requiring process, and hence its biosynthesis may be affected by lowered ATP metabolism as a result of phosphate stress and reduced glycolysis due to sugar depletion.

The increase in pyrethrin content in nitrogen-stress medium will find application in scaling up cell cultures for pyrethrin production using a two-stage culture method. Further work on scaling up of cell culture in airlift bioreactor is in progress.

1. Zieg, R. G., Zito, S. W. and Staba, E. J., *Planta Med.*, 1983, **48**, 88.
2. Khanna, P. and Khanna, R., *Indian J. exp. Biol.*, 1976, **14**, 630.
3. Ravishankar, G. A., Rajasekaran, T., Sarma, K. S. and Venkataraman, L. V., *Pyrethrum Post*, 1989, **17**, 66.
4. Murashige, T. and Skoog, F., *Physiol. Plant.*, 1962, **15**, 473.
5. Ravishankar, G. A., Sarma, K. S., Venkataraman, L. V. and Kadyan, A., *Curr. Sci.*, 1988, **57**, 381.
6. Sasse, F., Knobloch, K. H. and Berlin, J., *Proceedings of the 5th International Congress on Plant Tissue and Cell Culture*, (ed. Fujiwara, A.), Abe Photo Printing Co Tokyo, 1982, 343.

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Malathion-induced changes in biogenic-amine levels and acetylcholinesterase activity in the cockroach *Periplaneta americana*

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Acetylcholine is the major neurotransmitter in insects. Many insecticides inhibit the enzyme acetylcholinesterase, which degrades acetylcholine and prevents cholinergic toxicity. Other substances whose neurotransmitter function has been demonstrated in mammals have been reported in insects. Here we show that sublethal doses of malathion, which has high anticholinesterase activity in the cockroach *Periplaneta americana*, cause a decrease in endogenous levels of the putative neurotransmitters 5-hydroxytryptamine, dopamine and noradrenaline and an increase in the level of 5-hydroxyindoleacetic acid.

SYNAPTIC transmission via acetylcholine is an accepted mechanism of nerve transmission in insects[1-4]. A large number of acetylcholinesterase (AChE) blockers are therefore used as insecticides. Even though their role is far from clear, other, putative neurotransmitters such as 5-hydroxytryptamine (5-HT), dopamine (DA) and noradrenaline (NA) have also been reported in insects. Presence of 5-HT was reported in *Manduca sexta*[5] and *Periplaneta americana*[6,7], DA as reported in *Aedes togoi*[8], both 5-HT and DA were reported in *Drosophila melanogaster*[9], while 5-HT, DA as well as NE were found in *P. americana*[10]. Flangan and Allan[11] demonstrated that 5-HT may, in fact be a neurohormone in *Rhodnius prolixus*.

Butcher[12] showed that there was interaction between cholinergic, tryptaminergic and adrenergic nerves in rats. According to him, AChE facilitates the release of dopamine, which subsequently acts as a neurotransmitter. It appears logical that, if, in fact, there is a functional interaction between cholinergic and monoaminergic nerves in insects, levels of 5-HT, DA and NA would be affected by anti-AChE pesticides.

Here we report the effects of the anti-AChE pesticide malathion on activity of AChE and levels of 5-HT, DA and NA in the common cockroach *P. americana*.

Adult *Periplaneta americana* (2.75 ± 0.087 cm length, 0.86 ± 0.017 g weight) were treated with 20% (0.21 μg per insect) and 80% (0.84 μg per insect) LD_{50} of malathion (LD_{50} was determined for *P. americana* $LD_{50} = 1.05$ μg per insect). The pesticide (98% purity) was dissolved in acetone and 0.05 μl of the solution containing appropriate dose of pesticide was applied to the dorsal thoracic surface of each insect using a microsyringe; controls received acetone alone. Biochemical estimations were made 24 h and 96 h after treatment.

AChE activity was measured by the method of Ellman *et al.*[13] Brain, along with ventral nerve cord, was removed and homogenized in 0.1 M phosphate buffer, pH 8 (50 mg per ml of buffer), for 5 min in an ice bath. The homogenates were centrifuged at 1000 g for 30 min at -4°C and the supernatants used as enzyme source. Enzyme activity was measured at 25°C in a 10-mm-path-length cuvette using an incubation mixture consisting of 0.01 ml of 5×10^{-4} M freshly prepared acetylthiocholine iodide as substrate, 0.05 ml of 12.5×10^{-4} M DTNB (5,5′-dithiobis (2-nitrobenzoate)) solution dissolved in 0.1 M phosphate buffer (pH 7), 0.05 ml of the enzyme-containing supernatant and 1.45 ml of 0.1 M phosphate buffer (pH 8). Blanks contained 0.01 ml of buffer instead of substrate. Change in absorbance at 412 nm was monitored for 3 min. Enzyme activity was expressed as micromoles of substrate hydrolysed by comparing absorbance curves with a glutathione standard curve. Protein was estimated according to Lowry *et al.*[14]

