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EFFECTS OF THE NON-PROTEIN AMINO ACIDS L-CANAVANINE AND L-CANALINE ON THE NERVOUS SYSTEM OF THE MOTH *MANDUCA SEXTA* (L.)

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SUMMARY

Injection of L-canavanine, a naturally occurring arginine analogue, and of its metabolic derivative, L-canaline, induced almost continuous motor activity in adult tobacco hornworms, *Manduca sexta* (L.). Initially the moths flew normally, but after a time interval that depended both on the amino acid and on the dose (1–45 μmol/g fresh weight) the moths became disorientated and muscle activity was less patterned. Canaline produced its initial effects 12–30 min after injection, whereas activity in response to canavanine began after a delay of 1–2 h. Canaline (derived from canavanine by an arginase-mediated hydrolytic cleavage) is probably the biologically active factor.

Canaline did not affect axonal conduction of action potentials nor the activity of mechanoreceptors on the forewing. Canaline (22 μmol/g fresh weight) prolonged the postsynaptic potential of flight muscle fibres, but after 20–40 min. the electrical activity of muscle fibres was normal. The results show that canaline alters the activity of the central nervous system of adult *M. sexta*, but its mode of action is unknown.

INTRODUCTION

Many plants accumulate secondary metabolites that may curtail the feeding activity of phytophagous insects and other herbivores (Fraenkel, 1959, 1969; Ehrlich & Raven, 1965; Whittaker & Feeny, 1971; Feeny, 1975). The nature and efficacy of plant chemical defences have been of keen interest to those studying herbivore-plant interactions and plant resistance to various pests. Plant metabolites are also of interest because these compounds may have pharmacological properties useful in research. One such metabolite is L-canavanine, $H_2N—C(=NH)—NH—O—CH_2—CH_2—CH(NH_2)CO_2H$, a structural analogue of L-arginine produced by certain leguminous plants (Rosenthal, 1977*a*).

Previous studies on the mode of action of canavanine in insects have focused

primarily on developmental and biochemical effects. Severe and often fatal developmental abnormalities result when *Manduca sexta* or other larvae that normally do not feed on canavanine-containing plants ingest diets supplemented with canavanine (references in Dahlman & Rosenthal, 1976; Harry, Dror & Applebaum, 1976).

In the present study we examined the physiological effects of L-canavanine injected into *Manduca sexta* (L.) (Lepidoptera: Sphingidae). L-canaline, $H_2N—O—CH_2—CH_2—CH(NH_2)CO_2H$, an ornithine analogue, was also studied, since it is a metabolic derivative of canavanine and may be the biologically active factor.

MATERIALS AND METHODS

Manduca sexta were raised on agar-based artificial diet (modified after Yamamoto, 1969). Moths were used one day after pupal–adult ecdysis. L-Canaline and L-canavanine were prepared according to the methods of Rosenthal (1973, 1977*b*). L-[guanidinooxy-^{14}C]canavanine was prepared by enzymatic synthesis utilizing L-canaline and L-[guanidino-^{14}C]arginine after the method of Allende & Allende (1964). At the start of an experiment the appropriate amino acid was dissolved in physiological saline solution (pH 7·0) consisting of 25 mM-NaCl, 25 mM potassium methanesulphonate, 4 mM-$CaCl_2$, 33 mM-$MgCl_2$ and 150 mM Tris methanesulphonate (modified after Rheuben, 1972). For studies on intact moths, small volumes of the test solution (*ca.* 0·1 ml/g fresh wt) were injected into an anterior abdominal segment.

Behavioural observations were made on moths placed in individual containers after injection. Moths receiving either canavanine or saline were observed every 15 min, and moths receiving canaline were inspected every 5 min. Each moth was observed for 1 min at the stated intervals, and the percentage of this observation period spent in large-amplitude wing movements was measured. The length of time after injection, during which a moth spent at least 50% of an observation period making large-amplitude wing movements, will be referred to as 'duration of 50% flight'.

Extracellular muscle potentials were recorded by means of fine copper wires inserted into the flight muscles of a moth or pharate adult waxed to a support. After recording of the normal motor pattern had been obtained, the animal was injected with the test solution, as described above.

