STUDIES ON THE FREE RADICAL TURNOVER RATES DURING THE DEVELOPMENT OF *BOMBYX MORI*

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By

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Certificate

This is to certify that this thesis is an authentic record of work carried out by Miss. Mangalalaxmy Subramanian from November 1999 to August 2002 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.

Dr. K.V. Lazar

Declaration

I, Mangalalaxmy Subramanian do hereby declare that this thesis entitled "Studies on the free radical turnover rates during the development of *Bombyx mori*" 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 result of the bonafide research work carried out by me under the guidance of Dr. K.V. Lazar, Lecturer, Department of Zoology, University of Calicut. I further declare that the results presented in this thesis have not been submitted previously for any degree.

Station: Calicut University,
Date : 2/9/2002

Mangalalaxmy

Mangalalaxmy Subramanian

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Dedicated To

My not so educated grandmothers, for whom
this small achievement of mine would be
a dream come true...

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INTRODUCTION

INTRODUCTION

Aging can be functionally defined as an increase in the rate of mortality with age. Though aging research has a long history, the mechanisms that cause aging and result in species-specific life span have yet to be found.

Aging is not a disease and the distinction is central to an understanding of why the resolution of the leading cause of death in old age - cardio vascular disease, stroke and cancer - will tell us little about the fundamental biology of age changes. The resolution of all these conditions would result only in an increase of about 15 years in human life expectancy in the developed world (Hayflick, 2000), after which aging will be revealed as the leading cause of death. Hence more attention must be paid to basic research on aging.

A current hypothesis of aging postulates that deleterious reactions initiated by partially reduced oxygen species are an underlying factor in the causation of the aging process in multicellular organisms. This hypothesis is being refined and modified in response to the progress made in the field of free radical biochemistry, ever since it was postulated more than four decades ago by Gerschman *et al.* (1954) and Harman (1956).

The initial molecular species is the super oxide anion radical (O_2^-), which is converted to hydrogen peroxide (H_2O_2), either by spontaneous disproportionation

or enzymically by superoxide dismutase (SOD) activity. H_2O_2 is decomposed to oxygen and water by the enzyme, catalase, or peroxidases. Thus SOD, catalase and peroxidases constitute a mutually supportive team of defense against reactive oxygen species (ROS). While SOD lowers the steady state level of O_2^-, catalase and peroxidases do the same for H_2O_2 (Bandyopadhyay *et al.*, 1999). Though the free radical theory was not originally framed in terms of oxidative stress, that is, balance between oxidants and antioxidants in retrospect, its most essential prediction reformulated in current terms is that the level of oxidative stress is directly related to the rate of aging. Therefore on the basis of comparisons of the concentrations of the molecular products of oxy-radical reactions, levels of antioxidant defenses and rates of O_2^- and H_2O_2 generation, some insight can be gained into the tissue level of oxidative stress.

Although steady state level of oxidative stress depends on both prooxidant generation and antioxidant defenses, most of the studies pertaining to aging have focussed on antioxidant defenses. As a result, only limited and tentative information is available about the relationship between aging and rates of O_2^- and H_2O_2 generation.

Another prediction of the free radical hypothesis, according to Harman (1982) was that increased intake of exogenous antioxidants would slow down the rate of aging and lengthen life span. If antioxidants act as longevity determinants, then the over expression or under expression of SOD, catalase and glutathione

peroxidase (GPx), which together eliminate O_2 and H_2O_2, should prolong life span. Several studies have been conducted in this area utilizing transgenic procedures (Sohal, 1993).

Apart from the free radical theory, another theory that has formed the basis of the present work is the rate of learning theory by Pearl (1928). The main and the early prediction of Pearl's theory is that if the rate of metabolism of an organism is accelerated, it will have shortened life and vice versa. Several studies correlating the rate of metabolism in terms of physical activity, temperature, oxygen consumption, etc., and the rate of aging have been conducted but have not been successful in giving a clear picture (Rubner, 1908; Pearl, 1928; Miquel *et al.*, 1976; McArthur and Sohal 1982; Farmer and Sohal, 1987; Brierly *et al.*, 1996).

Biochemical and clinical studies show that certain compounds show antioxidant nature. Many of them have been studied for their effects on the activity of the antioxidant enzymes and thus in free radical quenching. Since ROS mediated oxidative stress is now regarded as a major factor leading to aging and age-related neurodegenerative diseases, suitable antioxidant therapies to control these processes have already attracted worldwide attention.

All the studies on aging have been done extensively on rats, mice, and mammals, including humans. Although insects are commonly thought of as 'lower' animals, nevertheless being highly complex organisms, they show surprisingly

similar, if not identical, biochemical physiological and even cytological features, comparable to those of higher animals. If we accept the principles of the universality of the aging process in multicellular animals, the several advantages of employing insects as experimental animals in studies of the aging process far outweigh any disadvantages, which their relatively small size may suggest. In insects, the fruit fly, *Drosophila melanogaster*, has been extensively used as an ideal model system. Of late, the houseflies, *Musca domestica*, have also been utilized in various studies. Insects are particularly useful for analyzing the effect of H_2O_2 removal on aging because they lack GPx, and thus catalase provides the main mechanism for H_2O_2 break down (Sohal, 1993).

Keeping in mind the various points discussed above, the main objective of the present work was to study the free radical turnover rates during the development of the fifth instar larvae of the silkworm, *Bombyx mori*. The hydrogen peroxide generation in the larvae was evaluated in the context of the larval development and metabolism. Several biochemical parameters were tested. Further the effects of a reducing amino acid, tyrosine, on the free radical turnover rates and metabolism during the development of the larvae was analyzed.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Any broad definitions of aging must include the element of time dependency of reproducible, observable changes in structure or function in an organism. It is usually defined as the progressive loss of function accompanied by decreasing fertility and increasing mortality with advancing age. Aging shows a broad phylogenetic distribution but is not universal, as some species shows no age-associated increase in mortality or decline in fertility (Kirkwood and Austad, 2000). Consideration of the process of aging must cover all three major phases of existence of any organisms, viz., the initial period of embryonic development, the middle-life stage of growth and development and finally, the period of senescence. Senescence as a more restricted part of the total aging process, can be defined as those reproducible time related alterations in structure and function in an organism which result in the decreasing capacity of that organism to survive and thus to result ultimately in its death.

General classification of the theories of aging

All the theories of aging can be expressed in terms of two general categories of factors governing and affecting the course of senescence (and therefore life span). These are (1) the controlling genetic factors which determine the time of onset, the course, and the duration of the process of senescence and (2) the

environmental factors, which cumulatively reduce the life span of an organism. It is more likely, however, that death represents an event resulting not by the unique action of one or the other, but rather by the interaction of the environmental factors with the genetically pre-determined, age-dependent, intrinsic involuntary processes (Clark and Rockstein, 1964).

Within each of two major categories of the theories of aging, a number of subdivisions have been proposed. Regarding genetic control of aging, these theories can be extended to such sub-categories as (1) cessation of growth and therefore failure to replace aging cells, (2) failure of a juvenile or growth substance, (3) the increasing production or accumulation of an aging factor or hormone, (4) depletion of essential substances and (5) accumulation of substances which may be chemically or mechanically harmful to the aging organism. As to the role of environmental influences, each of the following has been variously proposed as being the predominant factor in senescence: (1) cumulative radiation effect, (2) pathological effects and (3) physically traumatic influences of a changing environment. However, since organisms with different genetic constitutions may respond differently to different environmental conditions, it is difficult to envision the operation of a single limiting factor in the aging process.

One of the most important facts of the theory of aging is that each species has a characteristic life span. In other words, there is an over-riding genetic component passed on from generation to generation that restricts the life of each

animal. Aging can therefore be explained as a consequence of genetic diversity seen in all animals. For example, in the mouse there is an acceleration of the entire spectrum of age related processes in comparison with the human. A two and a half-year old mouse can be considered in many ways to be physiologically equivalent to a human approximately thirty times that of number of years. Any theory of aging, therefore, must be based on primary events that occur much more rapidly in short-lived animals than in long-lived animals.

The prospects for understanding aging

The critical questions in gerontology are what mechanisms cause an increase in mortality rate with age and which factors determine species-specific life span. Now there are numerous examples of plasticity of life span in the laboratory (Lithgow, 1996) presenting many ways to uncover mechanisms.

The role of genetics in determining life span is complex and paradoxical. Genes exert strong controls on life span and patterns of aging. Although the heritability of life span is relatively minor, some genetic variants significantly modify senescence of mammals and invertebrates, with both positive and negative impacts on age-related disorders and life spans. In certain examples, the gene variants alter metabolic pathways, which could thereby mediate interactions with nutritional and other environmental factors that influence life span (Finch and Tanzi, 1997). Genetic factors, lifestyle and societal investments in a safe and

healthful environment are important aspects of successful aging (Lamberts *et al.*, 1997). Traditionally the aging process including the development of physical frailty towards the end of life has been considered to be physiological and unavoidable.

Mechanisms of aging

About 300 different hypotheses have been proposed to explain the mechanisms of aging (Medvedev, 1990). The current dominant opinion among gerontologists seems to be that aging is a multi-causal phenomenon. Interplay among several different causal factors may determine the rate of aging and the achieved life span. Since virtually all the components in the body undergo some kind of alteration with age it is vital to separate cause from effect.

It is often mentioned that theories of aging fall almost exclusively within the domain of either the 'programmatic' or 'stochastic' schools of thought (Lints, 1971). According to the former view, aging 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. It is also implied that organisms have perfectly operative systems whose function deteriorates only by a specifically designed mechanism rather than as a result of inadequacies in the biological systems. The characteristic patterns of age-associated decline in function, disease and maximum species-specific longevity are believed to lend support to this view.

The 'stochastic' theories of aging can be said to advocate that aging is a result of random damaging events, which may be intrinsic or extrinsic in origin. Living systems are continuously exposed to potentially damaging reactions, the effect of which may not be totally reversible or correctable due to the limitations of repair mechanisms. Under such conditions putative damage would tend to accumulate resulting in gradual attrition of physiological functions and overall vitality of the organisms.

1. Free radical theory

The postulate that the net effect of the free radical induced deleterious reactions occurring in the cells either constitutes the aging process or is a major contributor to it was originally proposed more than 3 decades ago by Harman (1956) and Gerschman *et al.* (1954). This idea seems to owe its existence to the recognition that the mechanism of radiation and oxygen toxicity may involve, in common, the generation of free radical (Gerschman *et al.*, 1954). Although Gerschman's idea that oxygen was toxic and its use may be a casual factor in aging had great appeal, little was known then about the physiological origin of reactive oxygen species (ROS) or the nature of antioxidant defenses or mechanisms of reactions between ROS and biological molecules. Thus the above hypothesis has ever since been refined and modified in response to the progress made in the field of free radical biochemistry. The original evidence leading to this hypothesis was that X-ray irradiation, during or after exposure to hyperbaric oxygen, synergized

the acute lethal effects of oxygen. Further more, antioxidants provide protection against oxygen poisoning and also increase resistance to irradiation.

2. Somatic mutation theory

Exposure to ionizing radiation below the dosage causing an overt pathological syndrome has been known to cause premature onset of certain age-associated diseases and death in laboratory mammals. Since radiations accelerate the rate of mutations it was postulated that natural aging might also be due to mutations occurring randomly in the somatic cells (Curtis, 1963). The mutation load of cells would increase with age leading to dysfunction and altered expression of an ever-increasing number of genes. The consequences would be a decrease in the functional capacity of somatic cells. Curtis (1963) has reported evidences from regenerating liver, which he believed to support this theory.

3 Error catastrophe theory

Orgel (1963) proposed that alteration in gene expression can occur in the absence of somatic mutations, by a mechanism involving random errors in protein-synthesizing apparatus. Also known as Orgel's hypothesis, 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. In case an alteration leads to the reduced specificity on an information handling protein, it would result in an ever-increasing error frequency, which is cumulative, and in the

absence of an imposed selection for accurate protein synthesis, would lead to an error catastrophe, i.e. where the existences of viable cells become affected.

An argument, albeit circumstantial, against the error catastrophe theory is that the activity of many enzyme does not decline with age in insects or mammals (Wilson, 1973), nor does the total amount of soluble proteins within cells greatly increase with age, which would suggest that aging is unlikely to be a result of the accumulation of aberrant protein molecules.

4 Theory of clonal aging

A phenomenon which has aroused considerable interest among gerontologists was first reported by Hayflick and Moorhead (1961) who found that normal human embryonic fibroblasts (WI38), when cultured *in vitro*, undergo a finite number of about 50 ± 10 replications prior to death. The total number of cell doublings remains constant even if the cultures are preserved by freezing during an early passage for prolonged periods. The potential number of cell replications was later found to be inversely related to the age of the donor. Hayflick (1977) has interpreted the limited proliferative capacity of normal cultured cells to be a manifestation of aging at cellular level. He has proposed that the number of population doublings can be used as an index of the rate of cellular aging. The presumption behind this hypothesis is that the factors, which limit the proliferative

capacity of cells *in vitro*, are substantially the same that are responsible for aging *in vivo*.

Bell and co-workers (1978) have argued that cessation of cell proliferation in culture is a manifestation of cell differentiation rather than aging. They also drew attention to an interesting fact, which is left obscure in Hayflick's reports, that cells which have ceased to divide do not die and have in fact been kept alive for 12-14 months after cell divisions terminated. It was suggested that aging might not be a consequence of an intrinsic program of death but rather a product of extrinsic damage.

5. 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. A variety of mechanisms have been proposed to explain how deleterious transcriptional and translational changes could occur with passage of time. The distinct shifts in the pattern of protein synthesis during development and reduced themolability of chromatin in old animals have been claimed to constitute supportive evidence for the gene regulation theory of aging.

There is a paucity of concrete experimental evidence, which would strongly support any of the postulated mechanisms of aging. Age-associated alterations in

various biological systems have been described; however, the significance or the relationship of these alterations to the underlying causal factors remains obscure.

It is a known fact that telomeres get shortened with each cell division, unless their repeated sequences are replenished by telomerase. The telomere hypothesis of cellular aging (Bodnar *et al.*, 1998) proposes that cells become senescent when progressive telomere shortening during each division produces a threshold telomere length. The most compelling data supporting the view that telomere loss eventually restrains the proliferation of human cells arose from human tumors and immortal cell lines. If telomere shortening curbs the number of divisions allotted to primary human cells, then immortalization should somehow liberate cells from this restraint. Immortalization of human cells is invariably accompanied by a key change in telomere dynamics involving either the activation of telomerase or an alternative mechanism that maintains telomeric DNA (De Lange, 1998).

The tally of telomerase-positive human cancers is extensive, indicating that averting telomere loss is a common aspect of tumorigenesis. 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 recently assailed by studies of a knock out mouse lacking the essential RNA sub unit of telomerase (De Lange, 1998). Cells from these mice still could undergo malignant transformation and form tumors after transformation with viral oncoproteins contrary to the predictions of the telomere-clock model. However,

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

To resolve the controversy, Bodnar *et al.*, (1998) tested the effect of inappropriate activation of telomerase in normal human cells. They examined the proliferative potential of primary human cells that were forced to express telomerase from a transfected hTRT gene. This resulted in the addition of the TTAGGG repeats that normally cap human chromosome ends. The cells show spectacular growth potential and maintained a youthful morphology.

The recent data indicate that activation of telomerase in human tumors bypasses cellular senescence and is thus a requirement for tumor progression. It is hypothesized that one or more tumor suppressor genes prevent activation of telomerase in normal human cells (De Lange, 1998).

Mitochondrial function and aging

Changes in gene expression during aging are not limited to the nuclear genes. Several genetic disorders have been assigned to mitochondrial DNA and several studies show that age-dependent changes in mitochondria may be associated with the process of human aging (Cotton and Rogers, 1993). Aging has been reported to be associated with dramatically decreased expression of mitochondrial RNAs (Calleja *et al.*, 1993). Mitochondrial DNA accumulates

mutations during aging in *Drosophila* (Chen *et al.*, 1993) as well as in humans (Cortopassi *et al.*, 1992). *Drosophila* mitochondrial deletions and decreased mitochondrial transcription are likely to be related to the deterioration of mitochondrial structure and function observed during *Drosophila* aging (Fleming *et al.*, 1985). As the primary source of oxygen radicals, mitochondria are particularly subject to oxidative damage, which may be causing much of the aging related decline. There is now evidence that aging in higher animals may be due to accumulated mutations in the mitochondrial genome that reduce their function as respiratory units, and that once a mitochondrial mutation appears in a cell it seems to overgrow the "wild-type" mitochondria (Kadenbach and Muller-Hocker, 1990). It has been hypothesized that such oxidative damage to the mitochondria might be a major rate-determining factor in the aging of the organism. There is also indirect evidence that antioxidants may reduce mutationally relative events such as the development of malignant disorders (Troll, 1991).

Evolutionary theory of aging

Evolutionary theories of aging are based on the observation that the efficacy of natural selection decreases with age. This is because even without aging, individuals will die of environmental causes, such as predation, disease and accidents. Aging is thought to have evolved as the result of optimizing fitness early in life. A second process, namely the progressive accumulation of mutations with effects late in life, will reinforce this result. Longevity of a species is therefore also

determined by the amount of environmental mortality caused by the ecology of a species (Zwaan, 1999).

The favored theory for why aging occurs comes from evolutionary biology (Partridge and Barton 1993). The key to the evolutionary theory of aging 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 cause aging. The first phenomenon, 'mutation accumulation' is the random accumulation of inherited mutations that have adverse effect late in life. The second phenomenon, 'antagonistic pleiotropy', is the effect of a gene, which 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 characterize aging (Tower, 1996). Postponed senescence appears to result from the

selection for increases and decreases in the frequency of particular gene alleles pre-existing in the population.

Drosophila lines exhibiting postponed senescence are a valuable reagent for the analysis of aging. Physiological processes, which are altered due to postponed senescence, have been studied. The results were different when two independently derived sets of lines were studied. One set of lines showed that postponed senescence is associated with increased glycogen content and increased desiccation resistance, as well as increased lipid content and increased starvation resistance (Graves *et al.*, 1992) whereas in the second set they have been found to have increased resistance to oxidative stress (Arking *et al.*, 1991). For the former, it is hypothesized that the increased desiccation resistance may be due to the increased glycogen content, and it is likely that all or part of the increased starvation resistance is due to the increased lipid content. The significance of these alterations is supported by the fact that in independent selection experiments, selection on the basis of desiccation resistance and starvation resistance can in turn produce increased longevity (Rose *et al.*, 1992). Reversal of the selection for postponed senescence, i.e., now selecting for fertility at early ages, decreases longevity and, in parallel, decreases glycogen content and desiccation resistance and decreases starvation resistance and lipid content. Such reverse-selection experiments are particularly powerful because they support the conclusion that the alleles conferring increased stress resistance exhibit antagonistic pleiotropy with regard to early

fertility. In the postponed senescence lines these alleles confer increased stress resistance and, apparently, part of the increased life span, at the expense of early fertility. In the second set of lines, the observation is of particular interest because oxidative damage has been correlated with aging in a wide variety of organisms, including *Drosophila* and mammals.

Oxidative stress and aging

While there are a number of cellular sources of oxygen radicals, the majority originates as $\cdot O_2$ produced in mitochondria as a byproduct of normal metabolism. $\cdot O_2$ can be converted through several pathways to other types of oxygen radicals, such as the highly toxic $\cdot OH$ radical. Oxygen radicals such as $\cdot OH$ react with and damage macromolecules including lipids, proteins and nucleic acids. Such oxidatively damaged macromolecules have been found to accumulate in virtually every aging organism in which they have been looked for, including *Drosophila* (Orr and Sohal, 1994; Sohal *et al.,* 1995). Cells contain a variety of defenses against endogenous and exogenous oxidative stress, and three of the most important in *Drosophila* are: (1) superoxide dismutase (SOD), which converts, $\cdot O_2$ radicals to H_2O_2 and which is found in both the cytoplasm (Cu/Zn SOD) and the mitochondria (Mn SOD); (2) Catalase, which converts H_2O_2 to H_2O and O_2 and (3) Urate, which acts as an oxygen radical scavenger and requires the enzyme xanthine dehydrogenase (XDH) for synthesis. The accumulation of oxidative damage products with age demonstrates that oxidative stress defenses are not completely

efficient. These defenses may represent an important physiological process that is not adequate for the indefinite maintenance of the organism. The second set of postponed senescence lines exhibit increased expression of several oxidative stress resistance genes, consistent with their increased resistance to oxidative stress. It is hypothesized that this change in gene expression makes a major contribution to the postponed senescence phenotype in these lines (Dudas and Arking, 1995).

Oxidative damage to macromolecules – a candidate 'public' mechanism of aging

Oxidative damage to macromolecules has formed the basis of what is arguably the most popular current theory of a proximal mechanism of aging, the 'free radical theory of aging' (Harman, 1994). Even since Harman (1956) first proposed the free radical theory of aging, the molecular basis of aging and the role of ROS in this process have attracted considerable attention in recent years. A more generic nomenclature for this theory, however, would be the 'oxidative damage theory of aging' since some oxidative events, such as those associated with singlet oxygen, cannot be regarded as free radicals. It is now generally agreed that aging and age-related diseases result from ROS-mediated oxidative damage of lipid, protein and nuclear and mitochondrial DNA molecules (Harman, 1981). The concentration of oxidatively damaged proteins, lipids and DNA has been reported to increase with age (Sohal and Orr, 1995). The hydroxyl and peroxy radicals cause extensive damage of proteins resulting in aging and age-related degenerative

diseases. Other than O_2^- and H_2O_2 mediated oxidative damage, mutation in mitochondrial DNA also leads to the formation of defective respiratory enzymes, which not only result in decreased ATP synthesis but also generate more ROS to cause further oxidative damage

Some of the well-known consequences of generation of the free radicals *in vivo* are: DNA strand scission, nucleic acid base modification, protein oxidation and lipid peroxidation (Halliwell and Gutteridge, 1990). Oxidative protein damage may be brought about by metabolic processes, which degrade a damaged protein and prevent synthesis of a new protein. Free radicals stimulate protein degradation (Bandyopadhyay *et al.*, 1999).

