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Larvicidal Activity of Inhibitors of DOPA Decarboxylase on the Australian Sheep Blowfly, *Lucilia cuprina*

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Abstract

Inhibitors of DOPA decarboxylase, the key enzyme in the formation of the sclerotizing agent (*N*-acetyl dopamine) of the blowfly cuticle, have been tested for larvicidal activity against *L. cuprina*. A significant level of DOPA decarboxylase activity has been shown to be present throughout larval life in this species. Four potent *in vitro* inhibitors of *L. cuprina* larval DOPA decarboxylase (carbidopa, benserazide, methyl tyrosine and methyl DOPA) have been shown to be effective larvicides when fed to first- or second-instar larvae. However, no correlation is seen between the apparent K_i and LD_{50} values for these compounds. Treated larvae are observed to die at the next moult but death can be averted by the addition of *N*-acetyl dopamine to the food. Thus the toxic effects of the DOPA decarboxylase inhibitors appear to result from an inhibition of the formation of the sclerotizing agent in the cuticles of treated larvae.

Introduction

Lucilia cuprina is the major primary species of blowfly causing fly-strike of sheep (Shanahan and Roxburgh 1974), with the larval stages being capable of initiating strike lesions.

The larvicides used to date for controlling fly-strike have had their toxic effects by acting primarily on the larval nervous system. When first introduced these larvicides were very effective but have become much less so due to the development of resistance within the fly population (Shanahan and Roxburgh 1974). Hence, the development of new larvicides with totally different modes of action (thus reducing the possibility of cross-resistances with the earlier larvicides) is needed. One approach to the development of new larvicides could be to find compounds which inhibit enzymes essential for the larval development of the blowfly but which are less important to the metabolism of the sheep.

The insect cuticle provides not only physical protection against the environment but also an exoskeleton and a permeability barrier, and has been studied extensively by a variety of techniques (see articles in Hepburn 1976). The synthesis and secretion of the components of the cuticle, by the single layer of epidermal cells, represents a major biochemical difference between insects and mammals. The structure of the larval cuticle of *L. cuprina* has been examined by Filshie (1970) using the electron microscope. It is composed of an untanned, inner endocuticle (mainly chitin and protein) and an outer, complex epicuticle (protein and lipid). The epicuticle consists of four layers—the dense layer (closest to the endocuticle), the cuticulin, the outer epicuticle, and the superficial layer—and is totally resynthesized at each larval moult.

The insect cuticle does seem to be a promising target for insecticide action. Already some insect growth regulators are available which appear to act via effects on the larval cuticle. For example, the benzoylphenylureas (e.g. diflubenzuron) appear to interfere with chitin deposition, thereby weakening the endocuticle to such an extent that it is unable to withstand the muscular traction and increased turgor during moulting (Verloop and Ferrell 1977).

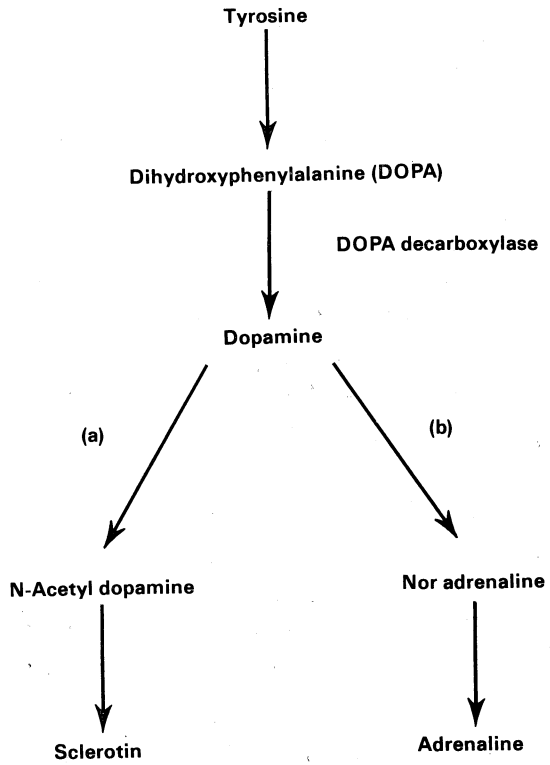

Fig. 1. The role of DOPA decarboxylase in the metabolic fate of tyrosine in the sclerotization of the blowfly pupal case (*a*) (from Karlson and Sekeris 1964) and in mammalian catecholamine biosynthesis (*b*) (from Molinoff and Axelrod 1971).

