CHAPTER IV

DISCUSSION

SUMMARY & CONCLUSION

DISCUSSION

Chromatographic analyses on free amino acids indicate that all of the amino acids commonly found in proteins have been identified in different regions of the alimentary canal, the fat body and the haemolymph of the two insects studied during present investigation. However, the pattern of free amino acids for each organ/tissue studied shows variations from each other. Among the three regions of the alimentary canal of <u>P.americana</u> the number of free amino acids is twelve in the foregut, thirteen in the midgut and again twelve in the hindgut. Though the number of amino acids ~~in~~ is more or less the same, but their pattern differs among these three regions of the gut. Of the several amino acids, only three of them i.e. alanine, phenylalanine and proline are seen in the foregut, midgut and the hindgut. The remaining amino acids are either common to foregut-midgut, midgut-hindgut or foregut-hindgut. Some amino acids are however, found to be organ-specific. For example, arginine, histidine, glutamine and lysine of the midgut are not seen in either foregut or the hindgut. In a similar way glutamate of the hindgut and valine of the foregut do not appear in either of the remaining organs. In spite of having the same number of amino acids as those of the foregut and the hindgut, the pattern of it varies in the fat body. Maximum number of free amino acids is seen in the haemolymph of the cockroach. Alanine, threonine and tryptophan are found to be common to both the fat body and the haemolymph. A similar variation in the pattern of free amino acids in different organs/tissues of S.gregaria is evident. The number of amino acids in the foregut and the midgut is twelve each while the hindgut was ten. It is interesting to note that among the two insects studied, the maximum number of amino acids i.e. fifteen is found in the fat body of S.gregaria but the haemolymph has only ten amino acids. Like cockroach, here also some amino acids are found to be more prevalent to some of the organs/tissues. Thus, a distinct organ/tissue-wise variation in the free amino acid pattern of the two insects is evident during present investigation. An abundant information is available on the occurence of free amino acids and their derivation among the different groups of insects. However, most of the information comes from the studies made on the haemolymph amino acids and very little is known about their distribution and metabolism in different tissues. A survey of twenty insect species belonging to seven orders has shown that, in general, all amino acids which are commonly contained in the protein have been identified either in tissue extracts or in the haemolymph (Chen,1962).

Some of the amino acids such as methionine, serine, hydroxyproline, tryptophan and ornithine, which are of much less common occurrence than the others, are found to be present in some organs/tissues of the insects studied presently. A similar accurrence of such rare components has been reported in a number of insect species (Wigglesworth, 1939, 1954; Buck, 1953; Wyatt, 1961; Gilmour, 1965). It has also been shown by several workers that some amino acid derivatives are characteristic of a single genus or a single order. Amino acid derivatives such as methionine sulfoxide has been found in P.americana (Stevens, 1961),ornithine, cystathionine and 3-hydroxykynurenine in Bombyx mori (Kondo, 1959, Makino et al, 1954), s-methylcysteine in Prodemia eridania (Prreverre and levenbook, 1960), α-tanthionine and α-cystatheonine in B. mori (Rajigopal Rao et al, 1967), α-amino butyric acid, homoarginine and hydroxyproline in Attacus ricini (Pant and Agrawal, 1964) and taurine in Musca domestica and Oncopeltus fasciatus (Pratt, 1950)

Both qualitative and quantitative changes in the contents of free amino acids have been followed in a large variety of insect species (Chen, 1966, 1985). However, most of the data deal with amino acid composition of the haemolymph (Wyatt, 1961). One of the characteristic features of the insect haemolymph is the high level of free amino acids and it is indeed highly variable with a given family or genus (Duchateau and Florkin, 1958). While abundent information is available on the free amino acids in the haemolymph, very little is known about their distribution in tissues other than the haemolymph.