Reactive oxygen species (ROS)

Oxygen is vital for aerobic life processes. However about 5% or more of the inhaled O_2 is converted to reactive oxygen species (ROS) such as $O_2^{\cdot}$, H_2O_2 and OH^- by univalent reduction of O_2. Thus cells under aerobic condition are always threatened with the influx of ROS, which however are efficiently taken care of by the cell without any untoward effect. When the balance between ROS production and antioxidant defenses is lost, 'oxidative stress' results which through a series of events deregulates the cellular functions leading to various pathological conditions including cardio-vascular dysfunction, neurodegenerative diseases, gastro duodenal pathogenesis, metabolic dysfunction of almost all the vital organs, cancer and

premature aging (Bandyopadhyay *et al.*, 1999). The free radical-mediated oxidative stress results in oxidation of membrane lipoproteins, glycoxidation and oxidation of DNA: which subsequently results in cell death. Various necrotic factors, proteases and ROS from damaged cell also attack the adjacent cells, resulting ultimately in tissue injury. Furthermore, tissue injury itself has been reported to cause severe oxidative stresses (Halliwell, 1997). Injury caused by ischemia reperfusion, heat, trauma, freezing, severe exercise, toxins, radiation or infection; leads to the generation of ROS and development of various disease processes (Halliwell, 1997).

The site of generation of ROS and their reactivity

Although O_2 can behave like a radical (a diradical) owing to the presence of two unpaired electrons of parallel spin, it does not exhibit extreme reactivity due to quantum-mechanical restrictions. Its electronic structure results in formation of water by reduction with four electrons, i.e.,

$$O_2 + 4H^+ + 4e^- \rightarrow 2H_2O$$

In the sequential univalent process by which O_2 undergoes reduction, several reactive intermediates are formed, such as superoxide (O_2^-), hydrogen peroxide (H_2O_2) and the extremely reactive hydroxy radical (OH^-) collectively termed as the reactive oxygen species (ROS). The process can be represented as:

$$O_2 \rightarrow O_2^- \rightarrow H_2O_2 \rightarrow OH \rightarrow H_2O$$

For the production of O_2^-, normally the tendency of univalent reduction of O_2 in respiring cells is restricted by cytochrome oxidase of the mitochondrial electron transport chain, which reduces O_2 by four electrons to H_2O without releasing either O_2^- or H_2O_2. However, O_2^- is invariably produced in respiring cells. This is due to the probable 'leak' of single electron at the specific site of the mitochondrial electron transport chain, resulting in inappropriate single electron reduction of oxygen to O_2^- (Bandyopadhyay *et al.*, 1999; Chance *et al.*, 1979). When the electron transport chain is highly reduced and the respiratory rate is dependent on the ADP availability, 'leakage' of electrons at the ubisemiquinone and ubiquinone sites increases so as to result in production of O_2^- and H_2O_2 (Bandyopadhyay *et al.*, 1999).

In the production of H_2O_2, peroxisomal oxidases and flavoproteins, as well as D-amino acid oxidase, L-hydroxy acid oxidase and fatty acyl oxidase participate (Chance *et al.*, 1979). Cytochrome P-450,. P-450 reductase and cytochrome b-5 reductase in the endoplasmic reticulum under certain conditions generates O_2^- and H_2O_2 during their catalytic cycles (Bandyopadhyay *et al.*, 1999). Likewise, the catalytic cycle of xanthine oxidase has emerged as an important source of O_2^- and H_2O_2 in a number of different tissue injuries. Xanthine oxidase, produced by proteolytic cleavage of xanthine dehydrogenase during ischemia, upon reperfusion in presence of O_2, acts on xanthine or hypoxanthine to generate O_2^- and H_2O_2 (Halliwell and Gutteridge, 1990).

The phagocytic cells, such as neutrophils when activated during phagocytosis, generate O_2^- and H_2O_2 through activation of NADPH oxidase. Neutrophil accumulation in inflammated tissue is one of the major reasons of oxidative damage due to generation of ROS. In addition, spontaneous dismutation of O_2 at neutral pH or dismutation by superoxide dismutase, results in H_2O_2 production. Various biological sources for the production of H_2O_2 have been reviewed (Bandyopadhyay *et al.*, 1999).

Except during abnormal exposure to ionizing radiation, generation of OH *in vivo* requires the presence of trace amount of transition metals like iron or copper. A simple mixture of H_2O_2 and Fe^{2+} salt forms $^{\cdot}OH$, as given by the following Fenton reaction.

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

Fe^{3+} can react further with H_2O_2 to form the following products:

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + O_2^- + H^+$$

Thus, a free-radical mechanism for the generation of $^{\cdot}OH$ may be deduced as follows.

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

The rate constant for the above reaction is very low but can be accounted for if reaction is catalyzed by traces of transition metal ions – the metal catalyzed Haber-

Weiss reaction (Halliwell and Gutteridge, 1984). The various steps of this reaction are:

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

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

and the net result is,

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

However, redox-active free iron or copper do not exist in biological systems, as these transition metal ions remain bound to proteins, membranes, nucleic acids or low molecular weight chelating agents like citrate, histidine or ATP (Halliwell and Gutteridge, 1984). During ischemic condition and cellular acidosis, transition-metal ions may be released from some metalloproteins (Bandyopadhyay *et al.*, 1999), 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: the Haber-Weiss catalyzed reaction (Halliwell and Gutteridge, 1984).

Primary defense against ROS: catalytic removal of ROS by antioxidant enzymes

Superoxide dismutase (SOD), catalase and peroxidases constitute a mutually supportive team of defense against ROS. While SOD lowers the steady-state level of O_2^-, catalases and peroxidases do the same for H_2O_2.

Superoxide dismutase

The first enzyme involved in the antioxidant defense is the superoxide dismutase: a metalloprotein found in both prokaryotic and eukaryotic cells. The iron containing (Fe-SOD) and the manganese-containing (Mn-SOD) enzymes are characteristic of prokaryotes. In eukaryotic cells, the predominant forms are the copper containing (Cu-SOD) and the zinc-containing (Zn-SOD) enzymes, located in the cytosol. Mn-SOD is found in the mitochondrial matrix of eukaryotes. The biosynthesis of SOD is mainly controlled by its substrate, the O_2^-. Induction of SOD by increased intracellular fluxes of O_2^- has been observed in numerous microorganisms well as in higher organisms (Bandyopadhyay *et al.*, 1999; Rister and Balchner, 1976).

Glutathione peroxidase

Glutathione peroxidase catalyses the reaction of hydroperoxides with reduced glutathione (GSH) to form glutathione disulphide (GSSG) and the

reduction product of the hydroperoxide (Chance *et al.*, 1979). This enzyme is specific for its hydrogen donor, GSH and non-specific for the hydroperoxides ranging from H_2O_2 to organic hydroperoxides. It is a seleno-enzyme, two third of which is present in the cytosol and one-third in the mitochondria (Bandyopadhyay *et al.*, 1999).

Heme peroxidase

Heme peroxidases such as horseradish peroxidase, 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 scavenges the endogenous H_2O_2 (Bandyopdhyay *et al.*, 1999).

Catalase

Catalase present in almost all the mammalian cells is localized in the peroxisomes or the microperoxisomes. It is a haemoprotein and catalyses the decomposition of H_2O_2 to water and oxygen and thus protects the cell from oxidative damage by H_2O_2 and $^{\cdot}OH$ (Bandyopadhyay *et al.*, 1999).

Antioxidant defense system in insects

Insects possess a suite of antioxidant enzymes and small molecular weight antioxidants that may form an effective response to an onslaught of dietary and endogenously produced oxidants. 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 the removal of H_2O_2 (Orr *et al.*, 1992).

Mathews *et al.*, (1997) have suggested a novel antioxidant enzyme, ascorbate peroxidase (APOX) in insects. Ascorbate peroxidase (APOX) activity, which catalyzes the oxidation of ascorbic acid with the concurrent reduction of hydrogen peroxide (H_2O_2), was found in larvae of *Helicoverpa zea*. According to them, since insects apparently lack a selenium-dependent glutathione peroxidase and as catalase has a low affinity for H_2O_2 this enzyme may be important in removing H_2O_2 (lipid peroxides) in insects. Another enzyme, dehydroascorbic acid reductase that may play a pivotal role in the elimination of hydrogen peroxide in insects was suggested by Summers and Felton (1993). Despite the presence of these enzymes it has been widely accepted that catalase provides the main mechanism for H_2O_2 break down (Sohal, 1993).

Ingestion of pro-oxidants exacerbates oxygen toxicity by increasing the production of these deleterious forms of oxygen (Ahmad, 1992). Water-soluble and lipid soluble antioxidants such as ascorbate, glutathione, tocopherols, and carotenoids have not been well studied in insects but may play very important antioxidant roles. Additionally, the peritrophic matrix and trehalose may possess

important antioxidant functions in insects. The enzymatic recycling of ascorbate, first noted in green plants, may also exist in insects (Felton and Summers, 1995).

Free- radical scavengers: the secondary defense against ROS

In addition to the primary defense against ROS by antioxidant enzymes, secondary defense against ROS is also offered by small molecules, which react with radicals to produce another radical compound, the 'scavengers'. These scavengers produce a lesser harmful radical species, and are called 'antioxidants'. For example, ∝-tocopherol, ascorbate and reduced glutathione (GSR) may act in combination as cellular antioxidants.

Biochemical and clinical studies show that certain compounds show antioxidant nature. Many of them have been studied for their effects on the activity of the antioxidant enzymes and prooxidant generation. Brack *et al.* (1997) have proposed that antioxidants may positively influence the aging process, protecting the organism against free radical induced damage. It has been suggested that supplemental intake of exogenous antioxidants extends average and, in some cases, maximum life span (Harman, 1968; Hochschild, 1971; Sharma and Wadhwa, 1983). Studies by other workers however do not corroborate this claim (Kohn, 1971; Sohal *et al.* 1991). From the theoretical point of view, it is also unlikely that exogenous antioxidants could provide significant protection against hydroxyl free radicals, which react with any surrounding molecules at virtually diffusion-

dependent rates. Sohal (1993) suggested that a more effective antioxidative strategy would be to reduce the possibility of ·OH generation, which is best achieved by the efficient elimination of O_2^- and H_2O_2 by enzymes. Studies on insects and mice have provided crucial information on the effects of many compounds on the antioxidant enzyme activity and prooxidant generation in these animals.

Acute ethanol administration was shown to affect primarily extra-peroxisomal catalase activity in rat liver thus rendering cytosolic superoxide dismutase more exposed to oxygen derivatives (Ribiere *et al.*, 1985). Roy *et al.* (1984) studied the effects of chlorpromazine on the activities of antioxidant enzymes and lipid peroxidation in the various regions of aging rat brain. Catalase did not change after chlorpromazine administration in any regions of the brain of rats from all age groups. The effect of centrophenoxine was also studied and similar results with regard to catalase activity were observed (Roy *et al.*, 1983). Iron is known to play a catalytic role in the generation of oxygen free radicals *in vitro*. Sohal *et al.* (1985) determined the *in vivo* effect of iron intake by administering 2mM ferrous chloride to adult male houseflies in their drinking water. The above treatment significantly shortened their lifespan, increased the concentration of inorganic peroxides and stimulated the activity of catalase. Sohal *et al.* (1984) have studied the biochemical basis for the effect of physical activity on the antioxidant enzyme activity and production of inorganic peroxides and

glutathione. They found that though the activities of SOD and catalase were not affected appreciably, the concentrations of inorganic peroxides and glutathione were higher in flies undergoing relatively high level of physical activity. Haloperidol treatment of mice brain mitochondria showed an enhancement of O_2^- and H_2O_2 production (Arnaiz *et al.*, 1999). Farmer and Sohal (1987) examined the life shortening effects of elevated ambient temperature in the adult housefly, *Musca domestica*. Effects of H_2O_2 administration on life span, activities of superoxide dismutase, catalase, concentration of endogenous H_2O_2 and glutathione in the housefly was described by Sohal (1988). Life span was shortened by H_2O_2 intake except in 10mM H_2O_2 administered flies, which exhibited the longest life span.

Zoccarato *et al.* (1990) studied the action of the glutathione transferase substrate, 1-chloro-2,4 dinitrobenzene (CDNB) on the synaptosomal production of H_2O_2. Comparisons of antioxidant defenses and mitochondrial prooxidant generation were made between short-lived insects and the rat tissues (Sohal *et al.*, 1990). Antioxidant defenses in insects were comparable to tissue in the rat but rate of O_2 and H_2O_2 generation were notably higher. Hai *et al.* (1993) showed the changes characteristic of 'oxidative stress' on treating two different fish species with an organophosphate insecticide (Dichlorvos). The activities of SOD, catalase and glutathione reductase were not affected by *in vitro* incubation with the intracellular proteinase calpain (Johnson and Hammer, 1994).

Sharma *et al.* (1997) studied the catalase activity of kinetin-fed *Zaprionus* fruit flies. Kinetin, a cytokinin plant growth hormone, retards senescence in plants, delays aging in human cells in culture, slows down development of insects and prolongs their lifespan. They observed that the increased longevity of kinetin-fed *Zaprionus* fruit flies was accompanied by an increase in the specific activity of catalase during developmental stages and in adult insects. Bolter and Chefurka (1996) investigated the production of H_2O_2 by mitochondria isolated from granary weevil on exposure to phosphine (PH3). The fumigant insecticide, PH3, is known to inhibit cytochrome oxidase *in vitro*. Production of H_2O_2 by PH3-treated insect mitochondria was increased significantly. Bains *et al.* (1998) studied the effect of butylated hydroxyanisole on catalase activity and malondialehyde content in the aging banana fruit fly, *Zaprionus paravittiger*. Their results suggested that BHA strengthened the defense mechanism of the insects by increasing catalase activity and reducing MDA content, which may be responsible for increased longevity of insects. Rao *et al.* (1997) investigated how salicylic acid (SA) enhanced H_2O_2 production and its influence on the H_2O_2 metabolizing enzymes.

Though the enhancement of antioxidant defenses through dietary supplementation would seem to provide a more reasonable and practical approach to reduce the level of oxidative stress, their mechanism of action *in vivo* are not elucidated. Simply adding a pharmacological agent may not be useful as most free radical scavengers act in oxidation-reduction reactions that are reversible, and

others such as ascorbate, can act both as antioxidants and pro-oxidants, depending on the conditions (Finkel and Holbrook, 2000).

Biochemical effects of tyrosine

Tyrosine an aromatic amino acid is essential for the tanning of the insect cuticle. Though this amino acid constitutes only a small proportion of the total free amino acids of the haemolymph of insect larva, it exhibits a conspicuous variation during the development of insects (Lazar and Mohamed, 1988).

Proteins are susceptible to oxidation by reactive oxygen species, where the type of damage induced is characteristic of the denaturing species. The induction of protein carbonyls is a widely applied biomarker, arising from primary oxidative damage. However when applied to complex biological and pathological conditions it can be subject to interference from lipid, carbohydrate and DNA oxidation products. More recently, attention was focused on the analysis of specific protein bound oxidized amino acids of the 22 protein forming amino acids. The aromatic and sulphydryl containing amino acid residues have been regarded as being particularly susceptible to oxidative modification, with L-DOPA from tyrosine, ortho-tryrosine from phenylalanine; and cysteine respectively; and kynurenine from tryptophan (Griffiths, 2000). Leeuwenburgh *et al.* (1999) have investigated the possibility of assaying the levels of o,o dityrosine and o-tyrosine which have been demonstrated as stable markers of protein oxidation in *in vitro* studies in urine.

They found that quantification of these compounds in urine could serve as a non-invasive measure of oxidative stress. Apart from using fluorescence markers for advanced glycosylation end products (AGE), protein, uric acid and creatinine concentrations in urine assays, di-tyrosine (di tyr) has also been used (Kirschbaum, 2001).

But, Rhemree *et al.* (2000) and van Overveld *et al.* (2000) have shown that tyrosine has a powerful antioxidant, capacity. They studied the total radical trapping antioxidant potential (TRAP) measurements of human seminal plasma and found that of the amino acids, only tyrosine possessed a slow TRAP and was present at a high concentration in seminal plasma. Therefore, this makes it an important contributor to the total antioxidant capacity of seminal plasma. Moosmann 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 acylated tyrosine and tryptophan, but not phenylalanine or short-chain acylated derivatives are potent inhibitors of lipid peroxidiation and oxidative cell death. Yen and Hsieh (1997) studied the antioxidant and free radical scavenging effects of dopamine, nor adrenaline, tyramine and tyrosine and compared with alpha-tocopherol. The antioxidant effect of dopamine and its related compounds on peroxidation of linoleic acid were in the

order of dopamine > alpha tocopherol / tyramine > tyrosine > noradrenaline as measured by the thiocyanate method. These amino compounds had reducing power, and a scavenging effect on reactive oxygen species, i.e., superoxide anion and hydroxyl radical. The results for reducing power and scavenging effect of these amino compounds had a similar trend as their inhibition of linoleic acid peroxidation. Kapiotis *et al.* (1997) showed that tyrosine exerted a strong inhibitory effect on O_2^-/NO^- initiated LDL oxidation as measured by thiobarbituric acid reactive substances (TBARS) formation and alteration in electrophoretic mobility of LDL and was thus able to protect human endothelial cells from O_2^-/NO^- cytotoxicity. Tyrosine has also shown to exert a protective effect on LDL modification by getting oxidized by HOCl (generated by the myeloperoxidase/H_2O_2/Cl^- system of activated neutrophils) to p-hydorxyphenyl acetaldehyde (p-HA), which in turn had the potential to act as an antioxidant in the lipid phase of LDL p-HA was also able to scavenge free radicals (Exner *et al.*, 2001). Ohkawa (1987) has shown that the supplementation of tyrosine, the amino acid precursor of catecholamines, during the stress maintains the content of catecholamines in the brain, which is considered to contribute to the development of the brain.

Most of the theories of aging are historic in nature; the genetic basis of phenotypic characters was not clear when they were proposed. With the advancements made in the field of molecular genetics, it has become clearer that

there is a genetic basis of all expressed characters of an organism. Any alteration in the genetic constitution will result in a phenotypic effect. The process of aging, therefore, can be attributed to the genetic constitution of an organism (other than the environmentally induced conditions such as climate, temperature, availability of food, etc.). There are several demonstrable effects or traits related to aging mechanism such as biochemical constituents, enzymatic activity, etc. and are consistent with aging processes in organisms. However the precise genetic cause (causes) and the metabolic pathways responsible for the phenotypic characters of organisms are yet to be elucidated.

MATERIALS AND METHODS

MATERIALS AND METHODS

Brief description of the experimental animal

The silkworm, *Bombyx mori* L. belongs to the phylum Arthropoda, class Insecta and the order Lepidoptera. They feed exclusively on mulberry leaf. The newly hatched larvae, undergo moulting four times, become mature silkworms and start spinning the cocoon. 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 moths usually comes out of the cocoon early in the morning, mate on that day itself and the females lay the eggs in the evening or the following morning. After oviposition, the moth weakens gradually and dies after 4-5 days. Each female lays about 400-700 eggs. Hatching occurs only in the early morning and the tiny larvae starts feeding on mulberry leaf.

Silkworm rearing

The bivoltine silkworm hybrid, Elite – CSR 2 x 4 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 rearing appliances were disinfected in advance with chlorine dioxide/bleaching powder to free the rearing environment and the surrounding from pathogens. These larvae were fed with fresh tender mulberry leaves cut into the size of half to one centimeter squares. Clean wet sand in trays were placed around the rearing bed to ensure 80-90% humidity. The worms were fed three times a day. The worms settle for the first moult on completion of 3 days and the duration for all the worms to come out of the first moult was about 24 h. The rearing beds were cleaned daily in the morning expanding the size of the bed and extending it to more trays corresponding to the growth of the larvae.

The larvae resume feeding after moulting and with 6-7 feeding the larvae were ready for the second moult. The above process of bed cleaning and feeding was done daily, except when feeding was stopped during moults. The third instar had duration of 3-3½ days and the duration of the third moult was about 24 h. The fourth instar took 4-4½ days followed by the fourth moult spanning about 30 h. The duration of the fifth instar was normally about 6 days and the larvae started to spin cocoons by the end of this stage.

The present work was done on the fifth instar larvae, beginning from the first day after its fourth moult, and continued till the last day of the instar, just before spinning began.

12.5 mM solution of tyrosine 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 distilled water while the other set of larvae were fed with leaves dipped in the tyrosine solution. The leaves were fed at least three times a day for both sets simultaneously. It was verified that larvae were fed *ad libitum*.

Determination of growth rate of the larva

The fifth instar larva were separated from the colony and used for the experiments. The 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 the larvae were noted at 24 h intervals during the development, in the case of both normal and treated larvae. The weight of food consumed was calculated using a reference value (of 1.5 for moths) derived by Mathavan and Pandian (1974), which is expressed as a ratio between dry weight of food consumed and dry weight of excreta voided. The rate of food consumption was expressed as mg dry leaf consumed per unit time per animal, for 1 hour and 24 hour intervals.

Fresh excreta was collected from a definite number of synchronous larvae at 24 hour intervals starting from the first day of the instar, till they began spinning. The excreta were dried in an oven at 100° C for 1 hour and then 60° C to a constant weight. The rate of excretion was calculated and expressed as mg dry excreta voided per unit time per animal, for 1 hour and 24 hour intervals.

