mitochondrial level due to electron leaks (Turrens *et al*., 1982; Alexandre and Lehninger, 1985; Konstantinov *et al*., 1987). If these free radical species are not immediately neutralized, they form highly reactive hydroxyl radical that damage the biomolecules causing oxidative injury (Kleinveid *et al*., 1989; Halliwell and Gutteridge, 1985)

The most frequently studied reactive oxygen species are given below:

Super oxide radical ($O_2^{\cdot}$)

This ROS is formed when oxygen takes up one electron leakes in the mitochondrial electron transport but its formation is easily increased when exogenous components (redox cycling compound) are present. The production of super oxide radicals at the membrane level (NADPH oxidase) is initiated in specialized cells with phagocytic functions and contributes to their bacterial action. Enhanced production of super oxide anion is considered to play a pivotal role in the pathogenesis of CNS neurons. $O_2^{\cdot}$ generated xanthine and xanthine oxidase triggers cell death associated with nuclear condensation and DNA fragmentation in cerebellar granule neuron (Satoh *et al*., 1998).

Superoxide anion radical ($O_2^{\cdot}$), if not scavenged can highly deleterious to the living cells. The mechanism by which $O_2^{\cdot}$ causes damage to biological molecules is not precisely known, but it is widely believed to be due to the combined action of $O_2^{\cdot}$, H_2O_2 and metal ions, which results in the production of highly reactive hydroxyl radicals (Halliwell, 1981). Further, while elucidating the chain of events leading to oxidative damage in human umbilical endothelial cells exposed to high glucose, Qualiaro *et al*. (2007) suggested that the superoxide anion generated at mitochondrial level had a starting role in the cascade of events leading to hyperglycemia generated apoptosis.

Increased superoxide generation associated with pulmonary hypertension was noticed in faetal lambs by Brennan *et al.* (2003). They found a two fold increase in superoxide formation in lungs from persistent pulmonary hypertensive lambs (PPHN) compare with controls after nine days of ductal ligation. They further investigated the potential sources of the increased superoxide generated in the PPHN lungs and identified an increase in the P67 phox subunit of the NADPH oxidase enzyme complex.

Hydrogen peroxide (H_2O_2)

H_2O_2 is mainly produced by enzymatic reactions. These enzymes are located in microsomes, peroxysomes and mitochondria. Even in normoxia conditions, the hydrogen peroxide production is relatively important and leads to a constant cellular concentration between 10^{-9} and 10^{-7} M. It was found that H_2O_2 alone and in combination with other substances cause deleterious damages in the living tissue and the direct interaction of transition metal (copper) to DNA in the presence of H_2O_2 caused destabilization and fragmentation of naked DNA (Prasad *et al*., 2006)

In plant and animal cell, superoxide dismutase is able to produce H_2O_2 by dismutation of O_2, thus contributing to the lowering of oxidative reactions. In most eukaryotic cells super oxide dismutase exists in two different forms, cytosolic type containing copper and zinc and a mitochondrial type containing manganese (Fridovich, 1978). The natural combination of dismutase and

catalase contributes to remove H_2O_2 and thus has a true cellular antioxidant activity. H_2O_2 is able to diffuse easily through cellular membranes. Chance *et al*. (1979) proved that catalase is highly effective in the breakdown of H_2O_2 at high concentrations and also has the unique characteristic that it can degrade H_2O_2 by catalytic or by peroxidatic mechanism.

Hydroxyl radical ($OH^{\cdot}$)

Hydroxyl free radical ($OH^{\cdot}$) is one of the most reactive chemical species and is believed to be capable of reacting with almost any contiguous molecule (Halliwell, 1981). The iron catalysed decomposition of hydrogen peroxide is considered the most prevalent reaction in biological systems and the source of various deleterious lipid peroxidation products. It may also be noticed that an important part of hydroxyl radical is produced (with NO_2) by the decay of peroxinitrite or peroxy nitrous acid.

A free radical mechanism for the generation of OH may be deduced as follows.

$$O_2^{\cdot} + H_2O_2 \rightarrow OH^{-} + OH^{\cdot} + O_2$$

The rate constant for the above reaction is very low but can be accounted for if reaction is catalysed by traces of transition metal ions – the metal catalysed Haber Weiss reaction (Halliwell and Gutteridge, 1985). The various steps of this reaction are

$$Fe^{3+} + O_2^{\cdot} \rightarrow \quad Fe^{2+} + O^2$$

$$Fe^{2+} + H_2O_2 \rightarrow \quad Fe^{3+} + OH^{\cdot} + OH^-$$

And the net result is

$$O_2^{\cdot} + H_2O_2 \rightarrow \quad O_2 + OH^{\cdot} + OH^-$$

Bandyopadhyay *et al.* (1999) pointed out the release of transition ions from some metalloproteins during ischemic condition and cellular acidosis, resulting in generation of OH, as shown in the above reaction. The extent of damage to the cells by $O_2^{\cdot}$ and H_2O_2 increase in presence of the transition metal ions due to the generation of more powerful $OH^{\cdot}$. Another reaction involving myelo peroxidase and Cl^- ions represent an important $OH^{\cdot}$ production process in neutrophils during phagocytosis.

Nitric oxide ($NO^{\cdot}$)

Nitric oxide is produced in various types of cells and is not too reactive (poorly oxidative function), even under physiological concentration (up to 100nm) it reacts rapidly with oxygen to yield nitrogen dioxide (NO_2) which inturn may react with NO to yield nitrogen trioxide (N_2O_3). The rapid reaction of O_2 produced in different pathological states, with nitric oxide gives the extremely reactive peroxy nitrite (Borek, 2001) which mediates oxidation, nitrosation and nitration reaction. Nitric oxide is naturally formed in activated macrophages and endothelial cells and considered as an active agent in

several pathological studies based on inflammation, organ reperfusion and also may play an important role in atherosclerosis. $NO^{\cdot}$ exerts neurotoxic effects on dopamine function in primary midbrain cultures (Canals *et al*., 2001).

One mechanism that the macrophages use to exert their cytotoxic effects on the target cells by releasing $NO^{\cdot}$ is a cellular functions including macrophages mediated cytotoxicity, neurotransmission and smooth muscle relaxation. (Palmer *et al*., 1988; Nathen and Xie, 1994). Enzyme nitric oxide synthase (NOS) was first identified as endothelium derived relaxing factor (EDRF) from vascular endothelial cells (Furchgott and Zawatdzki, 1980). It is now clear that NO has numerous role in biological system including vasodialation, regulation of blood pressure, inhibition of platelet aggregation and adhesion (Fledman *et al*., 1993; Mc Donald *et al*.,1993; Ignaro, 1996). Beckman (1996) studied the physiological and pathological chemistry of nitric oxide and clearly find out its antioxidant property.

Antioxidant protection

The purpose of antioxidants in a physiological setting is to prevent ROS concentrations from reaching a high level within a cell that cause damage. Antioxidants may offer resistance against oxidative stress by scavenging free radials, inhibiting lipid peroxidation and or some other mechanism. (Youdin and Joseph, 2001). All organisms possess antioxidant

defence system, that protect against oxidative damage and numerous damage removal and repair enzymes,that remove and repair damaged molecules. However, the natural antioxidant mechanism can be inefficient dietary intake of antioxidant compounds become important (Terao *et al.*, 1994; Duh, 1998; Espin *et al.*, 2000). Cellular antioxidants may be enzymatic (catalase, glutathione peroxidase and superoxide dismutase) or non enzymatic (glutathione, thiol, some vitamins and metals.)

Enzymatic antioxidants – primary defence mechanism

Superoxide dismutase (SOD)

Superoxide dismutases are mettallo enzymes that are essential for dismutation of $O_2^{\cdot}$ to H_2O_2 and O_2 (Sohal, 1985)

$$O_2^{\cdot} + O_2^{\cdot} + 2H \xrightarrow{SOD} H_2O_2 + O_2$$

SODs are important initial components in the cellular defence against oxygen toxicity. In mammalian tissues three superoxid dismutases designated as Cu. Zn-SOD, Mn- SOD and extracellular SOD are reported. These enzymes play an important role in the antioxidant defence system against superoxide anion ($O_2^{\cdot}$) generated *in vivo* and may be involved in various pathophysiological processes including inflammation, cancer, diabetes, ageing and ischemia (Ishikawa, 1993). In most eukaryotic cells SOD exists in two forms, a cytosolic type containing copper and zinc and a mitochondrial

type containing manganese (Fridovich, 1978). The biosynthesis of SOD is mainly controlled by increased intracellular fluxes of $O_2^{\cdot}$ and it has been observed in numerous micro organisms as well as higher organisms (Rister and Balchner, 1976; Bandyopadhyay *et al.,* 1999)

The activities of SOD were measured in fourth instar larvae of *Chironomous riparius* by Choi *et al.* in 1999. Three types of superoxide dismutase were identified. Cu-Zn SOD in haemolymph and post mitochondrial fraction, Mn SOD in mitochondrial fraction and presumably Fe SOD in post mitochondrial fraction. The latter could have an endosymbiotic or a parasitic origin. Cu- Zn SOD tended to increase in the last phase of larval development, independently of protein or haemoglobin content. Where as some investigators suggested that over expression of Mn-SOD and Cu-Zn SOD can extend the lifespan in *Drosophila* by increasing the rate of conversion of O_2 to H_2O_2 (Sun *et al*., 2002).

Glutathione peroxidase (Gpx)

The term peroxidase refers to a variety of specific and non - specific enzymes that catalyse the transfer of hydrogen from a donor to H_2O_2. Glutathion peroxidase is believed to play an important role in the breakdown of H_2O_2, because it is much more efficient than catalase at low concentrations of the substrate. Further glutathione peroxidase can also react effectively with organic hydro peroxides which are believed to be one of the main products of

free radical reactions in the cells and the glutathione system is thought to be one of the major cellular redox buffering system in mammals (Sohal, 1985). Hydrogen peroxide (H_2O_2) formed by the dismutation of superoxide ($O_2^{\cdot}$) catabolised at the expense of reducing equivalents from glutathione by glutathione peroxidase (GPx) to form glutathione disulphide (GSSG) or mixed protein disulphides (Morrison *et al*., 2005). Glutathion peroxidase is a soleno - enzyme, two third of which is present in the cytosol and one third in the mitochondria (Bandyopadhyay *et al*., 1999). Glutathione depletion associated with ageing was proved by Hu *et al*. (2000). According to Liu and Choi, (2000) the effect of old age on rodent liver glutathione appears to be strain dependent and the age related reduction in protein, mRNA and activity of glutamate cysteine ligase (GSL) - the rate limiting enzyme in glutathione synthesis - was associated with a 37% reduction in liver glutathione in aged F 344 rats. Caballero *et al.* (2000) presented substantial evidence on the antioxidant property of glutathion. According to him the ratio of oxidized and reduced glutathione (GSSG/GSH) is an accurate, specific and sensitive way of evaluating oxidative stress.

Catalase

Catalase is primarily localized with in peroxisomes and to a lesser extent in mitochondria which is the main site of H_2O_2 production from O_2 dismutation. Catalase is highly effective in the breakdown of H_2O_2 at high

concentrations and also has a unique characteristic that it can degrade H_2O_2 by catalytic mechanism (Chance *et al.*, 1979)

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

In a recent study on protective effects of catalase over expression on UVB - induced apoptosis in normal human keratinocytes by Rezvani *et al*. (2006) pointed out that catalase overexpression has a protective role against UVB irradiation by preventing DNA damage mediated by the late ROS increase.Catalase activity revealed differences in time related changes across a 24 h period that are more obvious in peak levels between the brain, kidney and liver tissues of mouse (Sani *et al*., 2006). Their results also showed significant organ differences with the highest catalase activity in liver compared with kidney and brain. This might be related to several factors such as their respective physiological function, the risk of exposure to oxidative damage and the balance between synthesis and degradation of proteins during normal metabolism.

Heme peroxidase

Heme peroxidases such as lactoperoxidase and other mammalian peroxidases have been studied most extensively. The enzyme catalyses the oxidation of a wide variety of electron donors with the help of H_2O_2 and thereby scavenge the endogenous H_2O_2 (Bandyopadhyay *et al*., 1999).

Non enzymatic antioxidants – second defence mechanism

In addition to the primary defence against ROS by antioxidant enzymes, secondary defence against ROS is also offered by non enzymatic antioxidants. They react with radicals to produce another radical compound, the 'scavengers'. These scavengers produce a lesser harmful radical species and are called antioxidants. Among this reduced glutathione (GSR), ascorbate, thiols, some metals like Se, vitamins like vitamin A, vitamin C, vitamin E, amino acids such as tyrosine, tryptophan *etc* are most important (EI-Demerdash, 2004). In a similar study, Ischikawa, (1992) and Kunze *et al*.(1992) have reported that metabolites produced by micro organisms may serve as antioxidants, lipoxygenase inhibitors, synergists or metal chelating agents. In addition, these metabolites can decompose lipid peroxides and use them as nutrients.

Among vitamins, vitamin C is the more effective antioxidant when compared to vitamin E, but it can not be stored in the body so that must be taken frequently. (Guney *et al*., (2007) studied the protective effects of vitamins C and vitamin E against the endometrial damage and oxidative stress during the fluoride intoxication in rats. They observed that vitamin administration along with fluorine (F) treatment causes a decrease in endometrial MDA but an increase in SOD, GSH-Px and CAT activities

significantly and the vitamins exhibit a histopathological protection against F-induced endometrial damage.

Selenium is also generally recognized to be a trace mineral and antioxidant. It has great importance in human health by protecting the cells from harmful effects of free radicals. EI Demerdash (2004) studied the antioxidant effect of vitamin E and selenium on lipid peroxidation, enzyme activities and other biochemical parameters in rats exposed to aluminium. It was found that vitamin E or Se alone significantly lowers the levels of free radicals, total lipids, cholesterol, urea and bilirubin whereas enhances the activities of glutathione-S-transferase, sulphydryl groups, total proteins and albumins and he concluded that vitamin E and selenium have beneficial antioxidant effects and could be able to antagonize aluminium toxicity.

Vitamin C is a natural antioxidant found abundantly in citrus fruits. In a recent study Yousef *et al.* (2007) tested the protective effect of ascorbic acid against the toxicity of stannous chloride on oxidative damage, antioxidant enzymes and biochemical parameters in male New Zealand white rabbits. Their results suggested that ascorbic acid alone significantly decrease the levels of thiobarbituric acid which is a marker of lipid peroxidation. They also noticed the increased activities of GST, SOD and CAT and the treatment with ascorbic acid could minimize the toxic effects of stannous chloride.

(Muthuvel *et al*., 2006) also proved the antioxidant capacity of vitamin C in a neurotoxic compound PCB induced oxidative stress in albino rats.

Co Q 10 is a fat- soluble vitamin like natural antioxidant present in every cell of the body and serves as a coenzyme for various enzymatic steps in the production of energy within the cells. It acts as a free radical scavenger, a stabilizer of the cell membranes and an intracellular scavenger of the free radicals to prevent lipid peroxidation (Farswan *et al*.,2005)

Selenium (Se) was accepted as an essential micronutrient in humans due to its occurrence as an essential component of enzyme, glutathione peroxidase. There appears to be some species variation in the level of association of Se with glutathione peroxidase (Beilstein and Whanger, 1983; 1986) . They reported that in erythrocytes of many animals, 75 to 85% of the Se was associated with glutathione peroxidase but in humans there is a much lower association. This research group has shown that these species difference may be due to different dietary forms of Se.

Carotenoids are a group of antioxidant nutrients found in many fruits and vegetables. Carotenoids include Beta carotene (provitamin A), alpha carotene, Cryptoxanthin, Lycopene, Lutein and Zea xanthin. Beta carotene (BC), a carotenoid pigment functions mainly as provitamin A in animals and also acts as a powerful free radical scavenger and chain breaking antioxidant (Smith, 1998). It protects against heart diseases (Kohlmeier and Hastings,

1995) may also provide prevention from heart attacks and strokes (Abbey *et al.*, 1995), slows down the progress of cataracts and prevents macular degeneration (Carson *et al*., 1994; Seddon *et al*., 1994)

Kumar *et al*. (2005) studied the hepato protective activity of Beta carotene (BC) on paracetamole induced hepatic damage in rats. Paracetamol administration significantly reduced hepatic glycogen, Glutathione (GSH), glutathione S-transferase (GST), glutathione peroxidase (GPx) and glutathione reductase (GSH-R). Pretreatment of rats with BC significantly increased the enzyme activities.

Garlic extract contain antioxidant phytochemicals such as unique water- soluble and lipid soluble organosulfur components and flavonoids notably allixin and selenium. Garlic extracts exerts antioxidant action by scavenging ROS enhancing the cellular antioxidant enzymes like superoxide dismutase, catalase and glutathione peroxidase and increasing glutathione in the cells. Garlic extract inhibit lipid peroxidation and reducing oxidative modification of low density lipoproteins LDL (Borek, 2001). Ratty et al. (1998) reported that Flavanoids are natural products, which have been shown to possess various biological properties related to antioxidant and anti-inflammatory mechanisms. Antioxidant and anti- inflammatory activity of flavonoids from extract of *Aspergillus candidus* was recently investigated by Malpure *et al*. (2006).

Antioxidant system in insects

Antioxidant enzymes and small molecular weight antioxidants perform an effective response against oxidants in insects. Antioxidant enzymes such as superoxide dismutase, catalase, glutathione transferase and glutathione reductase have been characterized in insects (Felton and Summers, 1995). Since insects lack glutathione peroxidase activity, catalase activity provides the sole enzymatic mechanism for removal of H_2O_2 (Orr and Sohal, 1992; Sohal,1993)

Other than catalase, ascorbic peroxidase (APOX), was found in larvae of *Helicoverpa zea* (Mathews *et al*., 1997) and another enzyme, dehydro ascorbic acid reductase suggested by Summers and Felton (1993), may play an important role in the elimination of H_2O_2 in insects. Antioxidant effects of different substances such as ascorbate, glutathione, thiol, tocopherol, carotenoids, reducing aminoacids *etc* are not yet been well studied in insects, but may play certain very important antioxidant role.

Tryptophan – biochemical effects

The reducing aminoacid tryptophan with the highest molecular weight of all aminoacids occurring in proteins, comprises an indolyl moiety, a system capable of donating electrons and therefore, liable to form complexes with a wide range of other molecules. The aromatic ring of tryptophan contain delocalised pi (π) electron clouds that enable them to interact with other π

systems and to transfer electrons. Molecular interactions have been observed between tryptophan and nucleic acid, and a variety of other molecules of biological importance (Linzen 1974). In the complexes of tryptophan, either the indole ring or its benzene nucleus are retained and the latter also being recast into the pyridine ring. Among these a plant growth hormone – indole acetic acid, a hormone - melatonin, an enzyme co factor - nicotinic acid and a neurotransmitter substance- serotonin are important. In insects, the major product of tryptophan degradation is a group of 10 to 15 pigments, the ommochrones (Linzen, 1966).

Tryptophan which emits only short wave length can be measured with high specificity at the nanomol level by a method first described by Egehaaf (1957) which is based on the formation of tryptochrome and iodotryptochrome on spraying with potassium iodide. Green (1949) estimated the tryptophan content in *Drosophila melanogaster* protein at 0.84 mole percent which is slightly higher than that of *Drosophila viridis*. In *Bombyx mori* rb mutant the average tryptophan content range between o.96 percent (in larvae) with filled spinning glands and 1.38 percent (Young pupae). In the pharate adults and moth just after emergence 1.16 percent (Linzen 1971)

Formyl kinurenin is a product of the tryptophan – oxygenase catalysed reaction. Green (1952) fed formyl- kynurenine to vermilion mutants of *Drosophila* and observed formation of brown age pigment. The kynurenine

determined in whole animals may be confined to individual tissues. This is certainly true in *Drosophila*, where the fat body present near the anterior region of the larval body accumulates kynurenine near the time of pupation, and exhibits a light blue fluorescence (Rizki, 1961). 3- hydroxyl kynurenine occupies the key position in tryptophan metabolism. Its natural L isomer isolated from the silk worm *Bombyx mori* mutant rb (Iwata and Ogata 1966), is subjected to known detoxification mechanisms since a phenolic compound. Recently Griffith (2000) also suggested that the aromatic and sulphydryl conaining aminoacid residues have been regarded well susceptible to oxidative modifications with L-DOPA from tyrosine, ortho- tyrosine from phenylalanine and cystein, kynurenin from tryptophan. Pryor (1940), Inagami (1958) and Linzen and Ishiguro (1966) suggested the participation of 3-hydroxyl kynurenine in the cuticular tanning of insects. According to the observations of Savvateeva *et al.* (1999), tryptophan kynurenine pathway is conserved in insects, rodents and humans and any alterations in the intermediate metabolites of this pathway put it effect on memory, synaptic and glial immuno reactivity.

Anthranilic acids and their conjugates were the main end products of tryptophan metabolism during larval development of the silkworm while in the pupa the formation of 3- hydroxyl- kynurenine and the pigments was favoured (Inagami 1958). Ommochromes are regarded as the end products of

tryptophan metabolism which are either excreted or stored until the death of the insect.

The most important fact with regard to tryptophan metabolism in insects is the failure to degrade 3-hydroxy-anthranilic acid to water, CO_2 and ammonia. As a consequence metabolites produced prior to this metabolic block will accumulate especially during embryonal development in the egg, in dormant and pupal stages and during moults. Accumulated metabolites may be subsequently excreted or used for some secondary function (Linzen, 1971).

Dietary effect of tryptophan were studied by several workers (Ashley and Curzon, 1981; Segall *et al*., 1983; Benedietti and Moja , 1993; Picard *et al*., 1993; Moosman and Bell, 2000; Hussain and Mitra, 2000). Moosman and Behl (2000) found that an astounding accumulation of tyrosine and tryptophan residues in the transmembrane domains of integral membrane proteins, especially in the region of the highest lipid density, perform vital antioxidant functions inside lipid bilayers and protect cells from oxidative destruction. They also showed that long- chain acetylated tyrosine and tryptophan, but not phenylalanine or short chain acetylated derivatives are potent inhibitors of lipid peroxidation and oxidative cell death. Tryptophan hydroxylase (TrPH) is the initial and rate- limiting enzyme involved in the biogenesis of the neuro transmitter serotonin (Jequier *et al*., 1967). It is also involved in the biogenesis of melatonin in the pineal gland. Hussain and Mitra

(2000) suggested that TrPH activity is affected by ageing process which can drastically affect the levels of serotonin in brain. They also added that, a combined effect of inefficient phosphorylation and oxidative damage of TrPH may be the cause of diminished activity. Reinikainen *et al.* (1990) have given considerable evidences for a decreased brain level of serotonin patients with Alzheimer's disease. In the study by Segall and Timiras (1976), it was hypothesized that tryptophan deficiency delays growth, development and maturation of the central nervous system, in particular by decreasing the levels of the neurotransmitter serotonin, and by interfering with central nervous system, protein synthesis or neurotransmitter metabolism. Ooka *et al.* (1988) noted that histological biomarkers of ageing were also delayed after tryptophan restriction in some organs.

MATERIALS AND METHODS

The experimental animal

The silkworm *Bombyx mori* L belongs to the Phylum Arthropoda, Class Insecta and Order Lepidoptera. They feed on mulberry leaves. The newly hatched larvae, in about four weeks time undergo moulting four times, become mature silkworms and start spinning the cocoon at the end of 6^{th} day of last instar. Fabrication of cocoon is completed in 2–3 days. The silkworm pupates inside the cocoon in another 2-3 days during which various organs are formed very rapidly to metamorphose to moth. The moth emerges after about 12 days of life 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, copulates on that same day itself and each females lays about 400-750 eggs. The newly hatched larvae come out by breaking off egg shell.

Silk worm rearing

The bivoltine silkworm hybrid, Elite- CSR 2X4 was used for the study. The silkworm rearing was undertaken by procuring newly hatched larvae immediately after their brushing from Serifed, Malappuram, Kerala. The rearing house and all the appliances were disinfected in advance with chlorine

dioxide/bleaching powder to free the rearing environment and the surroundings from pathogens. The rearing bed was made up by chopped leaves having the size of half to one cm. squares and covered with blue polythene sheet. Clean wet sand in trays were placed around the rearing bed to ensure 80-90% humidity and the rearing room temperature was maintained at 27^0C. Tender mulberry leaves with more moisture content, protein and carbohydrates were used for feeding the worms in the initial stages of rearing and were fed three times a day.