Different organs of the alimentary canal and the tissues like fat body and the haemolymph of the two insects studied presently exhibit different pattern of amino acids in them. Similar reports on different tissues are available indicating both qualitative and quantitative changes in the free amino acids. In Bombyx it has been shown that there is unequal distribution of individual amino acids even though the total concentration appears to be the same (Briecteux- Gregoire and Florkin, 1958). In Prodenia larvae the total content of free amino acids is about the same in the fat body and haemolymph but it is found to be much lower in the gut (Levenbook, 1962). A considerable difference in the amino acid pattern of the muscles of various species and also in various tissues of the same species has been reported by Duchateau and Florkin (1955 a). Quantitative results obtained for the salivary glands and haemolymph in Drosophila larvae indicate that the concentration of glutamate is more than ten times higher in the salivary gland tissue than in the haemolymph, although the values for glutamine are much closer to each other (Chen, 1966). The differences for many other amino acids are also evident (Singh, 1982; Anderson, 1984).

From the numerous data so far available and the results obtained during the course of present investigation, it appears that no general pattern can be recognised in the variation of individual amino acids since many of them found in one region of the alimentary canal do not appear in the other region, but may be seen in either the fat body or the haemolymph.

Such variation in the free amino acid pattern may reflect the balance between synthesis and degradation of amino acids and also their requirement to fulfil different functional demands. However, it is almost imposible to give a synthetic picture of the amino acid pool in different groups of insects since there exists a great variation, both quantitative and qualitative, in the free amino acid composition within a given family or genus and even in a given species at different stages of life cycle (Florkin and Jeuniaux, 1964). Perhaps the most important function of free amino acids is that of protein synthesis for which all twenty common amino acids are required simultaneously. Lack of any one of the essential amino acids prevents protein synthesis and leads to an increased degradation of other amino acids (Horie and Inokuchi, 1978). The amino acids required for protein synthesis are derived from hydrolysis of food proteins and from the turn over of cell proteins. The ratios of amino acids required for protein synthesis can be modified by selective degradation and by synthesis of non-essential amino acids (Candy, 1985). Thus, from the foregoing it seems that it is certainly an oversimplification to interpret the amino acid pattern merely in terms of protein synthesis and degradation.

Organ/tissue-wise changes in the amino acid pattern may be interrelated from the view point of the metabolic activities. There is sufficient evidence indicating that they fulfill a additional metabolic functions. It has been suggested that proline plays the role of a readily available metabolizable energy reserve for the flight and movements (Thayer and Terian, 1970, Balogun,1974). Various amino acids are known to function as neural transmitters (Chen, and Widmer, 1968; Neal, 1975). Several amino acids participate in the synthesis of phospholipids. Other amino acids like alanine, glutamine, glycine, tryptophan etc are known to have important roles in the synthesis of eye pigment, cuticle proteins, chitin etc. The functional significance of some of the important amino acids has already been described in the foregoingsection of chapter I.

The detailed metabolic pathways of different amino acids in different tissues especially in the fat body and haemolymph of many insects have been reviewed by Kilby (1966), Schoffeniels and Gilles (1970), Jeuniaux (1971) and Chen (1985).

The nature of the diet largely contributes to the alteration of the free amino acid pattern within a given species, both quantitatively and qualitatively. Insects utilized for the present investigation show altogether different feeding habits and perhaps it may be one of the reasons to have a different amino acid pattern in them.

The electrophoretic analysis of proteins demonstrates that a number of fractions appear in normal or untreated cockroaches and grasshoppers respectively. However, the number of fractions in each of the organs and tissues is different in both the insects. The individual fractions also exhibit variations in their staining intensity as well as in the range of migration. Besides different regions of the alimentary tract, the hepatic caeca and Malpighian tubules are also analysed for their electrophoretic pattern. They also possess distinct and reproducible fraction of proteins. Reports indicating different number of fractions in various insects are available (Wittacker, 1959; Duke and Pantelouris, 1963; Schmidt, 1965; Brunert, 1967; Wang and Patton, 1968; Patel and Schneiderman, 1969; Kubicz and Galuska, 1971; Schmidt and Hess, 1973; Schmidt and Schwankl, 1975; Turunen and Chippendale, 1980; Schmidt et. al.,1982; Arabale, 1985; Gourikumari and Habibulla, 1987).