Determination of volume of total haemolymph and weight of the total fat body

The measurement of the volume of haemolymph was made directly using a fine calibrated capillary tube. For extracting the haemolymph, the larvae were anaesthetized slowly with diethyl ether as described by Mohammed (1974) since reflex bleeding was observed in the larvae as in the case of *Mylabris pustulata*. 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 complete extraction of haemolymph, the larva was gently pressed from anterior and posterior ends simultaneously until no more haemolymph was oozing out of the wound. Changes in the volumes of haemolymph of the treated and normal larvae were observed during the developmental stages.

The fat body of the treated and the normal larvae were dissected out carefully in ice-cold insect ringer. Water adhering to the fat body lobes was wiped off carefully with a filter paper and weighed immediately. Variations in the fresh

weights of fat body in the developmental stages of treated and normal larvae were observed.

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

Biochemical analyses

Ten larvae each were collected for the normal and treated sets of larvae and dissected in insect ringer. 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

Total protein was estimated following the method of Lowry *et al.* (1951) using crystalline bovine serum albumin (fraction V, Sigma) as standard. The proteins were 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 was extracted with 0.5N perchloric acid at 90°C for 15 minutes. The residue left over the hot extraction was dissolved in 1N

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 acids

The total free amino acid content in the tissues was estimated by the method of Lee and Takahashi (1966). The homogenized tissues was precipitated 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 read at 540nm against the reagent blank in a spectrophotometer.

Estimation of glucose

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

Estimation of creatinine

Creatinine content in the tissue was estimated based on Jaffe reaction by McFate *et al.* (1954). The homogenates were precipitated with 10% sodium tungstate and 2/3 N sulphuric acid and centrifuged at 2000 rpm for 20 min. The filtrate was used for the estimation and the yellow-orange colour developed was read at 520 nm in a spectrophotometer.

Estimation of urea

Urea was estimated in the tissues using Fearon reaction, modified by Beale and Croft (1961). The homogenates were deproteinized with 0.3N 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 min. The colour developed was read after cooling, at 535 nm in a spectrophotometer.

Estimation of hydrogen peroxide and catalase

Both estimations were done using a novel based on where the formation of soluble coloured peroxotitanium complex by the reaction of hydrogen peroxide with potassium titanium oxalate and the absorption maxima, λ_{max} for the orange/yellow colour developed, was measured at 410 nm (Muhlebach *et al.*, 1970).

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

For the estimation of hydrogen peroxide, the homogenized tissue was deproteinised with 20% trichloroacetic acid (TCA) and centrifuged at 3000 g 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 read 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 1 ml. The mixture was incubated at 37°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 g for 10 min. and the supernatant was used for the estimation as described above. The difference in the values of the control and test give 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 ml of haemolymph and as mg/min/larva.

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

The activity of AAT 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 were directly taken for the enzyme assay. In the case of AAT, the substrate mixture of∝-oxoglutaric acid and L-aspartic acid in a phosphate buffer of pH 7.4 was incubated with the enzyme

source for one hour. 2,4-dinitrophenyl hydrazine solution was used to stop the reaction and 0.4 N sodium hydroxide was added to it. In the case of AlAT, substrate mixture was made of α-oxoglutarate and L-alanine in phosphate buffer of pH 7.4. The colour developed was read after 10 min at 520nm in a spectrophotometer. One unit enzyme activity corresponds to the formation of 1mole of keto acid per minute at 37°C under the experimental conditions.

RESULTS

RESULTS

Growth rate of the silkworm

The duration of the fifth instar larval period was found to be 6 days. The larval stages were identified by their food intake and size characteristics: Day 1, the newly moulted larva began feeding 12 hours after ecdysis; Day 2, the larva was in the active feeding and growing stage; Day 3, the larva acquired its body size and was still actively feeding; Day 4, the larval food intake showed a decline; Day 5, the larva had well-developed silk glands and had reduced feeding; Day 6, the larva had completely stopped feeding and was just about to start spinning the cocoon.

In the larvae treated with tyrosine, the larvae showed a light black pigmentation on the cuticle, more pronounced on the anterior region, that was visible 24 hrs after the first feeding began. These larvae were also comparatively bigger and stouter in size than the normal larvae, with an increase in the food consumption. This increase in general body size became more prominent as the larvae developed.

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

Table 1

Figure 1

The fresh weight of the normal larvae was minimum at the beginning of the instar but rose sharply up to Day 4, attaining its maximum weight on Day 5. The fresh weight of the treated larvae followed a similar pattern as that of the normal but showed an increase in body weight than the normal. The increase in body weight was only 5-6 % on the first two days, but later it showed almost an 8-14% increase during the next three days. The weight of the normal and treated larvae declined after Day 5 though maintaining a higher scale in the latter.

The rate of excretion calculated for 24 hr intervals of both normal and treated larvae are presented in Table 2 and Figure 2.

Table 2

Figure 2

The rates of food consumption of both normal and treated larvae are given in Table 3 and Figure3.

Table 3

Figure3

The rate of food consumption was low at the beginning of the instar, but rapidly increased and attained the peak by Day 3 and started declining from Day 4

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

Larval age	mg/larva, mean ± S.D.	
	Normal	Treated
Day 1	1.62 ± 0.15	1.72 ± 0.18
Day 2	3.35 ± 0.43	3.52 ± 0.31
Day 3	3.98 ± 0.33	4.52 ± 0.27
Day 4	4.34 ± 0.40	4.92 ± 0.26
Day 5	4.92 ± 0.37	5.32 ± 0.57
Day 6	3.92 ± 0.41	4.10 ± 0.32

Values are the means of 5 determinations.

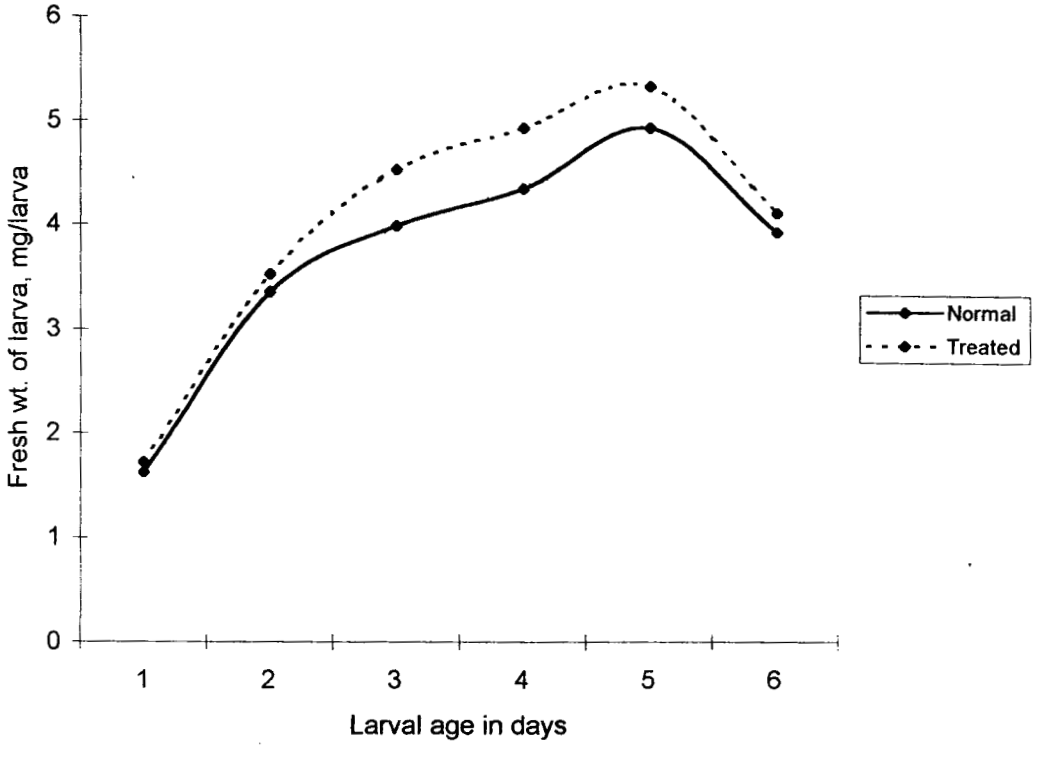

Figure 1. Changes in the total fresh weight of the larva

Table 2. Changes in the rate of excretion

Larval age	mg/24hr/larva, mean ± S.D.	
	Normal	Treated
Day 1	234.15 ± 0.25	265.42 ± 0.24
Day 2	479.03 ± 0.09	558.25 ± 0.03
Day 3	611.06 ± 0.04	713.90 ± 0.98
Day 4	589.90 ± 0.52	664.01 ± 0.87
Day 5	480.22 ± 0.17	404.09 ± 0.86

Values are the means of 5 determinations.

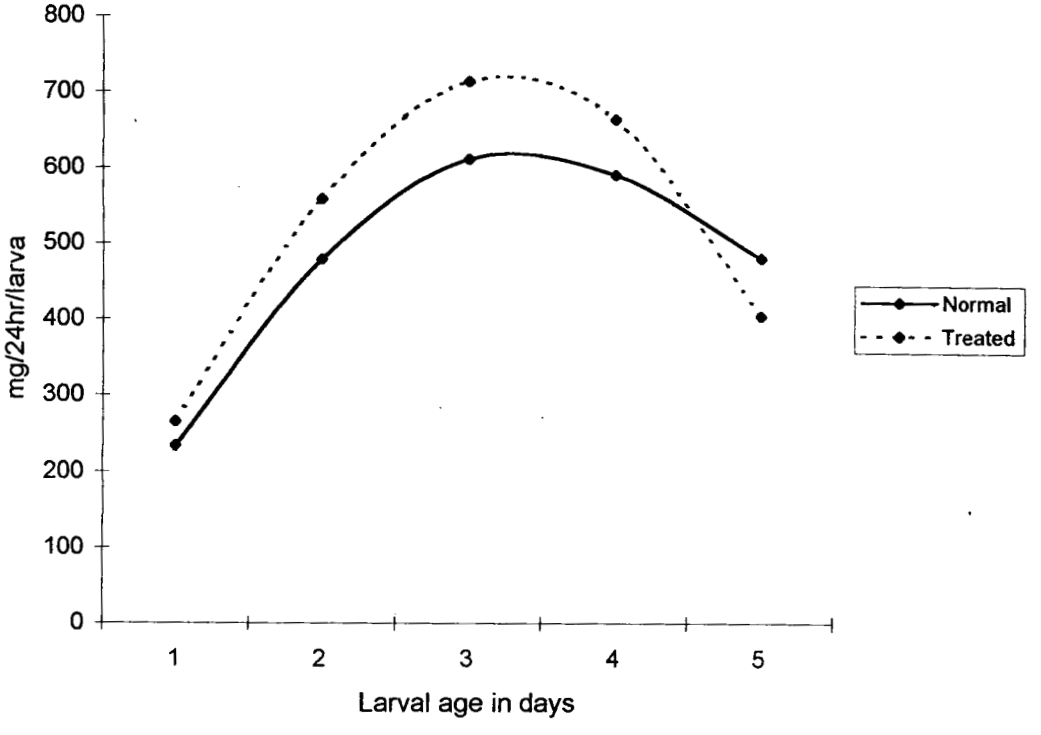

Figure 2. Changes in the rate of excretion

Table 3. Changes in the rate of food intake

Larval age	mg/24hr/larva, mean ± S.D.	
	Normal	Treated
Day 1	351.23 ± 0.38	398.13 ± 0.36
Day 2	718.55 ± 0.14	837.38 ± 0.04
Day 3	916.59 ± 0.07	1070.85 ± 1.47
Day 4	884.85 ± 0.78	996.02 ± 1.31
Day 5	720.32 ± 0.26	606.14 ± 1.29

Values are the means of 5 determinations.

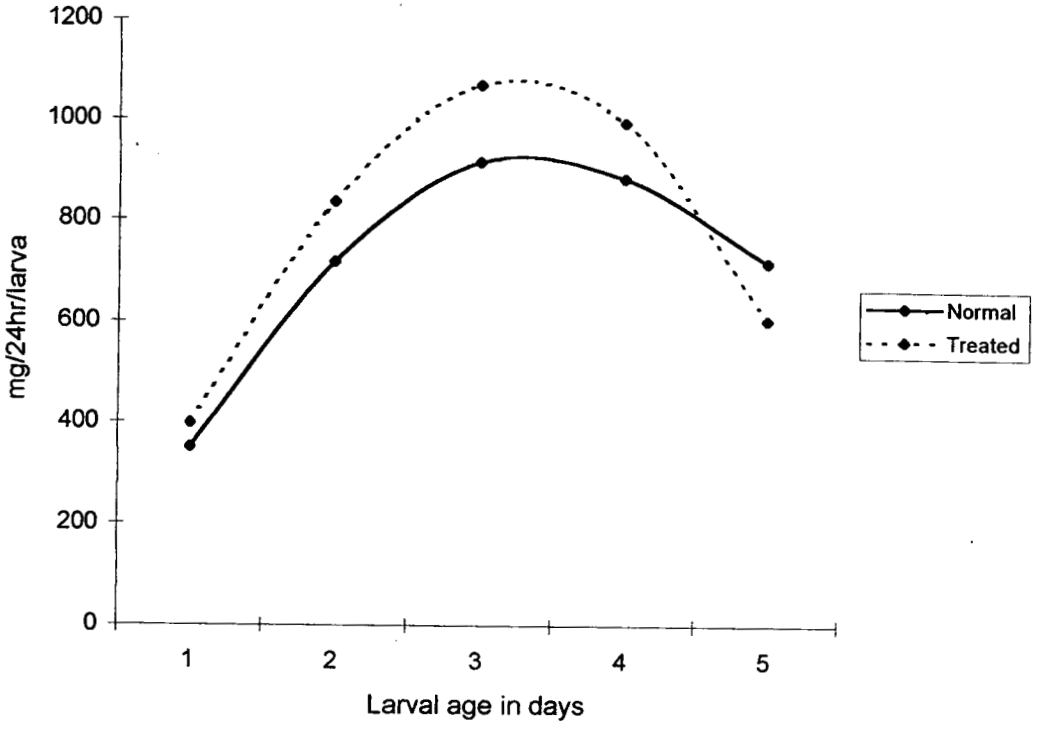

Figure 3. Changes in the rate of food intake

to Day 6. The pattern of variations in the rate of excretion corresponded to that observed for the rate of food consumption.

The treated larvae showed a higher rate of food consumption than the normal. There was almost a 13% increase in food consumption in the beginning to a 17% during Day 3.

The haemolymph was slightly greenish, which was less dense in the early stages of the instar but gradually became denser as the instar progressed. The haemolymph of the treated larvae was darker in coloration and appeared to be heavily pigmented. The changes in the volume of haemolymph in the normal larvae during the development are presented in Table 4 and Figure 4.

Table 4

Figure 4

The volume of haemolymph was low at the beginning of the instar but gradually rose and attained the peak during Day 5 followed by a decline to Day 6 towards pupation. The variation in the volume of haemolymph was similar in the treated larvae also.

The fat body appeared as creamy white and less dense at the beginning of the instar but became denser as growth proceeded. The variations in the weight of fat body in the normal and treated larvae during the development are presented in Table 5 and Figure 5.

Table 4. Changes in the volume of haemolymph

Larval age	ml/larva, mean ± S.D.	
	Normal	Treated
Day 1	0.18 ± 0.04	0.19 ± 0.08
Day 2	0.34 ± 0.07	0.32 ± 0.05
Day 3	0.50 ± 0.07	0.44 ± 0.04
Day 4	0.54 ± 0.07	0.52 ± 0.07
Day 5	0.70 ± 0.05	0.64 ± 0.05
Day 6	0.53 ± 0.03	0.53 ± 0.03

Values are the means of 5 determinations.

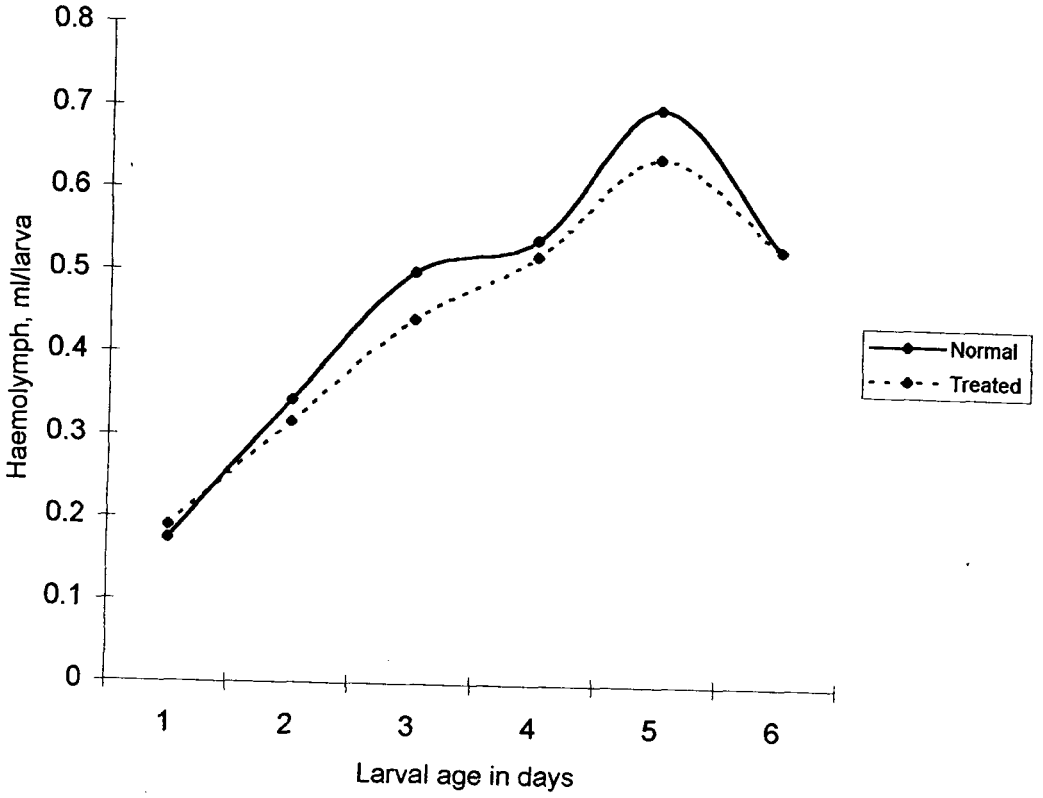

Figure 4. Changes in the volume of haemolymph

Table 5

Figure5

The fresh weight of the fat body was low initially but showed a sharp rise from Day 2 to Day 4, with the maximum in Day 5, which was followed by a decline on Day 6. The variation in the weight of fat body was similar in the treated larvae also, but showed an increase in the weight than the normal during each day. The increase was around 35-45%, reaching a peak on Day 5.

Hydrogen peroxide levels and catalase activity

Hydrogen peroxide levels in per unit fresh tissue and whole tissues were noted in the haemolymph and fat body of both normal and treated larvae and are given in Table 6a & 6b and Figure 6a & 6b.

Table 6a and 6b

Figure 6a and 6b

The amount of hydrogen peroxide in the per unit volume haemolymph of the normal larvae gradually increased from Day 1 and reached a maximum during Day 5 and then dropped down during Day 6 to values seen initially. The same pattern was seen for the total haemolymph though the increase is steeper here.

The treated larvae followed the same pattern as that of the normal but showed an increase in the amounts generated. The increase was initially 30-50%

Table 5. Changes in the weight of fat body

Larval age	mg fresh tissue/larva, mean ± S.D.	
	Normal	Treated
Day 1	35.55 ± 1.34	48.20 ± 0.20
Day 2	61.52 ± 1.82	89.20 ± 0.20
Day 3	147.44 ± 0.45	178.83 ± 0.18
Day 4	179.12 ± 0.89	222.02 ± 0.67
Day 5	244.13 ± 1.64	349.13 ± 0.58
Day 6	236.90 ± 1.88	242.34 ± 0.43

Values are the means of 5 determinations.

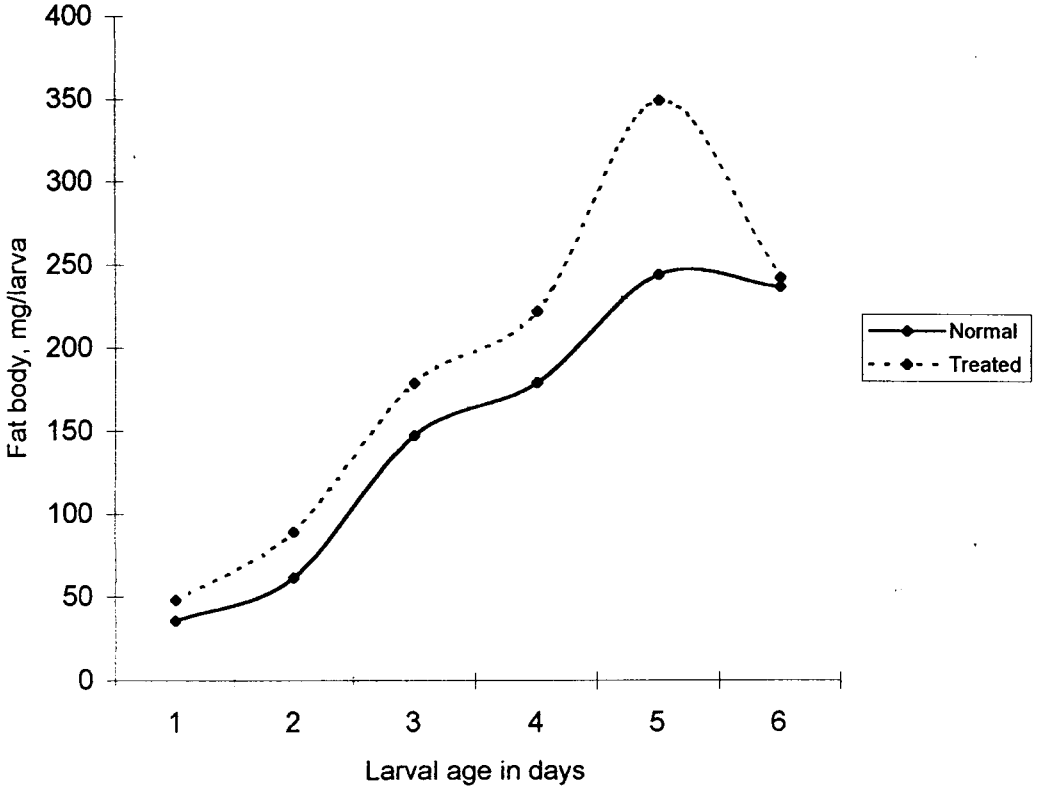

Figure 5. Changes in the weight of fat body

Table 6 a. Changes in the hydrogen peroxide levels in haemolymph

Larval age	mg/ml, mean ± S.D.		mg/total tissue, mean ± S.D.	
	Normal	Treated	Normal	Treated
Day 1	6.10 ± 0.21	5.61 ± 0.54	1.07 ± 0.24	1.07 ± 0.52
Day 2	5.50 ± 0.23	8.77 ± 0.35	1.89 ± 0.90	2.78 ± 0.21
Day 3	4.38 ± 0.84	5.59 ± 0.62	2.19 ± 0.18	2.94 ± 0.63
Day 4	7.42 ± 0.34	8.25 ± 0.45	3.71 ± 0.56	4.29 ± 0.35
Day 5	7.33 ± 0.59	9.12 ± 0.24	5.13 ± 0.80	5.84 ± 0.25
Day 6	3.34 ± 0.74	3.98 ± 0.21	1.77 ± 0.56	2.12 ± 0.31

Values are the means of 5 determinations.