By about 3 ½ days, all the worms have settled for moult uniformly. The duration for all the worms to come out of first moult was about 24h. The rearing beds were cleaned daily in the morning with expanding the size of the bed and extending it to more trays corresponding to the growth of the larvae. With 6-7 feeding the larvae were ready for 2^{nd} moult. In the third instar, the larvae were fed 9-10 times for 3-3 ½ days. On getting ready for moult the polythene cover was removed and the relative humidity brought down to 60-65%. The duration of 3^{rd} moult was about 24h. Following the regular procedure, the feeding was resumed and required spacing was provided. The 4^{th} instar took 4-4 ½ days followed by the 4^{th} moult spanning about 30 h. The duration of 5^{th} instar was normally about 6-7 days and the larvae started to spin cocoon by the end of this stage. During 5^{th} instar the larvae were fed with fully matured mulberry leaves and for the last two days it was coarse leaves.

The present work was done on the fifth instar larvae, beginning from the newly moulted stage and continued till the last day of the instar, just before spinning began. The larval period was divided into seven chronologically identified stages: i.e., 0h, 24 h, 48h, 72 h, 96 h, 120 h and 144 h.

5 mM solution of tryptophan in distilled water was used for treating the mulberry leaves. After the fourth moult the larvae were segregated into two sets. One set was fed with mulberry leaves dipped in 5mM tryptophan solution and drained in air for half an hour. The other set of larvae were fed with leaves dipped in distilled water and drained in air for half an hour. The experimental animals were fed *adlibitum*.

Determination of growth rate of the larva

The fifth instar larval period was counted from the time when it started feeding after the fourth moult, to the time when it began spinning. The fresh weights of both normal and treated larvae were noted at 24 h intervals during the period of development.

Determination of volume of haemolymph

Total volume of haemolymph was measured directly by using a fine calibrated capillary tube at 24 h intervals. For extracting the haemolymph, the larvae were anaesthetized slowly with diethyl ether as described by

Mohammed (1974). One of its thoracic legs was amputated with a sharp scissors and the haemolymph that oozed out was immediately drawn into a calibrated capillary tube and its volume found out. To ensure more or less complete extraction of haemolymph, the larvae was gently pressed from anterior and posterior ends simultaneously until no more haemolymph was oozing out of the wound.

Determination of weight of the total fat body

The fat body of the normal and treated larvae was dissected out in ice cold ringer. The adhering water was carefully wiped off from the fat body lobes with a filter paper and weighed immediately. Variations in the fresh weight of fat body during the developmental stages of treated and normal larvae were observed.

For conducting the various tests, haemolymph and fat body samples were extracted from appropriated number of normal and treated larvae separately. The analyses were carried out on all larval stages of the fifth instar, both for treated and normal ones.

Biochemical analyses

For various biochemical estimations pooled haemolymph samples were extracted from appropriate number of both normal and treated larvae separately. For the estimations of fat body samples the tissue was

homogenized and diluted to appropriate volume with water for all assays except enzymes. The analysis were carried out at 24 hour intervals in on the basis of unit volume and total volume in the case of haemolymph and on the basis of unit weight of fresh tissue and total tissue in the case of fat body. The pooled haemolymph and fat body samples were isolated from the larvae of each set. The tissues were stored at – 20° C until the estimations were carried out.

Estimation of total protein

The method of Lowry *et al*. (1951) was used for the estimation of protein by using crystalline bovine serum albumin (fraction V, Sigma) as standard. From the homogenized tissue, the protein was precipitated with trichloroacetic acid (TCA). The precipitate was then successively extracted with ethanol- chloroform, ethanol - ether and finally ether at room temperature. The final residue left was dissolved in 1 N sodium hydroxide. The blue colour developed was measured against a reagent blank at 540 nm in a Shimadzu UV 250 spectrophotometer.

Estimation of total free amino acid

The total free amino acid content in the tissues was estimated by the method of Lee and Takahashi (1966). The homogenized tissue was precitated with 10% sodium tungstate and 2/3 N sulphuric acid and centrifuged at 2000 rpm for 20 min. The resultant supernatant was used for the amino acid

estimation. The colour developed was measured at 540 nm against the reagent blank in a spectrophotometer.

Estimation of glucose

Glucose was estimated according to Morgan (1975). Homogenates of the tissues were deproteinised by 0.3 N barium hydroxide and 5% zinc sulphate and filtered. The filtrate was then used for the estimation and the blue colour developed was measured at 540 nm against a reagent blank in a spectrophotometer.

Estimation of creatinine

The estimation of creatinine was done according to McFate *et al*. (1954) based on Jaffe reaction. The homogenates were precipitated with10% sodium tungstate and 2/3 N sulphuric acid and centrifuged at 2000 rpm for 20 minutes. The filtrate was used for the estimation and the yellow orange colour developed was read at 520 nm in a spectrophotometer.

Estimation of urea

Fearon reaction, modified by Beale and Croft (1961) was used for the estimation of urea. The homogenates were deproteinized with 0.3 N barium hydroxide and 5% Zinc Sulphate and filtered. The filtrate was treated with diacetyl monoxime- phenyl anthranilic acid and then with activated acid

phosphate reagent and heated for 11 minutes. The colour developed was measured after cooling, at 535 nm in a spectrophotometer.

Estimation of Hydrogen peroxide and catalase

Both estimations were done based on the formation of soluble coloured peroxotitanium complex in a reaction of hydrogen peroxide with potassium titanium oxalate and the measurement of the orange/ yellow colour developed, at 410 nm (Muhlebach *et al*., 1970).

$$Ti^{4+}(aq) + H_2O_2 \rightarrow Ti(O_2)(OH)_{N-2}^{4-n+} + nH^+$$

For estimation of hydrogen peroxide, the homogenized tissue was deproteinised with 20% trichloroacetic acid (TCA) and centrifuged at 3000 rpm for 10 min. 0.5 ml of the protein- free solution was mixed with 1.5 ml of acidic potassium titanium oxalate reagent and the bright yellow colour formed was measured at 410 nm in a spectrophotometer.

The determination of catalase activity was based on estimating the amount of residual hydrogen peroxide in the assay mixture after incubation of a known amount of hydrogen peroxide with the enzyme extract for a fixed time interval. .

The assay system consisted of 0.5 ml of the homogenized tissue extract in 0.1 M phosphate buffer pH 7.0 or 0.5 ml of blood and 0.88 M H_2O_2 in a total volume of 1ml. The mixture was incubated at 37^o C for 30 min. The

reaction was terminated by adding 0.8 ml of 20% TCA. The assays for the controls were prepared by adding TCA before the incubation. The test and control assay mixtures were centrifuged at 3000 rpm for 10 min. and the supernatant was used for the estimation as described above. The test and control solutions gave different values. The difference in the values gives the amount of hydrogen peroxide oxidized in 30 min. This gives the rate of enzyme action expressed as mg hydrogen peroxide hydrolyzed per min per unit weight of fresh tissue or per unit volume of haemolymph and as mg/min/larva or ml/min/larva respectively. Specific activity of the enzyme also calculated from this and was expressed as unit activity per mg.crude tissue protein.

Estimation of aspartate amino transferase (AsAT) and alanine amino transferase (AlAT) activity

The activity of AsAT and AlAT was estimated following the method of Reitman and Frankel (1957) using pyruvic acid standard. The homogenates of the tissues were centrifuged and the clear supernatant was directly taken for the enzyme assay. The enzyme substrate mixture after incubation is treated with 2, 4-dinitrophenyl hydrazine solution and then 0.4 N sodium hydroxide. The colour developed was read after 10 min at 520 nm in a spectrophotometer. One unit enzyme activity corresponds to the formation of 1mole of keto acid formed per minute at 37º C under the experimental

conditions. The specific activity of AsAT and AlAT enzyme was expressed as unit activity per mg crude tissue protein.

Statistical Analysis

The data were subjected to statistical analysis to evaluate whether the variations are significant between normal and treated insects using student's t test method.

RESULTS

Growth rate pattern of the silk worm

The fifth instar larval period of silkworm was found to last for six days and the body of larvae attained a length of 6-7 cm. The larval stages were identified by their size and feeding characteristics. The fifth instar larval period is divided into feeding (0 - 120 h) and non feeding stage (120 - 144 h) The larva began feeding after 12 hours of its penultimate moult. From 48 h to 96 h the larva was in the active feeding and growing stage. After 120 h the larval food intake showed a decline. The larva attained a maximum body weight with well developed silkgland at 120 hours. After 144 h the larva completely stopped feeding and was ready to start spinning the cocoon. The fabrication of the cocoon was completed in two to three days.

The larvae administered with tryptophan showed a similar pattern in growth rate except a short delay in commencement of spinning. The tryptophan administered larvae were also comparatively bigger and stouter in size than the normal larvae.

The fresh weight of the larvae during the development of the fifth instar of both normal and treated are recorded in Table 1 and Figure 1.

Table 1

Figure 1

Table 1. Changes in the total fresh weight of the larva

Larval age	mg/ larva, mean ± SD	
	Normal	Treated
0h	1.09 ± 0.08	1.19 ± 0.14
24 h	1.62 ± 0.17	1.73 ± 0.14
48 h	3.81 ± 0.52	3.98 ± 0.28
72 h	4.28 ± 0.31	4.85 ± 0.28**
96h	4.47 ± 0.45	5.61 ± 0.41**
120 h	4.8 ± 0.36	5.99 ± 0.42**
144 h	4.1 ± 0.24	5.17 ± 0.29**

Values are the means of 5 determinations

*The values are significant at $P<0.05$ against the normal for n=5

**The values are highly significant at $P<0.01$ against the normal for n=5

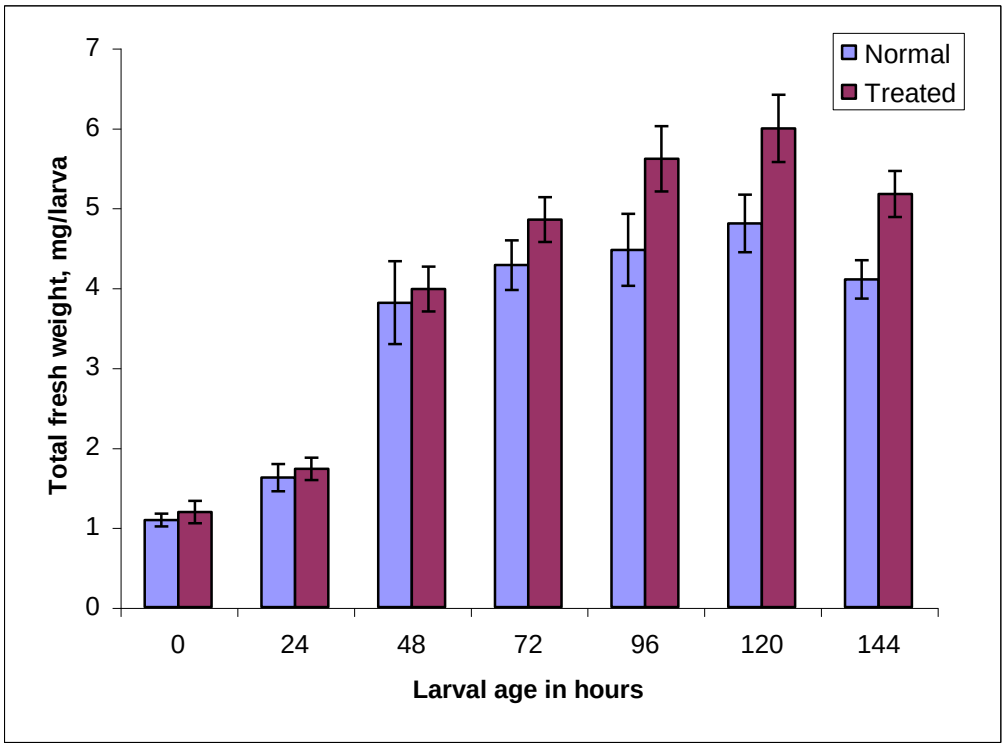

Figure 1. Changes in the total fresh weight of the larva

The fresh weights of normal larvae were minimum at 0h, which gradually increased and attained the maximum weight at 120 h followed by a decline. The fresh weight of the tryptophan treated larvae followed a similar pattern of changes to that of the normal but with a higher magnitude. The treated larvae showed 4 -13% increase in weight from the normal at the beginning and then recorded 25 - 26% increase during 72 h onwards. The weight of both normal and treated larvae declined after 120 h.

The changes in volume of haemolymph in the normal and treated larvae during fifth instar are given in Table 2 and Figure 2.

Table 2

Figure 2

The haemolymph was slightly green in colour in normal larvae but showed a brownish tinch in the treated, and became more viscous in the case of both normal and treated with development. The volume of haemolymph was low at the beginning of the instar but gradually rose and attained the peak at 120 h followed by a decline up to pupation.

The pattern of changes in the volume of haemolymph in the tryptophan treated larvae was the same as that of the normal but with a higher magnitude during the end of the final instar larval period.

Table 2. Changes in the volume of haemolymph

Larval age	ml/ larva, mean ± SD	
	Normal	Treated
0h	0.11 ±0.02	0.15 ± 0.03
24 h	0.18 ± 0.07	0.22 ± 0.06
48 h	0.36 ± 0.03	0.38 ± 0.03
72 h	0.51 ± 0.08	0.52 ± 0.07
96 h	0.55 ± 0.04	0.58 ± 0.06
120h	0.68 ± 0.05	0.77 ± 0.05*
144 h	0.55 ± 0.06	0.67 ± 0.04*

Values are the means of 5 determinations
*The values are significant at P<0.05 against the normal for n=5,
**The values are highly significant at P<0.01 against the normal for n=5

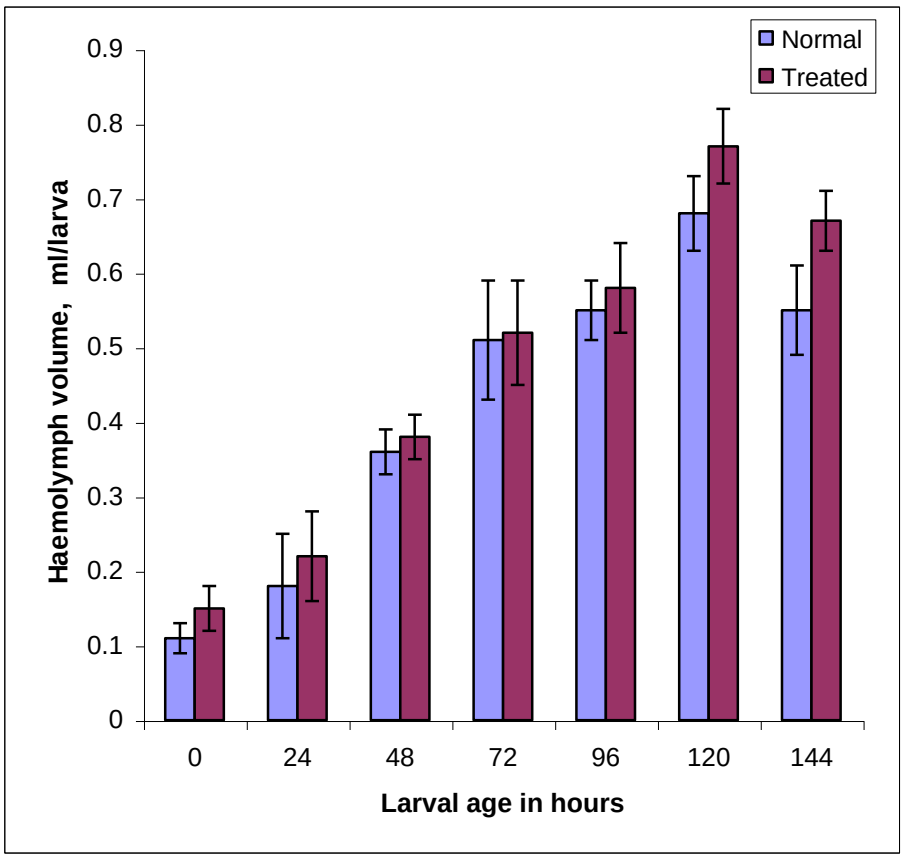

Figure 2. Changes in the volume of haemolymph

In the final instar larvae of silkworm the fat body cells were creamy white in colour, moderately aggregated and freely suspended in the haemocoel. The changes in the weight of total fat body of normal and treated larvae during development are presented in Table 3 and Figure 3.

Table 3

Figure 3

The fresh weight of the fat body of normal larvae showed a sharp increase between 48 h and 72 h followed by a gradual increase recording the peak value at 120 h and declined thereafter. In the case of treated larvae the changes in the fresh weight of fat body showed a 14-38% increase from normal values recording the peak value at 144 h in contrast to its slight reduction in normal larvae.

Total protein

The levels of total protein in the haemolymph and fat body of the normal and treated larvae during its developmental stages are given in Table 4.1 and 4.2 and Figure 4.1 and 4.2.

Table 4.1 and 4.2

Figure 4.1 and 4.2

Table 3. Changes in the fresh weight of fat body

Larval age	mg fresh tissue / larva, mean ± SD	
	Normal	**Treated**
0h	21.18 ± 2.96	20.09 ±1.81
24 h	34.60 ± 4.95	42.31 ± 4.08
48 h	62.31 ± 8.63	86.62 ± 10.92*
72 h	146.54 ± 15.25	167.14 ± 16.96
96 h	177.4 2± 21.05	198.08 ± 22.75*
120 h	241.5 3± 30.95	304.06 ± 36.35*
144 h	234.64 ± 28.85	306.13 ± 30.87*

Values are the means of 5 determinations
*The values are significant at $P<0.05$ against the normal for n=5,
**The values are highly significant at $P<0.01$ against the normal for n=5

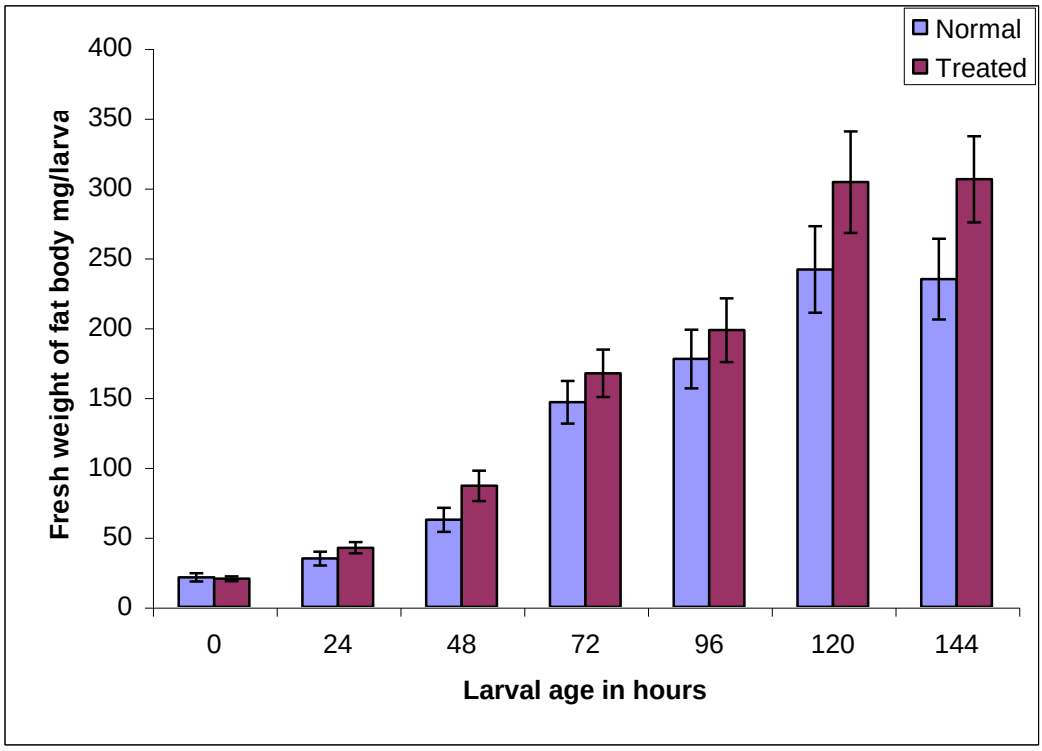

Figure 3. Changes in the fresh weight of fat body

Table 4.1. Changes in the total protein levels in the haemolymph

Larval age	mg/ml, mean ± SD		mg/ total tissue, mean ± SD	
	Normal	**Treated**	**Normal**	**Treated**
0h	10.45 ±1.05	11.02 ±1.54	1.22 ±0.13	1.61 ±0.23
24 h	15.38 ± 1.55	18.01 ± 1.76	2.69 ± 0.65	4.15 ± 0.61*
48 h	22.89 ± 2.38	39.39 ± 2.81**	7.87 ± 1.02	14.97 ± 2.32**
72 h	32.32 ± 3.01	57.23 ± 6.55**	15.81 ± 2.12	30.33 ± 3.01**
96 h	50.41 ± 6.56	88.44 ± 10.01**	25.21 ± 3.21	45.99 ± 5.98**
120 h	66.32 ± 7.89	80.91 ± 9.76	46.42 ± 5.02	48.54 ± 4.46
144 h	59.4 ± 7.01	74.62 ± 8.51	35.52 ± 3.61	47.62 ± 4.95*

Values are the means of 5 determinations

*The values are significant at $P<0.05$ against the normal for n=5,

**The values are highly significant at $P<0.01$ against the normal for n=5

Figure 4.1a

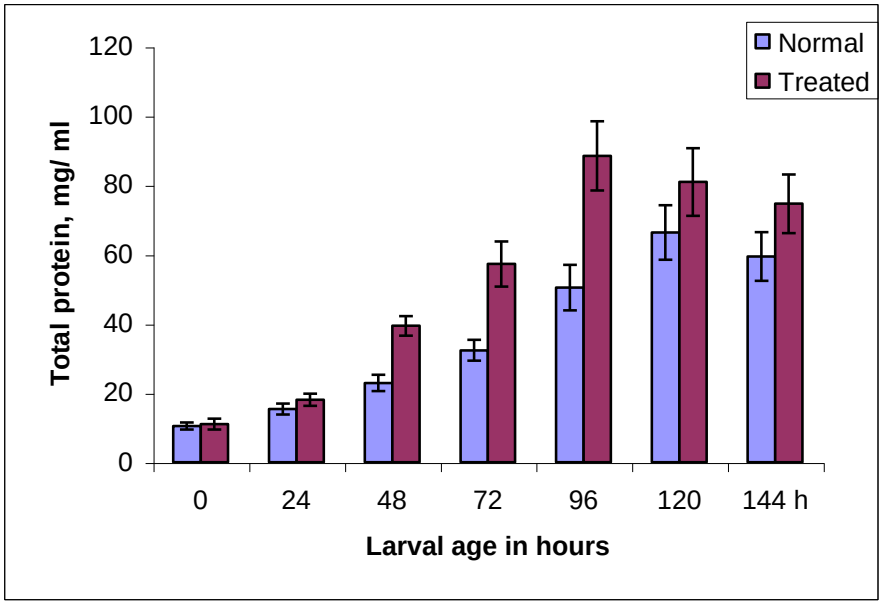

Figure 4.1b

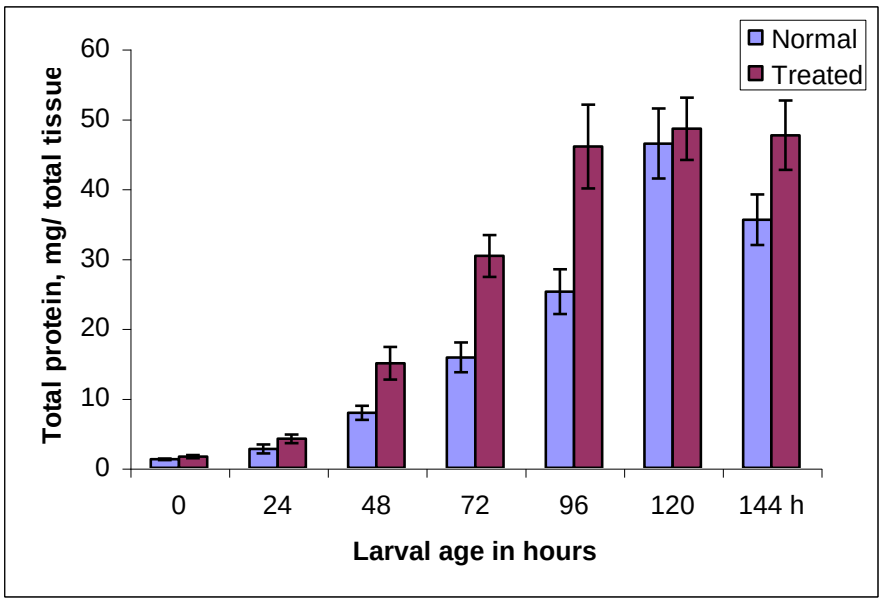

Figure 4.1. Changes in the total protein levels in the haemolymph

a. Changes per unit volume haemolymph

b. Changes per total haemolymph

Table 4.2. Changes in the total protein levels in the fat body

Larval age	mg/mg fresh tissue, mean ± SD		mg/total tissue, mean ± SD	
	Normal	Treated	Normal	Treated
0h	0.06 ± 0.01	0.05 ± 0.008	3.08 ± 0.43	2.98 ± 0.33
24 h	0.09 ± 0.01	0.11 ± 0.009*	4.76 ± 0.58	4.03 ± 0.53
48 h	0.12 ± 0.02	0.12 ± 0.02	3.26 ± 0.30	9.69 ±0.98**
72 h	0.13 ± 0.01	0.12 ± 0.01	17.61 ± 1.34	20.62 ± 3.03
96 h	0.14 ± 0.02	0.13 ± 0.01	23.42 ± 2.46	21.8 ± 3.04
120 h	0.16 ± 0.02	0.15 ± 0.016	29.13 ± 3.16	28. 51 ± 4.52
144 h	0.14 ± 0.02	0.15 ± 0.012	33.61 ± 3.28	35.65 ± 4.81