Some of the protein fractions from the foregut, for example, disappear in the midgut and again appear in the hindgut. Such fractions also show appearance and disappearance in either fat body or in the haemolymph or in both. They also exhibit variations in the staining reactions. Such changes in the behaviour of the fractions in different organs/tissues of the two insects understudy may be related to the different physiological conditions of various organ-systems. Proteins produced in the homolagous tissues or organs of closely related species may differ strikingly in their patterns and biochemical properties (Chen, 1985). Further, investigations of proteins in insects are handicapped not only by the complications such as physiological adaptations of various organ-systems but also due to the complication of hormonal control of protein synthesis. Wheather the changing patterns of the proteins in different organs/tissues studied presently is due to latter phenomenon can not be said at this juncture.

Results obtained from both qualitative and quantitative studies indicate that, in general, the fat body and the haemolymph of both the insects show higher concentration of proteins. Biochemical estimation of proteins from both the sexes also indicate a rather higher protein content in the females than in the males. Such differences in the protein concentration may be related to the various metabolic activities of the adult life. Further, proteins that are produced at an earlier stage can be stored in some tissues and utilized only later during adult development (Levenbook,1985). Insect fat body is the major site for the storage of proteins. The reason for storing protein in larval fat body is fairly obvious, namely as a build-up of material which can later be used at the time of metamorphosis when adult tissues are being assembled. The storage of proteins in the adult fat body is for a different reason, the fact that the adult stage is the reproductive stage in the life cycle. The high level of proteins in the adult female could be correlated with the stage of egg development. Ample of evidence is available in this context (Telfer,1960; Hill,1962; Englemann & Penney,1966; Scheurer, 1969 b; Adiyodi 1967; Pan, 1971; Price, 1973; Hagedorn and Kunkel, 1979; Pan & Wyatt,1976; Chen,1971; 1978; Bodnaryk, 1978). It has been pointed out that proteins of the haemolymph can be utilised directly as a source of material for protein synthesis (Hellery 1932, Chen 1956, Rober *et al.* 1977). But it is now generally accepted that nearly all the proteins are synthesized in the fat body (Wyatt.,1980). The haemolymph proteins can be sequestered by a number of tissues especially by the fat body where they are stored in the form of storage granules and are utilized as per the demand (Locke and Collins, 1966, 1967).

The diet on which the insects feed, may also influence the changing pattern and variations in the staining intensity of the fractions. This is in agreement with the view of Schmidt & Writh (1974) who have shown in F.proteinsis that the changes in the intensity of the fractions are related to the type of food. Similarly Bodnaryk & Morrison (1966) have demonstrated the influence of the diet on the protein pattern of the haemolymph in the developing overies in M.domestica. According to them, protein/carbohydrate food causes an increase/decrease in individual protein fractions. Possibly this might be one of the reasons in the changing pattern of proteins in the insects studied presently.

In order to study the insecticidal effect of the selected test oil, the insects understudy were exposed to different dietary concentrations. In general the neem oil did not yield any evidence of absolute antifeedant potential, However, the higher dietary concentrations of the neem reduced the amount of diet consumption. At a dietary concentration of 100 µl per insect a slight behavioral effect is seen after 24 hours feeding. Such insects were utilized for the analyses of amino acid and protein pattern. Nutritional effect of neem has been studied in different insects (Schlueter, 1984; Tare, 1984; Mala et.al.,1987; Karnavar and Dlamini, 1987; Barnby & Klocke, 1987).