Biogenic-amine levels in nervous tissue of treated and control insects were measured fluorimetrically. Twelve milligrams of nervous tissue pooled from two insects was taken for each replicate. Tissue was homogenized in 3.0 ml of acidified butanol for 5 min and the homogenate centrifuged at 2000 *g* for 10 min. Supernatants were shaken in capped tubes for 10 min with 5.0 ml of heptane and 1.0 ml of 0.1 N HCl. The mixture was centrifuged for 5 min at 2000 *g*. The aqueous layer was used for measuring 5-HT, NA and DA. NA and

DA were measured according to the method of Maickel *et al.*[15] and 5-HT according to Curzon and Green[16]. Five ml of the remaining organic layer was transferred into capped tubes containing 0.6 ml of 0.5 M Tris buffer (pH 7), and the mixture shaken for 10 min and centrifuged at 2000 *g* for 10 min. The aqueous layer was removed and used to estimate 5-hydroxyindoleacetic acid (5-HIAA) by the method of Curzon and Green[16]. Amine content is expressed as μg per mg (mean $\pm$ SE of six replicates). Student's *t* test was applied to identify significant ($P < 0.05$) differences. Two-way analysis of variance was applied to identify significant ($P < 0.05$) time- and dose-dependent changes.

The data in Table 1 show that min mg treatment with 20% (0.21 μg per insect) and 80% (0.84 μg per insect) LD_{50} of malathion reduced AChE activity to 91% and 65% of controls respectively after 24 h and to 58% and 52% after 96 h. Two-way analysis of variance showed that the malathion treatment caused a time-dependent inhibition of AChE. Table 2 shows that endogenous levels of 5-HT, DA and NA decreased significantly with both dose and time dependence after malathion treatment. Levels of 5-HIAA increased significantly after treatment.

Data presented earlier[17] showed that treatment of cockroaches with malathion not only reduced activity of AChE but also caused significant reduction in endogenous levels of 5-HT, DA and NA. The anti-AChE property of organophosphorus compounds is well known[18-21]; AChE is indeed considered to be the primary target of these pesticides. The changes in the levels of other biogenic amines are not so easily explained.

5-HT is present in the central nervous system of insects and is thought to have a neurotransmitter role[5,9,22]. The present results show that the decrease in level of 5-HT is accompanied by an increase in the endogenous level of 5-HIAA. Both changes were dose- and time-dependent. The observation of high levels of 5-HIAA in the nervous system of *P. americana* indicates that, in this insect at least, 5-HT is mainly catabolized by monoamine oxidase (MAO). An increase in the level of 5-HIAA also indicates increased utilization of 5-HT. Decreased 5-HT and increased 5-HIAA have also been reported in the snail *Planorbis*[23] after treatment with *p*-chlorophenylalanine, and in rat[24] after treatment with dichlorovos, fenitrothion and disyston.

Dihydroxyphenylalanine (DOPA), the precursor of catecholamines, is known to be present in a number of insects[25]. In general three principal enzymes, namely MAO, catechol-O-methyltransferase and phenol sulphotransferase, are responsible for degradation of catecholamines. In cockroach brain the presence of DA-specific MAO has been established[26]. Unfortunately, the two other enzymes still remain to be demonstrated. The catecholamines are also believed to be putative neurotransmitters in insects[8,25,27-29]. In cockroach DA is responsible for the release of salivary secretion[30] and NA stimulates the release of fatty acids from the fat body[31]. NA and DA are known to regulate carbohydrate metabolism in *P. americana*[32] and in the fruit fly *Drosophila*[33]. In vertebrates liver glycogen is decreased after administration of adrenaline[34-36]. Adrenaline also causes an increase in lactic acid and keto-acid production in perfused isolated frog liver[37]. It has been shown earlier that after malathion treatment glycogen level in *P. americana* is greatly reduced[38]. These findings, together with the present observations concerning the lowering of NA content in *P. americana* as a result of malathion treatment, suggest that malathion alters glucose metabolism through adrenaline. This result is in good agreement with those obtained for vertebrates and *drosophila*[33,34-36].