Table 6 b. Changes in the peroxide levels in fat body

Larval age	mg/mg fresh tissue, mean ± S.D.		mg/total tissue, mean ± S.D.	
	Normal	Treated	Normal	Treated
Day 1	0.028 ± 0.08	0.042 ± 0.03	0.99 ± 1.24	2.03 ± 0.68
Day 2	0.031 ± 0.04	0.043 ± 0.01	1.91 ± 2.68	3.81 ± 0.77
Day 3	0.060 ± 0.02	0.082 ± 0.04	8.84 ± 3.51	14.72 ± 1.28
Day 4	0.031 ± 0.01	0.072 ± 0.02	5.52 ± 2.41	15.94 ± 0.82
Day 5	0.014 ± 0.01	0.027 ± 0.01	3.42 ± 0.87	9.32 ± 0.43
Day 6	0.010 ± 0.02	0.024 ± 0.04	2.32 ± 0.94	5.76 ± 0.57

Values are the means of 5 determinations.

Figure 6a - I

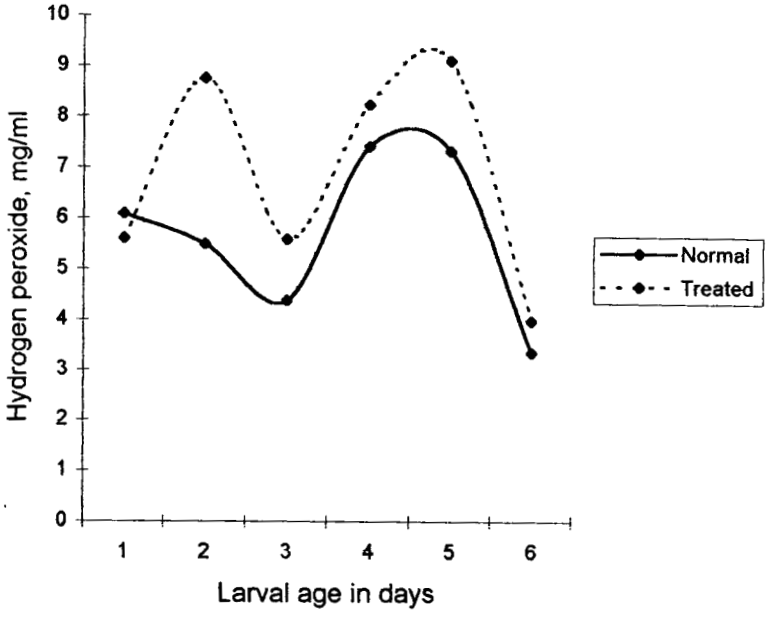

Figure 6a - II

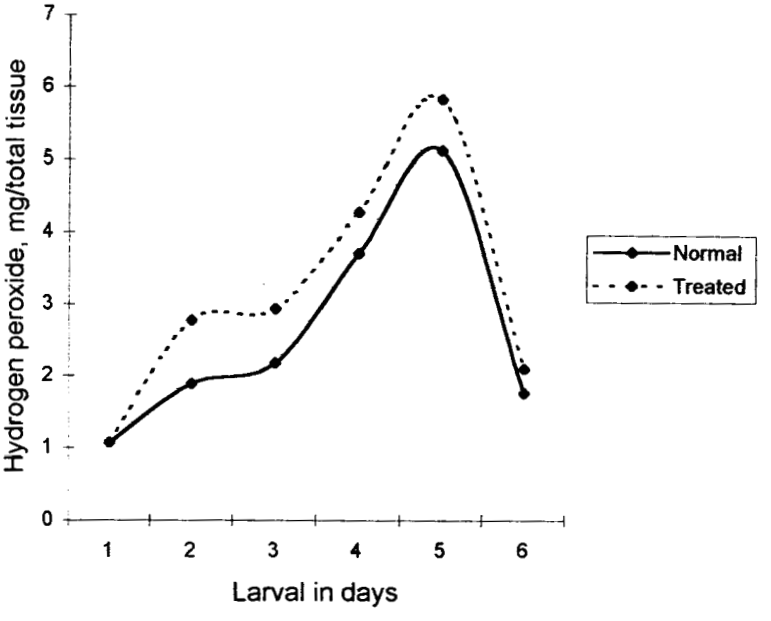

Figure 6a. Changes in the hydrogen peroxide levels in haemolymph

I - Changes per unit volume of haemolymph

II - Changes per total haemolymph

Figure 6b - I

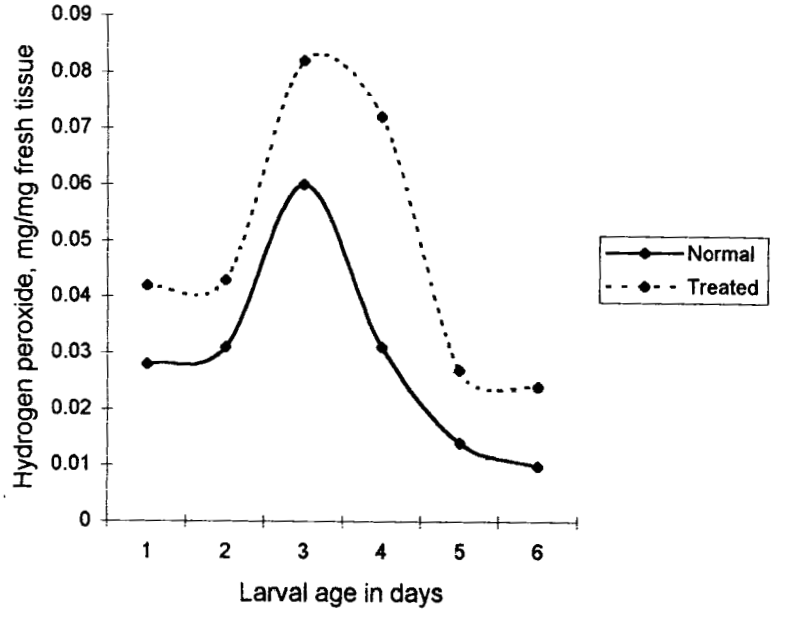

Figure 6b - II

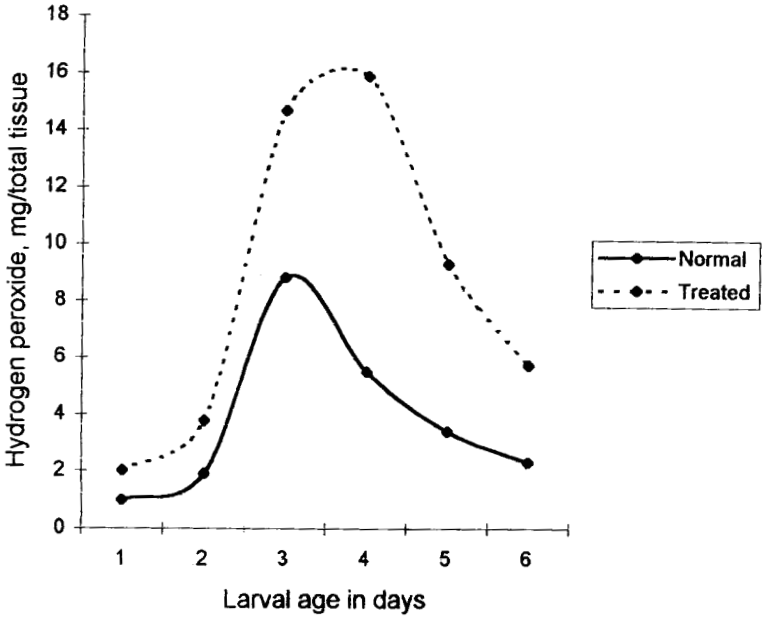

Figure 6b. Changes in the hydrogen peroxide levels in fat body

I - Changes per unit weight of fat body

II - Changes per total fat body

than normal but then reduced to 15-20% than normal, when whole tissues were considered. In general, the peroxide level is higher in the treated larvae.

On the basis of per unit weight of fat body of normal larvae, the level of H_2O_2 increased from Day 1 and reached its peak on Day 3 followed by a decline up to Day 6. When the levels in total fat body were considered the pattern followed was the same. The H_2O_2 levels in the treated larvae showed a similar pattern except that there was a 2- to 3- fold increase than in the normal larvae when whole fat body content were considered.

The activity of the antioxidant enzyme catalase per unit fresh tissue and total tissues were estimated in the haemolymph and fat body of both normal and treated larvae and are presented in Table 7a & 7b and Figure 7a & 7b.

Table 7a and 7b

Figure 7a and 7b

In the case of the haemolymph of normal larvae, the catalase activity increased from Day 1 and reached its maximum on Day 4 and then declined slowly to Day 6. But on the basis of total volume of haemolymph the rise in catalase activity from Day 1 was more compared to per unit tissue values, though the peak activity was still on Day 4. The catalase activity in the treated larvae followed the same pattern as that of the normal, except that there was a 5-10% increase in its activity than that of the normal.

Table 7 a. Changes in the catalase levels in haemolymph

Larval age	mg/min/ml, mean ± S.D.		mg/min/total tissue, mean ± S.D.	
	Normal	Treated	Normal	Treated
Day 1	83.71 ± 11.23	78.30 ± 10.22	14.65 ± 1.82	14.89 ± 2.03
Day 2	92.04 ± 10.58	103.12 ± 12.03	31.66 ± 1.67	32.71 ± 1.53
Day 3	81.80 ± 13.24	97.51 ± 11.52	40.92 ± 2.31	43.11 ± 3.21
Day 4	103.70 ± 12.51	106.94 ± 13.65	51.86 ± 3.01	55.59 ± 2.35
Day 5	61.92 ± 11.91	70.32 ± 14.21	43.33 ± 1.94	44.99 ± 2.14
Day 6	66.46 ± 11.01	66.01 ± 13.33	35.22 ± 5.42	34.98 ± 5.33

Values are the means of 5 determinations.

Table 7 b. Changes in the catalase levels in fat body

Larval age	mg/min/mg fresh tissue, mean ± S.D.		mg/min/total tissue, mean ± S.D.	
	Normal	Treated	Normal	Treated
Day 1	0.41 ± 0.21	0.45 ± 0.32	14.73 ± 2.81	21.54 ± 2.47
Day 2	0.53 ± 0.14	0.47 ± 0.28	30.80 ± 2.11	57.22 ± 2.66
Day 3	0.48 ± 0.33	0.47 ± 0.41	70.70 ± 2.64	83.20 ± 3.04
Day 4	0.54 ± 0.26	0.55 ± 0.55	97.11 ± 3.52	122.51 ± 2.42
Day 5	0.47 ± 0.11	0.42 ± 0.23	114.61 ± 1.95	146.73 ± 2.87
Day 6	0.48 ± 0.61	0.49 ± 0.40	112.30 ± 2.88	119.22 ± 3.22

Values are the means of 5 determinations.

Figure 7a - I

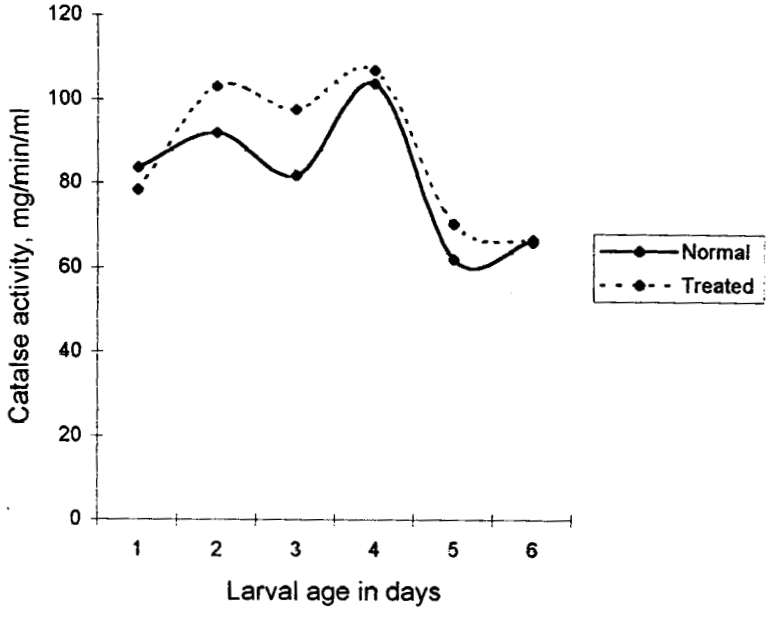

Figure 7a - II

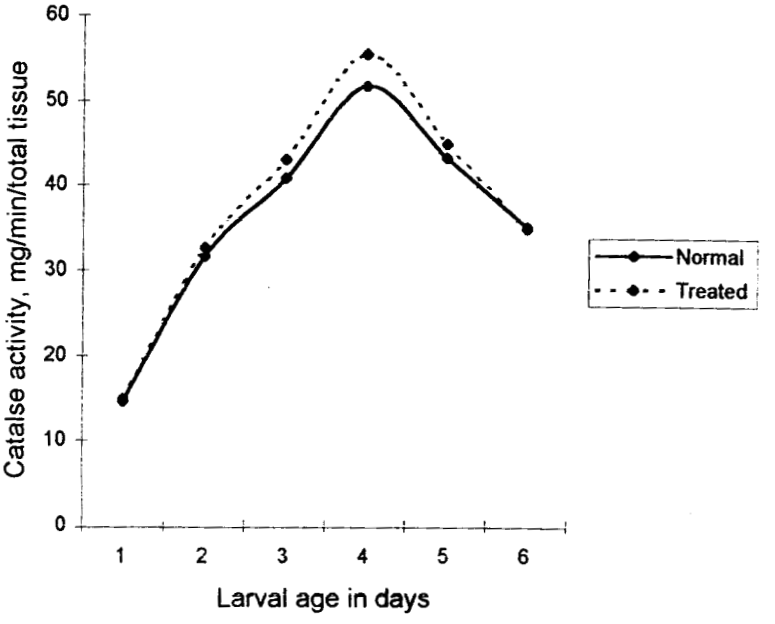

Figure 7a. Changes in the catalase activity in haemolymph

I - Changes per unit volume of haemolymph

II - Changes per total haemolymph

Figure 7b - I

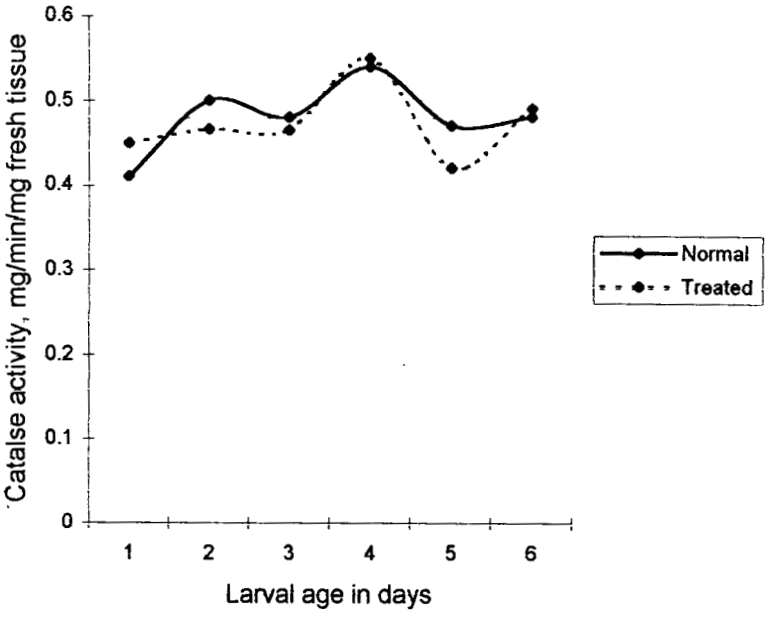

Figure 7b - II

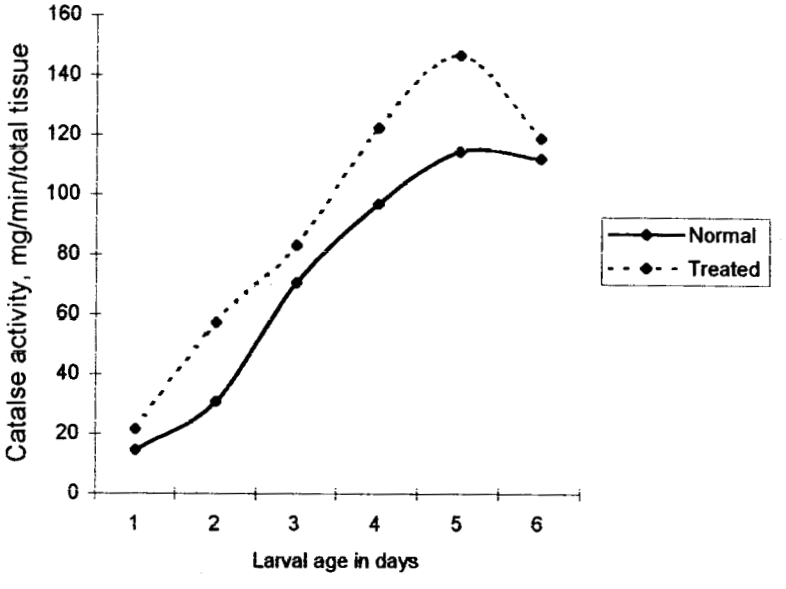

Figure 7b. Changes in the catalase activity in fat body

I - Changes per unit weight of fat body

II - Changes per total fat body

The catalase activity per unit weight of fat body was more or less consistent during larval development, with a slight increase on Day 4. However catalase activity of the total fat body showed a different picture. The catalase activity of the total fat body of normal larvae increased steadily from Day 4 and reached a maximum during Day 5, then declined slightly on Day 6. The catalase activity in the total fat body of the treated larvae followed the same pattern, but with an increase in its level. There was a 50-80% increase in catalase activity initially but then the increase dropped down to 20-30% and then to 6% by Day 6. The catalase activity per unit weight of fat body of the treated larvae did not show much difference from that of the normal.

Total protein

The content of total protein in the haemolymph and fat body of the normal and treated larvae during its development is given in Table 8a & 8b and Figure 8a & 8b.

Table 8a and 8b

Figure 8a and 8b

The total protein in the haemolymph of the normal larva increased with the development of the fifth instar and reached a peak on Day 5. This peak was more prominent when total tissue values were considered because there was a decline in the total protein in Day 6.

Table 8 a. Changes in the total protein levels in haemolymph

Larval age	mg/ml, mean ± S.D.		mg/total tissue, mean ± S.D.	
	Normal	Treated	Normal	Treated
Day 1	14.08 ± 1.84	20.03 ± 0.50	2.46 ± 0.33	3.81 ± 0.10
Day 2	23.13 ± 0.34	32.53 ± 4.31	7.96 ± 0.12	10.32 ± 1.36
Day 3	32.30 ± 6.92	44.28 ± 2.87	16.15 ± 3.46	19.57 ± 1.27
Day 4	48.80 ± 2.28	54.52 ± 1.88	24.40 ± 1.15	28.35 ± 0.98
Day 5	63.92 ± 3.39	72.43 ± 2.44	44.74 ± 2.38	46.35 ± 1.56
Day 6	67.71 ± 0.93	62.37 ± 5.37	35.89 ± 0.50	33.06 ± 2.85

Values are the means of 5 determinations.

Table 8 b. Changes in the total protein levels in fat body

Larval age	mg/mg fresh tissue, mean ± S.D.		mg/total tissue, mean ± S.D.	
	Normal	Treated	Normal	Treated
Day 1	0.13 ± 0.05	0.16 ± 0.01	4.74 ± 2.12	7.71 ± 2.25
Day 2	0.13 ± 0.02	0.15 ± 0.03	8.23 ± 1.03	13.38 ± 0.13
Day 3	0.13 ± 0.01	0.13 ± 0.02	18.85 ± 1.14	23.60 ± 0.13
Day 4	0.14 ± 0.01	0.14 ± 0.02	24.98 ± 1.67	31.81 ± 4.71
Day 5	0.12 ± 0.02	0.12 ± 0.01	28.95 ± 4.12	40.52 ± 1.49
Day 6	0.16 ± 0.01	0.08 ± 0.04	37.67 ± 2.34	19.38 ± 1.21

Values are the means of 5 determinations.