Values are the means of 5 determinations

*The values are significant at $P<0.05$ against the normal for n=5,

**The values are highly significant at $P<0.01$ against the normal for n=5

Figure 4.2a

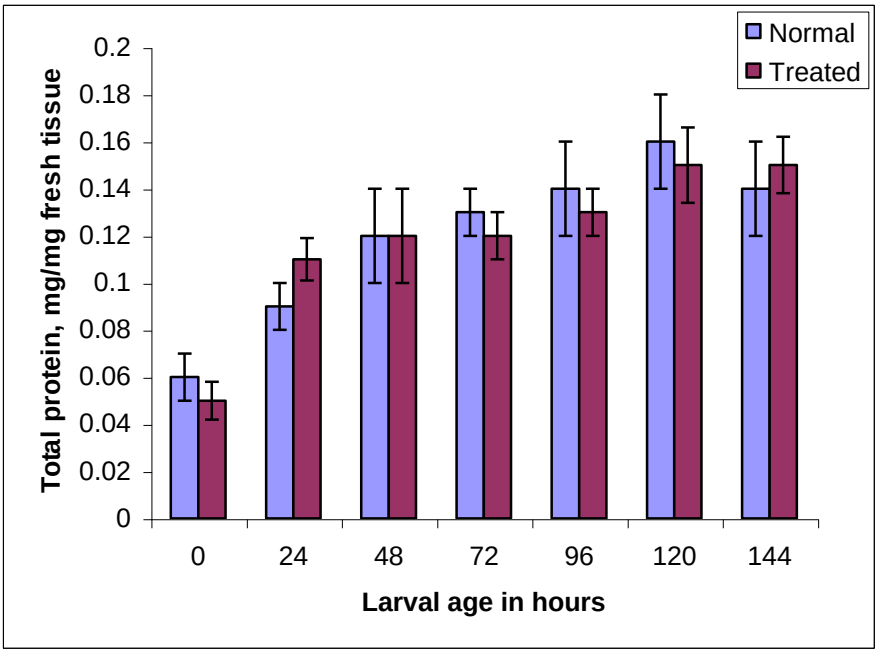

Figure 4.2b

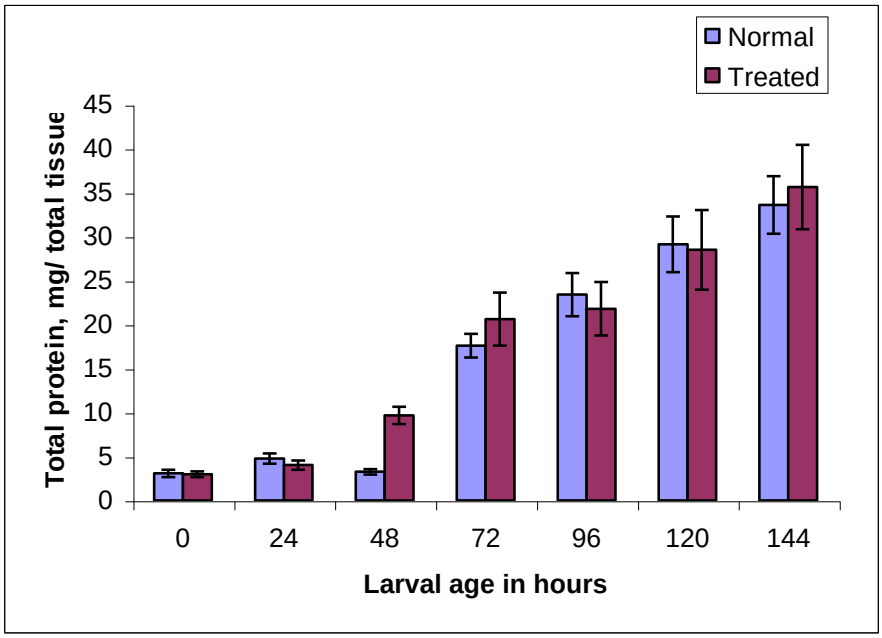

Figure 4.2. Changes in the total protein levels in the fat body

a. Changes per unit weight of fat body

b. Changes per total fat body

Total protein in the haemolymph of the normal and treated larvae showed a sharp increase up to 120 h followed by a gradual decline. The total haemolymph protein of treated larvae showed 34-94% increase when compared to normal and maintained a relatively steady level between 96 h and 144 h in contrast to a 10% reduction in protein content during final stages of the normal. The difference was more significant when the levels of protein per total tissue were considered.

The normal larvae showed 11 fold increase in total fat body protein recording the peak value at 120 h. In the treated laevae, the pattern of the changes in the levels of fat body protein was same but with a change of 12 fold.

Total free amino acids

The titre of total free amino acids per unit volume/weight and total volume/ weight of the haemolymph and fat body in the developmental stages of the normal and treated larvae are given in Table 5.1 and 5.2 and Figure 5.1 and 5.2.

Table 5.1 and 5.2

Figure 5.1 and 5.2

The total content of free amino acids in the haemolymph of the larvae gradually increased, recording the peak value at 96 h followed by a sharp dip.

Table 5.1. Changes in the total free amino acid levels in haemolymph

Larval age	mg/ml, mean ± SD		mg/total tissue, mean ± SD	
	Normal	**Treated**	**Normal**	**Treated**
0h	4.27± 0.51	4.18 ± 0.62	0.85 ± 0.15	0.79 ± 0.07
24 h	6.11± 0.65	6.97 ± 1.25	1.08 ± 0.13	1.60 ± 0.18**
48 h	12.40 ±1.9	16.33 ± 2.05*	4.4 2± 0.61	6.21 ± 0.85*
72 h	13.13 ±1.52	16.73 ± 1.93*	6.51 ± 0.96	8.82 ± 0.98*
96 h	16.20 ± 2.21	18.90 ± 2.21	8.70 ± 0.74	10.23 ± 1.29
120 h	12.91±1.20	12.70 ± 1.02	8.98 ± 0.91	8.62 ± 0.88
144 h	9.12 ± 0.93	9.86 ± 0.94	5.81 ± 0.87	6.80 ± 0.87

Values are the means of 5 determinations

*The values are significant at $P<0.05$ against the normal for n=5,

**The values are highly significant at $P<0.01$ against the normal for n=5

Figure 5.1a

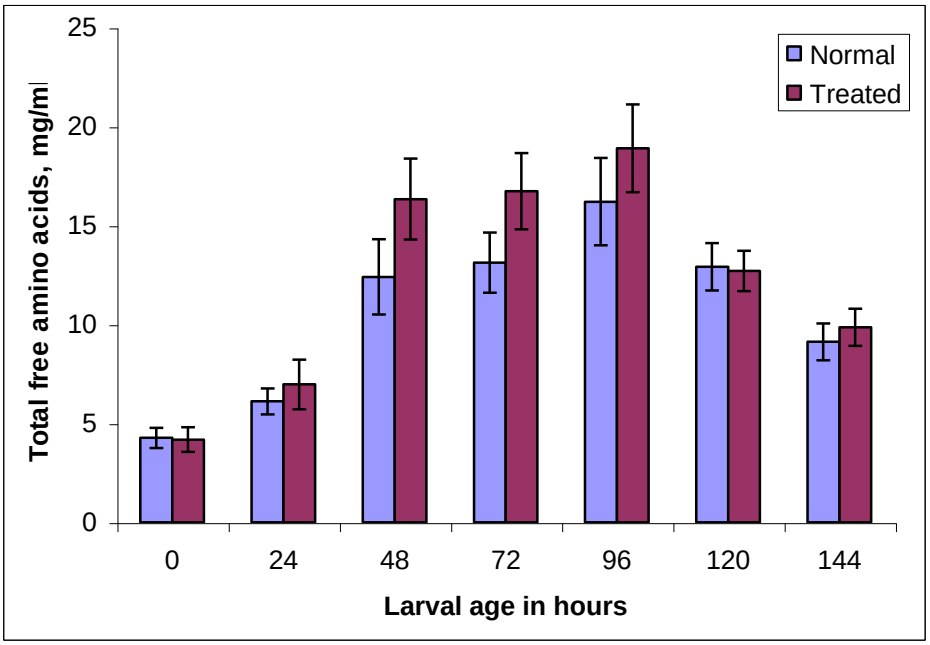

Figure 5.1b

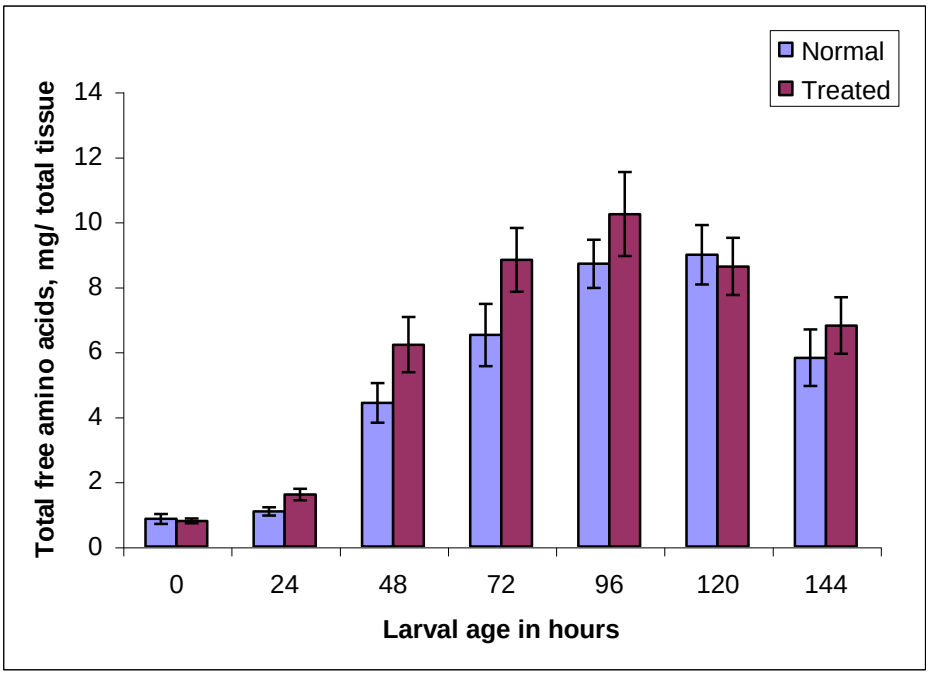

Figure 5.1. Changes in the total free amino acid levels in haemolymph

a. Changes per unit volume haemolymph

b. Changes per total haemolymph

Table 5.2. Changes in the total free amino acid levels in fat body

Larval age	mg/mg fresh tissue, mean ± SD		mg/total tissue, mean ± SD	
	Normal ($x 10^{-3}$)	Treated ($x 10^{-3}$)	Normal ($x 10^{-3}$)	Treated ($x 10^{-3}$)
0h	5.12 ± 0.61	4.98 ± 0.45	196.24 ± 21.58	187.18 ± 22.46
24 h	6.80 ± 1.09	6.95 ± 0.86	242.61 ± 26.52	324.14 ± 41.65*
48 h	7.58 ± 1.08	7.23 ± 1.1	468.40 ± 49.85	626.70 ± 73.87*
72 h	8.82 ± 0.97	7.53 ± 1.16	1297.72 ± 122.67	916.08 ± 117.45 *
96 h	6.79 ± 1.12	6.68 ± 0.93	1205.71 ± 117.46	1125.54 ±121.33
120 h	6.48 ± 0.89	8.92 ± 1.28*	1201.80 ± 116.55	2896.09 ± 320.48**
144 h	5.10 ± 0.61	7.18 ± 1.01*	1038.84 ± 121.03	2497.80 ± 323.28**

Values are the means of 5 determinations
*The values are significant at P<0.05 against the normal for n=5,
**The values are highly significant at P<0.01 against the normal for n=5

Figure 5.2a

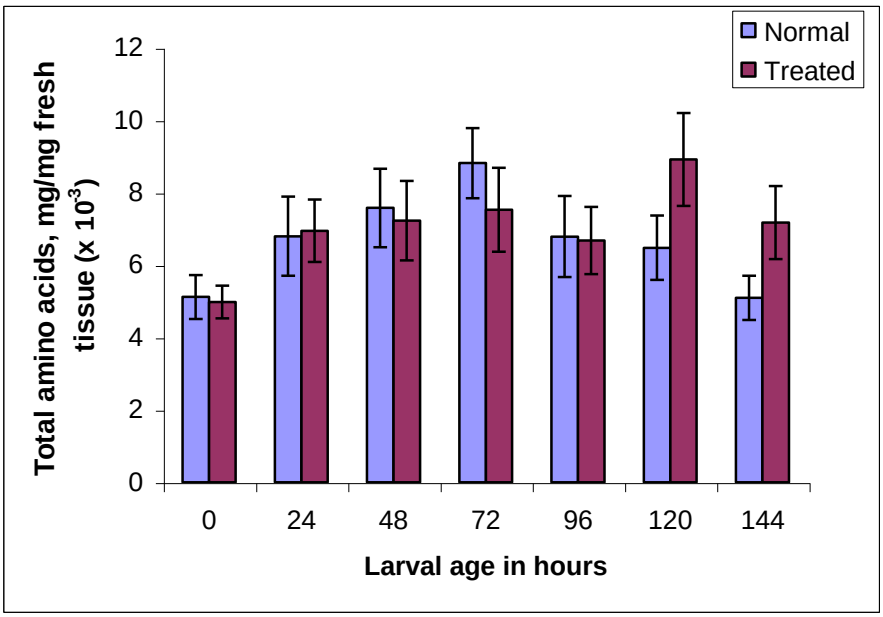

Figure 5.2b

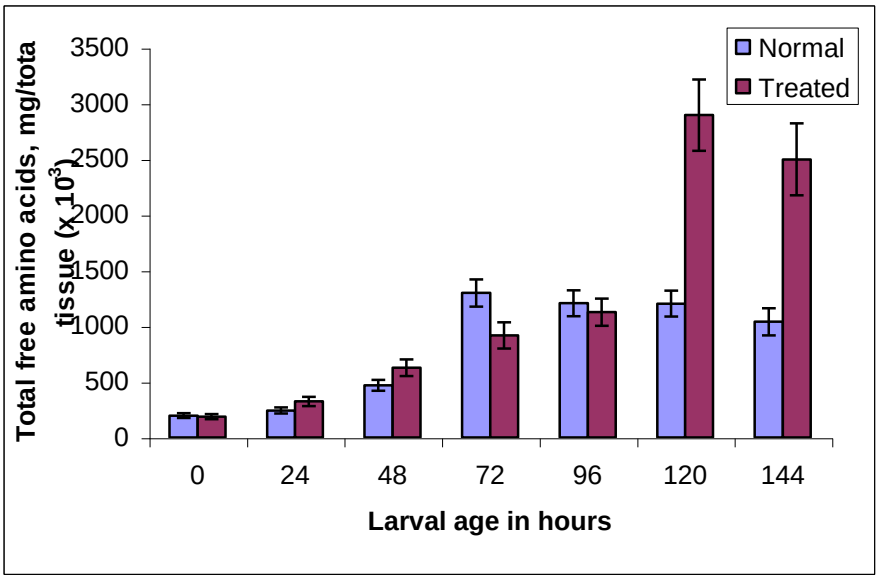

Figure 5.2. Changes in the total free amino acid levels in fat body

a. Changes per unit weight of fat body

b. Changes per total fat body

The total free amino acid levels in treated larvae showed a similar pattern of changes to that of the normal but with a higher magnitude. The increase was more significant during the feeding stage.

The total content of free amino acids in the fat body is much less than that found in the haemolymph. There was a slight difference in the pattern and magnitude of the changes between normal and treated larvae when compared to haemolymph. Free amino acid titre of treated larvae was high during the beginning and final stages of larval development. This difference was more striking when the estimations were done on the total tissue.

Urea

The concentration of urea in the haemolymph and fat body during the period of development of normal and treated larvae is presented in Table 6.1 and 6.2 and Figure 6.1 and 6.2.

Table 6.1 and 6.2

Figure 6.1 and 6.2

The amount of urea in the haemolymph of normal larvae was initially high, but exhibited a sharp reduction during the remaining larval period. At the end of the larval stage, the level of urea was less than10% of the initial value.

Table 6.1. Changes in the urea levels in haemolymph

Larval age	mg/ml, mean ± SD		mg/total tissue, mean ± SD	
	Normal	**Treated**	**Normal**	**Treated**
0h	1.12 ± 0.13	1.05 ± 0.12	0.12 ± 0.01	0.11 ± 0.02
24 h	1.09 ± 0.08	0.71 ± 0.006**	0.19 ± 0.03	0.16 ± 0.04
48 h	1.00 ± 0.10	0.64 ± 0.042**	0.36 ± 0.02	0.24 ± 0.02**
72 h	0.78 ± 0.06	0.36 ± 0.031**	0.43 ± 0.04	0.19 ± 0.04**
96 h	0.76 ± 0.04	0.25 ± 0.024**	0.39 ± 0.03	0.13 ± 0.03 **
120 h	0.04 ± 0.03	0.11 ± 0.043	0.07 ± 0.006	0.06 ± 0.004*
144 h	0.031 ± 0.004	0.10 ± 0.010**	0.01 ± 0.001	0.05 ± 0.004**

Values are the means of 5 determinations

*The values are significant at $P<0.05$ against the normal for n=5,

**The values are highly significant at $P<0.01$ against the normal for n=5

Figure 6.1a

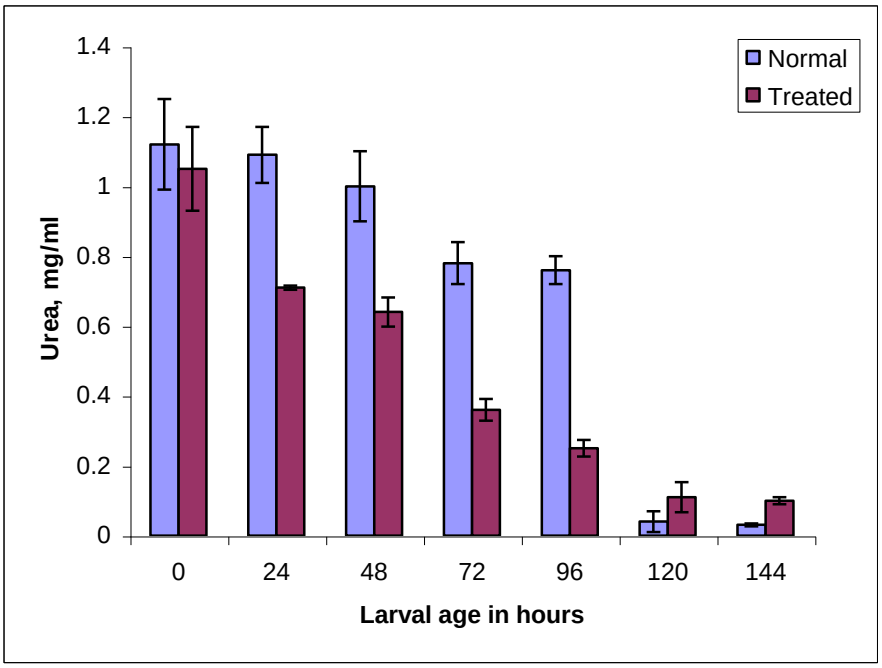

Figure 6.1b

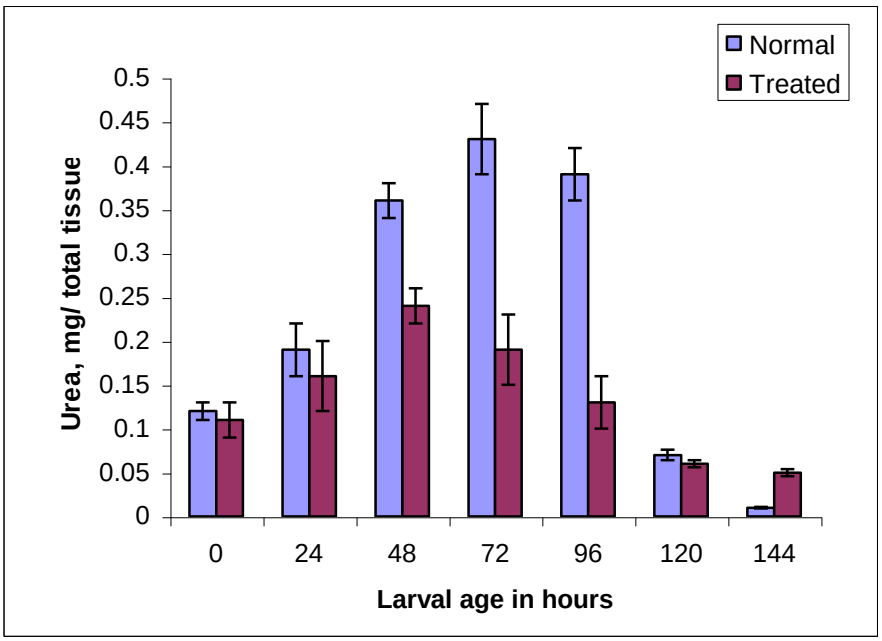

Figure 6.1. Changes in the urea levels in haemolymph

a. Changes per unit volume of haemolymph

b. Changes per total haemolymph

Table 6.2. Changes in the urea levels in fat body

Larval age	mg/mg fresh tissue, mean ±S.D		mg / total tissue, mean ± SD	
	Normal ($x 10^{-4}$)	Treated ($x 10^{-4}$)	Normal ($x 10^{-4}$)	Treated ($x 10^{-4}$)
0h	0.61 ± 0.05	0.68 ± 0.07	17.45 ± 2.09	21. 85 ± 2.40
24 h	0.54 ± 0.06	0.82 ± 0.07**	19.33 ± 1.62	31.12 ± 2.63**
48 h	0.52 ± 0.04	0.59 ± 0.06	32.12 ± 4.81	51.10 ± 5.42**
72 h	0.64 ± 0.05	0.62 ± 0.07	94.34 ± 12.20	70.18 ± 6.44*
96 h	0.67 ± 0.05	0.76 ± 0.08	118.81 ± 16.53	127.90 ±17.23
120 h	0.46 ± 0.04	0.66 ± 0.04	112.20 ± 12.34	116.61 ± 15.26
144 h	0.43 ± 0.04	0.62 ± 0.05**	101.86 ± 11.24	110.52 ± 15.85

Values are the means of 5 determinations
*The values are significant at P<0.05 against the normal for n=5,
**The values are highly significant at P<0.01 against the normal for n=5

Figure 6.2a

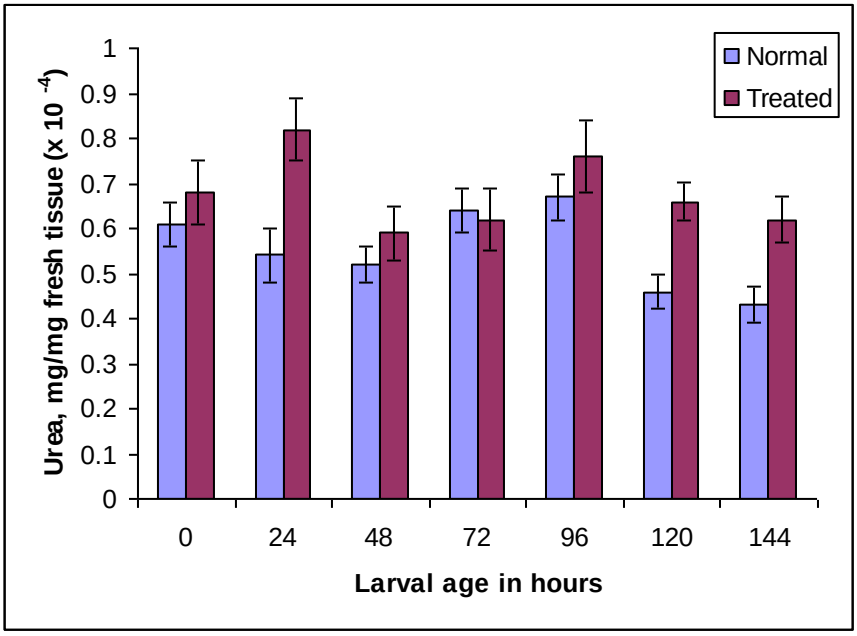

Figure 6.2b

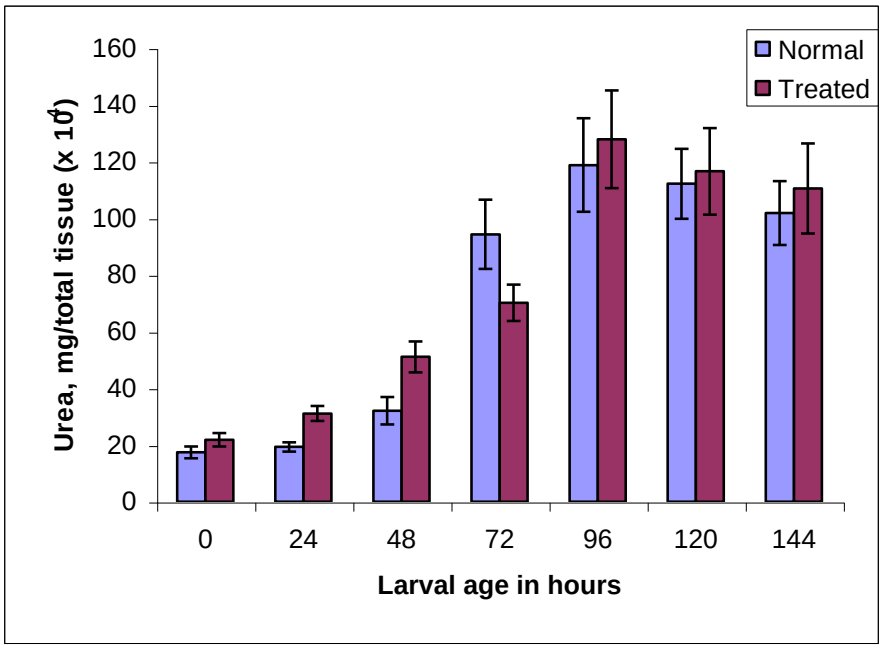

Figure 6.2. Changes in the urea levels in fat body

a. Changes per unit weight of fat body

b. Changes per total fat body

The level of urea in the haemolymph of the treated larvae also exhibited the same pattern of changes during the development of the larva but with a significant low magnitude.

