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EFFECT OF AZADIRACHTIN ON INCORPORATION OF ^{35}S-CYSTEINE INTO PEPTIDES IN THE BRAIN AND CORPUS CARDIACUM IN THE LOCUST *SCHISTOCERCA GREGARIA*

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Abstract: ^{35}S-cysteine was used as a tracer to examine the effect of azadirachtin on the incorporation of the amino acid into the peptides of the brain and the corpus cardiacum in the locust (*Schistocerca gregaria*). Both *in vivo* and *in vitro* experiments were carried out. The metabolism of ^{35}S-cysteine in the brain and corpus cardiacum was studied and analyzed by TLC and exclusion chromatography. The results demonstrate a higher amount of ^{35}S-cysteine being present in the corpus cardiacum than in the brain. The rate of ^{35}S-cysteine incorporation into the brain and the corpus cardiacum shows that the incorporation of ^{35}S-cysteine into the polypeptides, including glutathione, synthesized in the brain and corpus cardiacum is strongly inhibited by azadirachtin. This suggests that many of the biological effects of azadirachtin are due to interference with neuroendocrine processes, most of which are mediated by peptide hormones.

Key words: Azadirachtin, brain and corpus cardiacum, ^{35}S-Cysteine, *Schistocerca gregaria.*

INTRODUCTION

Azadirachtin, a tetranotriterpinoid and the major active principle from seeds of the neem tree (*Azadirachta indica*) has been studied for its biological activity on a number of insects. Azadirachtin, apart from its antifeedant effects, was also found to cause major disorders in some phytophagous insects. Treatment of the insects and/ or their food with the pure compound or with azadirachtin containing extracts caused growth inhibition, malformations, mortality and reduced fecundity.[1,2,3,4]

Similar morphogenetic defects could also be induced by synthetic hormone mimics and it was concluded that azadirachtin might function like the ecdysteroids, which are known to be present in many plants.[2] In 1987, Sieber *et al.*, reported that a major action of azadirachtin is to modify haemolymph ecdysteroid titres in fifth instar *Locusta migratoria*.[5] The studies on the uptake of the radio-labelled dihydroazadirachtin have further indicated that the compound is taken up, apparently with great specificity, into the corpus cardiacum and the brain.[6] Its accumulation in the brain and corpus cardiacum, however, may give a clue to one of the major sites of action, as noted by others.[7] Many of the effects of azadirachtin on

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various aspects of development are ultimately controlled by neurohormones.[3] There is ample evidence that most of these are peptides in nature.[8] Previous work has shown that azadirachtin has an effect on protein metabolism in the brain and corpus cardiacum. The neuroendocrine proteins from the corpus cardiacum have been characterized in order to elucidate the quantitative and qualitative differences in their biosynthesis due to azadirachtin treatment.[3]

The aim of the present work was to examine more precisely the effect of azadirachtin on the synthesis of possible neuropeptide material by the brain and corpus cardiacum, as this might be expected to indirectly affect a wide range of metabolic processes including protein synthesis in locust tissues. For this purpose, ^{35}S-cysteine was used, following the work of Mordue *et al.*,[9] who showed that this amino acid was abundant in neurosecretory peptides.[9]

The work *in vivo* was then followed by experiments done *in vitro* with brain and corpus cardiacum, in an attempt to distinguish between direct and indirect effects of the azadirachtin.

METHODS AND MATERIALS

Insects: Schistocerca gregaria were purchased from Blades Biological, Edenbridge, Kent, and were maintained under laboratory conditions in metal cages at a temperature of 28-30 °C, 40 % r.h. and a 12 h. light -12 h. dark cycle. The insects were fed on freshly washed spring cabbage leaves. Fresh tap water was also supplied. Cages were cleaned daily. Only seven days old adult female locusts weighing 2.5 - 3.0 g were used as test insects.