Figure 8a - I

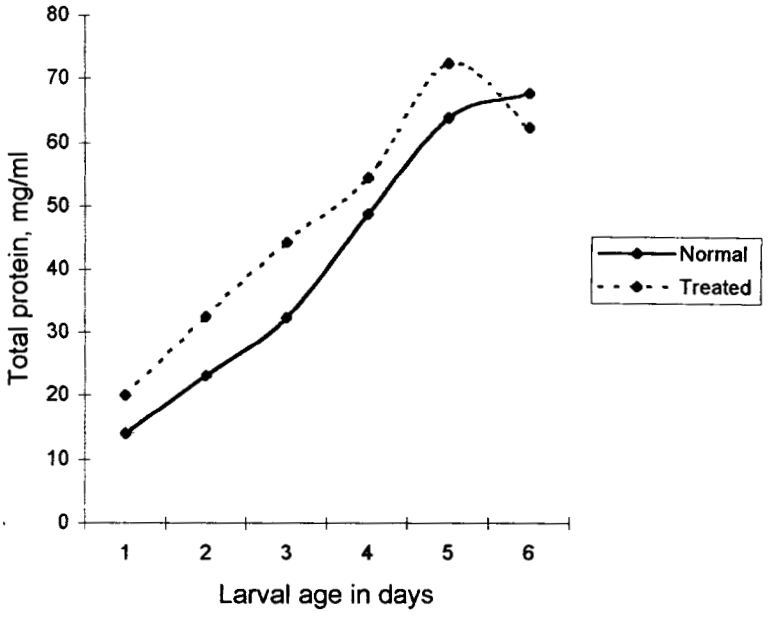

Figure 8a - II

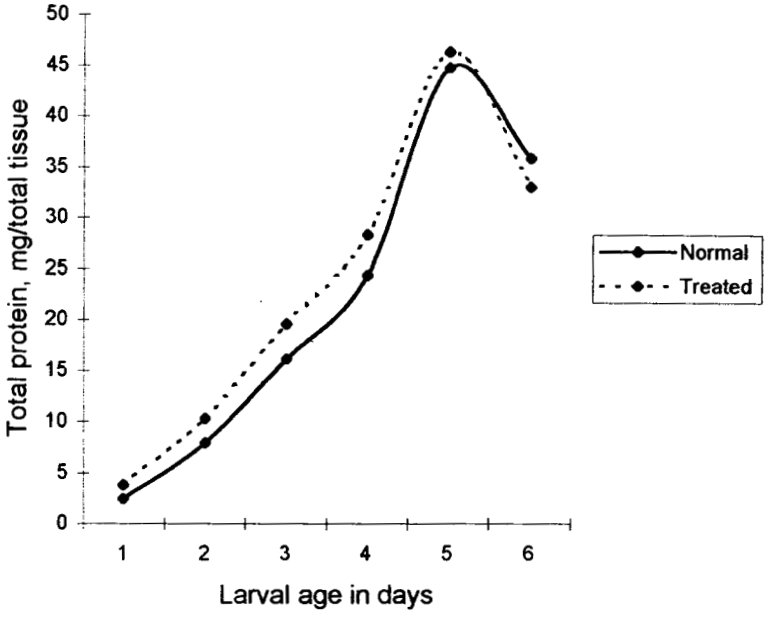

Figure 8a. Changes in the total protein levels in haemolymph

I - Changes per unit volume of haemolymph

II - Changes per total haemolymph

Figure 8b - I

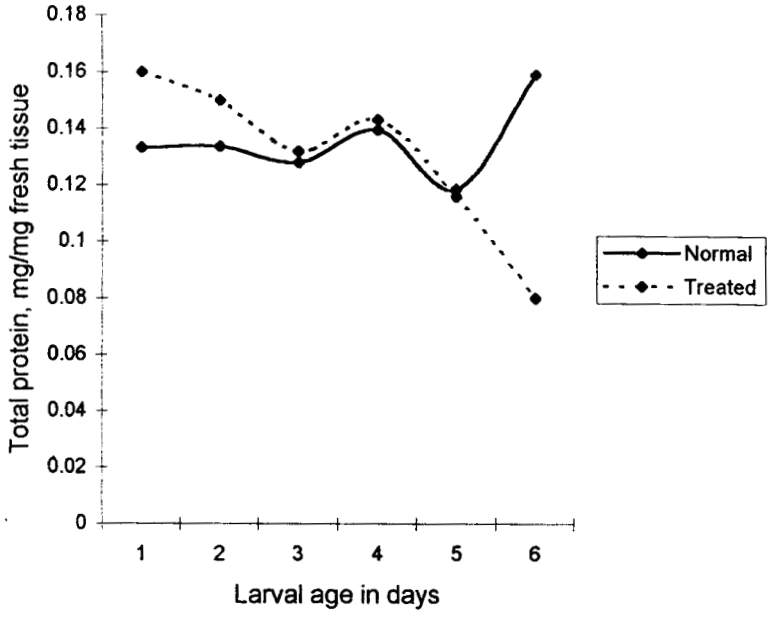

Figure 8b - II

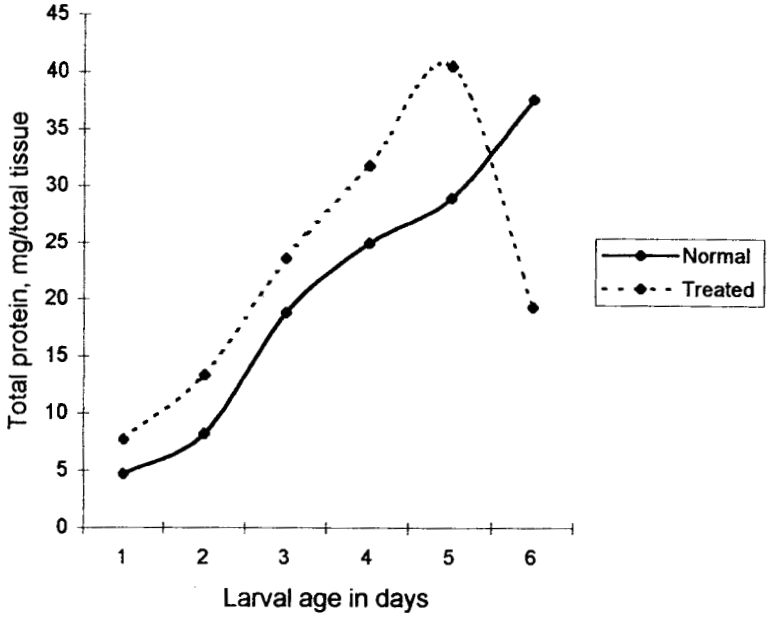

Figure 8b. Changes in the total protein levels in fat body

I - Changes per unit weight of fat body

II - Changes per total fat body

The treated larvae showed a similar pattern for the total protein content in haemolymph, though it had higher level than normal. On the basis of unit volume haemolymph there was an increase of about 30-40% initially and then reduced to 10-15% from Day 4. But this increase was reduced to 20-30% and 5-10% when total tissue values were considered.

The values per unit weight of fat body are more or less consistent from Day 1 to Day 5 with an increase on Day 6. The total protein in the fat body of the normal larva increased in a similar way as that in the haemolymph but showed no decline, reaching the peak on Day 6, when total tissue values are considered.

The treated larvae showed an increase in total protein content from Day 1 to Day 5and then declined on Day 6 when total tissue values were studied. It also showed a 30-40% increase than normal.

Total free amino acids

The total free amino acid content in the haemolymph and fat body of the normal and treated larvae during its development is given in Table 9a & 9b and Figure 9a & 9b.

Table 9a and 9b

Figure 9a and 9b

Table 9 a. Changes in the total free amino acid levels in haemolymph

Larval age	mg/ml, mean ± S.D.		mg/total tissue, mean ± S.D.	
	Normal	Treated	Normal	Treated
Day 1	5.38 ± 1.25	7.18 ± 2.87	0.94 ± 0.51	1.37 ± 0.94
Day 2	11.62 ± 0.95	10.97 ± 3.15	3.99 ± 0.22	3.48 ± 0.51
Day 3	11.84 ± 1.11	11.95 ± 1.12	5.99 ± 0.74	5.28 ± 0.47
Day 4	15.32 ± 1.65	12.39 ± 1.56	7.66 ± 0.34	6.44 ± 0.14
Day 5	13.12 ± 1.44	12.17 ± 2.44	9.18 ± 0.87	7.79 ± 0.32
Day 6	8.34 ± 2.56	10.25 ± 2.51	4.42 ± 0.68	5.43 ± 0.21

Values are the means of 5 determinations.

Table 9 b. Changes in the total free amino acid levels in fat body

Larval age	mg/mg fresh tissue, mean ± S.D.		mg/total tissue, mean ± S.D.	
	Normal (x 10^{-3})	Treated (x 10^{-3})	Normal (x 10^{-3})	Treated (x 10^{-3})
Day 1	6.78 ± 1.11	0.83 ± 0.21	241.35 ± 12.01	40.05 ± 9.51
Day 2	6.90 ± 0.91	2.91 ± 0.57	424.50 ± 11.38	259.57 ± 9.20
Day 3	6.17 ± 0.84	2.95 ± 0.32	910.00 ± 14.58	527.46 ± 11.23
Day 4	6.80 ± 1.44	3.33 ± 0.41	1216.98 ± 18.20	740.00 ± 13.01
Day 5	6.71 ± 1.51	3.80 ± 0.33	1638.89 ± 15.11	1180.00 ± 18.42
Day 6	4.77 ± 0.84	3.99 ± 0.52	1129.30 ± 16.54	966.78 ± 14.23

Values are the means of 5 determinations.

Figure 9a - I

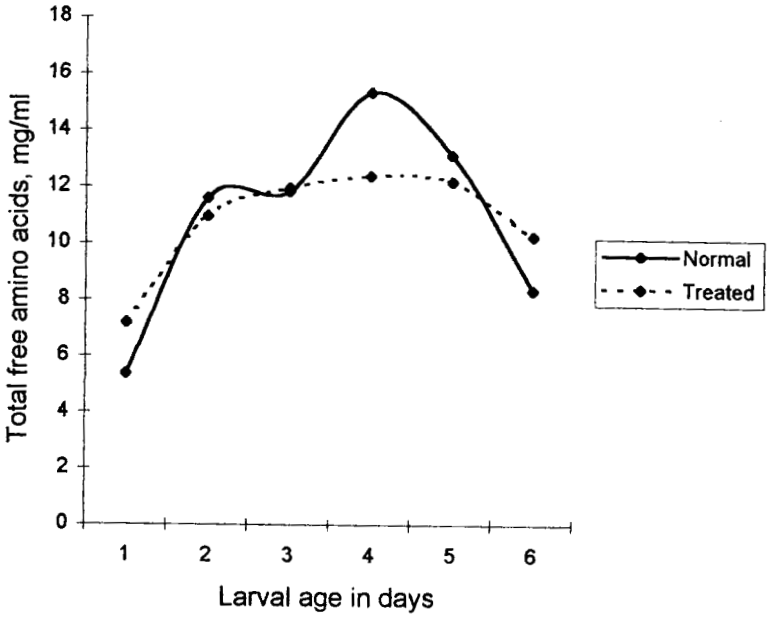

Figure 9a - II

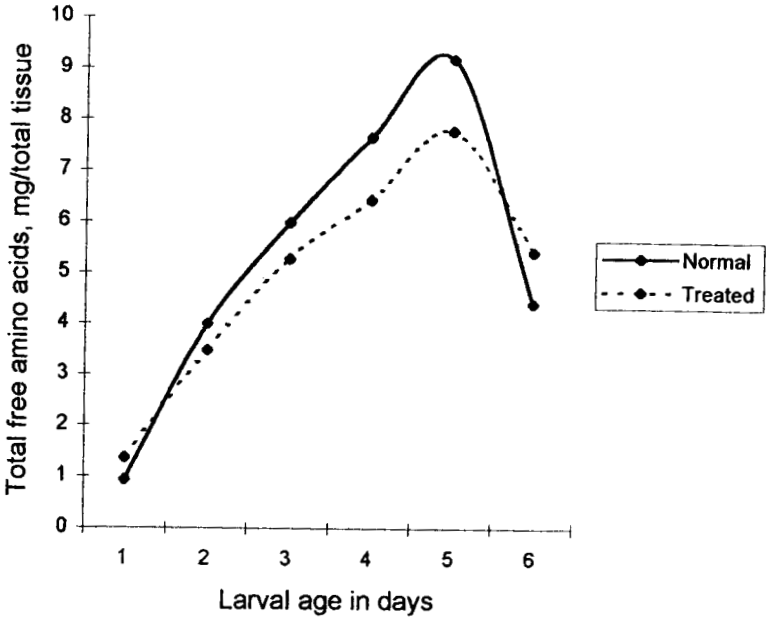

Figure 9a. Changes in the total free amino acids in haemolymph

I - Changes per unit volume of haemolymph

II - Changes per total haemolymph

Figure 9b - I

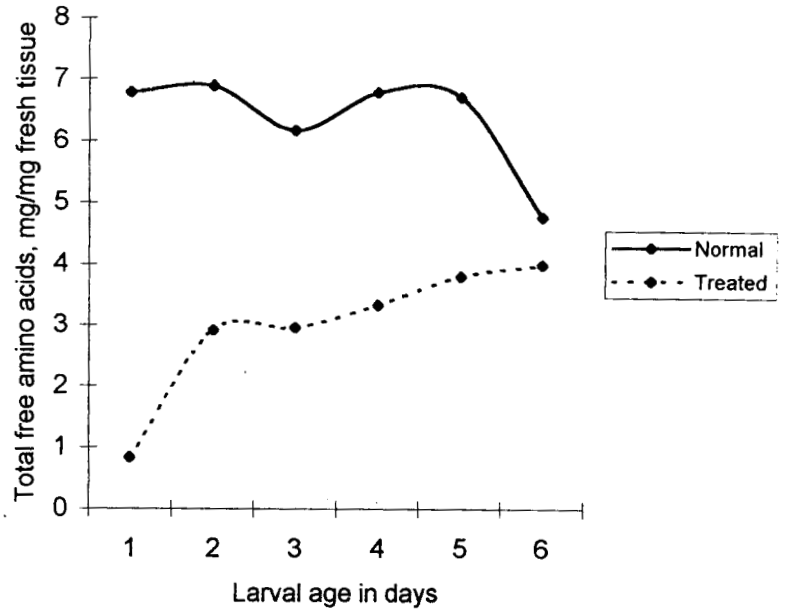

Figure 9b - II

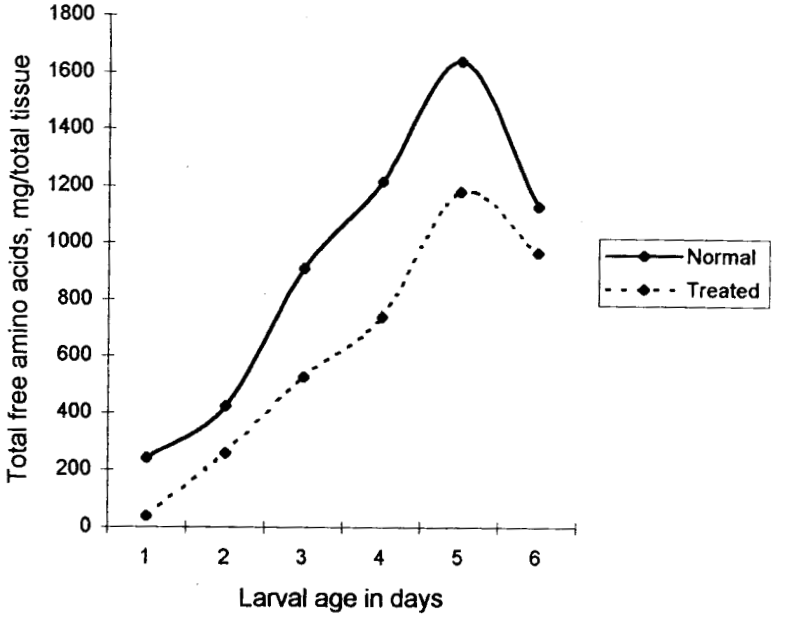

Figure 9b. Changes in the total free amino acids in fat body

I - Changes per unit weight of fat body

II - Changes per total fat body

The total free amino acid in the haemolymph of the normal larva showed a steep increase from Day 1 and reached a peak on Day 5 when the values per unit volume of haemolymph were studied. The increase was steeper with a peak on Day 5, when total haemolymph values were compared.

The values per unit volume of haemolymph in the treated larvae were lower than the normal and were more or less in a line from Day 2. They showed a similar pattern of total free amino acid though the levels were 15-20% lower than normal in total tissues.

The total free amino acid content of the total fat body of normal larvae showed an increase from Day 1 and reached its maximum on Day 5. The total free amino acid level per unit weight of fat body decreased from Day 1 to Day 6. The normal larvae showed almost 60% increase in fat body amino acid content than the treated, but this increase reduced to 20-30% towards the final days of the instar.

Aspartate amino transferase (AAT) activity

The AAT activity in the haemolymph and fat body of the normal and treated larvae during its development is given in Table 10a & 10b and Figure 10a & 10b.

Table 10a and 10b

Figure 10a and 10b

Table 10 a. Changes in the AAT activity in haemolymph

Larval age	AAT units/ml, mean ± S.D.		AAT units/total tissue, mean ± S.D.	
	Normal (x 10^{-3})	Treated (x 10^{-3})	Normal (x 10^{-3})	Treated (x 10^{-3})
Day 1	0.15 ± 0.01	0.14 ± 0.02	0.03 ± 0.01	0.03 ± 0.01
Day 2	0.13 ± 0.03	0.15 ± 0.02	0.05 ± 0.03	0.05 ± 0.01
Day 3	0.14 ± 0.03	0.17 ± 0.04	0.07 ± 0.02	0.08 ± 0.02
Day 4	0.20 ± 0.04	0.21 ± 0.01	0.10 ± 0.04	0.11 ± 0.04
Day 5	0.17 ± 0.02	0.20 ± 0.03	0.12 ± 0.03	0.13 ± 0.05
Day 6	0.16 ± 0.05	0.17 ± 0.02	0.08 ± 0.02	0.09 ± 0.01

Values are the means of 5 determinations.

Table 10 b. Changes in the AAT activity in fat body

Larval age	AAT units/mg fresh tissue, mean ± S.D.		AAT units/total tissue, mean ± S.D.	
	Normal (x 10^{-6})	Treated (x 10^{-6})	Normal (x 10^{-6})	Treated (x 10^{-6})
Day 1	2.14 ± 0.22	1.01 ± 0.15	7.62 ± 1.42	48.71 ± 3.99
Day 2	2.04 ± 0.25	0.80 ± 0.11	12.53 ± 2.33	71.42 ± 11.21
Day 3	1.48 ± 0.31	0.63 ± 0.08	21.75 ± 3.51	112.65 ± 15.50
Day 4	1.10 ± 0.10	0.69 ± 0.07	19.64 ± 2.68	153.24 ± 13.22
Day 5	0.67 ± 0.07	0.38 ± 0.05	16.38 ± 5.42	132.73 ± 15.11
Day 6	0.24 ± 0.02	0.34 ± 0.10	5.71 ± 1.52	82.44 ± 13.21

Values are the means of 5 determinations.