Intracellular potentials were recorded from the dorsal longitudinal muscle, which was exposed by dissecting away the overlying ventral cuticle and muscles. The nerve to this muscle (dl_1; Eaton, 1971) was stimulated via a glass suction electrode. Saline containing canaline sufficient to give 22 μmol/g body weight was added to the space around the thoracic ganglia and ventral aspect of the muscle. In other experiments the activity of a large sensory nerve that supplies the forewing (nerve IIN1b; Eaton, 1974) was recorded by means of a suction electrode. These moths were injected in the abdomen with 22 μmol canaline/g and then dissected quickly to expose the nerve.

Arginase activity was determined as follows. Individual, frozen insects (adults or pharate adults) were ground for 30 sec in a Sorvall Omni-mixer with 25 ml of 100 mM glycylglycine buffer (pH 7·6) containing 2 mM-$MnCl_2$ and saturated with phenylthiourea at 4 °C. After expressing the resulting slurry through cheesecloth, the homogenate was clarified further by centrifugation at 19000 *g* for 15 min. One ml of insect homogenate, supplemented with 4 mg of Sigma type III urease, was placed in each of eight 25 ml Erlenmeyer flasks. Each flask was sealed with a rubber septu[illegible]

supporting a plastic centre well containing 4 drops of Hydroxide of Hyamine, and placed at 37 °C for 45 min to metal-activate the arginase. The enzyme assay was initiated by injecting 1 ml of 100 mM-L-[guanidinooxy-^{14}C]canavanine (3750 cpm/μmol) into each of the sealed flasks. After 10, 20 and 40 min the reaction was terminated in duplicate flasks by injecting 2 ml of 2 N-HCl. Sixty min later the septum was removed and the evolved $^{14}CO_2$ was determined by placing the plastic centre well into 10 ml of Bray's scintillation fluid and assaying the radioactivity of the Hydroxide of Hyamine by liquid scintillation spectroscopy (Rosenthal, 1970). Duplicate zero-time samples served as the controls. Homogenate protein was determined by the method of Lowry *et al.* (1951). A unit of arginase is that amount of enzyme that forms 1 μmol canaline/min under the described assay conditions. Specific activity is defined as units/mg soluble protein.

RESULTS

Behaviour

Canavanine and canaline had comparable overall behavioural effects, although canaline acted more rapidly (Table 1). After a lag-time that varied with the compound and dose, the moths flew rapidly. They continued to fly although the tarsi contacted the substratum; in normal moths tarsal contact inhibited flight. Within 5–15 min after the initial response, treated moths lost their ability to right themselves. At about the time that they ceased making large-amplitude wing strokes during 50% of the minute

Table 1. *Responses of* Manduca sexta *adults to injections of canavanine or canaline* (*data are means* ± S.E.)

Conc. (μmol/g)*	Sample size	Time from injection to: First active wing movement (min)	Time from injection to: Loss of equilibrium (min)	Duration of 50% flight (min)	Mean time to death (days)
Canavanine					
11	13	126 ± 14†	182 ± 31†	34 ± 12†	4·2 ± 0·5
17	12	95 ± 6	102 ± 6	88 ± 8	2·1 ± 0·2
22	12	105 ± 6	122 ± 12	68 ± 6	2·2 ± 0·2
34	9	70 ± 4	88 ± 6	38 ± 4	1·9 ± 0·2
45	10	69 ± 3	88 ± 11	23 ± 5	1·6 ± 0·2
56	5	63 ± 3	66 ± 10	15 ± 0	1·2 ± 0·2
67	5	Never	33 ± 10	Never	1·0 ± 0·0
90	5	Never	Less than 5	Never	1·2 ± 0·2
Canaline					
0·6	5	28 ± 11‡	Never	Never	10·8 ± 1·0
1	6	29 ± 3	40 ± 3	43 ± 5	9·3 ± 0·8
3	9	24 ± 3	36 ± 4	32 ± 5	7·3 ± 0·7
6	7	16 ± 2	24 ± 2	34 ± 2	8·4 ± 0·9
11	6	12 ± 1	19 ± 4	23 ± 5	4·2 ± 0·4
22	7	12 ± 1	16 ± 2	12 ± 1	1·6 ± 0·3
45	6	12 ± 1	17 ± 2	11 ± 1	1·2 ± 0·2
Saline Control	20	Never	Never	Never	9·9 ± 1·1

* The canavanine dose selected for the initial experiments was in units of mg/g body weight. In subsequent studies a comparable dose of canaline, in units of μmol/g body weight, was administered. This fact accounts for the unusual doses, in μmol/g, reported throughout this study.