The prime concern of the present investigation is to demonstrate the insecticidal effect of neem oil on the chemical moieties such as amino acids and proteins. Perusal of the results described in the foreging chapter III clearly indicate that the neem oil tested in the present work exhibits marked effect on the distributional pattern of both free amino acids and proteins of both the insects. Of the several amino acids appeared in the different regions of gut of untreated cockroach, many of them either disappear in one organ and reappear in the other while some other are totally missing in any of the organs of treated insects. Such changes are also evident in the fat body and the haemolymph. The number of the aminoacids does not remain the same after exposure to the test oil. Either there is decrease or increase in them. In the normal or untreated cockroaches among the different organs/tissues the maximum number is evident in the haemolymph but after neem treatment this number goes down to ten in the same tissue. On the contrary, now the number is found to be increased in the hindgut of the treated insects. Likewise, there is also a organ/tissuewise change in the number and also appearance,disappearance of the amino acids is evident in the treated grasshoppers. Similar type of reports on several insects are available. However, most of these studies are carried out with the organic synthetic insecticides. Rambha Singh (1982) has studied the effect of malathion on the free amino acids in the haemolymph of <u>Dysdercus koenigi</u>, both qualitatively and quantitatively, of the fifteen amino acids, proline shows the maximum concentration, also by the glycine in the normal insects. After treatment with malathion for about 60 minutes a decrease in the concentration of all amino acids occurrs. The decrease in concentration is found to be more pronounced especially in the proline and glycine as compared to the other amino acids. After prolonged (105 minutes) malathion poisoning a further decline in the concentration, except glutamine, is seen. Thus, the magnitude of depletion of these amino acids is directly dependent upon the degree of toxicity. The decrease in the levels of amino acids is due to an imbalance between the rates of anabolism and catabolism in the poisoned insects. Under particular conditions proline can be mobilized as energy reserve. In DDT-poisoned cockroaches Corrigan & Kearns (1963) reported that there is distinct depletion of proline. Injection of labelled proline into these animals revealed a threefold increase in the oxidation of this amino acid to CO_2 compared to controls. Apparently there is an inhibition of certain glycolytic enzymes by the insecticide and the demand for oxidizable carbon is shifted to proline. In this connection it should be mentioned, that a sharp drop of the proline content in the thoracic muscle during flight of the tsetse fly Glossina morsitans has been observed by Bursell (1963). As suggested by Bursell the most likely mechanism is that proline is converted to glutamic acid by proline oxidase. Increase in the concentration of glutamine after second stage of treatment in the above mentioned work of Singh (1982) is difficult to explain since it enters into so many metabolic process (Meister, 1957).

A review by Florkin (1959) indicates that there is a high degree of variation of the haemolymph amino acid content among different insect species. According to him, the insecticidal intoxication as well as other environmental factors influence the level of free amino acids in the haemolymph. Josheph (1958), while analyzing the blood of T.molitor treated with DDT, found large increases in amino nitrogen, reducing substances and non-protein nitrogen but no change in protein nitrogen. Some amino acids such as alanine, glutamic acid, proline and histidine showed largest increase. Winteringham (1959) reported significant increases in the free glutamine in houseflies treated tropically with di-isopropylphosphoroforidate and with DDT. In contrast to the work of Joseph (1958) the concentration of most other amino acids remained unchanged. Pyrethrins and lindane caused only slight decreases in proline while dieldrin & TEPP produced no changes. More recently, Ramalingam (1984) reported the insecticidal effect of DDT & malathion in P. americana. According to him the number of free amino acids is found to be increased after treatment of these insecticides. A survey of the literature indicates that a possible explanation for these fluctuations may be found in the elevated rate of catabolism or other intermediary pathways of metabolism. From the foregoing literature it is apparent that there is every possibility of amino acid toxicity to insects. In a similar way there may be a likewise effect of neem oil in the insects studied presently.

Fluctuations in the free amino acids in different organs/tissues of the two insects studied presently could be related to the detoxification phenomenon. Several experiments indicate that the free amino acids play an important role in detoxification. In Locusta (Friedler & Smith, 1954), Aedes (Casida,1955) and Bombyx (Shyamala,1964) glycine is found to conjugate with benzoic acid to form hippuric acid, a detoxification mechanism similar to that in higher animals. The site of hippuricase which regenerates glycine from hippurate has been detected in both fat body and silk gland (Shyamala,1964). Limpel and Casida (1957 a, b) showed that radioactive iodine injected into the cockroach P.americana is excreted as monoiodohistidine. When labelled iodine is given in the form of monoiodohistidine, diiodohistidine appeared in the excreta. This points out that histidine like glycine also serves as a detoxicating agent in insects.