Figure 10a - I

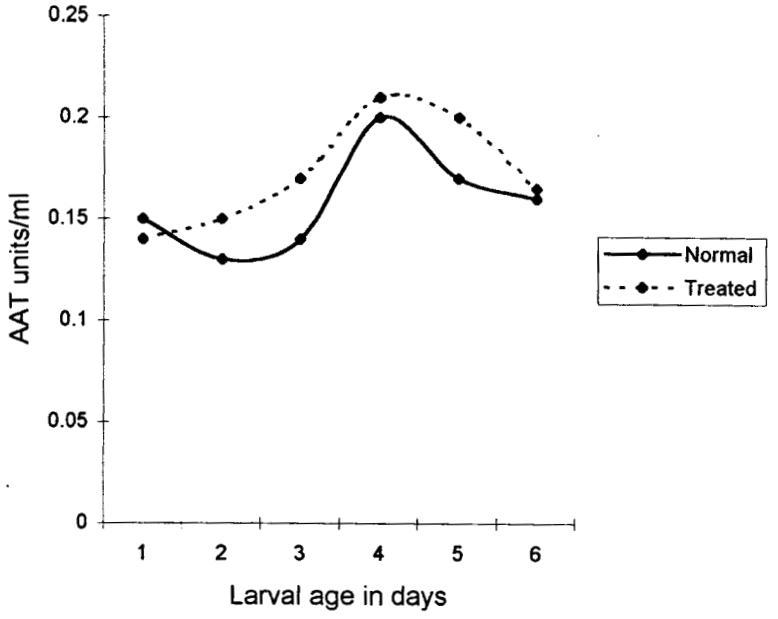

Figure 10a - II

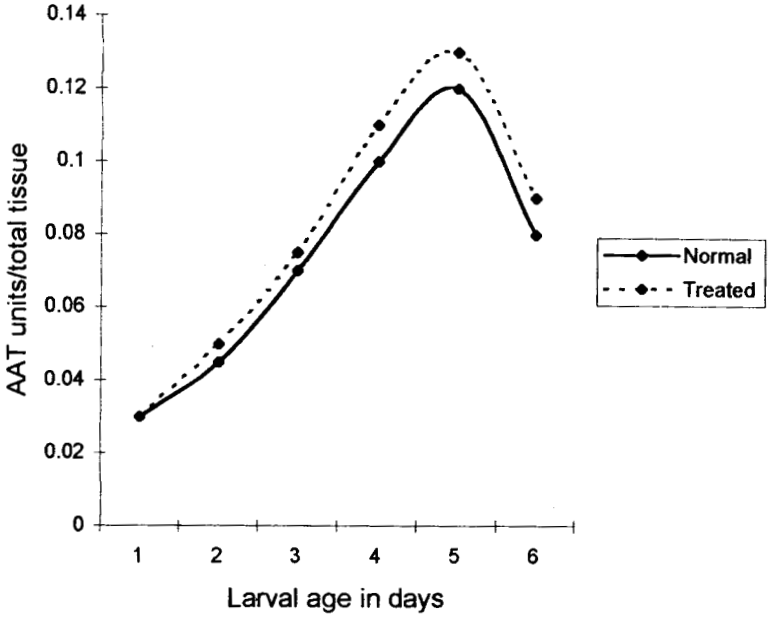

Figure 10a. Changes in the AAT activity in haemolymph

I - Changes per unit volume of haemolymph

II - Changes per total haemolymph

Figure 10b - I

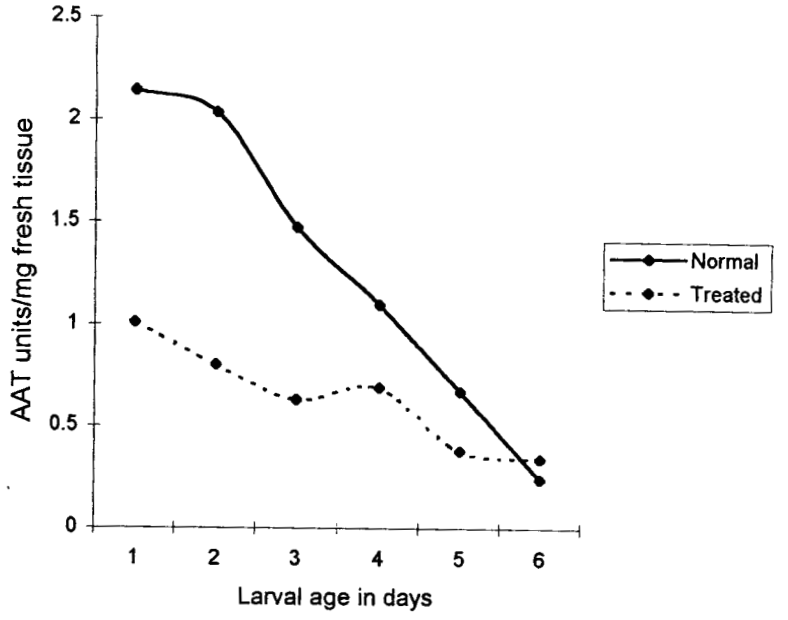

Figure 10b - II

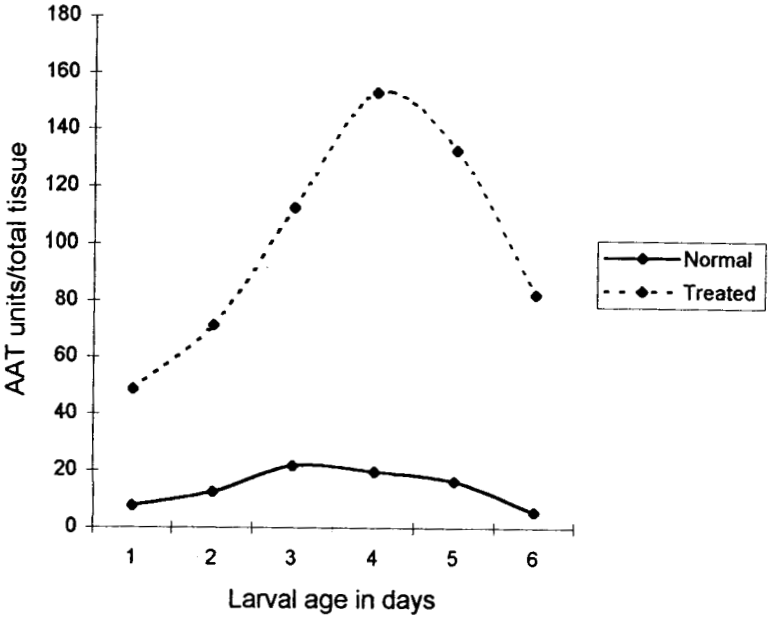

Figure 10b. Changes in the AAT activity in fat body

I - Changes per unit weight of fat body

II - Changes per total fat body

The AAT activity estimated per unit volume haemolymph exhibited a peak on Day 4 and the rise to this peak was more gradual. The value dropped down on Day 5 and steadied till Day 6. The AAT activity of the total haemolymph of normal larvae increased during the larval development and reached a peak on Day 5 but declined on Day 6.

The treated larva showed a similar pattern as that of the normal but with a slightly higher level of action. The activity of AAT on the basis of unit volume of haemolymph in the treated larvae was about 15-18% whereas; it showed a decrease from 5-8% in total tissue values.

The AAT activity per unit weight of fat body showed a sharp decline from Day 1 to Day 6. But, the fat body AAT activity of the total fat body of normal larvae increased from Day 1, reached a maximum on Day 3 and declined gradually towards pupation.

The treated larvae showed an elevated level of AAT activity than the normal for total tissue values. The AAT activity reached a peak on Day 4 and the increase in the activity was almost 6-12 fold than normal. The values per unit weight of fat body showed an opposite picture with the level of AAT activity less than the normal activity but with the same pattern. Here the values tend to remain steady from Day 4.

Alanine amino transferase (AlAT) activity

The AlAT activity in the haemolymph and fat body of the normal and treated larvae during the development of the fifth instar is presented in Table 11a & 11b and Figure 11a & 11b.

Table 11a and 11b

Figure 11a and 11b

The AlAT activity in the haemolymph of normal and treated larva increased form Day 1 and reached a maximum on Day 5 and declined on Day 6. The AlAT activity on the basis of unit volume of haemolymph went on increasing till Day 6 and were more or less similar.

In the fat body, the AlAT activity per unit weight of fat body decreased sharply from Day 1 to Day 6. But, for total values the AlAT activity increased from Day 1 and reached a peak on Day 3 and then declined gradually to Day 6. The treated larvae showed a very high activity of AlAT, though they followed the same pattern as that of the normal.

Glucose

The glucose content of haemolymph and fat body of normal and treated larvae during the development of the fifth instar is shown in Table 12a & 12b and Figure 12a & 12b.

Table 11 a. Changes in the AlAT activity in haemolymph

Larval age	AlAT units/ml, mean ± S.D.		AlAT units/total tissue, mean ± S.D.	
	Normal (x 10^{-3})	Treated (x 10^{-3})	Normal (x 10^{-3})	Treated (x 10^{-3})
Day 1	6.60 ± 0.51	8.32 ± 0.18	1.15 ± 0.09	1.61 ± 0.08
Day 2	7.51 ± 0.31	7.51 ± 0.20	2.58 ± 0.08	2.40 ± 0.17
Day 3	9.20 ± 0.47	9.64 ± 0.34	4.61 ± 0.18	4.24 ± 0.28
Day 4	14.22 ± 0.38	15.23 ± 0.31	7.12 ± 0.24	7.90 ± 0.35
Day 5	16.03 ± 0.42	18.55 ± 0.52	11.22 ± 0.38	11.83 ± 0.59
Day 6	19.31 ± 0.31	20.82 ± 0.75	10.23 ± 0.42	11.03 ± 0.71

Values are the means of 5 determinations.

Table 11 b. Changes in the AlAT activity in fat body

Larval age	AlAT units/mg fresh tissue, mean ± S.D.		AlAT units/total tissue, mean ± S.D.	
	Normal (x 10^{-6})	Treated (x 10^{-6})	Normal (x 10^{-6})	Treated (x 10^{-6})
Day 1	2.90 ± 0.51	1.50 ± 0.02	10.71 ± 1.23	72.33 ± 2.38
Day 2	2.69 ± 0.34	1.52 ± 0.01	15.93 ± 0.91	133.82 ± 3.45
Day 3	2.01 ± 0.28	1.31 ± 0.02	29.52 ± 2.12	232.41 ± 2.12
Day 4	0.13 ± 0.07	0.90 ± 0.01	23.81 ± 2.35	199.84 ± 2.68
Day 5	0.63 ± 0.01	0.37 ± 0.03	15.30 ± 1.24	129.22 ± 3.14
Day 6	0.22 ± 0.01	0.09 ± 0.02	5.22 ± 1.02	21.84 ± 2.10

Values are the means of 5 determinations.

Figure 11a - I

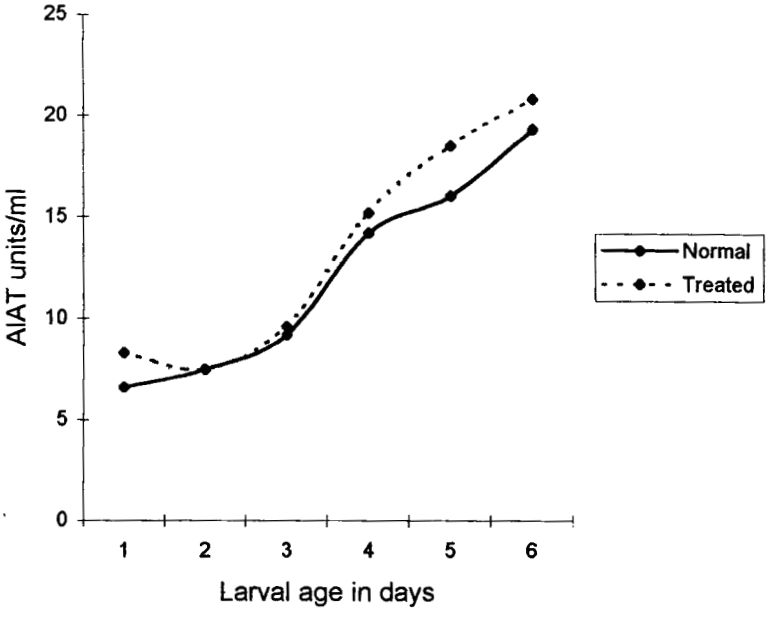

Figure 11a - II

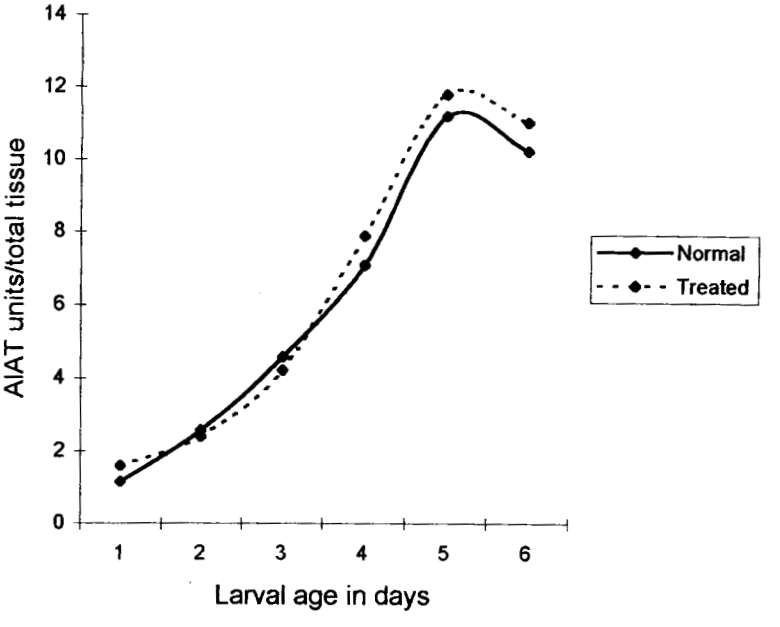

Figure 11a. Changes in the AlAT activity in haemolymph

I - Changes per unit volume of haemolymph

II - Changes per total haemolymph

Figure 11b - I

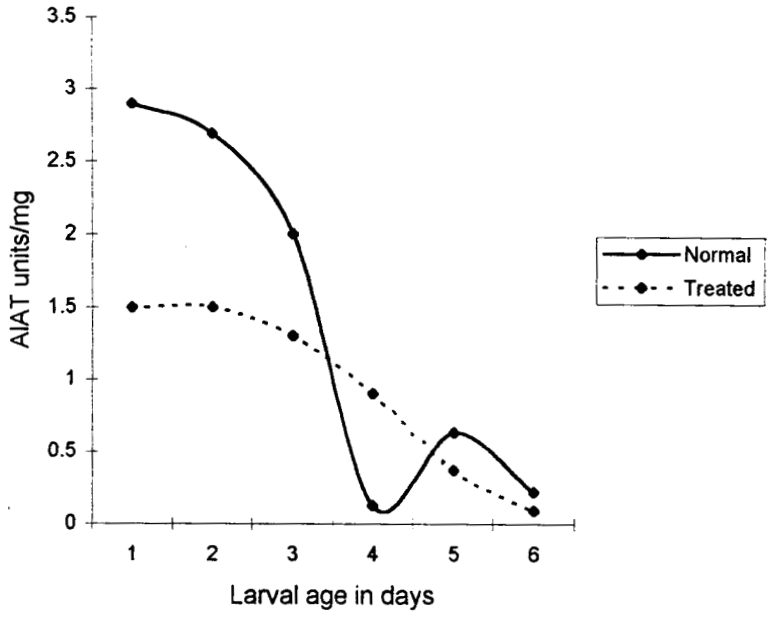

Figure 11b - II

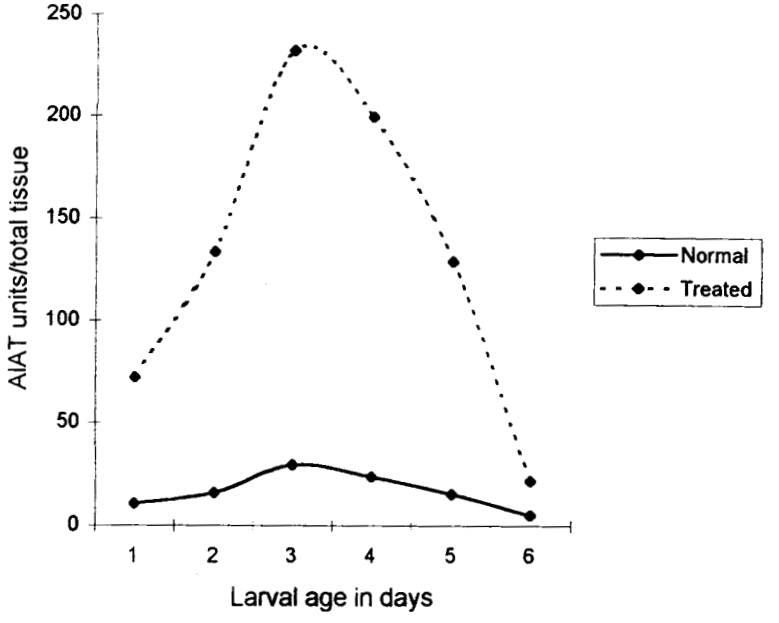

Figure 11b. Changes in the AlAT activity in fat body

I - Changes per unit weight of fat body

II - Changes per total fat body

Table 12a and 12b

Figure 12a and 12 b

The glucose content of the haemolymph of normal larvae increased gradually from Day 1 to Day 4 then rose sharply to Day 5 followed by a dip on Day 6. The treated larvae showed a similar pattern except that the levels were comparatively lower than normal. The glucose levels in the treated larvae were almost 30-40% reduced than in the normal larvae.

The glucose content in the fat body per unit weight of fat body in normal larvae rose sharply from Day 1 to Day 4 and then declined to Day 6. A similar pattern was observed when estimated in the total fat body. The values per unit weight of fat body in treated larvae increased sharply on Day 5, with a level greater than that of the normal for that day. From Day 1 to Day 4 the levels were lower than those in the normal larvae. The treated larvae showed a very reduced level of glucose content in the total tissues though a rise was seen from Day 1 to Day 5. There was a 3-5 fold reduction in the level of glucose when the total tissue values were observed.

Creatinine

The creatinine content of the haemolymph and fat body of normal and treated larvae is given in Table 13a & 13b and Figure 13a & 13b.

Table 12 a. Changes in the glucose levels in haemolymph

Larval age	mg/ml, mean ± S.D.		mg/total tissue, mean ± S.D.	
	Normal	Treated	Normal	Treated
Day 1	0.79 ± 0.04	1.01 ± 0.20	0.14 ± 0.02	0.19 ± 0.02
Day 2	1.06 ± 0.08	1.32 ± 0.34	0.36 ± 0.01	0.42 ± 0.02
Day 3	1.14 ± 0.06	1.13 ± 0.27	0.57 ± 0.03	0.51 ± 0.03
Day 4	1.55 ± 0.08	1.04 ± 0.36	0.78 ± 0.04	0.54 ± 0.01
Day 5	3.47 ± 0.71	2.78 ± 0.71	2.43 ± 0.09	1.78 ± 0.08
Day 6	3.00 ± 0.48	2.11 ± 0.83	1.50 ± 0.05	1.12 ± 0.09

Values are the means of 5 determinations.

Table 12 b. Changes in the glucose levels in fat body

Larval age	mg/mg fresh tissue, mean ± S.D.		mg/total tissue, mean ± S.D.	
	Normal (x 10^{-3})	Treated (x 10^{-3})	Normal (x 10^{-3})	Treated (x 10^{-3})
Day 1	1.15 ± 0.20	0.74 ± 0.04	40.97 ± 5.14	35.62 ± 4.32
Day 2	4.10 ± 0.22	1.41 ± 0.09	252.21 ± 8.56	125.77 ± 8.57
Day 3	4.97 ± 0.44	1.32 ± 0.07	733.01 ± 9.74	236.02 ± 11.14
Day 4	6.16 ± 0.68	1.28 ± 0.04	1103.12 ± 12.86	284.16 ± 12.52
Day 5	3.59 ± 0.54	9.34 ± 1.05	875.83 ± 13.98	326.06 ± 13.68
Day 6	4.26 ± 0.71	7.92 ± 1.14	1009.90 ± 15.74	191.92 ± 15.24

Values are the means of 5 determinations.

Figure 12a -I

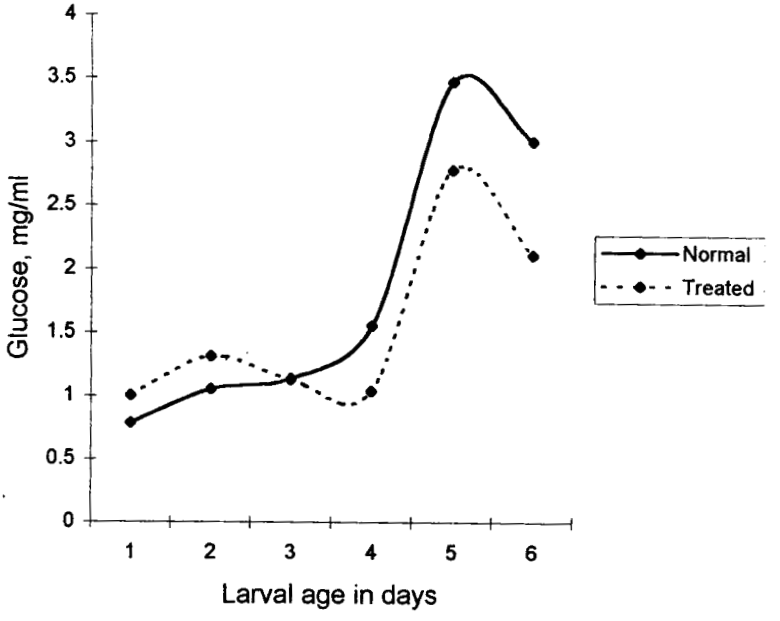

Figure 12a - II

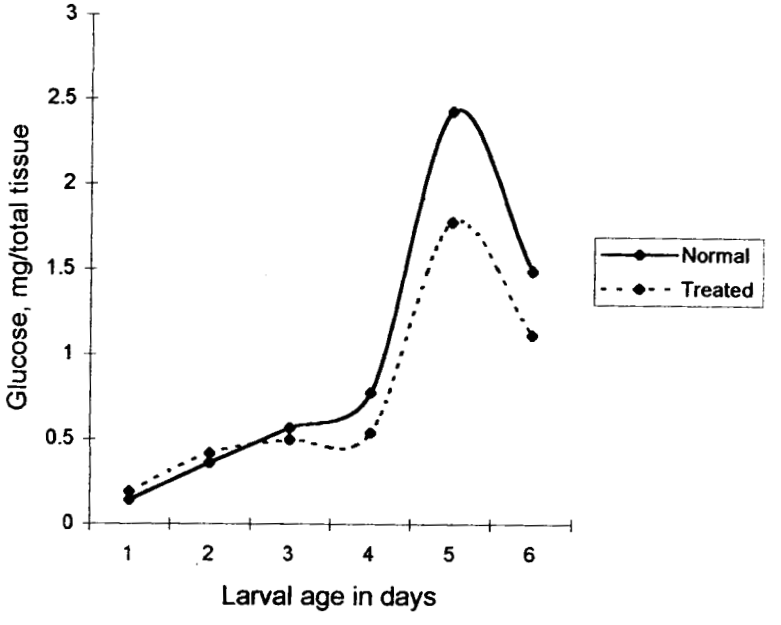

Figure 12a. Changes in the glucose levels in haemolymph

I - Changes per unit volume of haemolymph

II - Changes per total haemolymph

Figure 12b - I

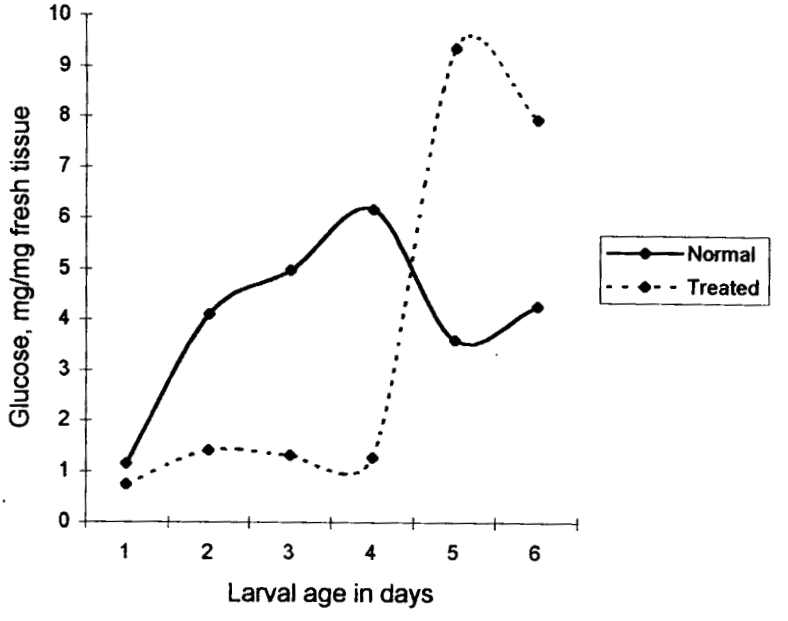

Figure 12b - II

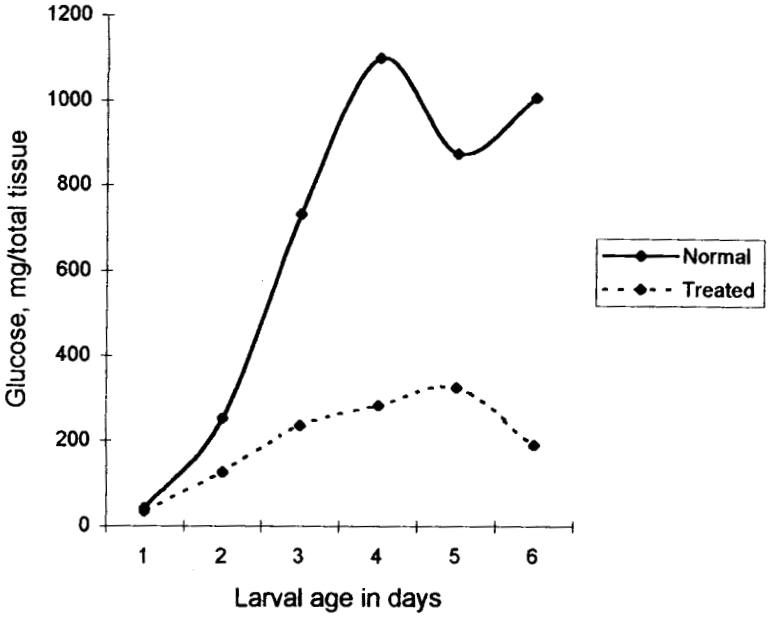

Figure 12b. Changes in the glucose levels in fat body

I - Changes per unit weight of fat body

II - Changes per total fat body

Table 13a and 13b

Figure 13a and 13b

In the haemolymph the creatinine content of the normal larvae steadily increased from Day 1 to Day 4 where it was seen at its maximum and gradually declined on Day 6. The treated larvae also showed a similar pattern for the creatinine content variation during the development but had lower levels than normal. The normal larvae had 30-50% higher levels of creatinine than the treated larvae in the case of total tissue values.