In addition to the chitin, stabilized protein enhances the strength and protective ability of the cuticle. In dipteran larvae, protein stabilization is seen most dramatically at the time of pupariation when the outer region of the larval endocuticle is hardened by the process of sclerotization (in association with tanning) to form the pupal case. Sclerotization is a process in which compounds produced in the epidermal cells diffuse into soft regions of the cuticle, cross-link the protein chains and harden the cuticular structure. The sclerotizing agent in the blowfly *Calliphora* sp. was found to be *N*-acetyl dopamine (Karlson and Sekeris 1964) and its biogenesis is under hormonal control. The pathway for the synthesis of *N*-acetyl dopamine from tyrosine is shown in Fig. 1*a*. Ecdysone stimulates the *de novo* synthesis of DOPA decarboxylase (aromatic-L-amino-acid decarboxylase, EC 4.1.1.28), which changes the metabolic fate of tyrosine from *p*-hydroxyphenylpyruvic acid to *N*-acetyl dopamine (Fragoulis and Sekeris 1975*a*). A possibly important role of sclerotization in the

cuticles of younger larvae is to stabilize and strengthen the cuticulin and perhaps the other epicuticle layers in newly forming and unfolding epicuticle. This was suggested by Wigglesworth (1947) for *Rhodnius prolixus*, and later by Locke and Krishnan (1971) as a result of their study of the cuticle of *Calpodes ethlius* using the electron microscope. Thus, chemicals that inhibit the sclerotization reaction might be expected to affect the viability of dipteran larvae at all stages of growth. Indeed, Sparrow and Wright (1974) have shown that α-methyl DOPA (a competitive inhibitor of DOPA decarboxylase) kills larvae of *Drosophila melanogaster* at the first and second larval moults.

In this paper are described experiments showing that a number of compounds that are potent *in vitro* inhibitors of DOPA decarboxylase from *L. cuprina* are also highly toxic when fed to the larvae. Death occurs at the moult and inhibition of cuticular sclerotization appears to be the primary cause of death.

Materials and Methods

Chemicals

DL-2-Methyl-3-(3,4-dihydroxyphenyl)alanine (DL-α-methyl DOPA) and DL-α-methyl-*m*-tyrosine were obtained from the Sigma Chemical Co. Clayton, Vic. Diflubenzuron [1-(4-chlorophenyl)-3-(2,6-difluorobenzoyl)urea; PH 60–40] was a gift from Philips-Duphar, Amsterdam, The Netherlands. Carbidopa (*S*-α-hydrazino-3,4-dihydroxy-α-methylbenzenepropanoic acid monohydrate; MK-486) was a gift from Merck, Sharp and Dohme (Aust.) Pty Ltd, South Granville, N.S.W., and benserazide [DL-serine 2-[(2,3,4-trihydroxyphenyl)methyl]hydrazine; Ro 4-4602] was a gift from Roche Products Pty Ltd, Dee Why, N.S.W. Other chemicals were of laboratory grade.

L-3,4-Dihydroxyphenyl[1-^{14}C]alanine (7·9 mCi/mmol) was obtained from the Radiochemical Centre, Amersham, U.K.

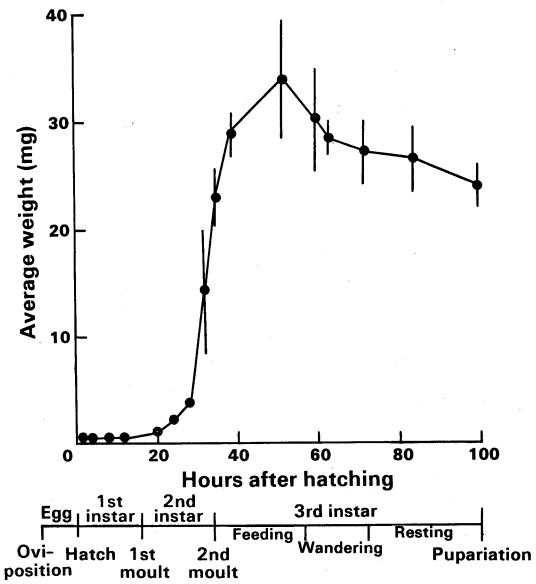

Fig. 2. The average liveweight of larvae of *L. cuprina* at various times after hatching when reared as described in the Materials and Methods. The different stages during larval life, the times of the larval moults and pupariation are also shown on the horizontal axis. Vertical bars show ± 1 s.d.

Larval Cultures of L. cuprina

Newly laid eggs were obtained from the Division of Entomology, CSIRO, Canberra, and were placed on fresh sheep's liver in a tray containing vermiculite. The time that the first larvae appeared was noted and unhatched eggs were removed from the liver about 1 h after this. Larval cultures were then maintained at 27°C. Hatching times for different cultures could be varied by placing

the eggs at 0–4°C. This delayed the development for the period of storage, but the larvae hatched as normal when returned to 27°C (Williams 1972).