† Five moths never responded. ‡ Three moths never responded.

of observation, they became unresponsive to external stimuli, such as a touch on an antenna or wing. Quivering wing movements of small amplitude (estimated 15–45°), interspersed with an occasional large-amplitude movement, continued with only brief interruptions until the moths died or, at low doses, recovered (Table 1). Other behavioural responses, such as ovipositional movements, egg deposition and spermatophore formation, were exhibited after injection of canavanine or canaline, but these responses were not analysed in this study. Control moths, injected with saline only, did not exhibit spontaneous activity, remained responsive to stimuli, and were able to right themselves.

At the minimum canaline or canavanine dose to which all moths exhibited spontaneous wing movement (1 and 17 μmol/g, respectively), canaline elicited initial wing movement in only one-third the time required by canavanine. The duration of 50% flight in response to canaline was only one-half as long as with canavanine. The mean time to death was four times longer for the minimum effective dose of canaline than for canavanine. However, when the effects of equimolar concentrations of these compounds were compared, the time to death did not differ (Table 1). These data suggest that canaline was the biologically active amino acid and that the delayed and prolonged reaction to canavanine probably resulted from the time lag associated with the production of canaline from canavanine.

Arginase activity

If the effects of canavanine were mediated by canaline, then *M. sexta* must have an arginase able to produce the required canaline. Arginase activity, determined under assay conditions that included an initial canavanine concentration of 50 mM, produced canaline at a rate of 4–6 μmol · B min^{-1} (Table 2).

Table 2. *Arginase activity of individual* Manduca sexta

Stage	Arginase activity/insect* Total activity (units)	Specific activity (units/mg protein)
Pharate adult 8–12 h preeclosion	5·86	0·0488 ± 0·0029
Adult	4·14	0·0434 ± 0·0017

* Each value is the mean of three determinations conducted with a single insect. A unit of arginase is that amount of enzyme that forms 1 μmol canaline/min under the assay conditions described in the text. Specific activity is defined as arginase units/mg soluble protein.

Motor patterns

Recordings of muscle potentials from tethered moths injected with either canavanine or canaline (22 and 45 μmol/g) initially showed a normal flight-motor pattern. Both treated and saline-injected moths produced a motor pattern characterized by (1) one or a pair of muscle potentials in each motor unit during each wing stroke, (2) elevator and depressor muscles excited alternately, and (3) a wingstroke period of 40–55 msec (Fig. 1 A–C). Subsequently the wingstroke period was longer, and bursts of potentials were produced. Later the motor output was more irregular and the

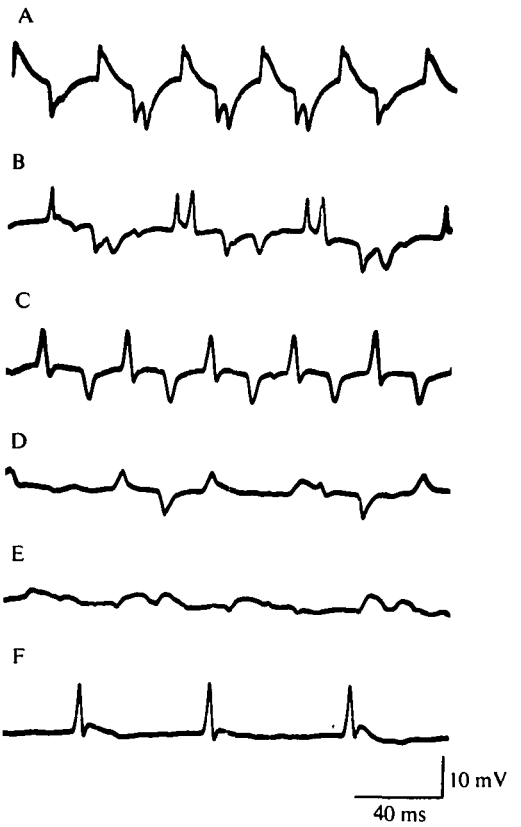

Fig. 1. Effect of 22 μmol canaline/g on the flight motor pattern. (A) Muscle potentials recorded extracellularly before injection. (B) Spontaneous activity 15 min after injection; the cycle time is longer than in normal flight. (C) Spontaneous activity 20 min after injection, same animal as in A and B; the flight motor pattern is normal. (D) 32 min after injection in another animal; cycle time is irregular and fewer units are excited. (E) 26 min after injection in same animal as in (A); flapping movements are produced by some units distant from the recording electrodes. (F) Same animal as in E, but 35 min after injection, showing a unit not activated earlier.

number of active motor units varied (Fig. 1 D–F). Activation of the flight pattern generator and the production of continuous motor output suggested that canavanine and canaline acted on the central nervous system.