In the fat body, on the basis of unit weight, the pattern takes the form of bell-shaped curve starting from Day 2. The creatinine content of normal larvae started at a minimum on Day 1 and increased steadily from Day 2 to reach a maximum on Day 5 after which the level dropped on Day 6 in the total fat body.

The treated larvae showed a steady increase in creatinine content starting from a minimum on Day 1 and rising sharply from Day 5. The levels of creatinine in the treated larvae were more or less same as that of the normal, though on Day 6 it increased almost 3- fold, for total tissue values. In the case of per unit weight of fat body values, the values were lower than normal till Day 4.

Table 13 a. Changes in the creatinine levels in haemolymph

Larval age	mg/ml, mean ± S.D.		mg/total tissue, mean ± S.D.	
	Normal	Treated	Normal	Treated
Day 1	0.096 ± 0.003	0.0033 ± 0.002	0.02 ± 0.002	0.00062 ± 0.008
Day 2	0.15 ± 0.002	0.1 ± 0.004	0.05 ± 0.004	0.032 ± 0.005
Day 3	0.20 ± 0.008	0.12 ± 0.004	0.09 ± 0.008	0.062 ± 0.007
Day 4	0.25 ± 0.005	0.22 ± 0.003	0.13 ± 0.02	0.096 ± 0.004
Day 5	0.15 ± 0.007	0.09 ± 0.001	0.11 ± 0.03	0.089 ± 0.003
Day 6	0.16 ± 0.005	0.06 ± 0.002	0.09 ± 0.01	0.057 ± 0.002

Values are the means of 5 determinations.

Table 13 b. Changes in the creatinine levels in fat body

Larval age	mg/mg fresh tissue, mean ± S.D.		mg/total tissue, mean ± S.D.	
	Normal (x 10^{-3})	Treated (x 10^{-3})	Normal	Treated
Day 1	0.15 ± 0.01	0.074 ± 0.04	0.01 ± 0.002	0.0036 ± 0.003
Day 2	0.067 ± 0.03	0.057 ± 0.01	0.0041 ± 0.006	0.0051 ± 0.008
Day 3	0.15 ± 0.02	0.11 ± 0.06	0.022 ± 0.001	0.019 ± 0.005
Day 4	0.25 ± 0.05	0.15 ± 0.03	0.044 ± 0.010	0.032 ± 0.010
Day 5	0.25 ± 0.02	0.184 ± 0.02	0.061 ± 0.008	0.064 ± 0.006
Day 6	0.12 ± 0.08	0.29 ± 0.05	0.027 ± 0.003	0.072 ± 0.002

Values are the means of 5 determinations.

Figure 13a - I

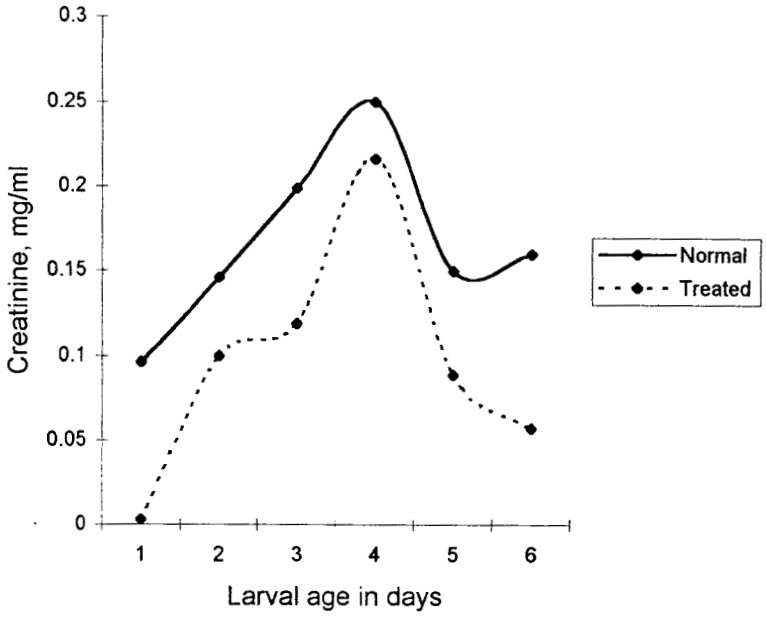

Figure 13a - II

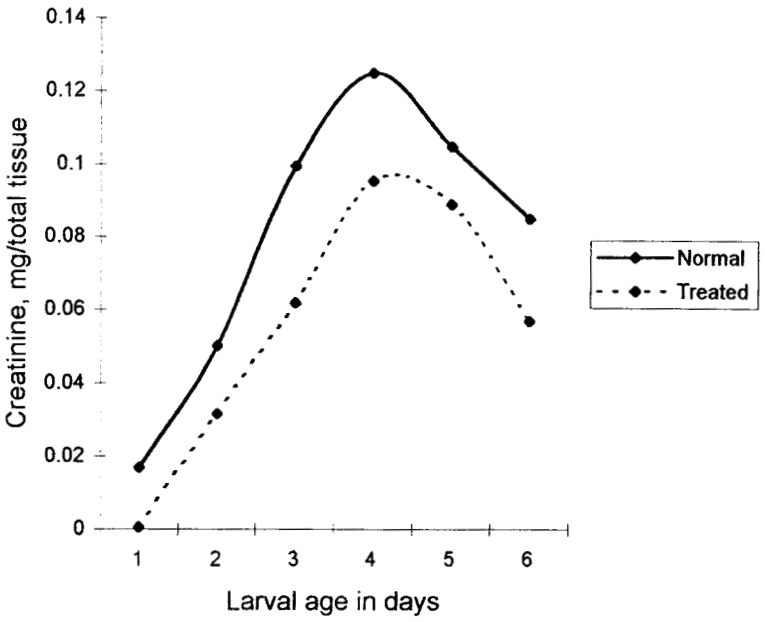

Figure 13a. Changes in the creatinine levels in haemolymph

I - Changes per unit volume of haemolymph

II - Changes per total haemolymph

Figure 13b - I

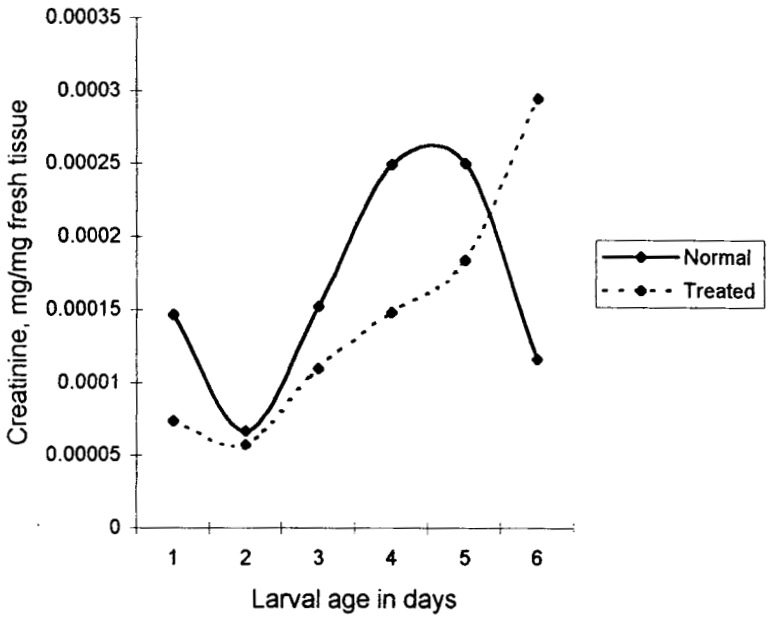

Figure 13b - II

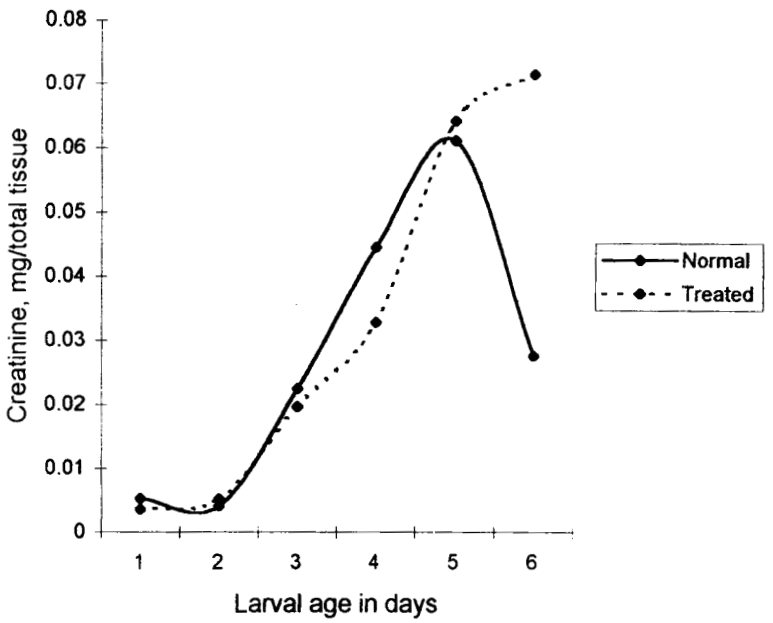

Figure 13b. Changes in the creatinine levels in fat body

I - Changes per unit weight of fat body

II - Changes per total fat body

Urea

The urea levels in the haemolymph and fat body of the normal and treated larvae is presented in Table 14a & 14b and Figure 14a & 14b.

Table 14a and14b

Figure 14a and 14b

In the haemolymph, the urea levels in the normal larvae decreased from Day 1 to Day 6 where it almost comes to zero on the basis of unit volume of haemolymph. The urea levels in the total haemolymph increased gradually from Day 1 to Day 3 and then decreased sharply to reach a minimum value by Day 6. The urea level in treated larvae was also lower than the normal larvae at its peak values by about 10-30% in the total tissue values. The treated larvae also showed a variation in their total tissue values similar to that of normal larvae, but the decline from Day 3 was comparatively gradual than the normal.

In the fat body, when the values per unit weight of fat body was considered, the urea level was seen to fall sharply from Day 1 to Day 6 but this increased from Day 2 steadily to reach a peak on Day 4 and then dropped sharply to reach a minimum value by Day 6 for total fat body.

Table 14 a. Changes in the urea levels in haemolymph

Larval age	mg/ml, mean ± S.D.		mg/total tissue, mean ± S.D.	
	Normal	Treated	Normal	Treated
Day 1	1.13 ± 0.10	1.12 ± 0.09	0.20 ± 0.02	0.21 ± 0.04
Day 2	0.91 ± 0.11	0.95 ± 0.04	0.31 ± 0.03	0.01 ± 0.009
Day 3	0.75 ± 0.09	0.73 ± 0.03	0.38 ± 0.05	0.02 ± 0.004
Day 4	0.70 ± 0.05	0.51 ± 0.02	0.35 ± 0.04	0.03 ± 0.009
Day 5	0.07 ± 0.01	0.22 ± 0.02	0.05 ± 0.009	0.06 ± 0.008
Day 6	0.0021 ± 0.009	0.09 ± 0.01	0.0011 ± 0.009	0.07 ± 0.01

Values are the means of 5 determinations.

Table 14 b. Changes in the urea levels in fat body

Larval age	mg/mg fresh tissue, mean ± S.D.		mg/total tissue, mean ± S.D.	
	Normal (x 10^{-3})	Treated (x 10^{-3})	Normal (x 10^{-3})	Treated (x 10^{-3})
Day 1	0.31 ± 0.02	0.13 ± 0.02	11.06 ± 0.24	6.25 ± 0.22
Day 2	0.16 ± 0.04	0.14 ± 0.03	9.98 ± 0.36	12.49 ± 0.45
Day 3	0.11 ± 0.05	0.15 ± 0.03	16.80 ± 0.44	26.14 ± 0.24
Day 4	0.12 ± 0.06	0.13 ± 0.04	20.61 ± 0.57	28.79 ± 0.73
Day 5	0.02 ± 0.04	0.04 ± 0.009	3.94 ± 0.25	14.33 ± 0.81
Day 6	0.01 ± 0.009	0.04 ± 0.01	2.90 ± 0.19	10.57 ± 0.52

Values are the means of 5 determinations.

Figure 14a - I

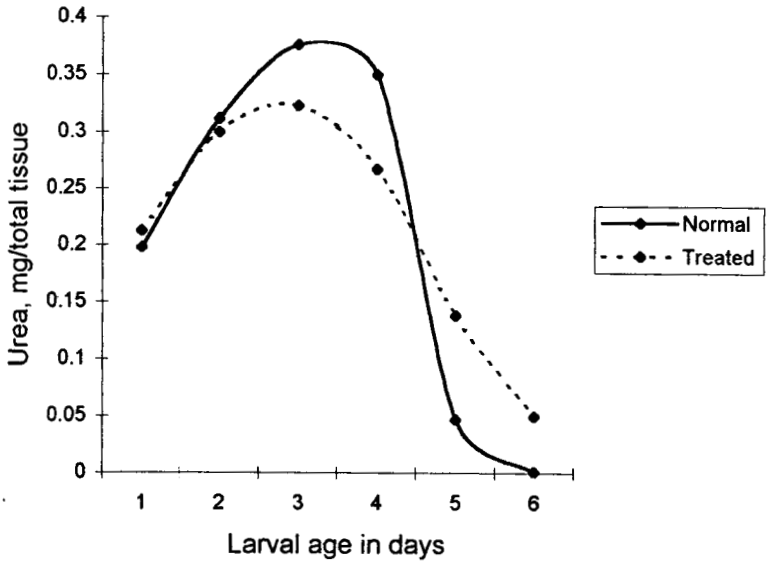

Figure 14a - II

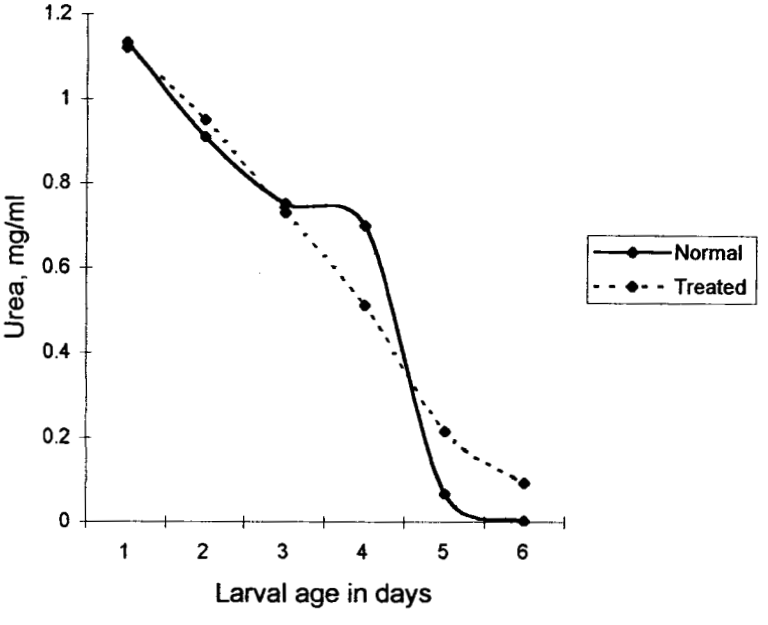

Figure 14a. Changes in the urea levels in haemolymph

I - Changes per unit volume of haemolymph

II - Changes per total haemolymph

Figure 14b - I

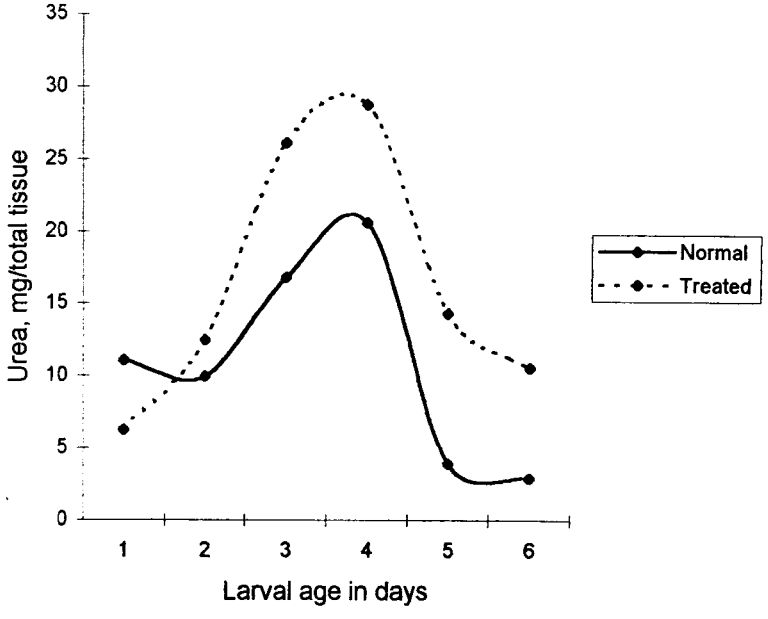

Figure 14b - II

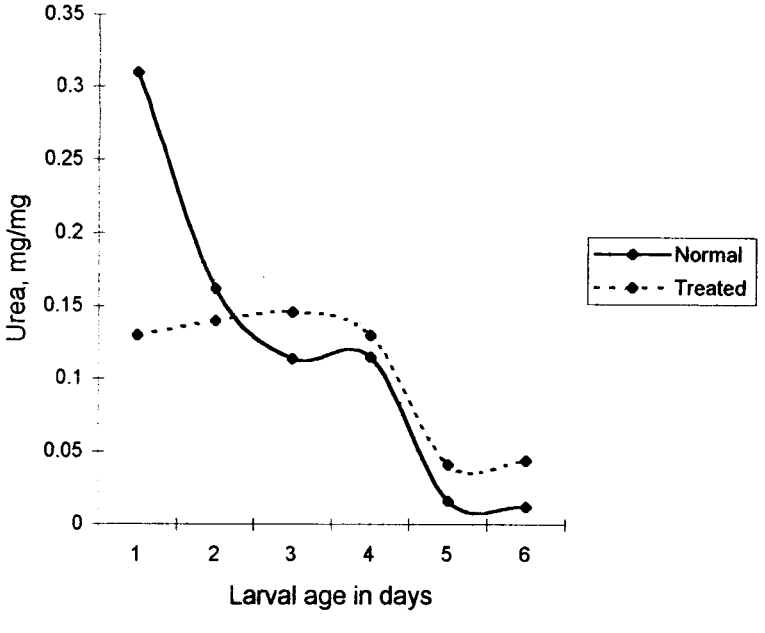

Figure 14b. Changes in the urea levels in fat body

I - Changes per unit weight of fat body

II - Changes per total fat body

The urea level, when per unit weight of fat body values were considered, is higher than the normal though it starts at a lower level on the first day. The same was observed when total tissue values were considered. The treated larvae showed an almost 40-80% increase in urea levels, and towards the final stages the increase was 4-fold.