The stages of larval development and the developmental profile for the increase in larval liveweight for *L. cuprina* are shown in Fig. 2. The times of the moults were estimated by periodically examining the cultures for cast-off cuticles, and for increased activity associated with moulting. Larvae were also staged into instars by examining the posterior spiracles, as described by Williams (1972). For cultures raised at 27°C, the first moult occurred at about 16 h and the second at about 34 h after hatching. Most of the feeding was done by late second- and early third-instar larvae, and by 56 h feeding had ceased. The larvae then entered a wandering stage during which they moved from the liver to the dry vermiculite. This is equivalent to the crop-full stage described by Dennell (1946) for *Sarcophaga faculata*. The crop was then progressively emptied with an equivalent loss in larval liveweight. By 72 h the crop was empty and the larvae were resting within the vermiculite. Pupariation occurred at about 100 h after hatching.

Larval Toxicity Experiments

The medium used for the toxicity tests was sheep's liver that had been blended with water to form a flowing, liquid paste. For dosage–mortality experiments, varying volumes of a solution or suspension of the chemical being tested were stirred into 25-ml samples of the blended liver and the mixtures placed in 150-ml glass jars. Fifty newly hatched larvae were placed in each jar and these cultures were then kept at 27°C. After 60–70 h the contents of each jar were emptied onto vermiculite to provide the conditions for pupariation. Second-instar feeding trials were carried out by the same method using 30 larvae selected from cultures 18–20 h after hatching.

Mortality percentages were calculated from the numbers of larvae forming normal pupae, using the Abbott's formula to correct for the natural mortality (Busvine 1971). Preliminary experiments established that adult flies emerged from $92 \pm 4\%$ of apparently normal pupae. LD_{50} values were calculated by linear regression analysis of the probit transformation of log dosage–mortality data (Busvine 1971), or established by inspection of the log dosage–mortality curves.

Larval Rescue Experiments

Rescue experiments were performed on a smaller scale since normally only first-instar larvae were used and the tests were terminated at mid second instar. Samples (2 ml) of blended liver containing known concentrations of both inhibitor and rescuing agent were placed in 30-ml vials. Twenty-five newly hatched larvae were placed on the liver in each vial and these were kept at 27°C. The number of larvae surviving the first moult in each vial were counted and the percentage survival calculated as 100 minus the percentage mortality (calculated as above).

DOPA Decarboxylase Activity

Preparation of tissue extracts from L. cuprina *larvae*

Crude extracts for developmental profile. At different developmental times, 0·2–0·5 g of larvae were taken from cultures, rinsed in distilled water and homogenized at 0°C with 1·5 ml of buffer [0·06 M TES, pH 7·3, and 0·1 mM phenylthiourea (PTU)]. The homogenate was centrifuged at 7700 *g* for 10 min and the supernatant collected, frozen and stored at −20°C for up to a week. Freezing and storage had no effect on the level of DOPA decarboxylase activity. A 0·1-ml sample of each preparation was used for each assay. Protein levels were determined by the Biuret method.

Partially purified extracts for kinetic studies. Frozen white pupae (about 10 g) were homogenized in buffer (0·06 M TES, pH 7·3, and 0·1 mM PTU) containing 3 mM 2-mercaptoethanol (10 ml/g) at 0°C. The homogenate was centrifuged at 7700 *g* for 10 min and the supernatant passed through four layers of cheese-cloth to remove lipids. The supernatant was then heated at 50°C for 3 min and centrifuged at 25 000 *g* for 30 min. Ammonium sulfate precipitation was performed on the heat-treated supernatant by the method used by Christenson *et al.* (1970) and the material precipitated between 40–70% ammonium sulfate saturation was obtained. This was dissolved in 2·5 ml of buffer (0·06 M Tris, pH 7·3, and 0·1 mM PTU) and the solution was dialysed against 2 litres of this buffer overnight. The dialysate was frozen and stored at −50°C. A 20-μl sample was used for each assay. Preparations had 1000–3500 units activity/ml. (One unit of DOPA decarboxylase activity is defined as the amount of enzyme which releases 1·0 nmole $^{14}CO_2$ from ^{14}C-DOPA per 20 min.) Preparations lost no activity during storage.