Table 1. Effect of 20% and 80% LD_{50} of malathion on acetylcholinesterase activity in nervous tissue of *Periplaneta americana*

Treatment (h)	AChE activity (μmol 'SH' hydrolysed/min/mg protein) 24 h	96 h*
None (control)	0.086 ± 0.0008	0.090 ± 0.002
20% LD_{50} (0.21 μg/insect)	0.078 ± 0.0001 (91)	0.052 ± 0.0008 (58)
80% LD_{50} (0.84 μg/insect)	0.056 ± 0.001 (65)	0.047 ± 0.001 (52)

Values are mean ± SE of six replicates. Numbers in parenthesis are per cent change with respect to appropriate control.
*Differences between 24-h and 96-h groups significant ($P < 0.05$) by two-way analysis of variance.

Table 2. Effect of treatment with 20% and 80% LD_{50} of malathion on levels of biogenic amines in nervous tissue of *Periplaneta americana.*

Treatment		Levels* of biogenic amines (μg per mg body weight) 5-HT	5-HIAA	DA	NA
None (control)	24 h	0.194 ± 0.009	0.130 ± 0.003	0.427 ± 0.002	0.147 ± 0.001
	96 h	0.205 ± 0.012	0.124 ± 0.001	0.411 ± 0.002	0.135 ± 0.009
20% LD_{50}	24 h	0.162 ± 0.002 (84)	0.145 ± 0.006 (112)	0.396 ± 0.005 (93)	0.093 ± 0.001 (63)
	96 h	0.020 ± 0.004 (10)	0.364 ± 0.075 (294)	0.352 ± 0.020 (86)	0.083 ± 0.002 (61)
80% LD_{50}	24 h	0.155 ± 0.002 (80)	0.175 ± 0.002 (135)	0.299 ± 0.002 (70)	0.078 ± 0.005 (53)
	96 h	0.014 ± 0.001 (7)	0.432 ± 0.001 (348)	0.254 ± 0.003 (62)	0.076 ± 0.002 (56)

Values are mean ± SE of six replicates. Numbers in parenthesis are per cent change with respect to appropriate control.
*Differences between 20%-LD_{50}, and 80%-LD_{50} and 24-h and 96-h groups significant ($P < 0.05$) by two-way analysis of variance.

In conclusion, it is clear from the present study that anti-AChE pesticides do not exclusively affect activity of the cholinergic system. They may also substantially affect the other, putative neurotransmitter systems and thereby make a multipronged attack on the nervous system of the target insect.