The hindgut of treated cockroaches has increased number of amino acids. Such increase could probably be attributed to the phenomenon of excretion. It has been demonstrated by Ramsay (1958) that in the stick insect Dixippus morosus amino acids from the haemolymph can enter the Malpighian tubules by passive diffusion. Some of them are probably absorbed in the rectum, but significant quantities may be eliminated with the faeces. However, in the present study, the hindgut of the treated grasshopper shows no significant change in the number of amino acids.

Both qualitative and quantitative analyses of different organ-systems and tissues of the treated insects studied presently indicate marked difference in the distributional pattern of protein fractions and soluble protein content as well. The number of the fractions is changed in each organ/tissue after exposure to the neem oil. According to the size (thickness) of the bands, the fractions are distinguished as major and minor (thin) ones. In general, the number of the major bands is increased in treated insects. The staining pattern is also changed. Further, in all the organs/tissues, the anodically migrating (low molecular weight proteins) fractions appeared more frequently. However, there are also changes associated with the slow migrating or high molecular weight proteins. Some of the fractions which are evident in the untreated insects disappear after the neem treatment. Among the different organ/tissues of treated insects the fractions from one organ or tissue show variations, in the staining intensity as well as in their individual number. The anodically migrating fractions show weaker staining reaction.

The results on the proteins of treated insects very well corraborate with the data obtained by other workers in the field. Ramalingam (1984) determined the insecticidal effect of DDT and malathion on the distributional pattern and the content of soluble and stractural proteins in the fat body, haemolymph and muscle of P.americana. According to him, the soluble protein in the fat body of DDT and malathion injected cockroaches showed a significant increase as compared to the control. Muscle showed in both the test groups an insignificant decline. Ovary in the test groups showed significant decline as compared to the control. The level of strucutural proteins increased in both the fat body and muscle of DDT & malathion treated cockroaches while in the ovary significant decline was noticed as compared to the control. The haemolymph protein fractions in both the DDT and malathion treated insects showed an increase in number of fractions with low molecular weight. In malathion treated cockroaches even the high molecular weight proteins are also increased as compared to the pattern obtained for the control. From the view point of the functional significance, Ramalingam (1984) states that the increase in the structural proteins in the two test groups, and also the soluble proteins in the fat body indicate either the synthesis or uptake of proteins from the haemolymph by the tissues subsequent to toxic stress. Possibly this may be one of reasons for the changing pattern of proteins in different organs/tissues of the insects studied presently. However, the synthesis can not be expected in view of the mechanism of these insecticides being inhibitory on synthase enzymes (O'Brien,1957). Hence the uptake of proteins from the haemolymph may be suggested. The decline of the two types of proteins in the ovary of treated cockroaches suggests that DDT and malathion may inhibit the transport of extra-ovarion proteins.

Schulz and Schluter (1983) investigated the protein composition in the haemolymph & oocytes of Epilachana varivestis by SDS-polyacrylamide gel electrophoresis after the treatement of neem. They found that there is much alteration in their protein spectrum before and after the treatment of neem and they state that such differences are due to the different physiological states and indicate more sensitive phase for neem's action.

Results obtained through the quantitative estimation of the proteins of two insects understudy are in agreement with the findings of Tare (1984). Protein contents of different organs/tissues of both the insects indicate that the admittedly [illegible] bioactive oil of A. indica causes an enhancement of the soluble proteins of males and females. The significance of an increase rather than a decrease in protein content can not be commented upon in a preliminary investigation of the present kind. However, it does afford basis of material for further highly instructive study.

Perusal of the available literature on the Indian neem tree indicates that the neem oil and its productus are utilized from the view point of different biological activities. Its medical uses have apparently been found. Besides this it has an antifeedent property. It has a distinct growth inhibitory property. Oviposition deterrant activity has also been shown and finally insecticidal properly of neem has been examined against some insects. The results obtained in the present work also indicate that the neem oil has some insecticidal effect as evidenced through the changed pattern of free amino acids and proteins. Though the present work forms a preliminary assessment, it may form a basis for further exploration and investigation incorporating still more such similar oils and insect species. Thus, the potential of neem might have been forgotten, were it not for increased public concern about the hazards of pesticides.