Partial purification of extracts from rat brain

Brains were collected from laboratory-bred Wistar rats and rapidly frozen in liquid nitrogen before being stored at −20°C. About 50 g of these brains were thawed, minced and homogenized with 150 ml of buffer (0·05 M Tris–HCl, pH 7·3, and 0·01 M 2-mercaptoethanol) at 0°C. The homogenate was centrifuged at 25 000 *g* for 30 min (4°C) and the crude supernatant collected. This was centrifuged in a Spinco L-50 rotor for 1 h at 45 000 rev/min (0–4°C). The high-speed supernatant was subjected to ammonium sulfate fractionation and the 30–70% pellet was obtained. This was dissolved in 5·0 ml of 0·05 M Tris–HCl buffer (pH 7·3) and the solution was dialysed against 2 litres of the buffer overnight. The dialysate was frozen and stored at −50°C, and 10–25-μl samples were used per assay. Preparations had approximately 400 units activity/ml initially; however, they lost about 60% of activity over a 6-week period of storage.

Standard assay procedure

Activity was measured by an adaptation of the $^{14}CO_2$ microdiffusion methods of Lunan and Mitchell (1969), Hodgetts and Konopka (1973), and Chen and Hodgetts (1974) using ^{14}C-DOPA as substrate. Glass scintillation-counter vials were used as the reaction vessels. The $^{14}CO_2$ was collected on a strip of filter paper 2 by 5 cm saturated with hyamine hydroxide. These strips were prepared by dipping each piece into a solution of hyamine hydroxide (1 M in methanol) and then air drying them for 10 min. Each was then rolled up, inserted in a cut-off Eppendorf microcentrifuge tube and placed upright in the reaction vessel. Following the addition of the assay mixture, each vessel was sealed with a Suba cap.

The assay mixture consisted of 0·06 M TES buffer, pH 7·3, 60 μM pyridoxal 5′-phosphate (PLP), 638 μM ^{14}C-L-DOPA (0·1 μCi) and enzyme extract to a final volume of 1·0 ml. Reactions were started by the addition of substrate, after a 15 min preincubation of the enzyme, PLP and inhibitor (if present). The vials were incubated at 37°C for 20 min and the reactions were terminated by the addition of 0·2 ml of 50% trichloroacetic acid from a syringe inserted through the Suba cap. Controls without enzyme (or with boiled enzyme) were run with each set of incubations.

After terminating the reactions, the vials were left for a further 45 min at room temperature to allow maximum absorption of the $^{14}CO_2$. (This was found to be 66% of the $^{14}CO_2$ released, using standard sodium [^{14}C]carbonate and HCl.) Each filter paper was then placed in a vial and 10 ml of butyl-PBD*/toluene/methoxyethanol scintillant was added. These vials were left for at least 90 min (to allow complete subsidance of the chemiluminescence due to reaction between hyamine hydroxide and the scintillant) before determining the level of radioactivity using a Beckman scintillation counter.

Results

Optimization of Assay Conditions and Characterization of the L. cuprina *and Rat Brain DOPA Decarboxylase Enzymes*

DOPA stability and controls

There was substantial non-enzymic breakdown of ^{14}C-DOPA under standard assay conditions and this increased sharply as the pH was raised. With crude *L. cuprina* extracts, boiled-enzyme and no-enzyme controls gave similar levels of breakdown (normally about 10% of the enzyme level) and these were subtracted from the test assay values. Boiled-enzyme controls using partially purified enzyme preparations were run in all experiments as the breakdown in these controls was usually less than that in no-enzyme controls. Breakdown was apparently reduced by some heat-stable component in these preparations.

Characteristics of the L. cuprina *enzyme*

The *L. cuprina* enzyme had a pH optimum of 7·3. Activity was increased (20–30%) by the addition of PLP and 60 μM was optimum. The reaction was linear with time

* Butyl-PBD, 2-(4′-t-butylphenyl)-5-(4″-biphenyl)-1,3,4-oxadiazole.

for at least 20 min and activity was linear with the concentration of enzyme extract (up to at least 0·1 ml with the crude extract). PTU caused some inhibition of the initial rate but was necessary to prevent darkening of the assay mixtures due to melanin formation. The apparent K_m for L-DOPA, using the partially purified preparation, was $2{\cdot}7 \times 10^{-4}$ M. This compares well with values of $2{\cdot}8 \times 10^{-4}$ M for an enzyme from *Calliphora vicina* (Fragoulis and Sekeris 1975*b*), and $8{\cdot}2 \times 10^{-5}$ M for that from *Drosophila melanogaster* (Clark *et al.* 1978).

Characteristics of the rat brain enzyme

DOPA decarboxylase from rat brain had a pH optimum of 7·3 and the activity could be enhanced by the addition of PLP up to 60 μM. The apparent K_m for L-DOPA was $6{\cdot}0 \times 10^{-5}$ M. This is similar to the value of $1{\cdot}9 \times 10^{-4}$ M for a hog kidney preparation (Christenson *et al.* 1970).