The forewing sensory nerve

The possibility that sensory input to the flight pattern generator was altered by canaline was evaluated by measuring the electrical activity of a large sensory nerve containing axons of mechanoreceptors located on the forewing (A. E. Kammer & G. F. Athey, in preparation). Both canaline-treated (22 μmol/g) and untreated wing nerves were active tonically and also responded with a brief burst of impulses when the wing was touched (Fig. 2). Wing nerve activity persisted apparently unchanged after the thoracic muscles of the dissected preparations exhibited the continuous rhythmic contractions induced by canaline treatment. Furthermore, mechanoreceptor activity continued beyond the time required for intact, canaline-treated moths to become unresponsive to stimuli.

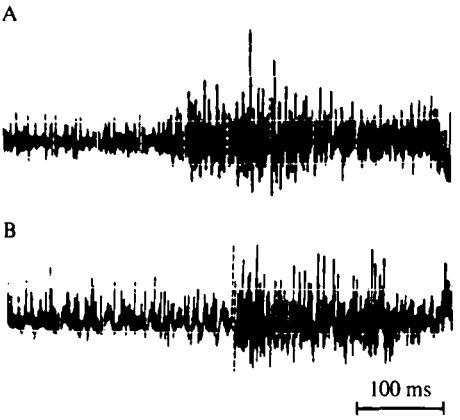

Fig. 2. Action potentials recorded from the sensory nerve supplying the forewing. The increase in activity was produced by touching the wing briefly with a probe. (A) In physiological saline. (B) 14 min after canaline injection.

To evaluate the possibility that canaline was not carried to the mechanoreceptors on the wing, moths were injected with ^{113}indium. After 20 min the radioactivity was assayed by placing a Geiger–Muller tube near the wing. The ^{113}indium had spread throughout the wing, suggesting that circulation of haemolymph was adequate to transport compounds from the abdomen throughout the wing.

Neuromuscular transmission

The possibility that canaline altered muscle membrane potentials or synaptic transmission was evaluated by recording intracellularly from the dorsal longitudinal muscle. Synaptic transmission at these neuromuscular junctions is probably mediated by glutamate (Rheuben, 1974). The resting potential of the muscle fibres was unaffected by 22 μmol canaline/g. Within 2–10 min after the application of canaline, the duration of the postsynaptic potential was 2–5 msec longer than normal (Fig. 3). In affected fibres the amplitude of the muscle potential ranged from 55 to 80 mV, but more spikes failed to overshoot 0 than in untreated fibres. The effect on the duration of the postsynaptic potential was transitory. In some preparations the fibres recovered quickly (Fig. 3C), but in other preparations a prolonged postsynaptic response was observed for 20–40 min. After this time most fibres gave normal postsynaptic responses. These results indicated that canaline did not excite muscle contraction directly, although it influenced synaptic transmission. They also provided further evidence that canaline did not interfere with axonal conduction.

Picrotoxin

Since canaline could affect the CNS by blocking the action of an inhibitory transmitter such as γ-aminobutyric acid (GABA), the effects of picrotoxin, a known antagonist of GABA, were evaluated. Moths injected with 50 μg picrotoxin/g (8 moths) or 100 μg picrotoxin/g (3 moths) behaved normally. Four of five moths receiving 200 μg picrotoxin/g body weight became active and flew within 4–5 min

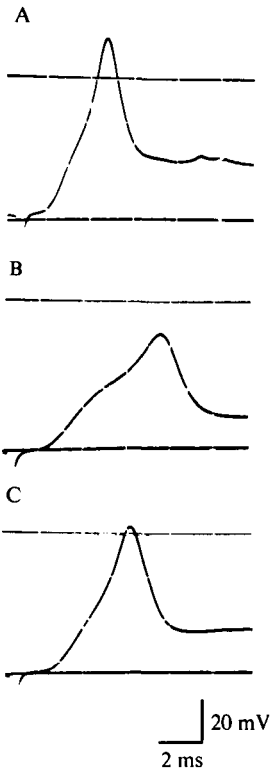

Fig. 3. Intracellular recording from the dorsal longitudinal muscle stimulated via its nerve. In each record the top straight line represents 0 potential and the bottom straight line the resting membrane potential. (A) In physiological saline. (B) 5 min after the addition of canaline to the saline; the postsynaptic response is prolonged and the active membrane response is reduced. (C) Another fibre, partial recovery 14 min after the addition of canaline.

after injection. Flight appeared normal in two of the moths, but two moths temporarily lost their ability to right themselves. After 1–8 min these four active moths were quiescent unless stimulated.