DISCUSSION

DISCUSSION

Growth rate pattern

In the silkworm, *Bombyx mori*, the final larval state 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 (Hugar and Kaliwal, 1998). In the present investigation the growth rate of the final instar was studied in both the normal and treated larvae, which consisted of six days, the sixth day being the time when the larvae stopped feeding and started spinning. Though there was no gross difference between the normal and treated larvae, it was found that there was a delay for the treated larvae to start spinning and is indicative of a tendency of the treated larvae to retain its juvenile form.

The changes in the total fresh weight of the larvae denoted that they attained their full size on Day 5 and no active growth takes place afterwards. In the treated larvae there was a 7-8% increase in body weight and the larvae appear bigger and stouter than normal. The larval cuticle shows black pigmentation, more on the thorax region than on the sides of the body in the treated larvae. This could be because of the formation of lipid pigments from tyrosine. Both prooxidants and

antioxidants may enhance lipid pigment formation, the former by enhancing lipid oxidation, the latter by enhancing copolymerization, which terminates the oxidation chain (Barr-Nea & Wolman, 1977). The black pigmentation persisted till the larvae begin to start spinning the cocoon.

The rate of food consumption and the rate of excretion (being proportional) showed a maximum on Day 3. Regardless of this, the fresh weight of the body is maximum on Day 5. The silk gland as an organ grows at a much faster pace than the rest of the body (Reddy and Benchamin, 1989; Reddy *et al.*, 1995). The rate of food consumption in the treated larvae is about 13-17% more than that in the normal larvae. This increase in food intake explains the increase in the fresh weight of the treated larvae.

The changes in the volume of haemolymph and the weight of the 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 of haemolymph and on the basis of unit weight and total weight of the fat body respectively. Since haemolymph is the immediate environment of the organs in the silkworm, the metabolic activity and the development are affected by the haemolymph Nakayama *et al.* (1990). Fat body 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.*, 1981). The functional role of the fat body in insects is

somewhat analogous to the combined functions of liver and adipose tissue in mammals (Sohal, 1985).

There was no significant change in the volume of haemolymph in the treated larvae. But, the weight of fat body showed a 20-45% increase in the treated larvae. This may be due to the storage of materials into the fat body at a higher rate in the treated larvae than in the normal.

Total proteins and total free amino acids

Proteins are the chief organic constituents of the cell. These macromolecules are concerned with the regulation of all biochemical events in the organism (Harper *et al.,* 1993). Accumulation of proteins in haemolymph and fat body during final instar insect development has been established by Chen (1985). It has been established that the proteins are synthesized in the fat body and released into the haemolymph, which are subsequently sequestered into the fat body and stored there depending upon the physiological condition of the animal.

The various aspects of the haemolymph proteins in insects have been studied by many investigators (see review, Buck, 1953; Wyatt, 1961; Chen, 1966, 1978; Jeaniaux, 1971; Price, 1973; Wyatt and Pan, 1978). 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 & Jeuniaux, 1974). The total protein levels in both the normal and treated larvae is seen to

increase during the development of the final instar and then decrease after reaching a maximum on Day 5. This is in conformation of the general observation that the concentration of protein increases during the larval stages and decreases at the pupal and adult stages (Engle and Woods, 1960; Wyatt, 1961; Chen, 1966, 1971; Jeuniaux, 1971; Florkin and Jeuniaux, 1974). When the total proteins per unit volume of haemolymph were observed, it showed a continuous increase in both the normal and treated larvae, during their development. This pattern changed when the amount was calculated per larva. This variation is approximated in time with the variation of the haemolymph volume. So, the total protein amount decreased at the end of larval life irrespective of their high concentration observed when estimated per unit volume. The sequestration of the proteins into the fat body from the haemolymph obviously results in a decrease in the concentration of the proteins in the haemolymph.

The treated larvae showed a higher level of total protein content in the haemolymph. There was a 20-50% increase in the protein levels initially but towards the end of the larval life the increase was only 10-20%.

A few studies have been made on the developmental variation of fat body proteins in relation to physiological changes in the larva of insects (Collins, 1969; 1974; 1975; Price, 1969; 1973; Chippendale, 1970; Collins and Downe, 1970; Martin *et al.*, 1971; Patel, 1971; Lafont *et al.*, 1975; Terra *et al.*, 1975; Dortland, 1978; Dortland and de Kork, 1978; Tojo *et al.*, 1978). The variation in the content

of fat body proteins during the developmental stages, when calculated per unit weight, showed that there was a slight increase till Day 4, then increased from Day 5. The variation was striking when the amount was calculated for total larval fat body where there was an 8-fold increase during the development. Accumulation of protein in the last larval fat body has been demonstrated in many insects (Kilby 1963; Karnavar and Nayar, 1973; Price, 1973; Wyatt, 1975; Chen, 1978).

The treated larvae also showed an increase in total protein content in the fat body when values for total larval fat body were seen. But, after Day 5, it showed a decline to a lower value than normal on Day 6. This shows that there is a reduction in the total protein content after Day 5. When calculated per unit weight, the variation in the content of fat body proteins was different from the normal. Contrary to the idea of protein accumulation, the protein levels showed a gradual decline from Day 1 to Day 6. This was masked when total larval fat body was considered because of the increase in the weight of fat body in the larvae during development. A decreased protein content in the fat body in general may be because some of the proteins except the ones stored for the future use of adult development might have been channelized to haemolymph through which it is transported to silk gland. Such high protein content according to the treatment of tyrosine also is obvious in the haemolymph.

A complementary picture of concentration variation was obvious in the free amino acid levels also. It has been generally observed that the free amino acids are

much higher in the larva than pupa or adult. Investigations reveal that the amount of free amino acids in insects is about 5 to 20 times higher than other invertebrates. The subject has been reviewed by Chefurka (1965), Corrigan (1970), Chen (1962, 1966, 1971), Jeuniaux (1971) and Florkin and Jeuniaux (1974).

The occurrence of a high titre of amino acid in the haemolymph is a universal phenomenon in insects. A change in the amino acid pool will directly influence the protein turnover, that is, the synthesis and degradation of proteins, and thus obviously, reflect the physiological state of the organism. The total free amino acid levels increased form Day 1 to Day 5 and then declined. The increase in the total amount of amino acid was proportional to the growth of the larva. The decrease in the material per unit volume of haemolymph after Day 4, apparently indicates that during this period there is a positive balance in protein storage. The observed changes in the total protein content in the normal larva were in tune with the above observations.

The treated larvae showed a similar pattern of total free amino acid during the development in haemolymph, but had comparatively lower levels than normal larvae. There was a 10-20% reduction in the level of total free amino acids, which could indicate for the higher protein turnover in the haemolymph of the treated larvae. This was also in tune with the changes observed in the total protein content in the treated larvae.

The interest in the investigation of free amino acids in the fat body is due to the fact that the tissue is an active site of the intermediary metabolism of amino acids (Kilby, 1963). On the basis of the unit weight of the tissue, the total free amino acid content in the fat body of normal larvae showed an increase up to Day 5 but declined thereafter. The changes in the total content of free amino acids in the total tissue (haemolymph and fat body) of the larvae of *Spodoptera mauritia* showed a similar pattern of variation during the development (Lazar and Mohamed, 1988). This decline corresponds with protein synthesis in the larvae. In the treated larvae, the total free amino acid content per unit weight fat body showed increase up to pupation. This indicates the breakdown of protein, which has been observed in the fat body of the treated larvae. However, experiments dealing with the biosynthesis of amino acids from [^{14}C] glucose in *Drosophila* suggest that the free amino acid pool is not identical with that available for protein synthesis as there are distinct differences in specific activities between free and protein - bound amino acids (Widmer, 1973). Free amino acids may also have some important functions other than serving as the precursors of haemolymph and fat body proteins.

Aspartate amino transferase (AAT) activity and Alanine amino transferase (AlAT) activity and Glucose levels

The activity of AAT and AlAT was studied because the proteins synthesis requires a balanced amino acid pool and transamination is one of the chief mechanisms which functions as a regulator of this pool (Reddy *et al.*, 1991).

Higher transaminase implies enhanced mobilization of fee amino acids into transamination activities. The AlAT activity forms a general index of amino acid breakdown and AAT marks the mobilization of amino acids into gluconeogenesis (Adibi, 1968; Davidson and Longslow, 1975). Both amino acid oxidases and transminases have been demonstrated in various tissues for a number of insects. Transaminase activity has been demonstrated in various insects (Price, 1961; Martignoni and Milstead, 1967; Kilby and Neville, 1957; Desai and Kilby, 1958 and Chen and Bachmann-Diem, 1964). The most extensive analyses of the transaminase reactions are those of on *Schistocerca*, on *Calliphora*, and on *Drosophila*. The *in vitro* studies of fat body homogenates have revealed that transamination involving glutamate, aspartate, alanine and their corresponding keto acids is always most active in insects. Compared to mammals, there is very little information on the structure and function of the amino transferases, which are known to play a key role in the intermediary metabolism of amino acids (Candy, 1985).

The haemolymph of normal larvae showed an increase in AlAT activity from Day 1 to Day 5 and then declined on Day 6. McAllan and Chefurka (1961) showed that the transaminase activity in the cockroach and the housefly increases during larval development and adult differentiation parallel to the increase in protein synthesis. AlAT activity increases rapidly during larval growth in *Drosophila nigromelanica* and then declines to a minimum at the middle of pupal

development. As both growth and differentiation are closely related to protein synthesis, the elevated activity of transaminase in the growing larva appears normal. The same profile has been reported for AAT during development of the housefly, *Musca domestica* (McAllan and Chefurka, 1961). AlAT activity was higher in the feeding insects than the non-feeding insects as suggested by Wadhwa *et al* (1986). So the increase in levels during development corresponds to the feeding stage of the larva in both normal and treated specimens. The treated larvae showed a similar pattern and their levels were more or less the same initially when total volume of haemolymph were observed. However on the basis of unit volume haemolymph, they showed a 5-10% increase than the normal larval activity. This suggests a marginal increase in amino acid breakdown, though not significant as seen earlier by the amino acid titre in the haemolymph of treated larvae.

In the fat body of the normal larvae, the AlAT activity showed a bell-shaped curve which suggests that amino acid breakdown after Day 3 declines. The AlAT activity of the treated larvae in the fat body showed a 7-8% increase in activity than normal, keeping in tune with the same pattern of the normal, when the total fat body values were analyzed. However AlAT activity on the basis of total fat body showed an increase with the growth of the larva. The most important physiological functions of L-alanine amino transferase are the maintenance of the amino acid pool at a proper level for protein synthesis (Meister, 1965), the supply of metabolites for

energy metabolism (Sacktor, 1974) and the catalysis of interactions between protein and carbohydrate metabolism (Katunuma *et al.*, 1968).

The AAT activity in the haemolymph of normal larvae increases from Day 1 to Day 5 then declines on Day 6. This peak is seen on Day 4 when per unit volumes haemolymph values are seen. In both cases, the AAT activity of the treated larvae is higher by about 7-13% than the normal larvae. This suggests increased amino acid breakdown for gluconeogensis.

As silk production advances, the activity of the enzymes such as AAT and AlAT involved in the transamination process increases accordingly (Klunova *et al.*, 1976). This is clear from the variation in the activity of these enzymes. The activity of these enzymes can be well accounted for when the glucose levels in the haemolymph and fat body of the larvae are analyzed. In the haemolymph, the glucose levels in both normal and treated larvae, increases gradually from Day 1 to Day 4 and then rises sharply to Day 5. This was followed by a decline on Day 6. This increase in glucose levels suggests an increase in gluconeogenesis, which corresponds with the increasing level of AAT/AlAT activity. Alanine amino transferase and aspartate amino transferase activities in the final instar larvae of *Spodoptera mauritia* showed an increase with the larval growth, accompanied by a sharp rise in the level of glucose in the larval fat body and haemolymph (Lazar and Mohamed, 1998). This indicated an elevated level of gluconeogenesis during development. The same can be said of the fat body glucose levels.

The treated larvae showed a 15-30% increase in glucose levels than normal larvae till Day 2 and then the levels reduced by 30-40% till Day 6. In the fat body, the reduction of the glucose levels in the treated larvae was more pronounced, i.e., almost a 3-4 fold reduction was observed. Hence, this does not correlate with the increased activity of AAT/AlAT in the treated larvae. This suggests that either the uptake of glucose has increased or the breakdown of amino acids.

Creatinine and urea

Though the excretion of creatinine has been studied in a few insects, its presence in the haemolymph has never been investigated. Creatinine has been reported in the final instar larvae of *Orthaga exvinacea* and *Spodoptera mauritia* (Lazar and Mohamed, 1991; Kuzhivelil and Mohamed, 1997). El Allaf *et al.* (1984) assessed the effects of age and sex on serum urea, serum creatinine and on creatinine clearance.

The creatinine levels in the haemolymph increased during the development of the larvae, reached a maximum on Day 4 and then started declining. In the fat body, the maximum level was seen on Day 4 and the treated larvae showed no decline in the level. Though the treated larvae follow the same pattern of variation of the normal, its levels in the haemolymph showed a 20-50% reduction. In the fat body, initially, this difference shortens as the level increases even after Day 4. Creatinine generation rate is a possible indicator of protein nutritional status

(Shinzato *et al.*, 1997). Creatinine, urea and glucose levels have been shown to increase in concentration with age in humans (McPherson *et al.*, 1978).

The increasing level of creatinine in haemolymph and fat body is suggestive of their relationship with the active anabolic phases of the larva. The reduced level of creatinine in the treated larval tissues indicates a reduced turnover of creatinine as product of protein metabolism.

The occurrence of urea has been demonstrated in the haemolymph of a few insects (Buck, 1953; Chefurka, 1965; Cochran, 1975). The variation of the material found during the larval development was very conspicuous. Both in the normal and treated larvae, the urea levels drop progressively from Day 3 and Day 4 in the haemolymph. In the fat body, the urea levels show a similar pattern. But, the urea levels in the haemolymph of the treated larvae were reduced than that of the normal by 10-15% while in the fat body it showed an almost 4-fold increase than normal. Sumida *et al.* (1995, 1990) have also shown the urea concentrations tend to decline toward larval-pupal transformation in the haemolymph of *Bombyx mori*. The observed results are also similar to that presented by Lazar and Mohamed (1989).

Urea is a protein-unfolding agent that can accumulate to locally high concentrations in tissues of many organisms (David *et al.*, 1999). The reduced level of urea in the haemolymph of the treated larvae may be due to its active degradation or its transport to the fat body and for accumulation, which explain the

higher urea levels there. The higher level of urea in the fat body of treated larvae may also be due to the absence of degradation of urea.

Hydrogen peroxide level and catalase activity

The rate of prooxidant generation has been found to be associated with life expectancy (Sohal, 1991). Sohal and Sohal (1991) have also shown that aging in the housefly is associated with an increase in the rate of H_2O_2 generation by mitochondria. In the present work, the peroxide level in the haemolymph of both normal and treated larvae increases steadily during development and reaches a maximum on Day 5 after which it falls. Results from at least three different laboratories have shown that the rate of different laboratories have shown that the rate of O_2^- &/or H_2O_2 generation by rat heart mitochondria increases as a result of aging (Sohal *et al.*, 1990; Nohl & Hegner, 1978; Muscri *et al.*, 1990). An increase in H_2O_2 production was observed in the midgut of the haematophagous hemiptera, *Rhodnius prolixus* during its development (Pace *et al.*, 2001).

Although steady state level of oxidative stress depends on both prooxidant generation and antioxidant defenses, most of the studies pertaining to aging have focused on antioxidant defenses.

Catalase activity has been studied in a variety of insects (Seslija *et al.*, 1999; Dudas and Arking, 1995; Durusoy *et al.*, 1995; Orr *et al.*, 1992). A similar result was observed in the haemolymph of the normal and treated larvae. The activity rose

from Day 1 to Day 4 and then began to decline. In the fat body, the activity increased till Day 5 and then declined.

The catalase activity has been studied to increase with age and decrease during the latter part of life (Sohal *et al.*, 1990). There is a general accord that catalase activity tends to decline with age in insects. An approximately 40% decline in catalase activity was previously observed in the adult housefly during aging (Sohal *et al.*, 1984; Nicolosi *et al.*, 1973). However, studies on correlating catalase activity and lifespan have shown that complete lack or reduced level of catalase activity does not affect life span of flies (Orr *et al.*, 1992; Mackay and Bewley, 1989). An over expression of catalase (about 50%) had no effect on the life span of flies, nor did it improve their viability to an experimentally enhanced level of oxidative stress induced by paraquat intake or hyperoxia (Orr & Sohal, 1992). Hence, it has been concluded that age-related changes in antioxidant defenses and in levels of oxygen free radical reaction products are selective in nature and are quite variable in different species and tissues, however the level of oxidative stress tends to increase during aging (Sohal *et al.*, 1990).

The peroxide levels are seen to increase in the haemolymph and fat body of the treated larvae when compared to that of normal larvae. In the haemolymph, there is initially a 30-50% increase, but towards the end of the instar, the level reduces closer to that of normal larvae. In the fat body, there is an almost 2-fold increase throughout the development. The increased peroxide levels in the tissues

of the treated larva suggest the prooxidant nature of tyrosine. But, the catalase activity of the tissues of the treated larvae also showed a higher level than normal larvae. In the haemolymph, this increase was only 5-10%, but in the fat body, there was a 20-50% increase in catalase activity. This suggests that there has been corresponding increase in the antioxidant activity with an increase in the prooxidant generation.

The general increase in body weight and fat body of the treated larva. corresponding to its increased food intake, suggests a general increase in its metabolic potential. The relationship between metabolic rate and aging has been largely studied on the basis of oxygen consumption (Miquel *et al.*, 1976), ambient temperature (Rubner, 1908; Pearl, 1928; Farmer & Sohal, 1987) and physical activity (Brierly *et al.*, 1996). McArthur & Sohal (1982) have suggested that in the milkweed bug, length of life is apparently related to the rate at which a fixed metabolic potential is expended. This modulation of rate of aging by the metabolic rate was studied by elucidating *in vitro* lipid peroxidation and accumulation of fluorescent age pigment (FAP). In the present work, the metabolic rate was studied during development by estimating the levels of various important biochemical constituents like total protein, total free amino acids, glucose, creatinine, urea and also the activity of transaminase enzymes – AAT & AlAT. Correlating these aspects with H_2O_2 level and catalase activity possibly indicates the protein metabolic activities and amino acid metabolism during development.

Generally, all the parameters studied, have shown a pattern similar to that of H_2O_2/catalase levels. They showed an increase from Day 1 to Day 4/Day 5 and then declined, except for urea where the declination was more pronounced. This decline of all parameters, in general, could be due to the changes taking place internally as the larva nears the pre pupal/pupal stage. More so, in the case of the silkworm, where silk production and spinning marks the beginning of prepupal phase. In the treated larvae, the pattern of variation of these parameters are almost similar but their levels show variation when compared to that of the normal larvae. The activity of AAT & AlAT and total protein levels are more than the normal while the levels of total free amino acids, glucose, creatinine and urea are less than normal. It is particularly interesting to note that hydrogen peroxide levels and catalase activity were higher on the treatment of tyrosine to the larvae in spite of its known antioxidant activity.

Tyrosine is present in thyroid compounds like monoiodo-L-tyrosine, diiodo-L-tyrosine, etc. and these derivatives have been shown to have LDL-antioxidant (low density lipid) properties, their importance being related to their 4'-hydroxy diphenyl ether structure and depending upon the nature and the position of substituents in this structure (Chomard *et al.*, 1998). Thyroxine has been shown to effect changes in insect development and maturation through changes in ecdysteroid patterns; silkworm larvae fed on thyroxine showed an increase in pupal haemolymph protein (Thyagaraja *et al.*, 1993). Though the effect of tyrosine

treatment appears to be similar in this case, thyroxine has also been studied to elevate the free amino acid pool in the haemolymph plasma of tasar silkworm, *Antheraea mylitta* (Reddy *et al.,* 1994) that is not seen in the case of the tyrosine treatment.

Treatment with tyrosine has generally shown a higher level of metabolic activity. Though this could indicate an increase in silk production, this area has not been studied. Moreover, the increase in amino acid and glucose levels could indicate a higher transportation of these substances during spinning. Study of the metabolism of these compounds in the pupal stage may elucidate their roles in silk production.

CONCLUSIONS

CONCLUSIONS

The following conclusions were drawn from the present study. The development of the larvae was marked by changes in the levels of metabolites and their enzymes. The treated larvae showed an observable increase in body weight and appeared bigger and stouter than normal. Their larval cuticle showed black pigmentation, possibly because of the formation of lipid pigments from tyrosine. This increase in body weight was explained by the increase in food intake shown by the treated larvae. There was also a corresponding increase in the weight of fat body in the treated larvae, though there was no significant change in the volume of haemolymph. This indicates storage of materials in the fat body at a higher rate in the treated larvae than the normal.

The increase in the growth of the larvae on the administration of tyrosine was accompanied by an elevated level of metabolites, viz., total protein, AAT activity and AlAT activity while glucose, total free amino acids, creatinine and urea showed an overall reduction. It is particularly interesting to note that hydrogen peroxide levels and catalase activity were higher on the treatment of tyrosine to the larvae in spite of its known antioxidant activity. Generally, all the parameters studied, have shown a pattern similar to that of hydrogen peroxide and catalase levels. They showed an increase from Day1 to Day 5 and then declined. This decline of all parameters, in general, could be due to the changes taking place

internally as the larva nears the pre-pupal/pupal stage. In general, dietary supplementation of tyrosine to silkworm showed a similar effect to that of thyroxine like hormonal compounds.

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