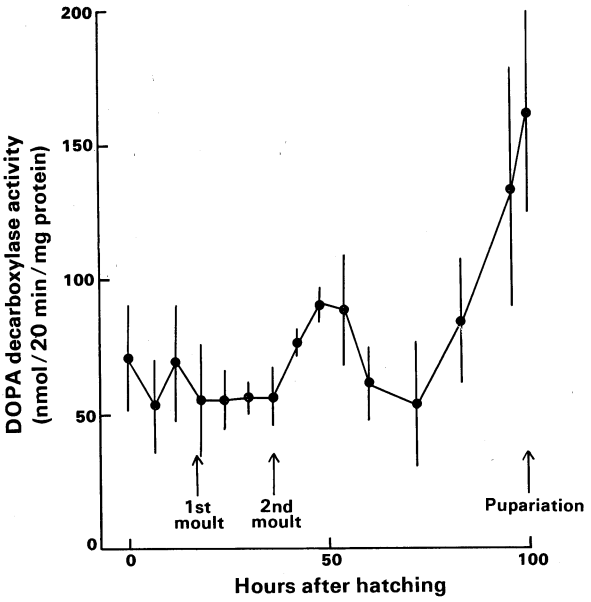

Fig. 3. Larval developmental profile for DOPA decarboxylase activity in *L. cuprina*. Specific activities of extracts of whole larvae were measured. Each point is the mean of 4–14 separate determinations. Vertical bars show ± 1 s.d.

Developmental Profile for DOPA Decarboxylase

The profile for the specific activity of DOPA decarboxylase throughout larval life is shown in Fig. 3. There is a basal level of activity throughout the larval stages of about 50 nmol/20 min/mg protein. This rises over the last day of larval life to a peak at the time of pupariation. The peak is at least three times the basal level. A second peak can be seen beginning soon after the second moult, which reaches a maximum of about twice the basal activity by 50 h after hatching (the time that feeding ceases). Variations in the specific activity may also occur around the time of the first moult but the data is too variable to be conclusive. As activity is present throughout larval life, it is possible that inhibitors of DOPA decarboxylase may have early larvicidal effects.

Inhibition Studies using the Partially Purified Enzymes

A number of very potent inhibitors of mammalian DOPA decarboxylase have been described, with particular reference to catecholamine synthesis (Fig. 1*b*) and

the treatments of Parkinson's disease and hypertension in humans. Those used in this study were DL-α-methyl DOPA (methyl DOPA) and DL-α-methyl-*m*-tyrosine (methyl tyrosine) (Sourkes and D'Iorio 1963), carbidopa (Porter *et al.* 1962) and benserazide (Burkard *et al.* 1964). All were found to be inhibitors of the partially purified enzymes from both *L. cuprina* and rat brain in the *in vitro* assay system. Inhibition constants (apparent K_i values) for these four inhibitors were determined for both enzymes by linear regression analysis of Lineweaver–Burk double reciprocal data and are shown in Table 1.

Table 1. Apparent inhibition constants and LD_{50} values for the DOPA decarboxylase inhibitors

Inhibition constants (apparent K_i values) were determined using partially purified enzymes prepared from white pupae of *L. cuprina* or from rat brain. LD_{50} values are the concentrations of the inhibitors in the food which killed 50% of the larvae

Inhibitor	Apparent inhibition constant (M)		LD_{50} (M)
	L. cuprina enzyme	Rat brain enzyme	
DL-α-Methyl DOPA	$3{\cdot}1 \times 10^{-6}$	$2{\cdot}9 \times 10^{-5}$	$1{\cdot}2 \times 10^{-4}$
DL-α-Methyl *m*-tyrosine	$1{\cdot}1 \times 10^{-4}$	$9{\cdot}3 \times 10^{-6}$	$2{\cdot}1 \times 10^{-4}$
Benserazide	$3{\cdot}2 \times 10^{-6}$	$1{\cdot}6 \times 10^{-6}$	$1{\cdot}1 \times 10^{-3}$
Carbidopa	$4{\cdot}5 \times 10^{-9}$	$8{\cdot}0 \times 10^{-9}$	$1{\cdot}6 \times 10^{-3}$

The results show that carbidopa was by far the most potent inhibitor of the *L. cuprina* enzyme having an apparent K_i ($4{\cdot}5 \times 10^{-9}$ M) about three orders of magnitude lower than that for the other compounds. Methyl DOPA and benserazide have similar apparent K_i's for this enzyme and methyl tyrosine (apparent K_i $1{\cdot}1 \times 10^{-4}$ M) is the least potent. Methyl DOPA is about 10 times more potent as an inhibitor of the *L. cuprina* enzyme compared with the rat brain enzyme while the reverse applies to methyl tyrosine. Carbidopa has much the same apparent K_i for both enzymes, as does benserazide.