Azadirachtin treatment: Azadirachtin was extracted and purified from Sri Lankan neem seeds using established methods.[6,10,11] Confirmation of purity of the azadirachtin was carried out by melting point (150 - 152 °C), TLC, reversed phase HPLC and NMR analysis.[6] Azadirachtin was dissolved in 10 % ethanol to give a final concentration of 1 mg/ml. The experimental locusts were injected with a dose of 3 μg azadirachtin/g body weight and the control insects starved for 12 h. before the experiment, received the same amount of 10 % ethanol / g body weight as the azadirachtin-treated locusts.

Application of ^{35}S-cysteine: ^{35}S-cysteine was obtained from Amersham International, Aylesbury, Bucks. The specific activity of the ^{35}S-cysteine was 1300 Ci/mmol. Each radiolabelled amino acid was dissolved in insect saline to give a final concentration of 1μCi/10μl. The amino acid (2 μCi/g) was injected into each locust 12 h. after injection of azadirachtin. Injections were made through the abdominal intersegmental membrane using a Hamilton 25 μl syringe.

Incorporation of ^{35}S-cysteine into the brain and corpus cardiacum

(a) *In vivo experiment*: Each locust was injected with 2 μCi ^{35}S-cysteine/g and the rate of incorporation was measured 4 h. after injection. The heads were removed and the endocrine system was quickly dissected under ice-cold saline. The brain and corpus cardiacum were isolated free of fat body and were washed with fresh insect saline before further analysis. Pooled samples from batches of 4 locusts were homogenized for 30 seconds in 500 μl of 70 % v/v aqueous methanol using an ultrasonic microprobe. The suspension was centrifuged at 10,000 g for 5 minutes and the pellet washed twice with 5 % TCA. The pellet was dissolved in 100 μl 0.1 M NaOH containing 0.1 % w/v SDS and kept overnight at 30 °C. The supernatant was freeze-dried and redissolved in 100 μl of 70 % v/v aqueous methanol. The samples (50 μl) of the supernatant and the redissolved protein were taken for estimation of radioactivity by scintillation counting. The rest of the protein-containing pellet was used to estimate the quantity of protein, and the quantity of labelled compound in the tissue was estimated with reference to the protein content.[12] Observations were repeated four times.

(b) *In vitro experiment:* Brain and corpus cardiacum were dissected separately and placed in 1 ml of the insect saline. Tissues from a batch of 12 locusts were pooled for each experiment. Each sample was pre-incubated for 30 minutes before adding 1 μCi of ^{35}S-cysteine. The rate of incorporation was measured after 4 h. The insect saline was maintained at a constant temperature of 30 °C and aerated throughout the experiment.

The samples were washed in fresh insect saline before being analyzed and then homogenized in 500 μl of 70 % methanol and centrifuged for 5 minutes at 10,000 g. The supernatant was freeze-dried and redissolved in 50 μl of 70 % aqueous (v/v) methanol. The analysis of pellet was as described in the *in vivo* experiment.

Direct effect of azadirachtin on the uptake of ^{35}S-cysteine into the brain and corpus cardiacum: A batch of four pre-incubated brains and the corpus cardiaca was placed in 1 ml of insect saline which contained 0.25 μCi of ^{35}S-cysteine. Samples of the medium (1 μl) were taken out to measure the radioactivity at different times. To investigate the effect of azadirachtin on the uptake, the tissues were pre-incubated with 12 μg of azadirachtin and the radioactivity in the medium was measured in the same way as the control experiment.

Metabolism of ^{35}S-cysteine in the brain and corpus cardiacum

(a) *Thin Layer Chromatography (TLC)*: The tissue extracts in 70 % methanol were chromatographed on a 20x20 cm cellulose TLC plate. The plate was double-developed in butanol:acetic acid:water (11:6:3 v:v:v). The position of the radiolabelled compounds

contained in the extract was identified using the following standards; a) glutathione (oxidised) b) cysteic acid c) cysteine d) cystine e) cysteine sulphonic acid and f) glutathione (reduced).

After resolution, these compounds were visualized by dipping in 1 % ninhydrin in acetone and heating for 5 minutes at 100 °C. The bands (1.0 cm) were scraped from the plate and were then transferred to scintillation vials for counting, so that the entire plate was analyzed for radioactive spots.