1. Mikalonis, S. J. and Brown, R. H., *J. Cell. Comp. Physiol.*, 1941, **18**, 401.
2. Metcalf, R. L. and March, R. B., *J. Econ. Entomol.*, 1950, **43**, 670.
3. Lewis, S. E., *Nature*, 1953, **172**, 1004.
4. Mehrotra, K. N., *J. Insect Physiol.*, 1961, **6**, 180.
5. Maxwell, G. D. and Hildebrand, *J. Comp. Neurol.*, 1981, **4**, 667.
6. Osborne, N. N. and Neuhoff, V., *Brain Res.*, 1974, **2**, 366.
7. Pandey, A., Mohammad, H. and Singh, R., *Brain Res.*, 1983, **273**, 67.
8. Shampengtong, L., Wong, K. P. and Ho, B. C., *Insect Biochem.*, 1987, **1**, 111.
9. Livingstone, M. S. and Tempel, B. L., *Nature*, 1983, **303**, 67.
10. Klemm, N., *Cell Tissue Res.*, 1983, **2**, 379.
11. Flangan, T. J. and Allan, B., *Brain Res.*, 1984, **806**, 243.
12. Butcher, L. L., *Life Sci.*, 1977, **21**, 1.
13. Ellman, G. L., Gourtney, K. D., Andres, V. Jr. and Featherstone, R. M., *Biochem. Pharmacol.*, 1961, **7**, 88.
14. Lowry, O. H., Rosenbrough, N. J., Farr, A. L. and Randall, R. J., *J. Biol. Chem.*, 1953, **193**, 265.
15. Maickel, R. P., Cox, Jr, R. H., Soillant, J. and Miller, F. P., *Int. J. Neuropharmacol.*, 1968, **7**, 275.
16. Curzon, G. and Green, A. R., *Br. J. Pharmacol*, 1970, **39**, 653.
17. Bose, C., *Biol. Mem.*, 1990, **16**, 66.
18. Blank, R. H. and Osborne, G. D., *N. Z. J. Agric. Res.*, 1979, **22**, 491.
19. Kapin, M. A. and Ahmad, S., *Insect Biochem.*, 1980, **10**, 331.
20. Pandey, G. C. and Agarwal, R. A., *Entomon*, 1982a, **7**, 123.
21. Pandey, G. C. and Agarwal, R. A., *Entomologia Gen.*, 1982c **4**, 235.
22. Klemm, N. and Sundler, F., *Neuro. Sci. Lett.*, 1983, **1**, 13.
23. Koe, B. K. and Weissman, A. J., *Pharmacol. Exp. Ther.*, 1966, **154**, 500.
24. Matsumura, F., *Toxicology of Insecticide*, Plenum Press, New York, 1976.
25. Nestler, C., Carolyn, S. B. and Wheeler, A. P., *Comp. Biochem. Physiol.*, 1981, **69**, 53.
26. Richter, V. D. and Reutschke, E., *Acta Histochem.*, 1977, **60**, 304.
27. Miller, T. A., *Annu. Rev. Entomol.*, 1975, **20**, 133.
28. Kerkut, G. A., *Br. Med. Bull.*, 1973, **29**, 100.
29. Evans, P. D., *J. Neurochem.*, 1978, **30**, 1009.
30. Maxwell, D. J., *Tissue Cell*, 1980, **12**, 703.
31. Bhakthan, N. M. and Gilbert, L. J., *Gen. Comp. Endocrinol.*, 1968, **11**, 186.
32. Ralph, C. L. and McCarthy, R., *Nature*, 1964, **203**, 1195.
33. Jacobs, M. E., *J. Insect. Physiol.*, 1970, **16**, 55.
34. Miller, M. R., *Comparative Physiology of Carbohydrate Metabolism in Heterothermic Animals*, University of Washington Press, Seattle, 1961, p. 125.
35. Plisetskaya, E. M., *Usp. Sovrem. Biol.*, 1964, **57**, 128.
36. Demael-Suard, A., Garin, D. and Peres, G. C., *J. Physiol.*, (*Paris*), 1968, **60**, 237.
37. Craig, A. B. Jr., *Am. J. Physiol.*, 1959, **196**, 969.
38. Bose, C., *Proc. Natl. Acad. Sci. India*, 1991, **61**, 25.

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Reactivity of carbon monoxide with haemoglobin *in vitro* and its spectrophotometric estimation

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Dilution and pH affect the affinity of haemoglobin in blood for carbon monoxide. We show by spectrophotometric estimation of carboxyhaemoglobin that haemoglobin in diluted blood and blood at slightly alkaline pH is carboxylated more quickly than haemoglobin in undiluted blood and blood at slightly acidic pH.

CARBON MONOXIDE (CO) is frequently encountered in forensic toxicology in accidental or intentional victims of fire, exhaust fumes, etc. Haemoglobin (Hb) containing bound CO, carboxyhaemoglobin (HbCO), is relatively stable, the binding constant for CO being 200–300 times that for oxygen[1–5]. CO in chemical combination with Hb in blood prevents oxygenation of cells throughout the human body. In the present study we have determined reactivity of CO with Hb in undiluted and diluted blood samples at different pH's by spectrophotometric estimation of HbCO.

Fresh blood was collected from a healthy adult in a heparinized tube. Two ml of this blood (12 g% Hb) was taken and CO gas was passed for different time periods between one second and 30 min at the rate of 0.5 ml (three bubbles) per second. Again 0.2 ml of fresh blood was taken and made up to 2 ml (10-fold dilution) with 0.4% ammonium hydroxide. Serial dilutions were then prepared, up to 100-fold. CO was passed for different time periods. Undiluted and diluted (with 0.4% NH_4OH at different pH's between 6.5 and 8.0) post-mortem blood was subjected to carboxylation in the same way.

Carbon monoxide was prepared from oxalic acid and sulphuric acid. Oxalic acid was taken in a round-bottom flask fitted with a cork with two inlets. One had a plain glass tube dipped into the solution of oxalic acid and sulphuric acid while other was L-shaped, so as to enable the collection of gases. As carbon dioxide is also formed during this reaction, the evolved gases were passed through potassium hydroxide solution to absorb carbon dioxide.

Carboxyhaemoglobin in the test samples was determined spectrophotometrically in a simulated double-beam spectrophotometer (Beckman DU-64) following the method of Siek and Rieders[6]. The formula used for calculating %HbCO was:

$$\%\mathrm{HbCO} = \left[\frac{A_{530} - A_{583}}{4.2} \times \frac{E^*}{A_{max}}\right] \times 100,$$