The level of urea in the fat body of normal larvae was gradually increased from the beginning of larval period recording the peak value at 96 h and then declined. The results obtained for treated larvae followed the same pattern as that of normal, but with a higher level.

Creatinine

The levels of creatinine in the haemolymph and fat body of normal and treated larvae are recorded in Table 7.1 and 7.2 and Figure 7.1 and 7.2.

Table 7.1 and 7.2

Figure 7.1 and 7.2

On the basis of unit volume of haemolymph the creatinine levels were low at the beginning of the larval development both in the normal and treated larvae, rose to the peak value in the fourth day of development and declined thereafter. The changes in the levels were not so prominent in the treated larvae. The pattern of changes in the levels of creatinine in the normal and treated larvae estimated on the basis of total volume of haemolymph was the same as that obtained for unit volume of haemolymph except that in the treated larvae the level was low during the entire period of final instar larval stage.

Table 7.1. Changes in the creatinine levels in haemolymph

Larval age	mg/ml, mean ±S.D		mg / total tissue, mean ± SD	
	Normal	Treated	Normal	Treated
0h	0. 06 ± 0.004	0.05 ± 0.006	0.02 ± 0.002	0.02 ± 0.003
24 h	0.10 ± 0.007	0.08 ± 0.012*	0.03 ± 0.003	0.02 ± 0.002**
48 h	0.16 ± 0.019	0.12 ± 0.014*	0.06 ± 0.005	0.04 ± 0.006**
72 h	0.24 ± 0.024	0.15 ± 0.019**	0.10 ± 0.010	0.07 ± 0.006**
96 h	0.28 ± 0.032	0.16 ± 0.022**	0.15 ± 0.022	0.08 ± 0.007**
120 h	0.16 ± 0.021	0.14 ± 0.015	0.13 ± 0.024	0.09 ± 0.013*
144 h	0.18 ± 0.026	0.14 ± 0.016	0.12 ± 0.013	0.10 ± 0.009

Values are the means of 5 determinations

*The values are significant at $P<0.05$ against the normal for n=5,

**The values are highly significant at $P<0.01$ against the normal for n=5

Figure 7.1a

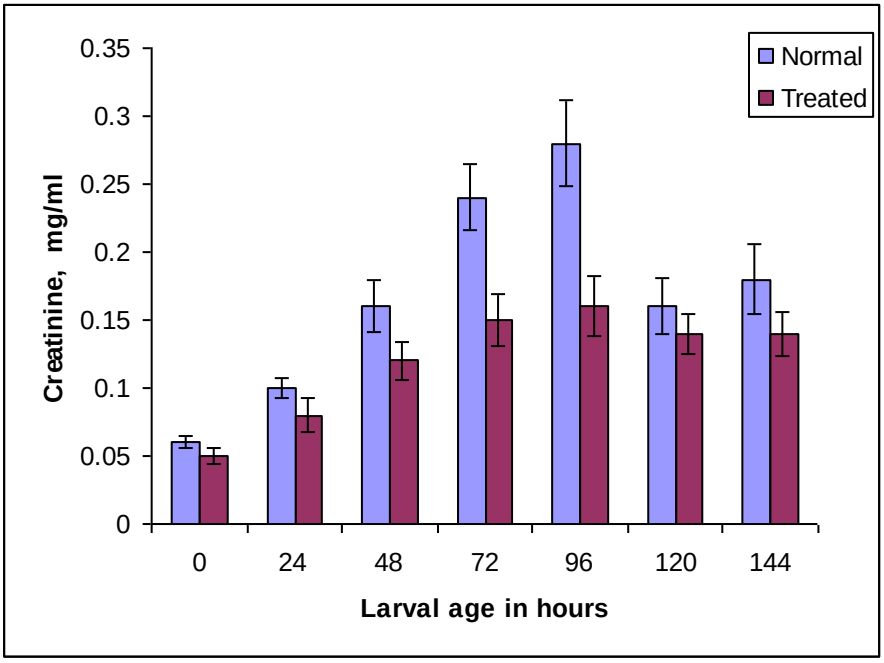

Figure 7.1b

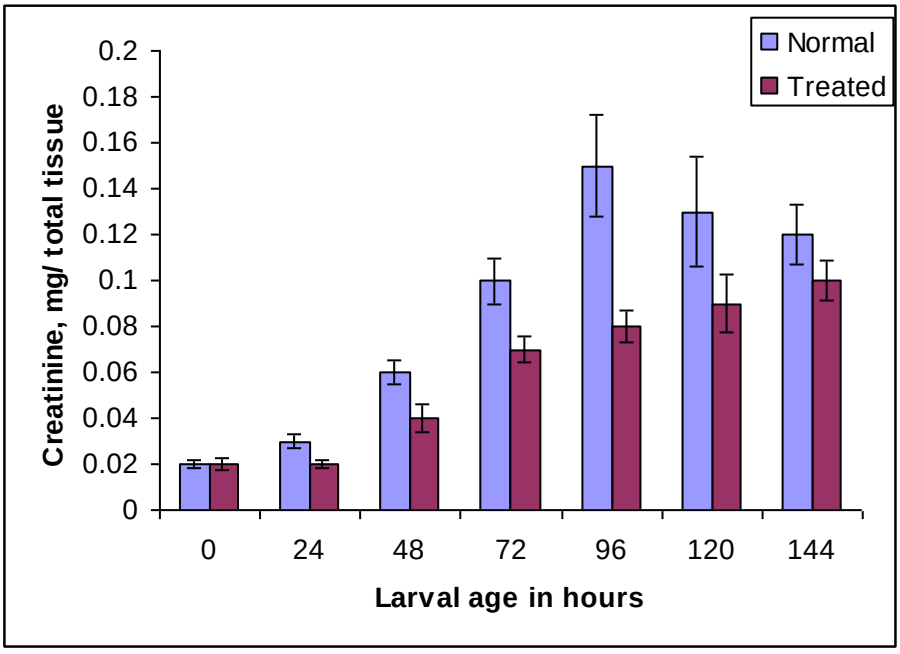

Figure 7.1. Changes in the creatinine levels in haemolymph

a. Changes per unit volume of haemolymph

b. Changes per total haemolymph

Table 7.2. Changes in the creatinine levels in fat body

Larval age	mg/mg fresh tissue, mean ±S.D		mg / total tissue, mean ± SD	
	Normal (x 10^{-4})	Treated (x 10^{-4})	Normal (x 10^{-4})	Treated (x 10^{-4})
0h	0.41 ± 0.04	0.48 ± 0.71	28.38 ± 3.14	34.09 ± 4.77
24 h	0.61 ± 0.05	0.62 ± 0.05	32.46 ± 2.80	51.19 ± 4.61**
48 h	0.59 ± 0.07	0.98 ± 0.08**	43.27 ± 5.24	85.13 ± 9.36**
72 h	1.61 ± 0.13	1.21 ± 0.12**	231.20 ± 27.71	96.34 ± 11.56**
96 h	2.48 ± 0.28	2.37 ± 0.31	451.11 ± 49.65	383.66 ±49.87
120 h	2.19 ± 0.19	1.19 ± 0.16 **	590.20 ± 70.83	698.4 2± 43.82
144 h	1.20± 0.11	1.10 ± 0.14	290.64 ± 32.11	741.63 ± 81.58

Values are the means of 5 determinations
*The values are significant at P<0.05 against the normal for n=5,
**The values are highly significant at P<0.01 against the normal for n=5

Figure 7.2a

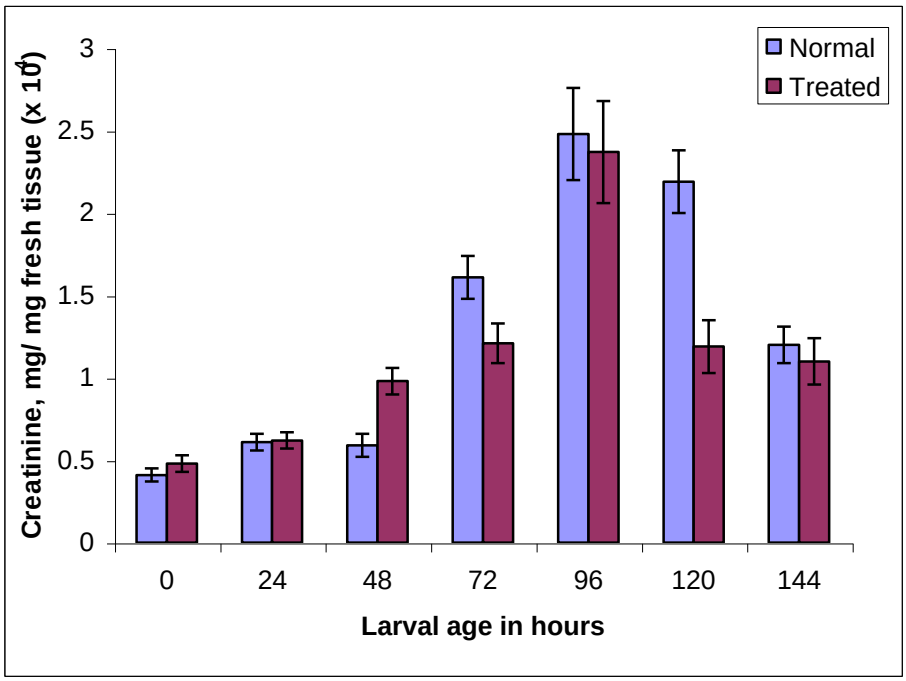

Figure 7.2b

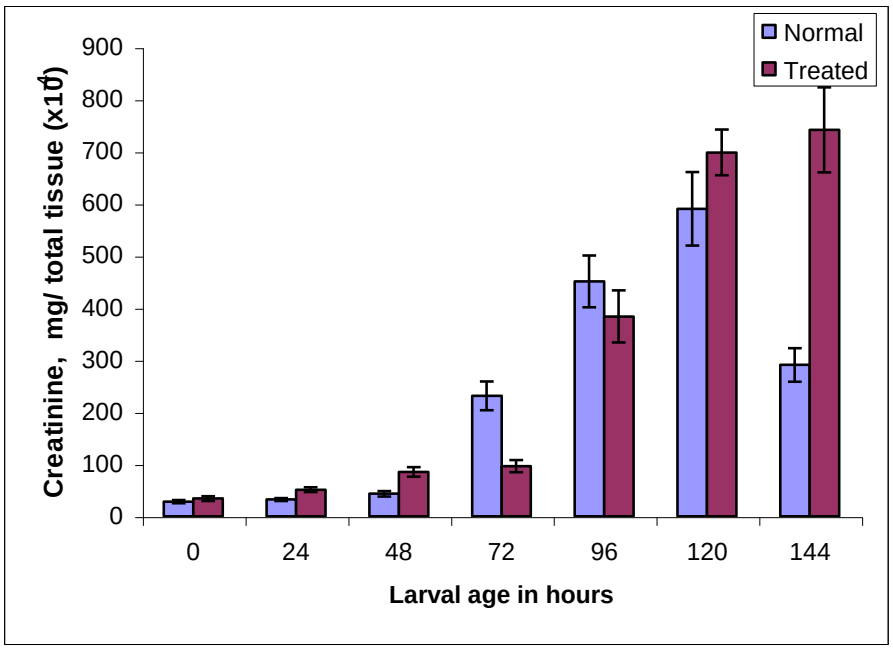

Figure 7.2. Changes in the creatinine levels in fat body

a. Changes per unit weight of fat body

b. Changes per total fat body

The changes in the level of creatinine in the larval fat body of both normal and treated larvae estimated per unit weight of fat body exhibited a similar pattern observed for haemolymph. The total tissue creatinine levels in treated larvae exhibited a peak value at 144 h.

Glucose

The changes in the concentration of glucose in the haemolymph and fat body of both normal and treated larvae during the developmental stages of the fifth instar larvae are presented in Table 8.1 and 8.2 and Figure 8.1 and 8.2.

Table 8.1 and 8.2

Figure 8.1 and 8.2

The levels of haemolymph glucose of both normal and treated larvae recorded a marked increase during the developmental period recording a peak value at 120h followed by a slight reduction towards pupation. The peak glucose levels in the total larval haemolymph was almost 28 times to that found at the early stage in normal larvae and approximately 12 times in tryptophan treated larvae.

The fat body glucose levels also exhibited similar variation during the development of the larvae as seen for haemolymph.

Table 8.1. Changes in the glucose levels in haemolymph

Larval age	mg/ml, mean ±S.D		mg / total tissue, mean ± SD	
	Normal	Treated	Normal	Treated
0h	0.54 ± 0.05	0.62 ± 0.09	0.09 ± 0.01	0.12 ± 0.01
24 h	0.69 ± 0.06	0.81 ± 0.07	0.15 ± 0.02	0.18 ± 0.02*
48 h	1.05 ± 0.09	1.42 ± 0.20*	0.38 ± 0.04	0.56 ± 0.08*
72 h	1.44 ± 0.12	1.61 ± 0.13*	0.61 ± 0.08	0.73 ± 0.06
96 h	1.56 ± 0.23	1.73 ± 0.25	0.79 ± 0.06	0.89 ± 0.09
120 h	3.56 ± 0.53	2.60 ± 0.39*	2.60 ± 0.21	1.58 ± 0.13**
144 h	2.98 ± 0.31	1.92 ± 0.15**	1.68 ± 0.14	1.18 ± 0.14**

Values are the means of 5 determinations
*The values are significant at $P<0.05$ against the normal for n=5,
**The values are highly significant at $P<0.01$ against the normal for n=5

Figure 8.1a

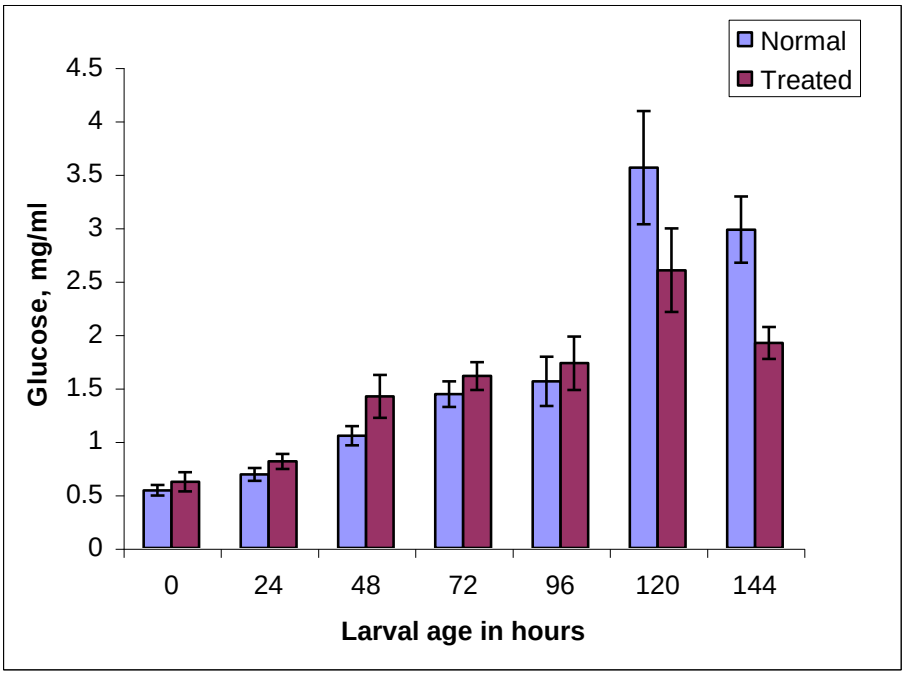

Figure 8.1b

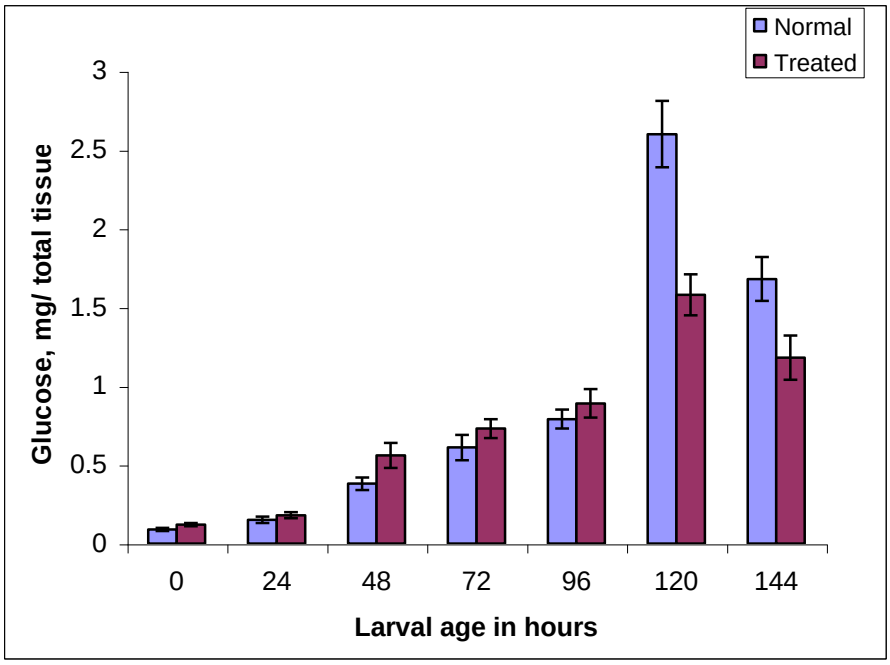

Figure 8.1. Changes in the glucose levels in haemolymph

a. Changes per unit volume of haemolymph
b. Changes per total haemolymph

Table 8.2. Changes in the glucose levels in fat body

Larval age	mg/mg fresh tissue, mean ±S.D		mg / total tissue, mean ± SD	
	Normal (x 10^{-3})	Treated (x 10^{-3})	Normal (x 10^{-3})	Treated (x 10^{-3})
0h	0.86 ± 0.10	0.82 ± 0.09	29.38 ± 3.71	27.64 ± 3.94
24 h	1.08 ± 0.16	0.92 ± 0.07	41.20 ± 4.94	35.78 ± 3.9
48 h	4.21 ± 0.38	2.61 ± 0.21**	221.22 ± 18.56	204.42 ± 24.53
72 h	4.98 ± 0.55	3.10 ± 0.25**	778.25 ± 68.97	521.35 ± 62.56**
96 h	6.53 ± 0.54	2.91 ± 0.35**	1058.66 ± 126.34	489.52 ± 53.84**
120 h	3.42 ± 0.32	4.90 ± 0.39**	876 .38 ± 72.10	906.71 ± 81.60
144 h	4.17 ± 0.61	4.22 ± 0.58	1003.19 ± 120.38	859.90 ± 94.58

Values are the means of 5 determinations

*The values are significant at P<0.05 against the normal for n=5,

**The values are highly significant at P<0.01 against the normal for n=5

Figure 8.2a

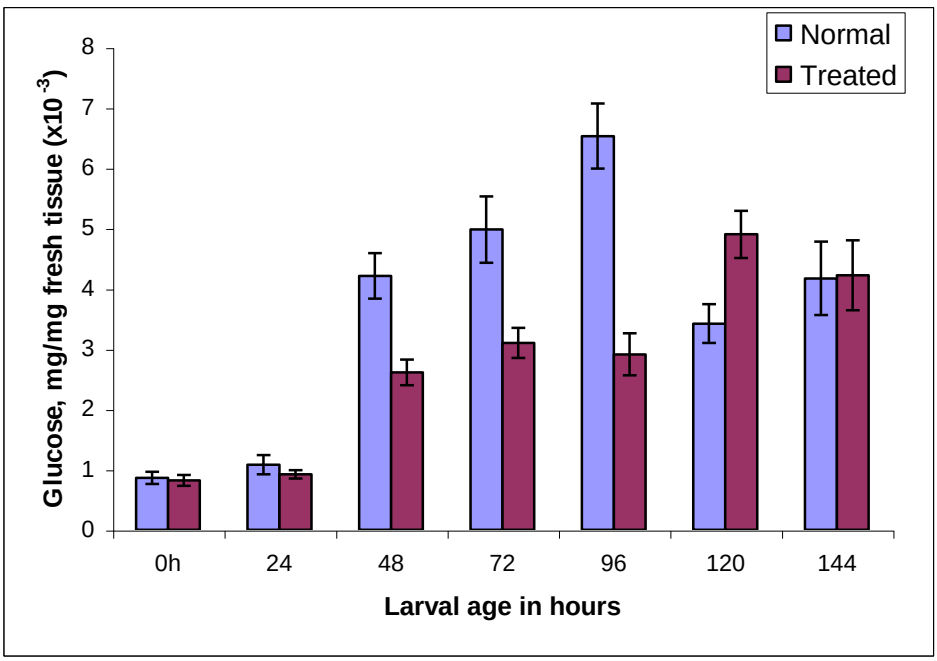

Figure 8.2b

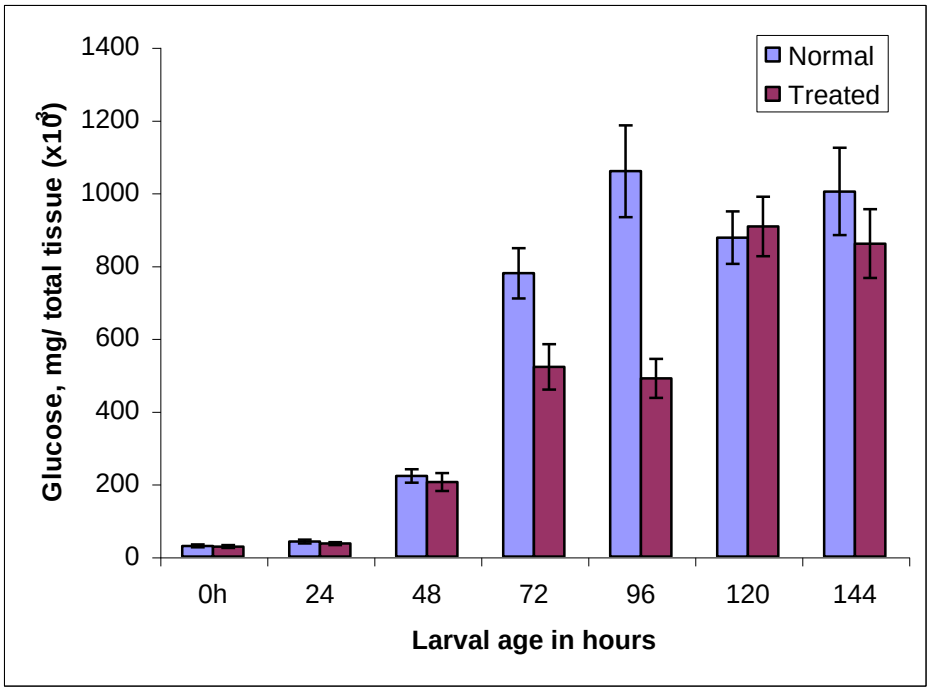

Figure 8.2. Changes in the glucose levels in fat body

a. Changes per unit weight of fat body

b. Changes per total fat body

It was observed that treated larvae maintained a higher level of glucose than that of normal during the feeding stage followed by a dip on non feeding stage. The fat body glucose level showed a consistent reduction in the treated larvae.