(b) *Derivatization of thiol group:* The extracts of brain and corpus cardiacum were reduced using 2 mM sodium borohydride ($NaBH_4$) and then reacted with 10 mM N-ethylmaleamide (NEM) to form NEM derivatives. The standards were also converted to a NEM derivatised form and analyzed by TLC as above.

(c) *Exclusion chromatography:* Exclusion chromatography was carried out to separate the low (Mr<1300) and high (Mr>1300) molecular weight peptides of the 70 % methanol-soluble fraction of both the brain and corpus cardiacum.

(i) *Preparation of the column*: Sephadex G-10 was used as the stationary phase and 70 % methanol-water was used as the solvent. To obtain good separation and satisfactory flow rates, the gel was allowed to swell in excess solvent and left to stand for 2 h. A syringe (1 ml) was used as a column and the material was packed without any air bubbles.

(ii) *Preparation of the sample*: The pooled samples of the 70 % methanol-soluble fraction of both brain and corpus cardiacum were used separately. First, they were reduced with sodium borohydride and then converted to the NEM derivatives. Two coloured markers with different molecular weights were used to identify the fractions. They were vitamin B_{12} (cyanocobalamin), red in colour, with molecular weight 1333 and 2,4, DNP-L- lysine hydrochloride, yellow in colour, with molecular weight 348. A sample (5 μl) of each marker (10 mM in 70 % methanol) was mixed with 50 μl of the extract and this sample was added to the top of the column and eluted with 1ml of 70 % methanol.

RESULTS

Excretion of injected radioactivity

The excretion of ^{35}S-cysteine from azadirachtin-injected females was compared in the 12 h. starved locusts and the normal fed locusts. Figure 1 shows the excretion profile of injected radiolabelled cysteine over 2 days. The excretion pattern of normal locusts was different from azadirachtin-injected locusts and starved locusts. Only 40 % of the applied radioactivity was excreted during the first 24 h. in normal locusts but both in the starved and azadirachtin treated locusts, more than 60 % of the

total radioactivity was excreted. Very little radioactivity was excreted after 24 h., although there was a small constant, daily excretion, about 2 % of the total. The balance remained in the body. The results were very similar to each other in all three groups after 24 h.

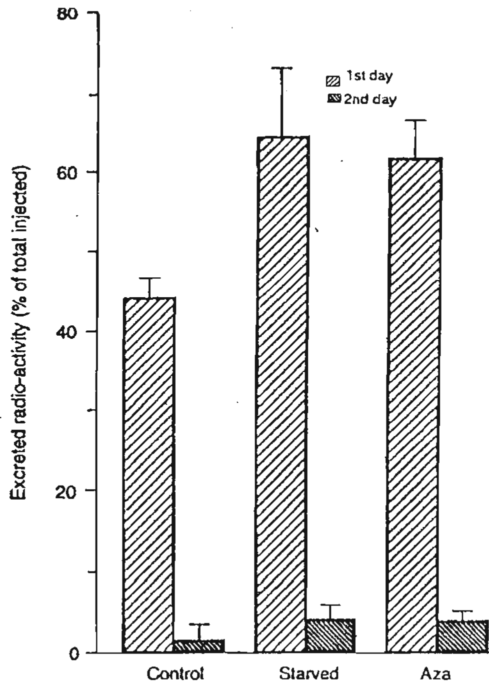

Figure 1: Excretion of radio-activity from the locust *Schistocerca gregaria* after injection of a dose of ^{35}S-cysteine.
Each locust receives 2 μCi of ^{35}S-cysteine with a specific activity of 1300 Ci. mmol^{-1}. The results are the means from 4 locusts, and the bars indicate the standard deviation from the mean.