Bosin *et al.* (1978) showed that various analogues of tryptophan were potent inhibitors of DOPA decarboxylase prepared from hog kidney. Tryptophan analogues with a methyl group at position α, 4, 5, 6 or 7 were tested as possible inhibitors of the *L. cuprina* enzyme. None, however, gave inhibition greater than 20% when present at 10^{-3} M, so they were not tested further.

Since carbidopa, methyl DOPA, methyl tyrosine and benserazide were all effective *in vitro* inhibitors of *L. cuprina* DOPA decarboxylase, their larval toxicity was tested. Methyl DOPA was used most intensively in these larval toxicity studies because of its greater availability.

Larval Toxicity Experiments

DL-α-*Methyl DOPA*

This compound was found to be highly toxic to *L. cuprina* larvae. Early first- or early second-instar larvae were placed on blended liver containing various levels of methyl DOPA. Death was observed to occur at around the time of the next moult.

Probit analysis of the data from a number of feeding trials gave an LD_{50} for first-instar larvae of $1{\cdot}2 \times 10^{-4}$ M, and for second-instar larvae of $1{\cdot}8 \times 10^{-4}$ M (Fig. 4). The second-instar larvae show a greater variance in lethal dosages within the population than do first instars. This is indicated by the shallower slope of the log dosage–probit mortality line (6·1 probit units/log unit of concentration for first-instar larvae compared with 3·0 for second-instar larvae).

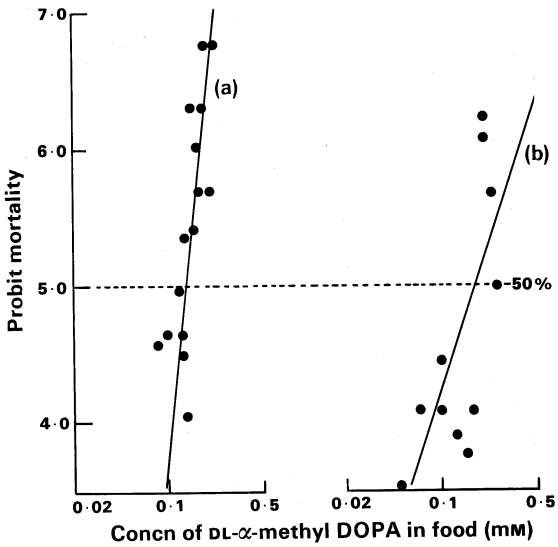

Fig. 4. Dosage–mortality regression lines for *L. cuprina* larvae fed α-methyl DOPA during the first instar (*a*) and the second instar (*b*). - - - - 50% larval mortality.

Other DOPA decarboxylase inhibitors

Similar toxicity trials were performed with first-instar larvae using methyl tyrosine, carbidopa and benserazide. They were all toxic to the larvae and death occurred at the time of the next moult. LD_{50} values, estimated by direct inspection of the log dosage–mortality curves for these inhibitors, are shown in Table 1. Methyl DOPA and methyl tyrosine had the highest toxicities (LD_{50} $1{\cdot}2 \times 10^{-4}$ M and $2{\cdot}1 \times 10^{-4}$ M respectively) while carbidopa and benserazide were an order of magnitude less potent. As can be seen in Table 1, there is no apparent correlation between the effectiveness of each compound as an inhibitor of the isolated enzyme (the apparent K_i value) and its larvicidal activity (the LD_{50} value). Methyl tyrosine alone had an LD_{50} comparable to its apparent K_i value.

Diflubenzuron, an inhibitor of chitin deposition

For comparison, toxicity trials were also carried out with diflubenzuron, a benzoylphenylurea larvicide that is thought to kill by interfering with chitin deposition (Verloop and Ferrell 1977). Probit analysis of the data for first-instar larvae gave an LD_{50} of $2{\cdot}1 \times 10^{-6}$ M and a slope for the line of 5·0 probit units/log unit of concentration. Therefore this compound is at least 100 times more toxic than the DOPA decarboxylase inhibitors tested. Death was observed to occur at the next

moult, in a manner apparently similar to that of larvae treated with the DOPA decarboxylase inhibitors.