Aspartate amino transferase (AsAT) activity

The total activity and specific activity of AsAT in the haemolymph and fat body of the normal and treated larvae during its development are given in table 9.1, 9.2 and 9.3 and Figure 9.1, 9.2 and 9.3.

Table 9.1, 9.2 and 9.3

Figure 9.1, 9.2 and 9.3

The total AsAT activity estimated per unit volume of haemolymph in normal and treated larvae exhibited gradual increase from 0h onwards and recorded the peak value at 96 h followed by a dip thereafter. The AsAT activity estimated per total volume of haemolymph also showed more or less the same pattern of variation but the magnitude of the changes were high.

The AsAT activities in the fat body of both normal and treated larvae estimated per unit weight of fat body were high in the early stages of the instar but showed a decline in the later stages. On the basis of total fat body the AsAT activities in the normal and treated larvae were initially low, gradually rose to its peak at 72 h followed by a significant reduction thereafter.

Table 9.1. Changes in the AsAT activity in haemolymph

Larval age	AsAT, units/ ml, mean ±S.D		AsAT, units /total tissue, mean ±SD	
	Normal ($x\ 10^{-5}$)	Treated ($x\ 10^{-5}$)	Normal ($x\ 10^{-5}$)	Treated ($x\ 10^{-5}$)
0h	12.01 ± 0.96	13.75 ± 1.24	1.63 ± 0.13	1.98 ± 0.22
24 h	13.03 ± 1.12	15.43 ± 1.26*	2.28 ± 0.18	4.24 ± 0.45 **
48 h	14.52 ± 1.18	17.81 ± 1.51*	4.99 ± 0.68	8.08 ± 1.32 *
72 h	13.70 ± 1.13	20.72 ± 1.71**	6.85 ± 0.51	9.01 ± 0.81*
96 h	20.44 ± 2.05	25.41 ± 2.5*	10.20 ± 1.20	11.37 ± 1.62
120 h	17.12 ± 2.5	17.19 ± 2.4	11.97 ± 1.71	10.31 ± 0.95
144 h	15.33 ±2.03	11.69 ± 1.6*	8.11 ± 1.11	6.05 ± 0.87 *

Values are the means of 5 determinations

*The values are significant at $P<0.05$ against the normal for n=5,

**The values are highly significant at $P<0.01$ against the normal for n=5

Figure 9.1a

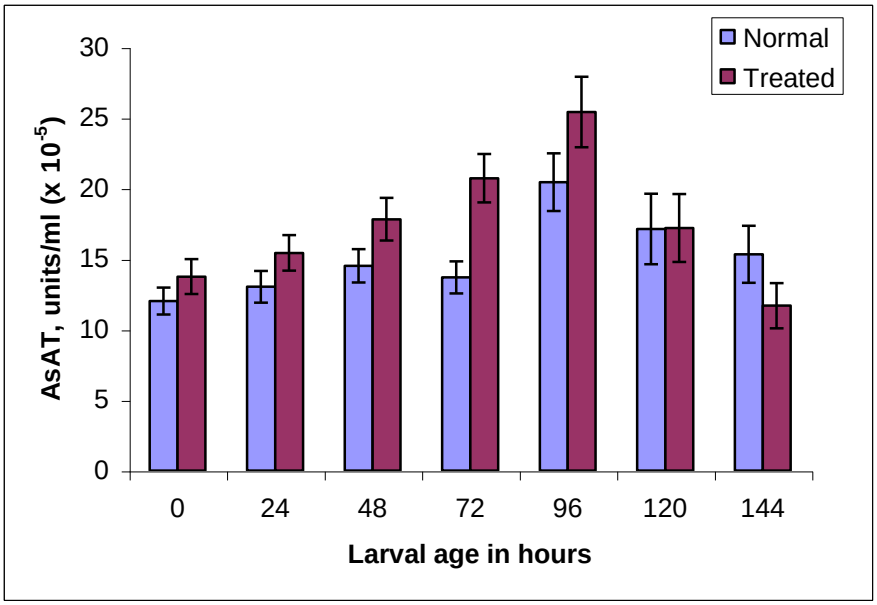

Figure 9.1b

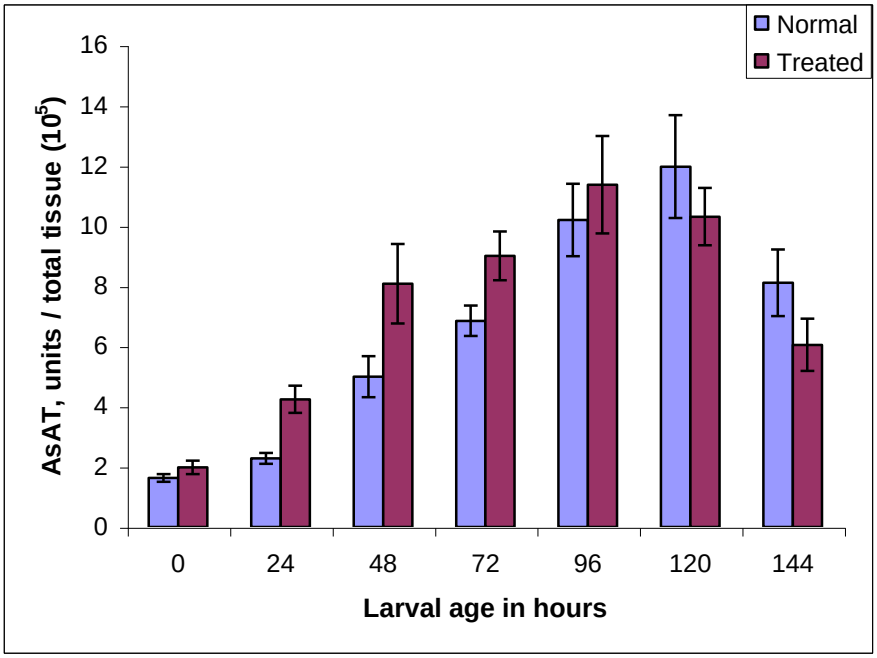

Figure 9.1. Changes in the AsAT activity in haemolymph

a. Changes per unit volume of haemolymph

b. Changes per total haemolymph

Table 9.2. Changes in the AsAT activity in fat body

Larval age	AsAT, units/ mg fresh tissue, mean ±S.D		AsAT, units / total tissue, mean ± SD	
	Normal (x 10^{-5})	Treated (x 10^{-5})	Normal (x 10^{-5})	Treated (x 10^{-5})
0h	0.17 ± 0.02	0.16 ± 0.02	5.09 ± 0.55	4.91 ± 0.59
24 h	0.21 ± 0.02	0.17 ± 0.03	7.68 ± 0.95	6.46 ± 0.85
48 h	0.25 ± 0.04	0.21 ± 0.02	12.60 ± 1.02	18.18 ±2.06**
72 h	0.29 ± 0.04	0.41 ± 0.05*	21.81 ± 3.14	32.17 ±2.95**
96 h	0.18 ± 0.03	0.32± 0.07*	19.51 ± 2.12	13.51 ± 1.5**
120 h	0.10 ± 0.009	0.11 ± 0.02	16.35 ± 1.31	16.21 ± 1.71
144 h	0.08 ± 0.006	0.06 ± 0.007	10.68 ± 1.49	8.91 ± 0.71

Values are the means of 5 determinations
*The values are significant at P<0.05 against the normal for n=5,
**The values are highly significant at P<0.01 against the normal for n=5

Figure 9.2 a

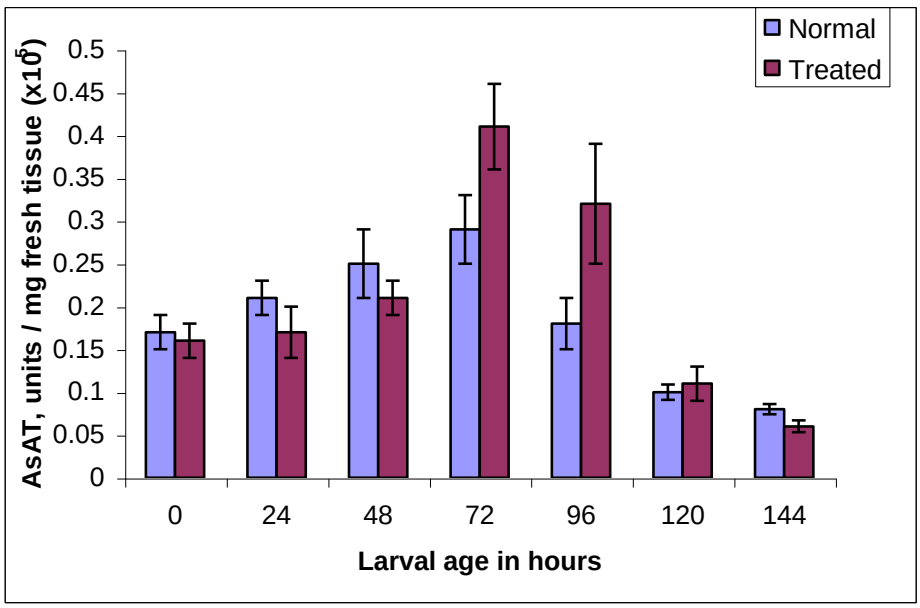

Figure 9.2b

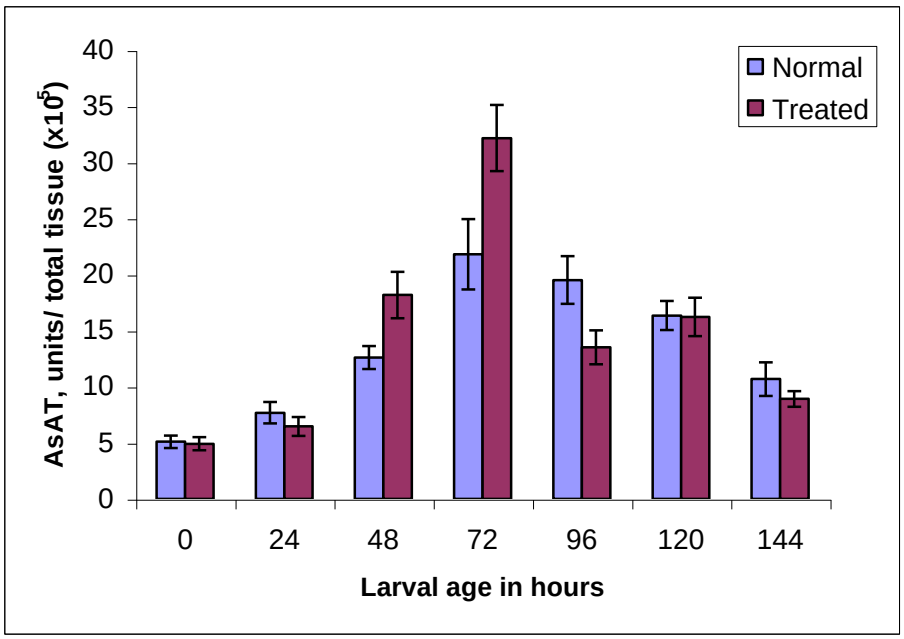

Figure 9.2. Changes in the AsAT activity in fat body

a. Changes per unit weight of fat body

b. Changes per total fat body

Table 9.3 a. Changes in the specific activity of AsAT in haemolymph

Larval age	Specific activity (units/ mg protein), mean ± SD	
	Normal (x 10^{-6})	Treated (x 10^{-6})
0h	2.95 ± 0.32	2.75 ± 0.49
24 h	3.15 ± 0.38	2.43 ± 0.34*
48 h	0.83 ± 0.07	0.36 ±0.04**
72 h	0.25 ± 0.02	0.1 ±0.008**
96 h	0.17 ± 0.014	0.05 ± 0.002**
120 h	0.06 ± 0.005	0.047 ± 0.004*
144 h	0.07±0.006	0.031±0.003**

Values are the means of 5 determinations

*The values are significant at P<0.05 against the normal for n=5,

**The values are highly significant at P<0.01 against the normal for n=5

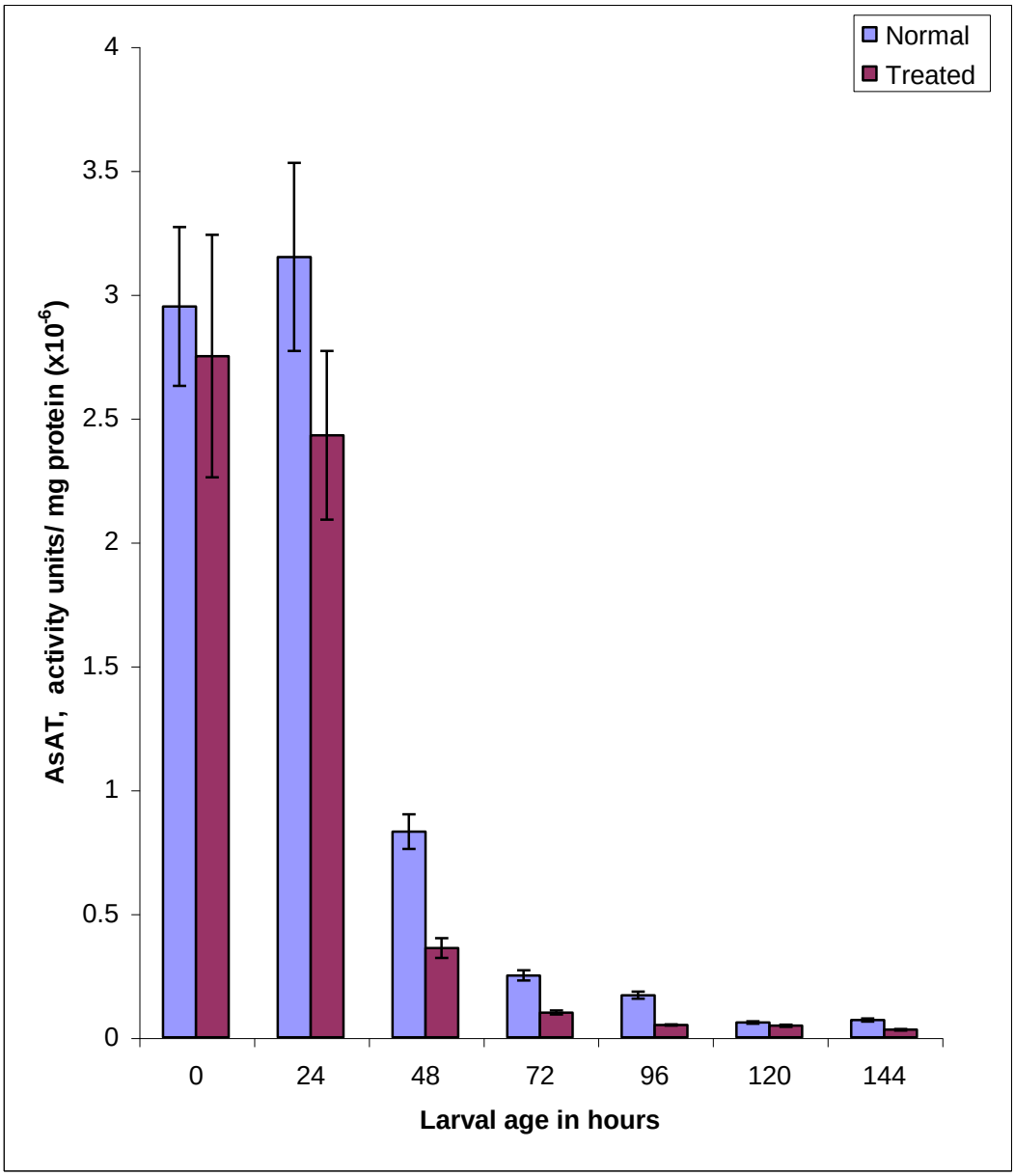

Figure 9.3a. Changes in the specific activity of AsAT in haemolymph

Table 9.3 b. Changes in the specific activity of AsAT in fat body

Larval age	Specific activity (units/ mg protein), mean ± SD	
	Normal ($x 10^{-6}$)	**Treated ($x 10^{-6}$)**
0h	0.28 ± 0.044	0.32 ± 0.052
24 h	0.30 ± 0.027	0.37 ± 0.04*
48 h	0.12 ± 0.014	0.20 ± 0.024**
72 h	0.07 ± 0.006	0.30 ± 0.04 2**
96 h	0.06 ± 0.005	0.03 ± 0.005*
120 h	0.02 ± 0.002	0.02 ± 0.002
144 h	0.03 ± 0.002	0.02 +0.002**

Values are the means of 5 determinations

*The values are significant at $P<0.05$ against the normal for n=5,

**The values are highly significant at $P<0.01$ against the normal for n=5

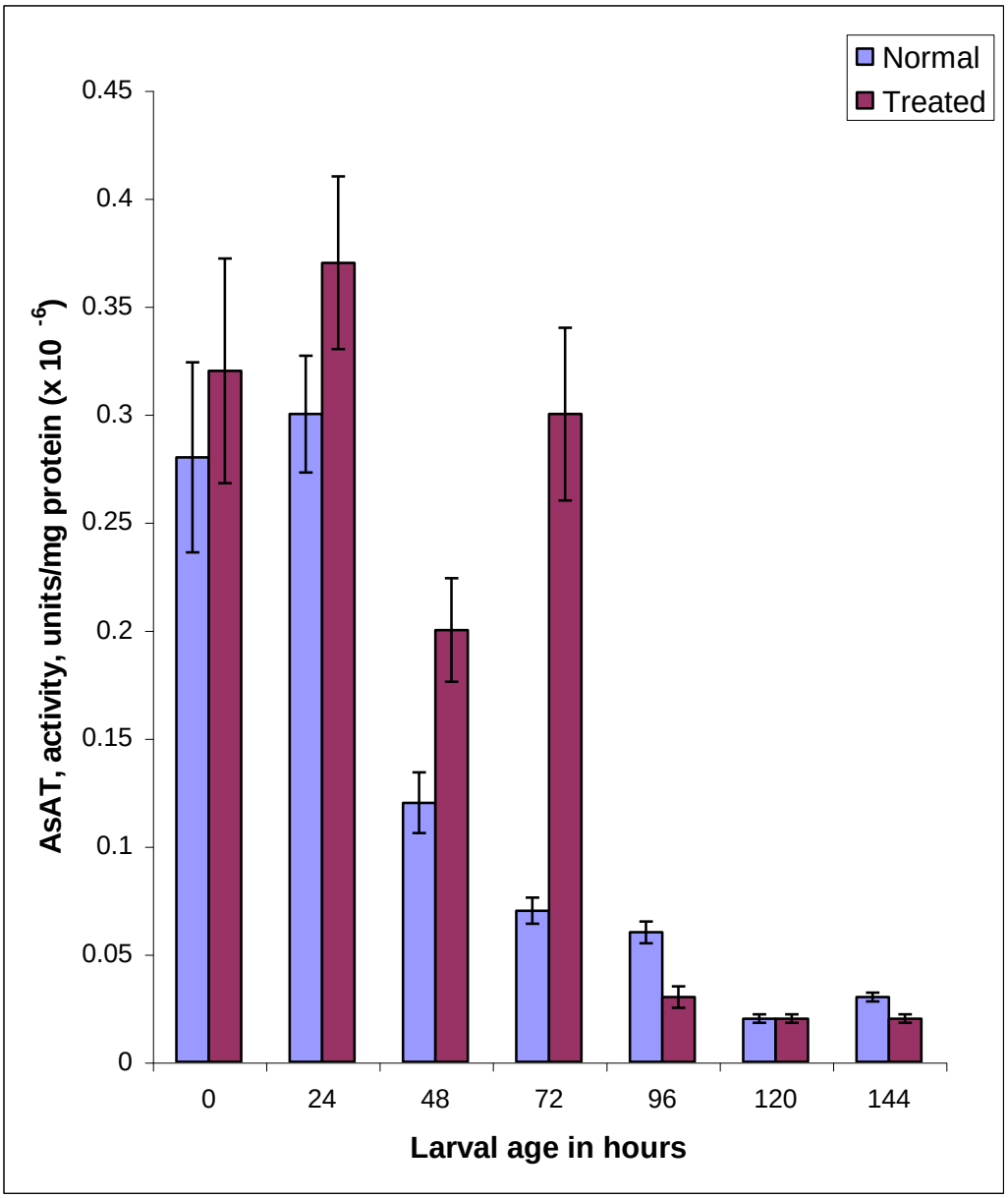

Figure 9.3b. Changes in the specific activity of AsAT in fat body

The AsAT in the haemolymph of both normal and treated larvae exhibited high specific activity during the initial developmental stages with a gradual and marked reduction during the later stages of larval life. The specific activity of AsAT in the fat body of normal and treated larvae were also follows the same pattern. Though the total activity was high in haemolymph of treated larvae, the specific activity recorded more than a 50% reduction.

Alanine amino transferase (AlAT) activity

The total and specific activity of AlAT of the normal and treated larvae during the development of the fifth instar are presented in Table 10.1, 10.2 and 10.3 and Figure 10.1, 10.2 and 10.3.

Table 10.1, 10.2 and 10.3

Figure 10.1, 10.2 and 10.3.

AlAT in haemolymph of normal larvae showed a sharp increase with the growth. The variation in the AlAT activity was about 2.7 fold and about 11 fold when estimated per unit volume and total volume of haemolymph respectively.