Incorporation of ^{35}S-cysteine into the brain and corpus cardiacum

(a) *In vivo* experiment

The incorporation level of ^{35}S-cysteine into the proteins of brain and corpus cardiacum 4 h. after injection is presented in Table 1. The 70 % methanol-soluble fraction contained peptides and the free amino acids. The corpus cardiacum showed a higher level of incorporation than the brain. The incorporation patterns of ^{35}S-cysteine into the brain and corpus cardiacum reveal striking differences between the azadirachtin-injected locusts and the control locusts. In the brain and the corpus cardiacum, total radioactivity (both the methanol soluble fraction and the insoluble fraction) was higher in the control locusts than in the azadirachtin-treated locusts. In the methanol-soluble fractions of the brain and the corpus cardiacum in

azadirachtin-treated locusts, the incorporation level was inhibited by 55 %, demonstrating that the rate of incorporation was rather poor and slow in the azadirachtin-treated locusts.

Table 1: Incorporation of ^{35}S-cysteine into the corpus cardiacum and the brain *in vivo*.

Tissue	Specific activity (dpm, mg^{-1} of protein)	
	70% methanol-soluble material	70% methanol-insoluble material
Control		
Brain	1625±131	553±62
Corpus cardiacum	2130±260	1732±92
Azadirachtin treated		
Brain	755±56	152±31
Corpus cardiacum	928±91	631±52

The results are the means (±SD) of 5 insects and the effects of azadirachtin were significant at below p=0.01

(b) *In vitro* experiment

The incorporation of ^{35}S-cysteine into the corpus cardiacum and the brain was measured 4 h. after addition of the radiolabelled amino acid into the medium. The incorporation pattern of radiolabel into the protein of 7 day old females reveals striking differences between the azadirachtin-treated tissues and the controls. The 70 % methanol-soluble material of both control and azadirachtin-treated tissues was separated into two clearly resolved fractions on the gel filtration column. All the higher molecular weight compounds were eluted with vitamin B_{12} and this fraction contained the compounds of molecular weight higher than 1300. The low molecular weight fraction, eluted with the lysine derivative, contained the compounds of molecular weight less than 1300, including all free amino acids and reduced and derivatised glutathione.

Although the incorporation of radiolabelled amino acid into the protein of the corpus cardiacum was higher than that of the brain, both the brain and the corpus cardiacum showed that 70 % of the total radioactivity incorporated into the protein was in the low molecular weight fraction, 27 % of the total was detected in the high molecular weight fraction and only 3 % was found in the precipitable protein

(Table 2). In general, the uptake of the radiolabel into the protein of the corpus cardiacum of the azadirachtin treated locusts was not significantly higher than that of into the brain in the control experiment. This confirmed that the incorporation was rather poor in the azadirachtin treated tissues. Although azadirachtin does not affect the uptake of radiolabel in the neurosecretory proteins, the total ^{35}S-cysteine retained in the brain and the corpus cardiacum was comparatively low in azadirachtin-treated locusts. These results are comparable with the *in vivo* results.

Table 2: Incorporation of ^{35}S-cysteine into the corpus cardiacum and the brain *in vitro*

Tissue	Specific activity (dpm, mg^{-1} of protein)		
	70% methanol-soluble material		70% methanol-insoluble material
	Low molecular weight fraction	High molecular weight fraction	
Control			
Brain	223.1±4.4	91.0±1.8	15.1±1.8
Corpus cardiacum	314.8±2.2	141.8±3.8	33.1±3.8
Azadirachtin treated			
Brain	80.5±3.7	38.1±4.1	8.9±1.0
Corpus cardiacum	84.3±9.1	53.9±5.8	18.4±1.1

The radioactivity of each fraction was measured with respect to the precipitated protein. The results are means (±SD) of 4 samples and the effect of azadirachtin was significant at below p=0.05

Uptake of ^{35}S-cysteine into the brain and corpus cardiacum

Figure 2 shows the rates of clearance of ^{35}S-cysteine from the medium into the brain and the corpus cardiacum during a 6 h. incubation. The results show that the rate of uptake is more rapid into the brain than into the corpus cardiacum but that azadirachtin did not affect the rate of uptake. For both tissues, the effect of azadirachtin showed an identical curve to the control experiment and therefore only one is shown in Figure 2.