Effects on pharate adults

Pharate moths produce a flight motor pattern like that of adults (Kammer & Rheuben, 1976); however, 8–12 h before eclosion the thoracic muscles are rarely active (Kammer & Kinnamon, 1977). Nine pharate moths in this inactive stage were injected with 17 μmol canavanine/g and two with 22 μmol/g. Of these 11 animals, 2 produced a flight motor pattern, 2 produced brief bursts of unpatterned potentials, and 7 (including the higher dose) were unaffected. Seven pharate adults were injected with 22 μmol canaline/g; 6 exhibited a flight motor pattern and the 7th produced unpatterned potentials. It is not known why the majority of the treated pharate moths were unaffected by canavanine whereas adult moths were susceptible to canavanine at these concentrations. These results cannot be rationalized in terms of a decreased arginase activity of the pharate moth (Table 2).

DISCUSSION

The results show that an injection of canaline or canavanine initially activates the flight pattern-generator in *M. sexta* and subsequently causes less patterned but continual motor activity. In the following discussion possible mechanisms of action of these amino acids are considered.

Canaline as the biologically active factor

Several observations support the hyposthesis that the effects of canavanine result primarily from the action of canaline. (1) Both amino acids produce essentially the same sequence of behavioural effects. (2) Except at the highest doses, the effects of canavanine do not occur until 1–2 h after injection, whereas canaline acts within 15 min. Although the *in vivo* formation of canaline from canavanine was not determined, it is reasonable to propose that arginase activity in the adult moth is sufficient to produce adequate canaline during the observed time delay. (3) Canavanine-treated moths exhibit the initial response of coordinated large-amplitude flight movements for a longer time than do the canaline-treated moths; the flight behaviour of the latter soon degenerates to quivering. If canaline is slowly produced from canavanine, the consequences of a canavanine injection will be delayed.

Effects on the central nervous system

The results suggest that canaline alters the activity of central neurones and thus causes the production of continuous motor output. The central processing of sensory input is also impaired, as indicated by three observations: (1) the moths become unresponsive to tactile stimulation, although the activity of wing mechanoreceptors appears normal; (2) the tarsal reflex that normally inhibits flight is inoperative; (3) the ability to remain upright is lost.

The mode of action of canaline on the CNS is not known. It may alter synaptic transmission, since it prolongs the postsynaptic potential at the neuromuscular junction. However, the transitory nature of this effect is puzzling, particularly in comparison with the long-lasting effects of canaline on the CNS. Furthermore, the efficacy of canaline is difficult to explain by comparison of its structure with the structure of putative neurotransmitters. Possible transmitter substances include acetylcholine, GABA, glutamic acid, dopamine, noradrenaline, and 5-hydroxytryptamine (Lunt, 1975). Another neuroactive amino acid is L-leucine, which blocks the activity of isolated abdominal nerve cords of *Periplaneta americana* (Tashiro, Taniguchi & Eto, 1972). A high dose of picrotoxin, an antagonist of GABA, produces behavioural changes similar to those caused by canaline, but the effects are transitory, possibly because the picrotoxin is metabolized rapidly. In contrast, the effects of canaline on the CNS are more prolonged than the effects of either picrotoxin on the CNS or canaline on the neuromuscular junction.

Another possible explanation for the results is that canavanine or canaline may act non-specifically or indirectly. For example, nicotine stimulates the release of factors that facilitate initially, and then depress, transmission from the cercal nerves to the giant fibres in *Periplaneta americana* (Flattum & Sternberg, 1970*a*, *b*). Non-specific

depression of neural activity by canaline may first affect inhibitory pathways, allowing the flight pattern generator to become active. Subsequently, canaline would depress other neurones, impairing coordination and reducing the number of active motor units. Alternatively, the active factor may influence the neurones indirectly by altering properties of the blood–brain barrier that actively regulates cation concentrations around the neurones in the CNS (Treherne, 1974, 1976). These speculations await testing by direct examination of the central nervous system.

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