Table 10.1. Changes in the AlAT activity in haemolymph

Larval age	AlAT, units/ ml, mean ±S.D		AlAT, units / total tissue, mean ± SD	
	Normal (x 10^{-5})	Treated (x 10^{-5})	Normal (x 10^{-5})	Treated (x 10^{-5})
0h	6.78 ± 0.74	7.08 ± 0.99	0.95 ± 0.08	1.18 ± 0.16
24 h	7.20 ± 0.95	17.05 ± 2.4**	1.08 ± 0.09	3.92 ± 0.57**
48 h	8.13 ± 1.2	16.06 ± 2.3**	2.81 ± 0.22	6.10 ± 0.65**
72 h	9.69 ± 1.35	11.06 ± 1.58	5.01 ± 0.45	5.86 ± 0.72
96 h	15.22 ± 1.6	7.51 ± 1.1**	7.45 ± 0.61	3.93 ± 0.38**
120 h	16. 80 ± 1.7	5.20 ± 0.78**	10.22 ± 1.2	2.92 ± 0.29**
144 h	18.79 ± 2.1	6.35 ± 0.66**	10.71 ± 1.42	4.52 ± 0.39**

Values are the means of 5 determinations
*The values are significant at P<0.05 against the normal for n=5,
**The values are highly significant at P<0.01 against the normal for n=5

Figure 10.1a

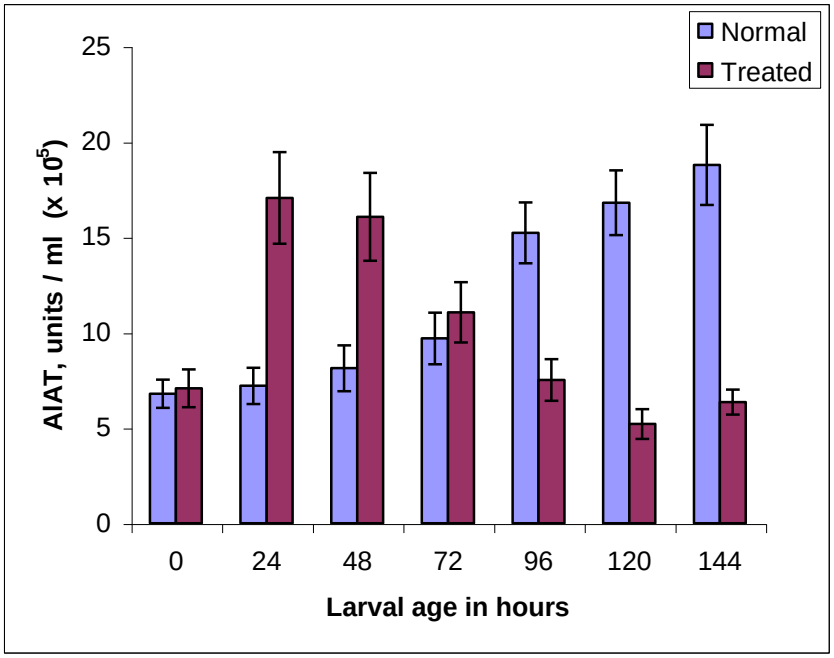

Figure 10.1b

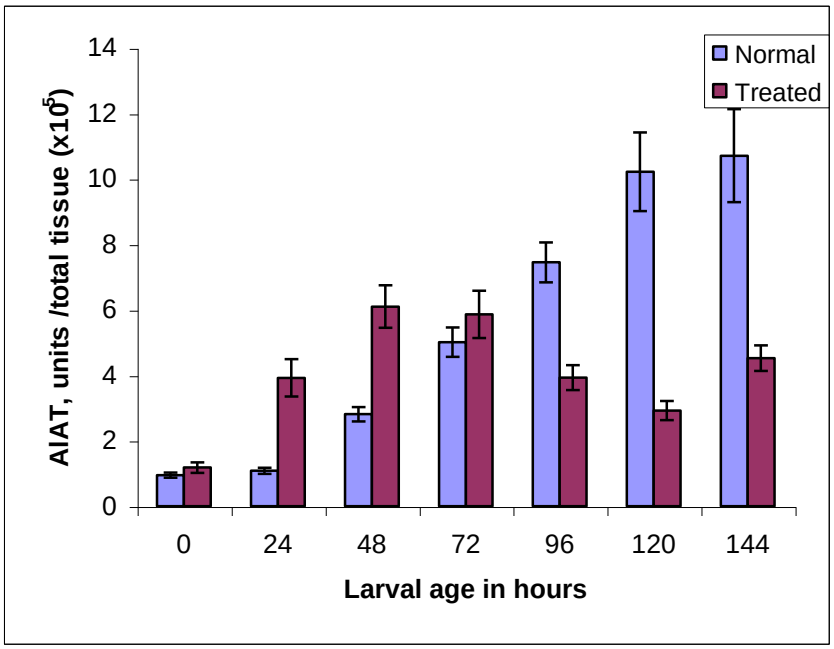

Figure 10.1. Changes in the AlAT activity in haemohelymph

a. Changes per unit volume of haemohelymph

b. Changes per total haemohelymph

Table 10.2. Changes in the AlAT activity in fat body

Larval age in hour	AlAT units/ mg fresh tissue, mean ± S.D		AlAT, units / total tissue, mean ± SD	
	Normal (x 10^{-6})	Treated (x 10^{-6})	Normal (x 10^{-6})	Treated (x10^{-6})
0h	0.28 ± 0.027	0.26 ± 0.025	8.98 ± 0.07	8.07 ± 0.89
24 h	0.31 ± 0.024	0.25 ± 0.022*	10.72 ± 1.52	9.69 ± 1.25
48 h	0.26 ± 0.031	0.23 ± 0.019	16.08 ± 2.08	19.91 ± 2.80
72 h	0.24 ± 0.020	0.17 ± 0.014**	35.01 ± 4.18	28.41 ± 2.92
96 h	0.12 ± 0.010	0.11 ± 0.009	16.03 ± 2.32	25.68 ± 3.22**
120 h	0.06 ± 0.005	0.06 ± 0.007	14.65 ± 1.90	20.89 ± 2.51*
144 h	0.02 ±0.003	0.02 ± 0.003	4.74 ± 0.58	8.31 ± 1.10**

Values are the means of 5 determinations

*The values are significant at P<0.05 against the normal for n=5,

**The values are highly significant at P<0.01 against the normal for n=5

Figure 10.2a

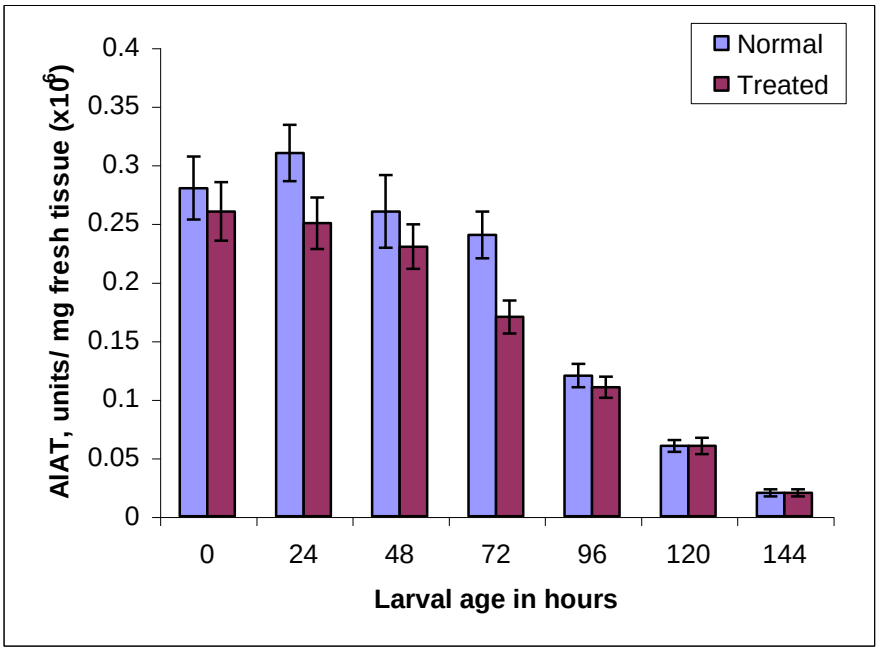

Fig. 10.2b

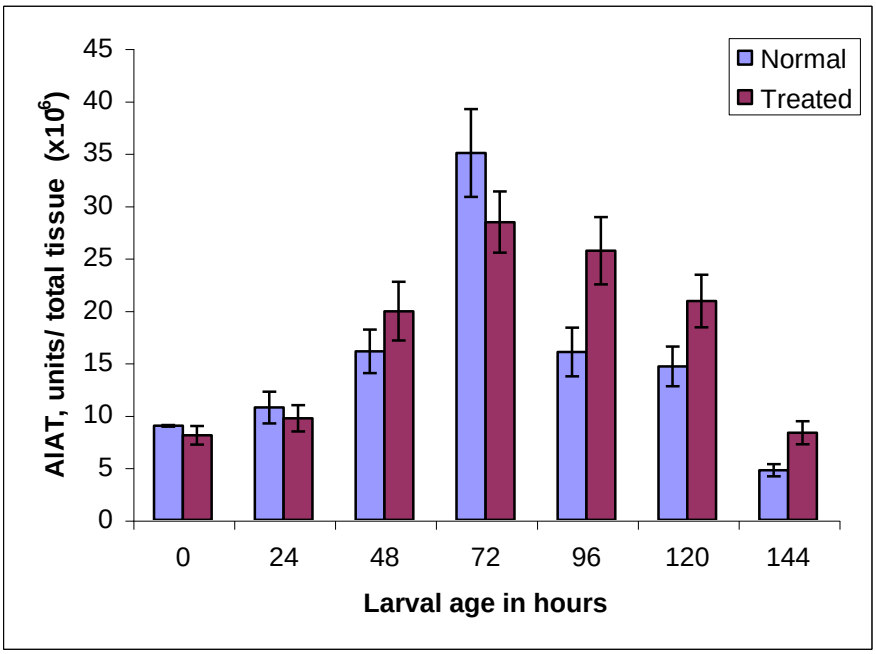

Figure 10.2. Changes in the AlAT activity in fat body

a. Changes per unit weight of fat body
b. Changes per total fat body

Table 10.3a. Changes in the specific activity of AlAT in haemolymph

Larval age	Specific activity (units/ mg protein), mean ± SD	
	Normal (x 10^{-5})	**Treated (x 10^{-5})**
0h	0.09 ±0.008	0.13 ± 0.014
24 h	0.13 ± 0.020	0.20 ± 0.030*
48 h	0.2 ± 0.018	0.03 ± 0.004**
72 h	0.02 ± 0.002	0.006 ± 0.0005**
96 h	0.01 ± 0.001	0.002 ± 0.0003**
120 h	0.005 ± 0.0006	0.002 ± 0.0002**
144 h	0.009 ± 0.001	0.003 ± 0.0004**

Values are the means of 5 determinations
*The values are significant at P<0.05 against the normal for n=5,
**The values are highly significant at P<0.01 against the normal for n=5

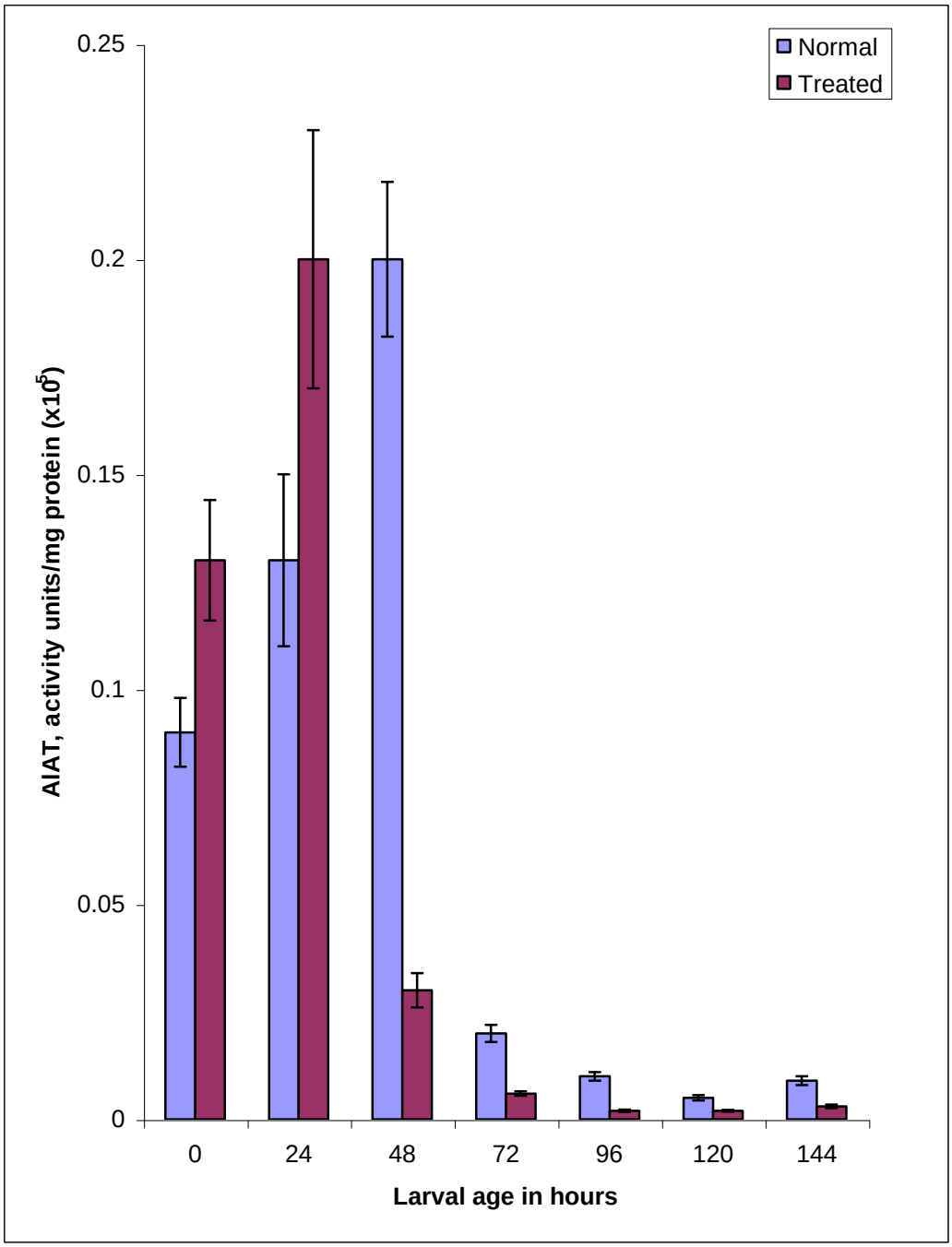

Figure 10.3a. Changes in the specific activity of AIAT activity haemolymph

Table 10.3b. Changes in the specific activity of AIAT in fat body

Larval age	Specific Activity (units/ mg protein), mean ± SD	
	Normal (x 10^{-5})	Treated (x 10^{-5})
0h	0.31 ± 0.034	0.38 ± 0.045
24 h	0.40 ± 0.050	0.61 ± 0.071**
48 h	1.60 ± 0.130	0.21 ± 0.030**
72 h	0.11 ± 0.021	0.05 ± 0.006**
96 h	0.03 ± 0.004	0.06 ± 0.005**
120 h	0.02 ± 0.002	0.024± 0.002*
144 h	0.04 ± 0.003	0.06 ± 0.0008**

Values are the means of 5 determinations

*The values are significant at $P<0.05$ against the normal for n=5,

**The values are highly significant at $P<0.01$ against the normal for n=5

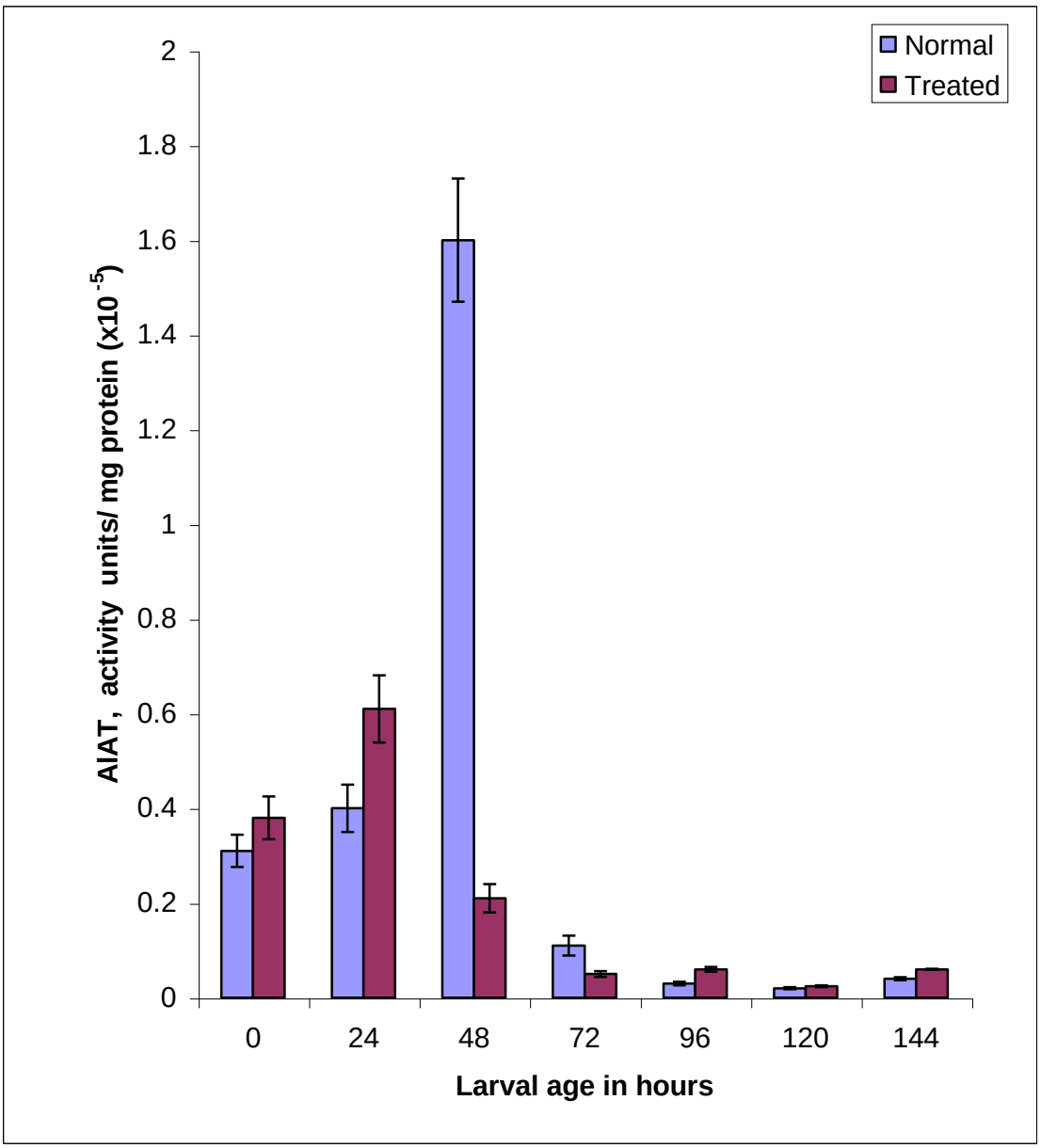

Figure 10.3b. Changes in the Specific activity of AIAT in fat body

In the case of treated larvae, the AlAT activity in haemolymph showed growth dependent changes with a marked difference in its pattern and magnitude between unit and total volume and from normal larvae. This pattern of changes in the levels of AlAT per unit volume of haemolymph of the treated larvae showed an approximate inverse relationship with the normal. The total activity of AlAT in the haemolymph of the treated larvae was high in the feeding stage followed by a sharp decline in the non feeding stage.

AlAT activity of the fat body of the normal and treated larvae per unit weight was high in the early stages of final instar larval period which declined sharply as the larval development progressed. The magnitude of reduction was 14 times and 13 times in the case of normal and treated larvae respectively from the initial value during development. The total AIAT activity of the fat body of the normal and treated larvae showed a different pattern, which was low initially, but rose sharply, recording the peak value at 72 h and followed by a steep decline.

The specific activity of AlAT in the haemolymph of the normal larvae was high initially but showed a sharp decrease with the development of the larvae, depicting a 10 fold variation. The changes in the specific activity of AlAT in the haemolymph of the treated larvae showed a similar pattern: the activity was high initially and declined thereafter with a consistent reduction from the normal.

Hydrogen peroxide and catalase activity

Hydrogen peroxide levels estimated per unit fresh tissue and total tissue in the haemolymph and fat body of both normal and treated larvae are given in Table 11.1 and 11.2 and Figure 11.1 & 11.2

Table 11.1 & 11.2

Figure 11.1 & 11.2

H_2O_2 levels per unit volume haemolymph of the normal larvae increased gradually and recorded a peak value at 72 h and decreased thereafter. H_2O_2 level at the final stage of the larvae was almost one half of that recorded in the previous stage. A different pattern of change was found in treated larvae where the values reached a maximum at 48 h and then dropped down sharply. The pattern of change in H_2O_2 levels of the normal and treated larvae more or less similar to that observed per unit volume of haemolymph.

The changes in the levels of H_2O_2 estimated per unit weight of fat body were more or less the same. When expressed per larva the amount of H_2O_2 showed a gradual increase as the larval growth progressed. The normal larvae recorded a peak value at 120 h which was 14 times greater than initial value. H_2O_2 levels in the treated larvae recorded its peak value at 72 h which was about 8.5 fold to that of initial value. On the basis of unit volume tissue and the total tissue the level of H_2O_2 was low in the treated larvae during the entire period of final instar and the magnitude of reduction was more than 85%.

Table 11.1. Changes in the hydrogen peroxide levels in haemolymph

Larval age	mg/ml, mean ± SD		mg/total tissue, mean ± SD	
	Normal	**Treated**	**Normal**	**Treated**
0h	3.71 ± 0.33	3.01 ± 0.45	0.39 ± 0.06	0.31 ± 0.05
24 h	5.90 ± 0.52	3.50 ± 0.28**	0.98 ± 0.14	0.35 ± 0.04**
48 h	5.40 ± 0.49	4.40 ± 0.38*	1.76 ± 0.21	1.68 ± 0.14**
72 h	5.91 ± 0.56	2.25 ± 0.21**	1.80 ± 0.14	0.66 ± 0.08**
96 h	6.80 ± 0.64	0.88 ± 0.12**	3.10 ± 0.35	0.46 ± 0.05**
120 h	6.10 ± 0.58	0.46 ± 0.06**	3.90 ± 0.41	0.27 ± 0.03**
144 h	2.91 ± 0.38	0.67 ± 0.11**	1.20 ± 0.18	0.25 ± 0.03**

Values are the means of 5 determinations

*The values are significant at $P<0.05$ against the normal for n=5,

**The values are highly significant at $P<0.01$ against the normal for n=5

Figure 11.1a

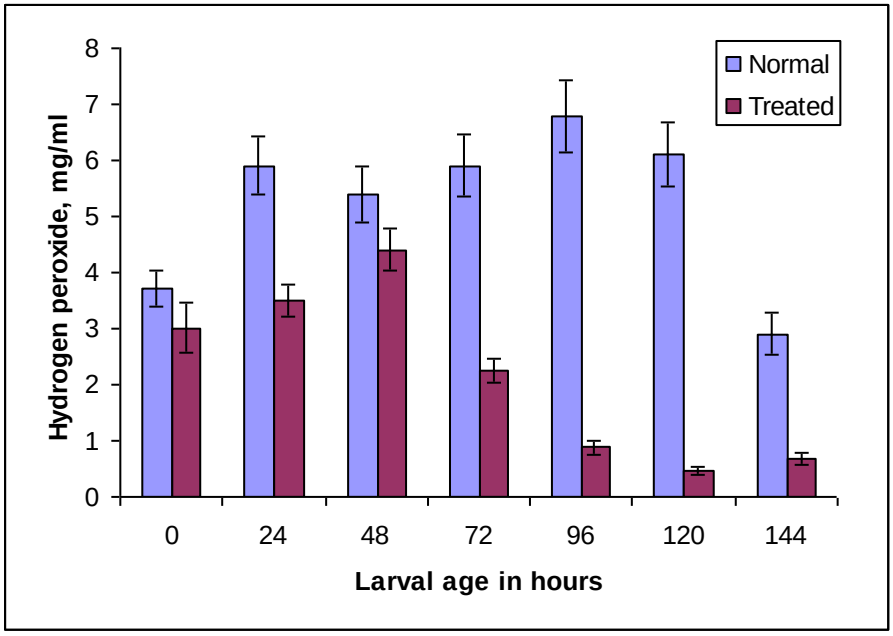

Figure 11.1b

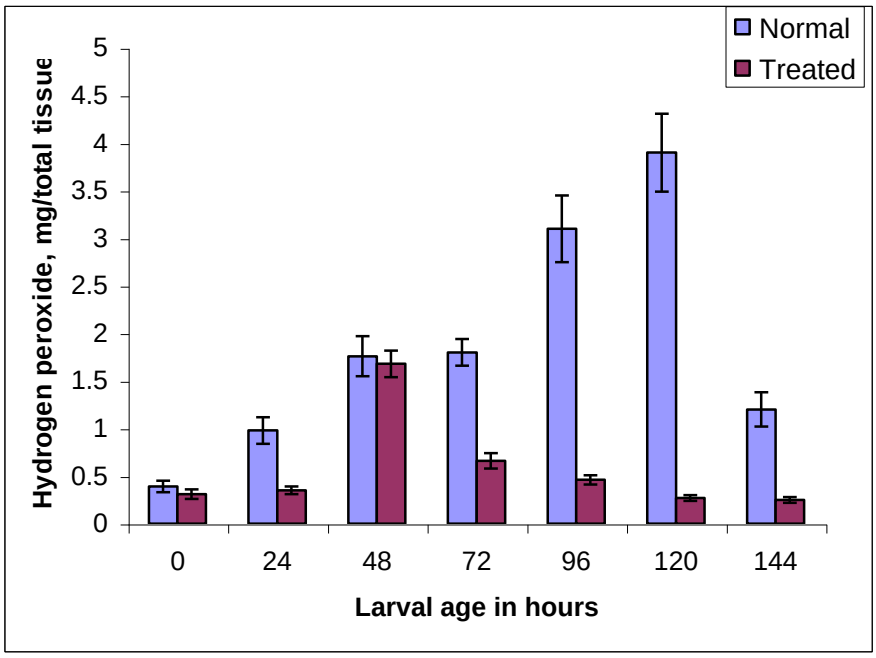

Figure 11.1. Changes in the hydrogen peroxide levels in haemolymph

a. Changes per unit volume of haemolymph

b. Changes per total haemolymph

Table 11.2. Changes in the hydrogen peroxide levels in fat body

Larval age	mg/mg fresh tissue, mean ± SD		mg/total tissue, mean ± SD	
	Normal ($x10^{-3}$)	Treated ($x10^{-3}$)	Normal ($x10^{-3}$)	Treated ($x10^{-3}$)
0h	0.91 ± 0.11	0.88 ± 0.15	68.71 ± 10.31	77.02 ± 13.86
24 h	1.01 ± 0.08	1.01 ± 0.15	71.08 ± 10.1	90.01 ± 7.5*
48 h	1.01 ± 0.09	1.01 ± 0.11*	80.04 ± 7.1	96.07 ± 8.8*
72 h	8.02 ± 1.0	6.06 ± 0.48*	320.06 ± 25.6	460 .12± 41.4**
96 h	5.01 ± 0.4	4.04 ±0.38*	720.11 ± 64.8	670.16 ± 53.6
120 h	4.04 ± 0.32	3.01± 0.28**	940.16 ± 112.8	620.21 ± 55.8*
144 h	3.02 ± 0.26	3.02 ± 0.31	800.24 ± 88.1	610.33 ± 67.1*