Metabolism of ^{35}S-cysteine in the brain and corpus cardiacum by TLC

Radioactivity in the peptides was assessed using TLC. Radioactivity was detected in the band corresponding to cysteine, cystine, glutathione (oxidised), and the origin,

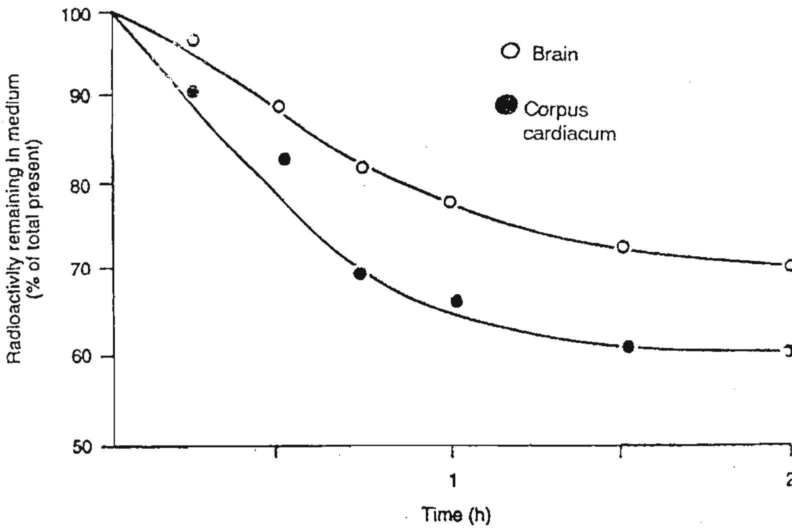

Figure 2: Direct effect of azadirachtin on uptake of ^{35}S-cysteine into the brains and corpora cardiaca of the locust *in vitro*.

The graphs represent the radioactivity of ^{35}S-cysteine in the medium. The results are the means of duplicates.

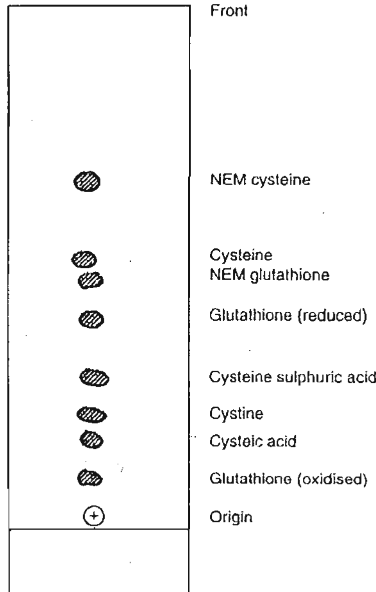

Figure 3: The TLC chromatogram of standard 'S' containing compounds.

The figure represents a TLC of standard sulphur-containing compounds, oxidized and reduced forms of glutathione as well as the N-ethyl maleimide (NEM) derivatives of cysteine and glutathione on cellulose plates after double development in butanol:acetic acid:water (11:6:3). After resolution, the chromatogram was stained with ninhydrin.

which was indicated by addition of carrier quantities of standards of sulphur-containing amino acids, visualized with ninhydrin. Then the plate was divided into 1cm bands, which were scraped off. There was, therefore, evidence for ^{35}S-cysteine metabolism in the brain and the corpus cardiacum but this was higher in the brain. In order to estimate the potential ^{35}S incorporated peptides that might interfere

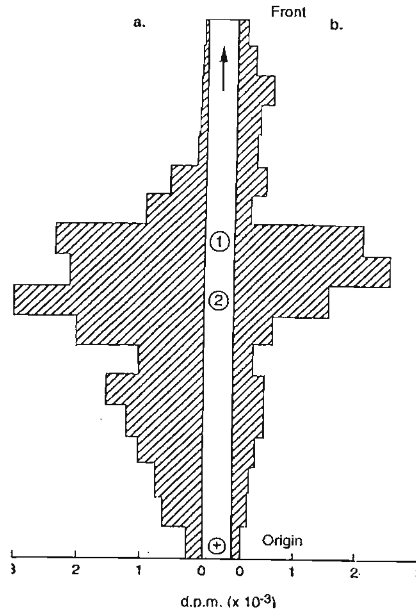

Figure 4: Incorporation of radiolabel from ^{35}S-cysteine into 70 % aqueous methanol-soluble material in the brain and corpus cardiacum *in vitro*.