Larval Rescue Experiments

If DOPA decarboxylase inhibitors are killing by interfering with the sclerotization pathway, then the addition of *N*-acetyl dopamine (the immediate sclerotization agent) or dopamine (the product of the DOPA decarboxylase reaction) should be able to prevent death by bypassing the blocked section of the pathway and providing substrate for sclerotization.

Rescue from methyl DOPA poisoning

N-Acetyl dopamine, mixed into blended liver containing LD_{100} levels of methyl DOPA, could prevent the death of larvae placed on it. Fig. 5 shows this rescue of larvae at the first moult. At a methyl DOPA concentration of 1 mM (Fig. 5*a*), with no addition of *N*-acetyl dopamine, there was 100% mortality. This could be reduced to zero (100% survival) by levels of *N*-acetyl dopamine greater than 1 mM. As the concentration of methyl DOPA in the food was increased, the capacity of *N*-acetyl dopamine to rescue the larvae was decreased. At 5 mM methyl DOPA, the maximal rescue level obtained with *N*-acetyl dopamine was only 70% (Fig. 5*b*), while at 20 mM methyl DOPA no rescue was obtained (Fig. 5*c*). These results suggest that high concentrations of methyl DOPA produce secondary lethal effects that are unrelated to sclerotization.

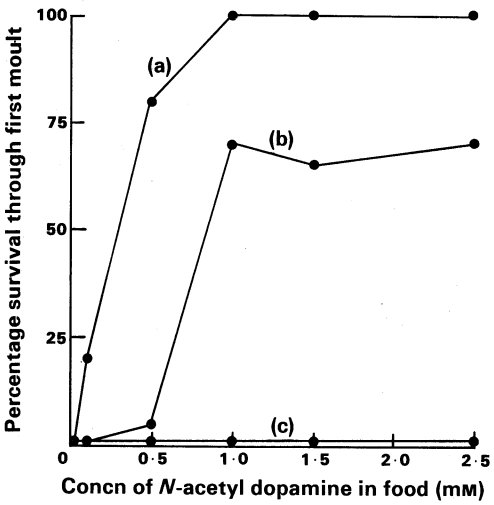

Fig. 5. Rescue of first-instar larvae from α-methyl DOPA poisoning by the simultaneous addition of various levels of *N*-acetyl dopamine to the food. The three curves show the effect of *N*-acetyl dopamine when different levels of α-methyl DOPA were used: (*a*) 1·0 mM, (*b*) 5·0 mM and (*c*) 20·0 mM.

N-acetyl dopamine was also found to rescue larvae fed methyl DOPA during the second instar. Additionally, dopamine was found to rescue first-instar larvae in a similar manner and at a similar concentration to *N*-acetyl dopamine.

Effective doses of N-*acetyl dopamine*

From sigmoidal dosage–percentage survival curves for *N*-acetyl dopamine, like those given in Fig. 5, ED_{50} values (i.e. the concentration of this compound which

prevents the death of 50% of the maximum number of rescuable larvae) can be estimated. Table 2 shows the ED_{50} values obtained from eight separate rescue experiments with different methyl DOPA concentrations. While the concentration of methyl DOPA varied over a 13-fold range, the ED_{50}'s only varied within a twofold range. This suggests that the level of *N*-acetyl dopamine effective in rescuing larvae is essentially independent of the methyl DOPA concentration (within the range 0·4–5 mM).

Table 2. Concentrations of *N*-acetyl dopamine effective in rescuing *L. cuprina* larvae from poisoning by different levels of α-methyl DOPA

ED_{50} (effective dose-50) is the concentration of *N*-acetyl dopamine in the food which prevents the deaths of 50% of the rescuable first-instar larvae. These values are calculated from dosage–survival curves (see Fig. 5)

Concn of DL-α-methyl DOPA (mM)	ED_{50} for *N*-acetyl dopamine (mM)	Concn of DL-α-methyl DOPA (mM)	ED_{50} for *N*-acetyl dopamine (mM)
0·4	0·55	2·0	0·75
0·8	0·65	4·0	0·83[A]
1·0	0·53[A]	5·0	0·75

[A] These values are the average of two independent experiments. Other values are from single, separate experiments.

Rescue from poisoning by other inhibitors

The data in Table 3 demonstrate that 100% of larvae raised on blended liver containing LD_{90}–LD_{100} levels of benserazide, carbidopa or methyl tyrosine could be rescued from death at the first moult by 5·0 mM *N*-acetyl dopamine. By comparison, larvae fed liver containing diflubenzuron (5·0 or 8·0 μM) could not be rescued by the addition of 5·0 mM *N*-acetyl dopamine.