Values are the means of 5 determinations

*The values are significant at $P<0.05$ against the normal for n=5,

**The values are highly significant at $P<0.01$ against the normal for n=5

Figure 11.2a

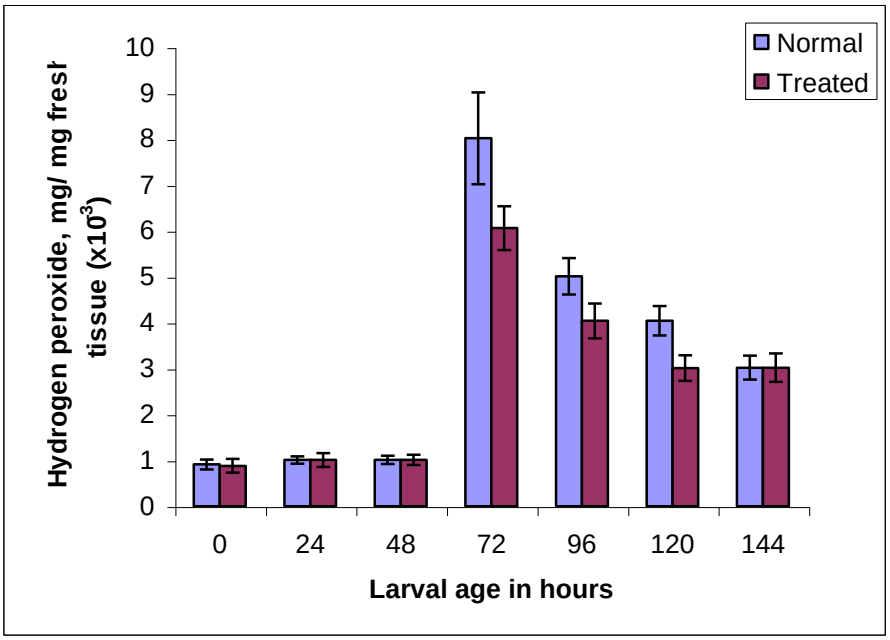

Figure 11.2b

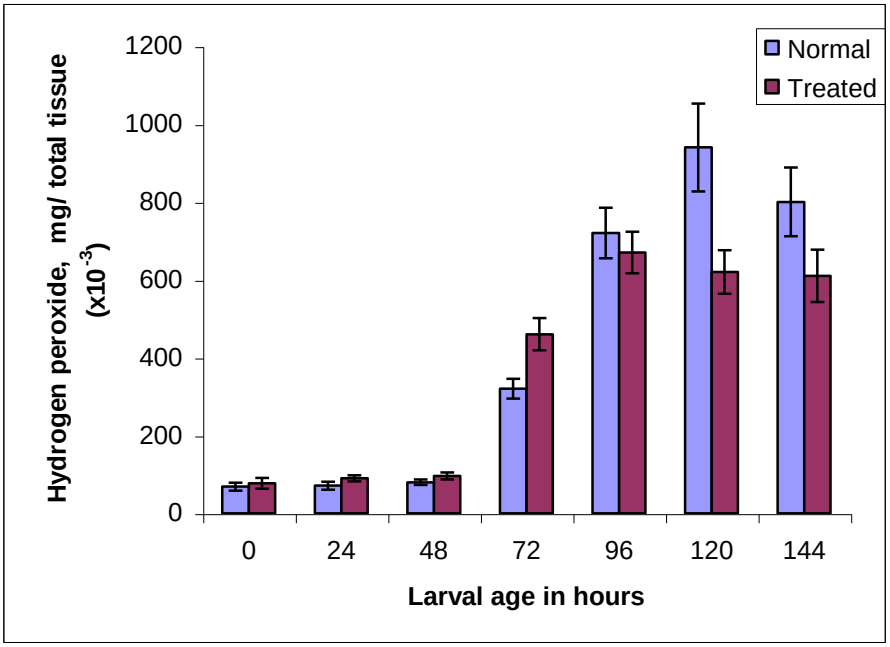

Figure 11.2 Changes in the hydrogen peroxide levels in fat body

a. Changes per unit weight of fat body

b. Changes per total fat body

The activity of the antioxidant haemoprotein catalase per unit fresh tissue and total tissue were estimated in the haemolymph and fat body of both normal and treated larvae and are presented in Table 12.1 and 12.2 and Figure 12.1 and 12.2. The specific activity of catalase was also estimated and given in Table 12.3 and Figure 12.3.

Table 12.1, 12.2 and 12.3

Figure 12.1, 12.2 and 12.3

Catalase activity of per unit volume haemolymph of normal larvae significantly increased from the beginning of fifth instar larval period and recorded a peak value at 72 h followed by a sharp dip. The pattern of changes in H_2O_2 levels in the total haemolymph was exactly similar to that observed per unit volume of haemolymph. There was a significant reduction in catalase activity in the treated larvae when compared with normal. The pattern of changes was same as that observed for total activity.

The activity of catalase in fat body of normal larvae remained more or less the same throughout its development except that it was low in its initial stage. The activity of catalase in the treated larvae was high in the active feeding stages of the larvae but declined sharply towards the end of larval development.

Table 12.1. Changes in the catalase levels in haemolymph

Larval age	mg/min/ml, mean ± SD		mg/min/total tissue, mean ± SD	
	Normal	**Treated**	**Normal**	**Treated**
0h	73.08 ± 10.23	70.98 ± 10.64	12.08 ± 1.69	13.28 ± 1.99
24 h	82.61 ± 8.50	80.13 ± 11.50	13.52 ± 2.10	23.71 ± 2.50**
48 h	91.83 ± 12.02	83.21 ± 7.22	30.83 ± 3.28	26.13 ± 3.81
72 h	80.50 ± 9.31	104.57 ± 16.83	39.71 ± 3.32	29.76 ± 2.75**
96 h	101.82 ± 17.80	96.71 ± 14.71*	49.90 ± 4.75	31. 83 ± 3.41**
120 h	59.90 ± 5.61	67.31 ± 9.51	41.52 ± 3.95	28.71 ± 2.80**
144 h	64.72 ± 7.82	56.24 ± 8.13	33.83 ± 3.61	26.91 ± 3.10*

Values are the means of 5 determinations

*The values are significant at $P<0.05$ against the normal for n=5,

**The values are highly significant at $P<0.01$ against the normal for n=5

Figure 12.1a

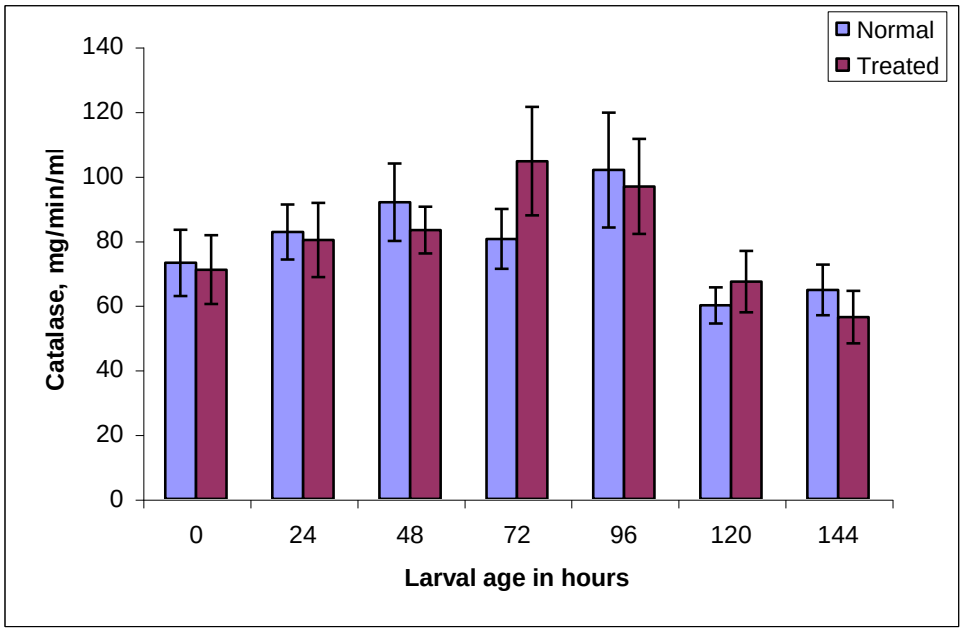

Figure 12.1b

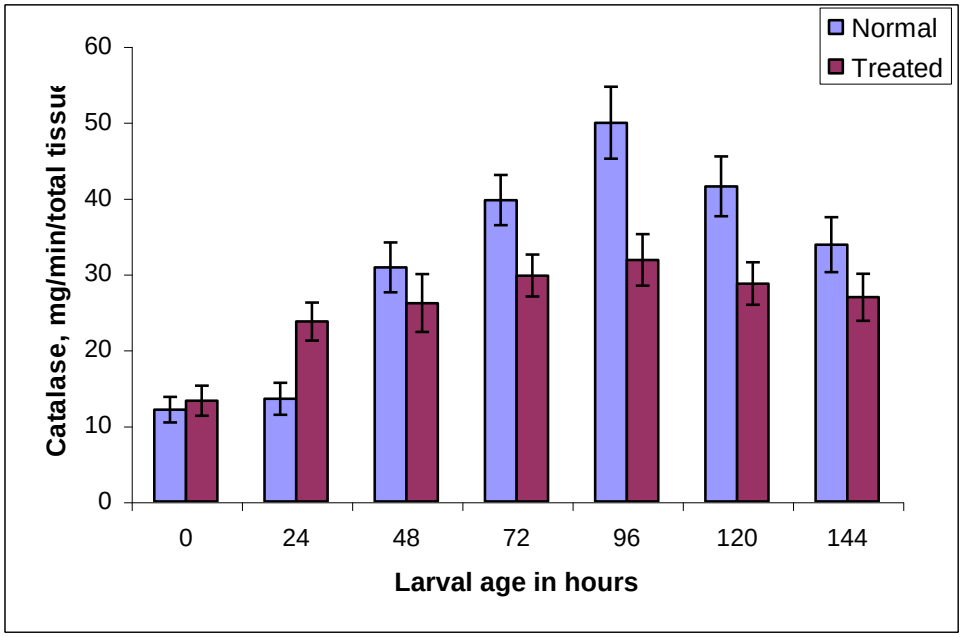

Figure 12.1Changes in the catalase levels in haemolymph

a. Changes per unit volume haemolymph

b. Changes per total volume haemolymph

Table 12.2. Changes in the catalase levels in fat body

Larval age	mg/min/mg fresh tissue, mean ± SD		mg/min/total tissue, mean ± SD	
	Normal	Treated	Normal	Treated
0h	0.31 ± 0.04	0.33 ± 0.05	25.69 ± 4.92	24.89 ± 2.98
24 h	0.36 ± 0.04	0.40 ± 0.06	30.78 ± 3.81	26.69 ± 2.51
48 h	0.46 ± 0.06	0.49 ± 0.05	28.80 ± 2.70	42. 89 ±3.76**
72 h	0.47 ± 0.05	0.47 ± 0.06	70.71 ± 9.19	62. 09 ± 5.05
96 h	0.51 ± 0.05	0.59 ± 0.07	91.04 ± 8.21	100.75 ± 9.75*
120 h	0.46 ± 0.06	0.29 ± 0.03**	114.63 ± 12.81	106.52 ± 12.78
144 h	0.47 ± 0.04	0.29 ± 0.04**	112.27 ±11.85	112.31 ± 14.42

Values are the means of 5 determinations

*The values are significant at $P<0.05$ against the normal for n=5,

**The values are highly significant at $P<0.01$ against the normal for n=5

Figure 12.2a

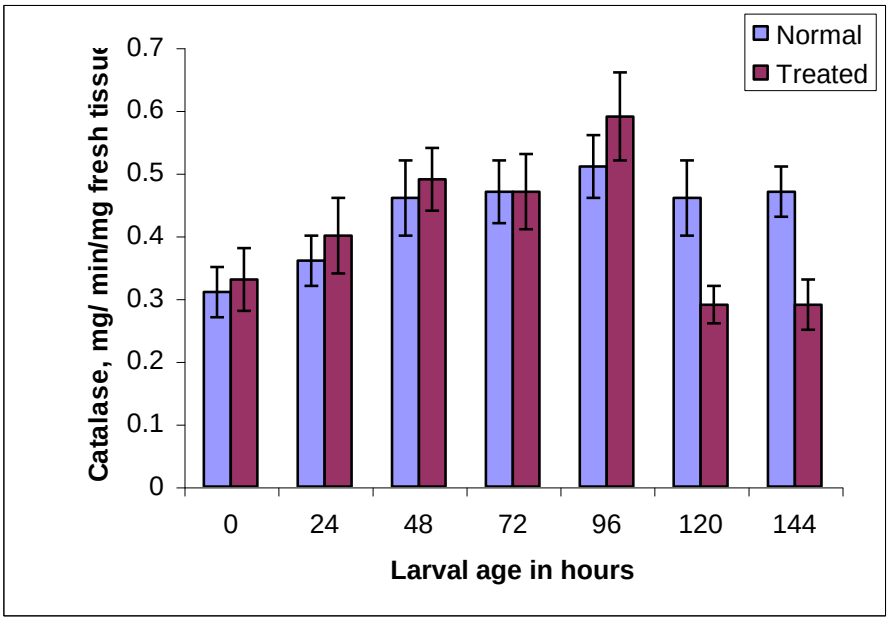

Figure 12.2b

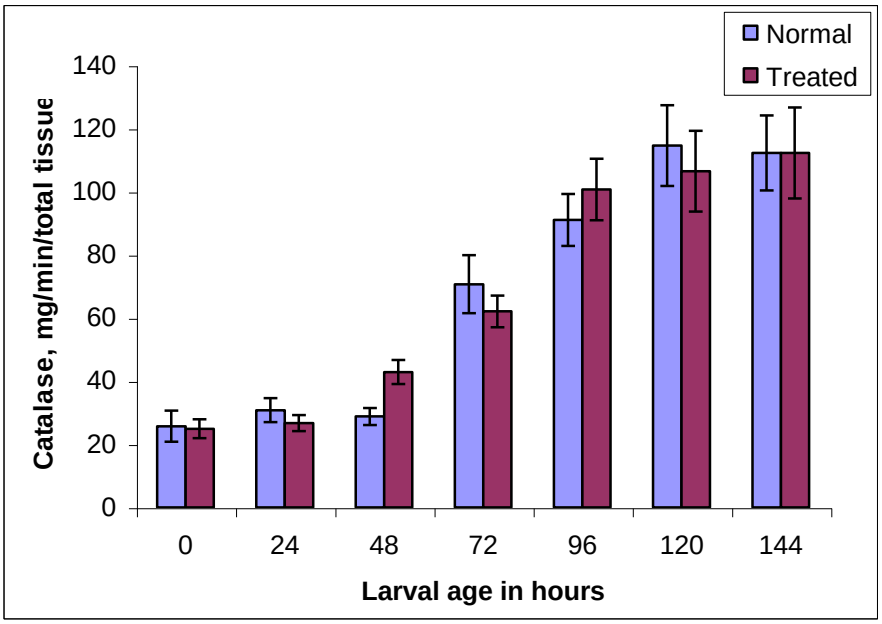

Figure 12.2 Changes in the catalase levels in fat body

a. Changes per unit weight of fat body

b. Changes per total fat body

Tble12.3a. Changes in the specific activity of catalase in haemolymph

Larval age	Specific Activity (units / mg protein), mean ± SD	
	Normal (x 10^{-3})	**Treated (x 10^{-3})**
0	1.51 ± 0.18	1.42 ± 0.23
24 h	1.90 ± 0.150	1.31± 0.142**
48 h	0.50 ± 0.051	0.12± 0.013**
72 h	0.13 ± 0.022	0.03± 0.003**
96 h	0.08± 0.006	0.02 ± 0.002
120 h	0.02 ± 0.002	0.01± 0.009
144 h	0.03 ± 0.003	0.01 ± 0.008**

Values are the means of 5 determinations

*The values are significant at P<0.05 against the normal for n=5,

**The values are highly significant at P<0.01 against the normal for n=5

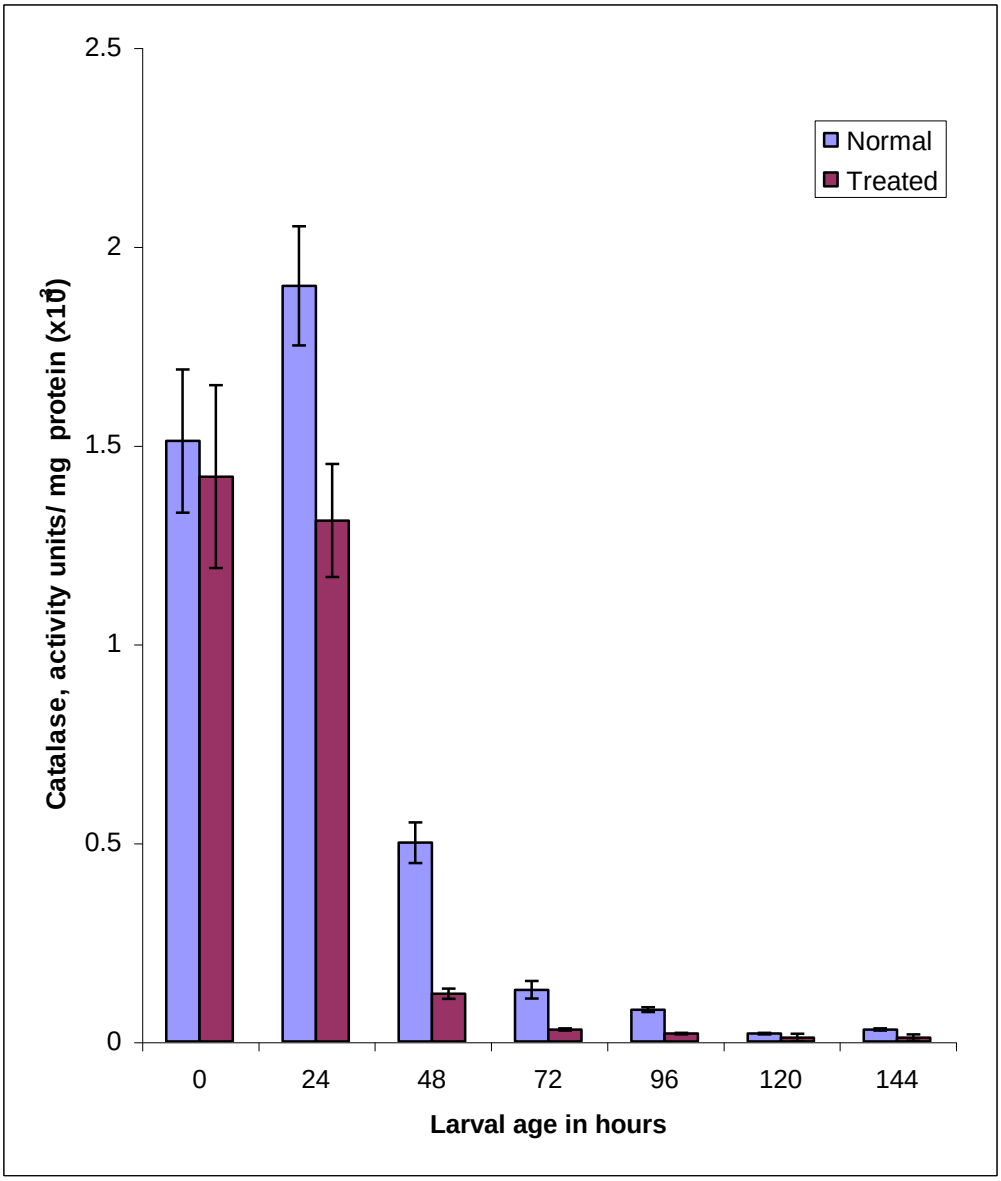

Figure 12.3a. Changes in the specific activity of catalase in haemolymph

Table12.3b. Specific activity of catalase in fat body

Larval age	Specific activity (units/ mg protein), mean ± SD	
	Normal	Treated
0h	1.28 ± 0.029	1.31 ± 0.183
24 h	1.18 ± 0.112	1.63 ± 0.182*
48 h	0.41 ± 0.030	0.51 ± 0.051*
72 h	0.18 ± 0.021	0.13 ± 0.012*
96 h	0.12 ± 0.012	0.18 ± 0.023**
120 h	0.13 ± 0.013	0.11 ± 0.009*
144 h	0.09 ± 0.007	0.09 ± 0.007

Values are the means of 5 determinations

*The values are significant at P<0.05 against the normal for n=5,

**The values are highly significant at P<0.01 against the normal for n=5

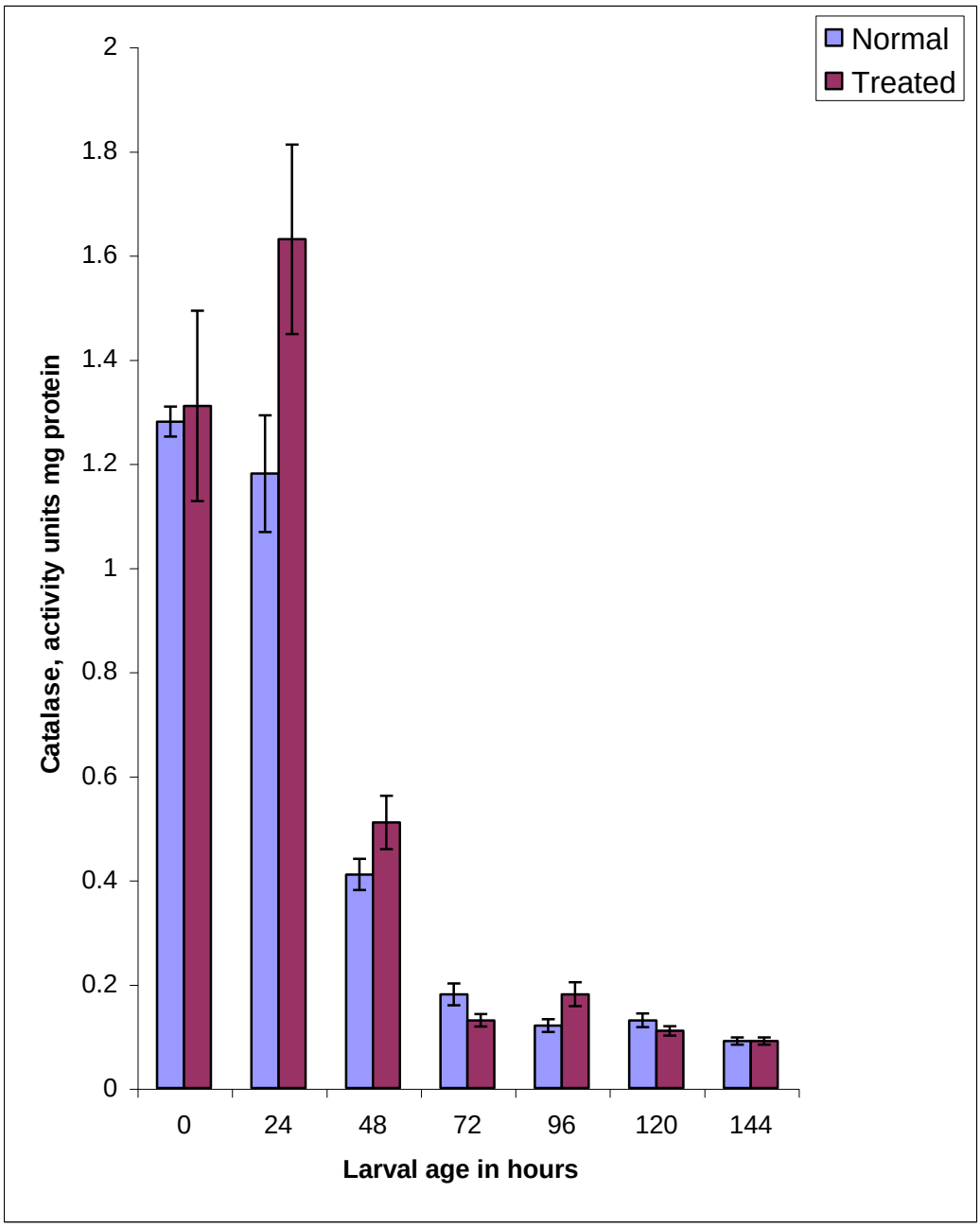

Figure 12.3.b. Changes in the specific activity of catalase in fat body

The change in the specific activity of catalase was found to be similar in the haemolymph and fat body of normal and treated larvae. High specific activity was recorded at the first half of fifth instar stage with a comparative low activity in the second half. Treated larvae showed 52 to 77% increase when compared to normal during the later half.