The figure represents the results of chromatography on cellulose of the 70 % methanol-soluble extract of the brain (a) and corpus cardiacum (b) incubated *in vitro* with $NaBH_4$ and NEM. The spots represent the location of (1) NEM cysteine; (2) NEM glutathione. After double development in butanol:acetic acid:water (11:6:3) (v:v:v), 1 cm strips of the cellulose were scraped into scintillation vials, and the radio-activity counted.

with an oxidized glutathione, the extracts were derivatised with NEM. Figures 3 and 4 show resolution of sulphur-containing amino acids on a cellulose TLC plate after staining with ninhydrin and TLC of the 70 % methanol-soluble fraction of the brain and the corpus cardiacum, after reaction with $NaBH_4$ and NEM respectively. The TLC indicated that the activity on the base line was contaminated by ^{35}S labelled oxidized glutathione, but this was solved by reduction with $NaBH_4$. Oxidized and reduced standards of glutathione were included since these are known to incorporate ^{35}S after administration of ^{35}S-cysteine to insects.

DISCUSSION

The experiments reported here were performed to examine the effects of azadirachtin on peptide synthesis in the brain and corpus cardiacum of adult female locusts by following the incorporation of ^{35}S-cysteine into peptides. Cysteine is unstable in aqueous medium and easily oxidizes to form the dimer cystine, or forms dithiol links with cellular proteins containing cysteine residues.[13] This will tend to suggest artificially high rates of incorporation. It was for this reason that the studies on the incorporation into "neuropeptides" were done with extracts, which had been reduced with $NaBH_4$ and then derivatised.

The two methods of protein precipitation employed in the present study were intended to achieve only a crude separation of polypeptide fractions, and they were used for different purposes. TCA (5 %) will precipitate polypeptide material with molecular weights greater than 5000. Thus the pellets are likely to contain most labelled amino acids which have been incorporated into a polymeric form. These experiments were mainly concerned with the possible precipitated material, i.e. the incorporation into "proteins". On the other hand, various proportions of aqueous methanol were employed to ensure that the possible neuropeptides remained in solution. Seventy percent aqueous methanol was used here following the work of Girardie *et al.*,[8] and Subrahmanyum *et al.*,[3] in extracting neuropeptide material from the neurohaemal organ of locusts.[3,8] These workers found that some polypeptides of very high molecular weight (Mr40,000-120,000) were soluble in the methanol. Thus the methanol method cannot achieve a separation between high molecular weight "proteins" and low molecular weight "peptides". It was for this reason that the methanol-soluble fraction was further resolved by exclusion chromatography.

One of the problems in trying to determine the primary actions of azadirachtin is that it has high general toxicity that is also complicated by the appearance of secondary effects. For instance, the secondary antifeedant effect of azadirachtin causes the insect to cease feeding and thus produces an effect of starvation. Highnam *et al.*,[14] showed that the uptake from the haemolymph of an injected radiolabelled amino acid was much slower in locusts starved for 5 days than in the normally-fed controls.[14] This may partly explain the results of Subrahmanyam *et al.*,[3] who found that the uptake of ^{35}S-cysteine from the haemolymph of locusts was also very slow in those insects which had been treated 7 days before with azadirachtin (3 μg/g).[8] These effects were avoided in the work reported here by using short time-courses for the experiments and by ensuring that control insects were starved to mimic the antifeedant effects of azadirachtin. The uptake of labelled amino acids was the same in 12 h. starved and azadirachtin treated locusts as in the fed control, eliminating this aspect as one of the possible reasons for an effect on protein synthesis. In both *in vivo* and *in vitro* experiments, no evidence was obtained for a direct effect of azadirachtin on the uptake of ^{35}S-cysteine into the brain and corpus cardiacum.