Table 3. Rescue by *N*-acetyl dopamine of first-instar larvae from poisoning by DOPA decarboxylase inhibitors

Percentage survival is 100 minus the percentage mortality, calculated as described in the Materials and Methods

Concentration of inhibitor	Percentage survival for *N*-acetyl dopamine concn (mM) of: 0	2·0	5·0
α-Methyl-*m*-tyrosine (3·0 mM)	0	21	100
Benserazide (2·5 mM)	8	5	100
Carbidopa (2·0 mM)	10	84	100

Discussion

Developmental Profile for DOPA Decarboxylase in L. cuprina *Larvae*

No distinct peaks of DOPA decarboxylase activity coincide with the larval moults, as would be expected if only the cuticulin was being sclerotized. The basal level throughout larval life suggests that some sclerotization is occurring continuously as the epicuticle unfolds. However, the enzyme extract is from whole larvae, so it is possible that some of the activity is due to DOPA decarboxylase involved in catecho-

lamine synthesis in the nervous tissues, as was shown for *Drosophila* sp. by Dewhurst *et al.* (1972). The peak of activity at 50 h after hatching coincides with the cessation of feeding and a colour change in the larvae from white to cream. There may be some sclerotization associated with preparing the larval cuticle for the imminent change of habitat that occurs under natural conditions: from the moist, host environment to the harsher, drier soil environment. The peak at pupariation is associated with sclerotization of the pupal case.

Toxicity of Inhibitors of DOPA Decarboxylase

The ability of *N*-acetyl dopamine (and dopamine) to rescue larvae from the lethal effects of DOPA decarboxylase inhibitors indicate that they are indeed killing by inhibiting the formation of the sclerotizing agent. The time of death, just after the moult, indicates that failure to sclerotize regions of the newly formed cuticle probably weakens it in such a way as to cause death. The fact that *N*-acetyl dopamine could not rescue diflubenzuron-treated larvae suggests that the former compound does not simply act non-specifically to rescue all larvae with weakened cuticles. With diflubenzuron, for some species of insect, death has been inferred to be due to the rupturing of the weakened cuticle during the moult (Mulder and Gijswijt 1973). Since the symptoms of death in *L. cuprina* larvae treated with methyl DOPA are similar to those seen in larvae treated with diflubenzuron, death with both compounds may be due to rupturing of the new cuticle during the moult. Alternatively, the organized structure of the cuticle might be changed by the failure of sclerotization, interfering with its effectiveness as a permeability barrier.

The lack of correlation between the LD_{50} and the apparent K_i values for the various DOPA decarboxylase inhibitors may possibly be explained by a failure of the compounds to successfully penetrate to the enzyme within the epidermal cells. It is interesting that benserazide and carbidopa (which show the greatest differences between LD_{50} and apparent K_i) are useful in treatment of Parkinson's disease in humans because they are unable to penetrate the blood–brain barrier. Thus they act only as DOPA decarboxylase inhibitors in the peripheral tissues and not in the central nervous system (Bartholino *et al.* 1967; Lotti and Porter 1970). Alternatively, effective enzyme inhibitors *in vitro* may be ineffective *in vivo* because they are inactivated within the tissues.

Inhibitors of DOPA decarboxylase may not appear to be ideal insecticides from the point of view of host–parasite selective toxicity. As noted earlier, DOPA decarboxylase is involved in the mammalian pathway for catecholamine synthesis. As shown in Table 1, the various inhibitors of the *L. cuprina* DOPA decarboxylase that were tested were just as effective against the enzyme from rat brain. Despite these observations, DOPA decarboxylase inhibitors are much less toxic to mammals than we have shown them to be to blowfly larvae. For example, the level of toxicity of methyl DOPA (oral LD_{50}) for rats is 5000 mg/kg (Christensen and Luginbyhl 1975), 250-fold higher than that for *L. cuprina* larvae. This difference in toxicity is probably a reflection of the great importance of a normal cuticle for larval survival. As a further illustration of this difference in toxicity between insects and mammals, it should be noted that the average daily dosage of methyl DOPA for therapeutic use in humans is 1·0 g (about 14 mg/kg, Goodman and Gilman 1970), about the LD_{50} level for *L. cuprina* larvae.

The LD_{50} values for the most effective DOPA decarboxylase inhibitors (methyl DOPA and methyl tyrosine) are about two orders of magnitude higher than that for diflubenzuron. Therefore further testing of these compounds as larvicides for the control of blowfly-strike of sheep is probably not warranted. The importance of this work is that DOPA decarboxylase and the sclerotization pathway have clearly been demonstrated to be potential sites for larvicide attack. It would seem to be worthwhile to attempt to formulate more effective inhibitors of this pathway.

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