Catalase activity estimated per total fat body of the normal larvae was low initially but increased gradually recording the peak value at 120 h. The pattern of changes in the activity of catalase in the treated larvae was more or les the same that observed for normal larvae.

The specific activity of catalase in the haemolymph and fat body of the normal and treated larvae were high in the beginning of the instar which declined sharply with the larval development.

DISCUSSION

Growth rate pattern of the larva

The final instar larvae of holometabolous insects undergo important physiological modifications associated with somatic growth and reorganization of tissues in the feeding and non- feeding stage respectively. Hugar and Kaliwal (1998) suggested that the final instar larval state of the silkworm *B. mori* is the most active feeding period during which the larvae accumulate large quantity of biomolecular reserves in various tissues and are endowed with unique biochemical adaptation to conserve nutritional resources for cocoon spinning, metamorphosis and reproduction. The pattern of growth of the final instar larvae of *B. mori* observed in the present study is in tune with the above findings. Though the pattern of growth was similar in both normal and tryptophan treated larvae, an increase in body weight and delay in spinning was observed in the later. This delay in spinning clearly indicate its tendency to retain the juvenile form. Similar life extending effect of dietary anti oxidants was previously reported by Brack (1997) in *Drosophila* on the administration of N-acetyl cysteine (NAC). Tsiropoulos (1983) made similar observations on the lifespan of *Ducus oleae* where the survival of the male and female flies were significantly shortened when the amino acid tryphophan was individually omitted from their diet.

The treated larvae appear bigger and stouter with a 12 to 17% of increase in body weight indicating that tryptophan supplements in the diet enhance the size of silkworm larvae. Inagami (1958) observed that anthranilic acids and their conjugates were the main end - product of tryptophan metabolism in the larva of silk worm which are either excreted or used for some secondary function.

The blood of insect is not devoted to the transport of respiratory gases and exists in a largely unbounded non vascular state. It is a medium bathing all the organs and is often a convenient tissue to evaluate the metabolic processes. So insects are depot organisms since they accumulate various compounds in their body fluids. Corrigan (1970) considered insect blood as more of a tissue than a liquid with regards to its high concentration of many metabolites.

Haemolymph of both normal and treated larvae attained a peak volume at 120h and then dropped down. The changes in the volume of haemolymph between normal and treated larvae found between 120 and 144 h corresponds with the increase of body weight of the larvae. The increase in the haemolymph volume also help the animal to overcome the osmorality changes due to the accumulation of metabolites during the development. Edney (1968) has pointed out that the osmolarity of the haemolymph remains relatively constant and is subject to strong osmoregulation. A group of 10 to

15 pigments were reported by Linzen (1966) as a result of tryptophan degradation in insects. The brownish tinch observed in the haemolymph of treated larvae may be due to the presence of pigments produced by tryptophan degradation.

Insect fat body is the major site of the synthesis and storage of haemolymph proteins (Chen, 1985). Fat body of insects also plays a vital role in the storage of biomolecules and responds to the fluctuation of the metabolites in the haemolymph fairly quickly (Tojo *et al*., 1978). The fresh weight of fat body in normal and treated larvae increases as the larval growth progresses. The increase in weight of the fat body observed in the present study is in accordance with the growth of the larva recorded during its development. The changes in the volume of haemolymph and the weight of fat body during the development of the larva point to the importance of the evaluation of the results of the tissue analysis on the basis of unit volume and total volume and unit weight and total weight of the haemolymph and fat body respectively.

Total proteins

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 insects (Chen,1985). From the morphogenetic point of view,

investigations of the haemolymph proteins are of particular interest because they provide us with an adequate background to judge the synthetic activity associated with the differentiation process in the developing organism.

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 origin and fate of major haemolymph proteins was demonstrated in *Calliphora* (Kinnear and Thomson 1975). They proved that four quantitatively major proteins are found in the plasma of haemolymph which are immunologically, electrophoretically and structurally distinct and do not share any common subunits. They also showed that these proteins are specific for different stages of development such as larval, pupal and adult. Different developmental patterns of proteins were identified in different animals including mammals (Steinhaur and Stephan, 1959; Laufer, 1960b; Terra *et al*., 1975; DeBlanchi and Terra, 1976; Sharma and Sharma, 1979: Fleming *et al.*, 1988; Koopmanschap *et al*., 1992; Tagawa *et al*., 1994).

The results of the present study demonstrate that the total protein levels in both normal and treated larvae increase during the development of the final instar with a slight decline before pupation. Similar findings have been recorded in *Popillia japonica* (Ludwig, 1954), *Drosophila* (Chen, 1956), *Bombyx* (Wyatt *et al.*, 1956; Subramanian, 2002), *Galleria mellonella*

(Denuce, 1958), *Samiya cynthia* (Laufer, 1960a). The magnitude of the changes in the titre of total protein in the whole haemolymph vary between normal and treated larvae: there was a 38-fold increase in the total protein of total haemolymph of the normal larvae as against 30-fold in the treated. The increase in concentration of protein in the early stages of larval period is in accordance with the active feeding and increased growth rate of the larva. It has been found that tryptophan is converted to a wide range of biologically active proteinaceous compounds (Linzen, 1971). The physiological significance of the temporary storage of protein in the haemolymph of the larva of *S. mauritia* was suggested to be partly a mechanism to maintain the osmotic pressure of the medium (Lazar and Mohamed, 1986). In the case of spinning insects it is necessary to maintain a high level of tissue protein in the final instar for it is the main source for cocoon production (Terra *et al.,* 1975).

The fat body of insects play a central role in the synthesis, storage and translocation of proteins. It has already been shown that in larvae the fat body functions both as a storage centre for fat, carbohydrate and protein and is the principal site for intermediary metabolism (Kilby, 1963). Insect haemolymph is an extracellular fluid bathing the fat body and therefore allow an easy exchange of metabolites with the latter.

Metabolism of proteins in the final instar larval fat body of insects has been studied by many investigators (Shigematsu, 1958; Kilby, 1963;

Loughton and West, 1964; Chippendale and Kilby, 1969; Tojo *et al*., 1978). In general, the level of fat body protein increased as the larval development progressed. The increase in the total fat body protein in the silkworm was about 11-fold and 12-fold in the normal and treated larvae respectively. The difference in protein content between haemolymph and fat body can be explained in the context of its synthesis in the fat body and released into the haemolymph and are subsequently sequestered into the fat body and stored there depending upon the physiological condition of the animal. Martin *et al.* (1971) suggested the possibility of conformational change in structure of the protein during its temporary retention in the haemolymph which is necessary before being stored in the fat body.

Total free amino acids

Insects are known to contain an unusually large amount free amino acids whose total concentration in some species has been found to be more than thirty times higher than that in other groups of animals (Florkin, 1959). Estimates of the protein content of other animals in the plasma showed that it is almost equal to that found in the haemolymph of insects, but in the case of free amino acid concentration of the blood of insects it is about 20 to 50 times higher than that in mammals (Lazar and Mohamed, 1988a).

Age related changes in amino acid pool sizes were estimated by different investigators (Buck, 1953; Chen, 1958; 1960; 1962; House, 1962;

Lipke and Frankel, 1966; Mohsin, 1979; Lazar and Mohamed, 1988a). In the present study, the total content of free aminoacids in the haemolymph of normal and treated larvae increased gradually from the period of 0 h to 96 h with a peak value at 96 h and then decreased. This is in conformation of the general observation that the concentration of free amino acid increases during the growth of the larva followed by a decline towards pupation (Chen, 1958; 1960; Wyatt, 1961; Lazar and Mohamed, 1988a). According to Buck (1953), the high titre of free amino acids in the haemolymph is correlated with active protein degradation and synthesis which occurs during growth. The change in the amino acid pool will directly influence the protein turnover and thus obviously reflects the physiological state of the organism. The decline in the level of free amino acid towards pupation observed in the present study apparently indicates a positive balance in protein storage during larval development.

The treated larvae showed a high level of amino acid titre in haemolymph than that of normal. The difference was more prominent during the feeding stages of larvae which clearly indicate a positive correlation with the high protein titre in the same period. A significant increase in the free amino acid levels in the liver of wistar rats which is treated with selenium, an antioxidant was reported by Padmaja and Raju (2003). Similar observation was also reported by Reddy *et al.* (1994) in thyroxine treated fifth instar larvae of tasar silkworm, *Antheraea mylitta* .

The interest in the investigation of free amino acids in the fat body is also due to the fact that the tissue is an active site of intermediary metabolism of amino acids, similar to the midgut gland or hepatopancreas in molluscans and crustaceans and the liver in vertebrates (Kilby, 1963).

It was noticed that the total content of free amino acids in the fat body of silkworm is much less than that found in the haemolymph. In the larvae of *S. mauritia* (Lazar and Mohamed, 1988a) observed that that the free amino acid content of the fat body was only about one-tenth of that found in the haemolymph. Though there was no much variation in the titre of free amino acids estimated per unit weight of fat body, its total content of the tissue showed about 6.1 fold and 15 fold variation in the normal and treated larvae respectively. Any depletion or repletion of amino acids during the development of the larvae indicate a shift in the equilibrium between synthesis and degradation of body proteins.

Glucose

Glucose form a predominant carbon source of chitin, a participant in energy metabolism and the substrate for protein and lipid synthesis in insects. The results of the present study revealed that the feeding stage of larva was characterized by low level of glucose in the tissue, which was increased sharply in the non-feeding stage. In the feeding stage, the larva undergo rapid growth and glucose is utilised for anabolic purposes, whereas in the non-

feeding stage, glucose is stored as an energy resource for the synthesis and degradation of tissues. Similar results have been reported during the larval development of *S. mauritia* by Lazar and Mohamed (1998). Downer and Mathews (1977) observed rapid removal of radio labelled glucose from the haemolymph of flying cockroach *Periplanetta americana* and concluded that haemolymph glucose is serving directly as a fuel for muscle metabolism .

The fat body levels of glucose also exhibited similar variation to that of the haemolymph during the larval development. Insect chitin, which accounts for a major part of cuticle, is a polymer of N- acetylglucosamine units. The final instar larvae of silkworm undergo larval – pupal transformation at the end of the instar. During this stage rapid reorganisation of integumentary structures takesplace for which a ready availability of its precursor molecules such as glucose is required. The high titre of glucose in tissues of the larvae at the end of the instar may be explained in the above context.

Aminotransferases

Aminotransferases are important links between carbohydrate and protein metabolic pathways (Knox and Greengard, 1965). The protein synthesis requires a balanced amino acid pool and transamination is one of the chief mechanisms which functions as a regulator of the same (Reddy *et al*., 1991). Aminotransferases have been studied in relation to the larval metabolism of insects under normal and experimental conditions (Scheider

and Chen, 1981; Halarnkar and Scooley, 1995; Nath *et al*., 1997). In the larvae of *Musca domestica* (McAllen and Chefurka, 1961) and *Drosophila nigromelanica* (Schneider and Chen, 1981), an increase in aminotransferases has been reported during development. An increase in aminotransferase activity was also observed in the larvae of *D. melanogaster* fed with carbohydrate free diets (Geer and Zacharias, 1974), in aestivating *Xenopus laevis* (Balinsky *et al*., 1987) and in mammals during post-natal development (Miller, 1969) indicating that the enhanced activity of the enzyme is linked with gluconeogenesis.

Relatively high level of aminotransferases and a high level of glucose in the larval tissues with subsequent decline in the amount of free aminoacids during larval pupal transformation was observed in *S. mauritia* by Lazar and Mohamed (1998), showing the increased utilization of free amino acids and glucose during metamorphosis. The results of the present study are in tune with the above. The high levels of AsAT activity is observed with a high titre of glucose in the haemolymph during the non-feeding stage.

The AlAT activity of the haemolymph showed a marked difference in its pattern and magnitude between normal and treated larvae. The activity of the enzyme was high during the feeding stage of treated larvae followed by a decline, whereas the normal larvae showed a reverse pattern. It was suggested that AlAT forms a general index of amino acid breakdown and AsAT marks

the mobilization of aminoacids for gluconeogenesis (Adibi, 1968; Davidson and Longslow, 1975).

The AlAT activity in the fat body decreases with the development of normal and treated larvae. Its activity in the fat body of the treated larva showed a significant increase compared to the normal at the end of the larval development. AlAT perform three important physiological functions, *viz.*, the maintenance of the amino acid pool at a proper level for protein synthesis, the supply of metabolites for energy metabolism (Sacktor, 1974) and the catalysis of interactions between protein and carbohydrate metabolism (Katunuma *et al*., 1968). Oral administration of an antioxidant curcumin in albino rats along with a pro-oxidant selenium resulted in a decreased transaminase activity in selenium + curcumin treated rats than control which are treated by selenium only (Padmaja and Raju, 2003). The decrease in transaminase activity observed in curcumin + selenium co-treated animals is supported by protective effect of curcumin in rat liver injury induced by carbon tetrachloride. Long term caloric restriction has been shown to extend maximum lifespan in laboratory rodents (Hagopian, *et al*., 2003). Their results demonstrate increased gluconeogenic activity in caloric restriction mice and are consistent with a state of increased hepatic gluconeogenesis and protein turnover during caloric restriction.

Urea

Urea is a significant excretory product of insects (Cochran, 1975). The occurrence of urea has been demonstrated in the haemolymph of numerous insect species (Wang and Patton, 1969; Corrigan, 1970; Yamada and Inokuchi, 1985; Lazar and Mohamed, 1989). Urea is an important nitrogenous compound, that play a significant role in osmoregulation in animals (Cochran, 1975). Though urea synthesis in animals was primarily considered to be a mechanism of the detoxification of ammonia. Its known function in terrestial and semiterrestrial animals include its role in the control of blood volume. The urea accumulates in the blood and reduces the vapour pressure and thus reduces the evaporative water loss. Though never substantiated on the bais of the total turnover of urea in the whole animal, its accumulation during periods of water storage (stress) has been demonstrated in molluscs, lung fishes and amphibians (Campbell, 1973). Joshi *et al*., (1996) reported that urea induced increased longevity is mediated through a reduction in reproduction in adult flies of *D. melanogaster*, maintained on urea supplimented food.

The results of the present study revealed a high haemolymph titre of urea during the early stages of fifth instar larvae with sharp dip during the later stages. The level of urea in the treated larvae also exhibited the same pattern of changes to that of the normal. Similar results have been observed

in the developing larvae of the moth *S. mauritia* (Lazar and Mohamed, 1989) and *Orthaga exvinacea* (Kuzhivelil and Mohamed, 2002).

The changes in the titre of urea in the haemolymph of *B. mori* larvae indicate that it functions as an osmotic component of the haemolymph. In the early stage of the larva, the animal can maintain a high level of urea for its osmoregulation without much problem of toxicity as it is more accessible to water through food. On the other hand, at the end of the instar the larva has to face desiccation, consequent to the cessation of feeding activity.

In the case of tryptophan treated larvae there was a drastic reduction in the titre of urea in the haemolymph. This may be either due to its increased transfer to fat body for balancing osmolarity of blood in differential stress conditions or due to the less availability of arginine as a product of protein degradation than that of normal. Arginase activity, which cause higher production of urea was studied in *B. mori* by Osanai and Yonezawa (1984). They found that urea is not present in the body of the silkmoth when reared at optimal temperature of 25^{o}C, but when reared at higher temperature, an extraordinary accumulation of urea occurs accompanied by a reduction in lifespan by one half. Their results indicate that conditions due to extrinsic factors influence the blood urea level.

Though the urea levels in fat body of the larva was much reduced when compared to haemolymph, the concentration increased sharply with the larval

development. similar observation where made in the pupating larva of *S. mauritia* where there is an accumulation of urea in the fat body in tune with its reduction in haemolymph (Lazar and Mohamed, 1989). The sequestration of the material from the haemolymph and its subsequent storage in the fat body in the non-feeding stage is obviously linked to the maintenance of the internal environment of the larva. The removal of urea results in a reduced osmotic pressure developed due to the compound in the haemolymph. On the other hand, the storage of the material in the fat body may facilitate the animal to maintain an osmotic balance with that of the internal environment.

Creatinine

Excretion of creatine and creatinine has been studied in a few insects (Bursell, 1967; and Cochran, 1985). Studies on the excretion of creatine and creatinine in *S. mauritia* revealed that their origin in the larval excreta is partly endogenous and that there is an ontogenic variation in the pattern of their excretion (Lazar, 1983). It was also concluded that their metabolism in the larva is similar to that of vertebrates. Further studies in the final instar larvae of *S. mauritia* Lazar and Mohamed (1988b; 1991) suggested that creatinine of insects is a metabolite of creatine as in vertebrates.

Creatinine generation rate is a possible indicator of protein turnover status (Shinzato *et al*., 1997). The changing level of creatinine in haemolymph

and fat body indicate the turnover of proteins during the development of the larva.

Hydrogen peroxide and catalase levels

Accumulation of hydrogen peroxide and a decline in catalase activity is associated with ageing process of organisms. Moosman and Behl (2000) found that accumulation of tryptophan residues in the transmembrane domains of integral membrane proteins perform vital antioxidant functions. Ooka *et al.* (1988) noted that the accumulation of histological biomarkers of ageing were also delayed after tryptophan restriction in some organs. (Hussain and Mitra (2000) suggested that tryptophan hydroxylase activity is affected by ageing process, which can drastically affect the levels of serotonin in brain. The dietary effects of non-enzymatic antioxidant such as carotenoides, selenium and beta carotenoids have been demonstrated in organisms (Smith, 1998; EI-Demerdash, 2004; Kumar *et al*., 2005).

Cytosolic enzyme catalase is a component of antioxidant defense system that reduces hydrogen peroxide to water and protect the cell from oxidative damage (Bandopadhyay *et al*., 1999). Generation of H_2O_2 associated with life expectancy is proved by different workers (Sohal, 1991; Sharma *et al*, 1995, Colavitti *et al*., 2002). H_2O_2 is normally eliminated from the cells by the activity of catalase and peroxidases. Catalase is primarily

localized within peroxisomes, and to a lesser extent in mitochondria, which is the main site of H_2O_2 production (Chance *et al.*, 1979).

In vitro studies on the role of reactive oxygen species on fibroblasts from patients with systemic sclerosis revealed that H_2O_2 act as a mediator of a positive autocrine feed back mechanism of ROS generation (Sambo *et al,* 2001). Cardoso *et al*. (2006) investigated the effects of H_2O_2 and H_2O_2 with the antioxidant agent N-acetyl-1-cystein or catalase on mean arterial pressure and heart rate of rats and found that H_2O_2 produced as a result of oxidative reaction causes misfunction of organs. They also concluded that even 1.0 to 1.5 μ mol doses of H_2O_2 can produce long lasting bradycardia.

The development of the final instar larvae of *B. mori* is marked by distinctive feeding and non- feeding stages. At the end of the non- feeding stage, the larva is transformed into a pupa with entirely different morphological features. The larva of *B. mori* maintain a high level of H_2O_2 in its feeding stages and showed a sharp dip in the non- feeding stage. H_2O_2 levels in the tryptophan treated larvae showed a conspicuous reduction apparently indicating its scavenging action on the free radicals. Similar reduction in peroxide level on the administration of mixed antioxidant supplemented diet was reported by Sharma *et al*. (1995) in adults of *Zaprionus paravittiger*, after the administration of mixed antioxidant supplemented diet.

Although steady state level of oxidative stress depends on both pro-oxidant generation and antioxidant defenses, most of the studies pertaining to ageing have focused on antioxidant defences. Catalase activity has been studied in a variety of insects (Nicolosi *et al*., 1973; Orr *et al*., 1992; Dudas and Arking , 1995 ; Seslija *et al*., 1999). Sohal *et al*. (1990) showed that catalase activity increased with age and decreased during the latter part of life. Studies on catalase activity and lifespan of flies have shown that complete lack or reduced level of catalase activity does not affect their lifespan (Orr *et al* ., 1992). An over expression of catalase (about 50%) had no effect on the lifespan of flies, nor did it improve their viability to an experimentally enhanced level of oxidative stress induced by paraquat intake or hyperoxia (Orr and Sohal, 1992). The increased levels of catalase activity in the haemolymph of normal and treated larvae in the active feeding stages and its decline in the non-feeding stages observed in the present study can be explained in the above context.

The treated larvae exhibited a significant reduction in haemolymph catalase activity which is in tune with the reduced H_2O_2 levels in the tissue. The results of the present study also demonstrate that there is a corresponding increase in the antioxidant activity with an increase in pro-oxidant generation. The high specific activity of the enzyme in normal and treated larvae, during the first half of the fifth instar larval period, in turn, provides an explanation for the total reduction of H_2O_2 titre during the same period. Studies on the

variation of catalase activity in brain, kidney and liver of adult male mice showed that the activity was highest in the liver compared with kidney and brain (Sani *et al*., 2006). The difference in the levels of enzymes in different tissues have been attributed several factors such as their respective physiological functions, the risk of exposure to oxidative damage and the balance between synthesis and degradation of proteins during normal metabolism. The reduced levels of catalase activity in the treated larvae compared to the normal also indicate a reduced pro-oxidant generation in the latter.

CONCLUSIONS

1. Tryptophan administered larvae showed a significant increase in their body weight compared to the normal indicating its potential as a supplement in the larval feed material.
2. Tryptophan fed larva exhibit a short delay in the commencement of spinning indicating the tendency of the larva to retain the juvenile form.
3. There was an increase in the volume of haemolymph and total weight of the fat body in the case of treated larvae compared to the normal.
4. The increase in weight of the fat body in treated larvae can be explained on the basis of increased accumulation of metabolites in the tissue.
5. There was a drastic increase in the levels of total protein in the haemolymph and fat body of the tryptophan treated larvae; the levels were 34 to 94% high in the haemolymph and 15 to 47% in the fat body.
6. An inverse correlation between the levels of protein in the haemolymph and fat body was observed in the larvae pointing to an extensive sequestration of the material from the haemolymph.

7. It has been suggested that the high level retention of proteins in the haemolymph of treated larvae and their sequestration into the fat body are in a dynamic equilibrium synchronized with the gross physiological state of the organism, in different stress conditions.

8. The larval tissues exhibited a high titre of free amino acids in the treated larvae compared to the normal.

9. There was a positive correlation between the titre of free amino acids and total protein in the larval haemolymph and fat body.

10. The maintenance of a high amino acid titre in the haemolymph and fat body of treated larva was suggested to be a major reason for its increased growth rate and body weight.

11. The rapid increase in the level of glucose in the tissues during the end of larval development was interpreted in the context of its use as a precursor molecule for the synthesis of integumentary structures in the pupa.

12. There was a correlation between levels of transaminases and glucose.

13. A marked reduction in the urea content in haemolymph was noticed in the treated larvae indicating and increased transfer of the material to fat body for balancing osmolarity of blood in different stress conditions.

14. The reduced levels of creatinine in the tryptophan treated larval tissues indicate a reduced turnover of creatinine as a result of protein catabolism.

15. The accumulation of H_2O_2 in the blood of treated larvae depicted an 85% reduction, when compared to the normal.

16. The levels of hydrogen peroxide recorded a sharp decline in the tryphotphan treated larvae indicating its role as a scavenger of free radicals.

17. The changing levels of catalase activity during larval development was in tune with the generation of pro-oxidants in the larvae.

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