SUMMARY AND CONCLUSIONS

The available literature on the neem tree. A. indica suggests that its products especially the seeds and oils have been utilised in biological studies from different angles. Efforts have also been made to utilize the neem as an insecticidal agent. However, perusal of the data indicates that there are hardly any reports regarding the insecticidal effect of the neem on the important chemical moeities like amino acids and proteins. Though, there exists an abundant information with reference to the application of various insectides among the insects, most studies are dealt with the synthetic organic insecticids. No doubt, these chemicals have proved to be most efficient in controlling the target pests but there are many disadvantages too, since they cause intrinsic toxicity not only to the target pests but also bring hazardous effects in other useful animals. Therefore, a different approach would be hunt for the bioactive principle from naturally occurring source like neem trees. Keeping this approach in mind the present investigation was undertaken to utilize the neem as an insecticidal agent and to see its effect on the distributional pattern of free amino acids and proteins from the different organs and tissues of the two insects. Insects selected for the present work are important from the socio-economic point of view, one being the pest of common household commodities and the another, being of agricultural crops. Both free amino acids and proteins were studied by employing well known methods and some interesting results were obtained, the salient features of which are as follows :

Amino acids :

1) Free amino acids were studied from the different regions of the alimentary canal, fat body and haemolymph by ascending paper chromatography and the results indicated that the pattern of free amino acids showed distinct organ/tissuewise variations in both the insects.

2) Nearly all of the amino acids commonly found in the proteins have been identified.

3) Each region of the alimentary tract possessed its own pattern of free amino acids and it differed from that of the fat body and the haemolymph as well.

4) Most of the amino acids were found to be common in the different organs/tissues studied presently.

5) Some amino acids which are of rare occurrence among the insects were also evident in the insects studied presently.

6) Organs and tissuewise variation in the number of amino acids was related and discussed with reference to the different metabolic pathways occurring during insect life.

7) A change in the amino acid pattern was evident after the treatment of neem oil.

8) The number of the amino acids was found to either increased or decreased in some organs/tissues.

9) The changed pattern of amino acids after the dietary treatment of neem was indicative of its toxic or insecticidal potential.

Proteins :

1) Qantitative analysis of proteins was made by employing polyacrylamide disc electrophoresis while quantitative studies were carried out by bioassay method.

2) Electrophoretic study exhibited distinct and reproducible protein fractions among the different parts of the alimentary tract and tissues like fat body and haemolymph.

3) A total of thirteen protein fractions appeared in the cockroach while twelve in the grasshopper.

4) Distinct organ and tissuewise variation in the fractions was evident in both the insects.

5) Fractions exhibited variations in their staining reaction. Most of the fractions were exhibited similarity in their rate of migration.

6) Some of the fractions found in one particular organ or tissue were found to be missing in some other organs.

7) Appearance and disappearance of the fraction, their variation in the staining reaction and also variations in the number was discussed from the view point of physiological activities and compared with the available literature.

8) After neem treatment the distributional pattern of proteins exhibited a marked change in that, in general the number of fractions was found to increased comparatively in most of the organs/tissue.

9) The staining intensity was also changed and the anodically migrating fractions increased.

10) Quantitative results also indicated an increase in protein content after treatment.

11) Though a clearcut physiological significance of such increase is not clear but certainly it shows that the neem has some toxic effect on these macromolecules.

From the results obtained it can be concluded that the tree products like neem oil present a distinct evidence of pest control potential for developing safe biological insecticides that can be used in programmes of Integrated Pest Management, or biological control of pests with minimal use of pesticides. The present investigation, though a preliminary work with limited scope, provides an excellent subject matter for further studies, especially from the view point of embryogenesis, growth and development of the insects. Because the development of a multicellular organism, like that of an insect, is a complex process and it is genetically controlled one. It envisages the synthesis of macromolecules like proteins and involves many intermediate reactions. Further, the morphological changes are a result of changing game of hormonal conditions which influence the amino acid and protein patterns of many tissues. Therefore, for better understanding such changes during development, studies involving organ and tissue specific amino acids and proteins especially are necessary since, proteins are the first conceivable products of gene activities.