The turnover of ^{35}S-cysteine in the brain and corpus cardiacum was compared between azadirachtin-injected and control locusts starved for 12 h. The rate of ^{35}S-cysteine incorporation demonstrates that the azadirachtin-treated locusts have a very low turnover of neuro secretory protein in the brain and the corpus cardiacum. In control locusts, in which the oocyte development is rapid, the transport of ^{35}S-cysteine labelled protein from the brain to the corpus cardiacum and its subsequent release are at significantly higher levels. The neurosecretory activity of the female *S. gregaria* was studied under various physiological conditions[9] and, in relation to feeding, following the incorporation of ^{35}S-cysteine into the products of neurosecretory cells.[14] These studies demonstrated that in maturing females (10 days old), synthesis and release of neurosecretory material is fast compared to immature females. Mature females have a much less active system than maturing or immature females. Incorporation of ^{35}S-cysteine into the median neurosecretory cells of starved females was low. Transport and release of neurosecretory material are minimal. The effects of azadirachtin are not comparable to starvation-induced effects on neurosecretory turnover, because the incorporation of ^{35}S-cysteine into the brain and the corpus cardiacum of azadirachtin-injected locusts was significantly lower than in starved insects.

In the azadirachtin-treated locusts, the transport of labelled protein from the brain to the corpus cardiacum and its release are at a very low level, though not completely inhibited. Hence, inhibition or disturbance of ovarian development in azadirachtin-treated females is mainly due to the changes induced in the endocrine system by the poor turnover of neurosecretory proteins. Synthesis and release of neurosecretory material are very rapid in maturing females.[14] The study of this process by labelling the protein clearly differentiates the transport and the release phases. As the ^{35}S-cysteine concentration of the haemolymph rapidly falls, it becomes limiting as early as 2 h. after injection due to adsorption, interchange of label with other tissues and excretion of the amino acid.[9]

The incorporation level of ^{35}S-cysteine in the brain and the corpus cardiacum was measured *in vitro*. These results were very similar to the *in vivo* results. The metabolism of ^{35}S-cysteine was studied using TLC and exclusion chromatography of 70 % methanol soluble fractions of the brain and the corpora cardiaca. The TLC results indicated that most (60 %) of radiolabel is due to oxidized glutathione and this obscured incorporation into other peptides which remained on the origin of the TLC plate. To solve this problem, oxidized and protein-bound thiols were converted to their reduced counterparts by the use of $NaBH_4$, followed by derivatization with NEM. Previous workers have failed to confirm that most of the incorporation of cysteine into "peptide" was into glutathione.[3]

In order to modulate true incorporation into high molecular weight peptides, the reduced 70 % methanol fractions of the brains and the corpora cardiaca were further analyzed by exclusion chromatography. Glutathione was eluted with the

low molecular weight fraction which contained all the other free amino acids and small peptides with molecular weight less than 1300. According to the results published by Girardie *et al.*,[8] the high molecular weight fraction contains all the neurosecretory proteins.[8] The molecular weight of these proteins is approximately 54,000-123,000. In agreement with these investigations, it was observed that the rate of incorporation is higher in the corpus cardiacum than in the brain. However, the incorporation of the level in the high molecular weight fraction is reduced in the azadirachtin-treated locusts. Thus, in the treated tissues, the appearance of labelled peptides is quantitatively reduced 4 h. after administration of ^{35}S-cysteine into peptide material of the azadirachtin-treated locusts, the same six fractions were present in the extracts of corpus cardiacum of both control and azadirachtin-treated locusts. More than 75 % inhibition due to azadirachtin can be seen in the corpus cardiacum but the brain shows only 65 % inhibition. Of the various endocrine centers investigated, azadirachtin is highly concentrated in the corpus cardiacum relative to the brain suggesting that the lower level of synthesis of peptides and the accumulation in the corpus cardiacum are due to this high concentration of azadirachtin in the corpus cardiacum.[15]

CONCLUSION

In summary, the results reported here suggest that azadirachtin interferes with formation of the peptides of brain and corpus cardiacum, which control so much of the metabolism and biology of the insects. It has yet to be proved, however, that azadirachtin lowers the titre of any identified peptide neurohormone in the haemolymph.

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