Destruxins: insecticidal compounds from the entomopathogenic fungus Metarhizium anisopliae

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DESTRUXINS : INSECTICIDAL COMPOUNDS FROM THE ENTOMOPATHOGENIC FUNGUS *METARHIZIUM ANISOPLIAE*

Submitted by R.I. Samuels

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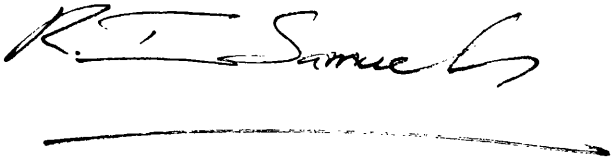

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To my Mother and Father

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ABSTRACT

The pathogenicity of a range of isolates of the entomopathogen *Metarhizium anisopliae* to the tobacco hornworm *Manduca sexta* was investigated. The highly virulent isolate ME1 appeared to kill by toxicosis; caterpillars became paralysed and died before hyphal invasion of the haemolymph. In contrast the less virulent RS549 apparently killed by massive hyphal invasion and disruption of host tissues.

Novel techniques for isolation and purification of Destruxin (DTX) from culture filtrates and infected larvae were developed.

Virulence was correlated to *in vitro* DTX production *viz*. ME1 produced more DTXA▲ than RS549. DTXA was found only in the haemolymph of ME1 infected larvae. These findings are consistent with the hypothesis that for certain insect/fungus combinations, toxin production is a key determinant of pathogenicity.

A survey of DTX toxicity using representatives of a number of insect orders showed that only Lepidoptera and to a certain extent Diptera were sensitive.

Mode of action studies were therefore performed using *Manduca sexta* larvae. Caterpillars injected with DTX immediately became tetanic due to muscle contraction. Tetany was followed by flaccid paralysis when the muscles became completely relaxed. Recovery was dose dependent.

Perfusion of DTX in saline over denervated caterpillar body wall preparations caused immediate depolarization of the muscles, accompanied by contraction. The effect could be reversed by prolonged washing (2 h) in DTX-free saline. Depolarization was not due to transmitter release since although desensitisation to the putative transmitter

▲ Destruxin A

glutamate blocked neuromuscular transmission, it failed to affect DTX induced depolarization. DTX therefore primarily acts directly on the muscle membrane. DTX depolarization is probably due to an increase in membrane permeability to one or more of the major cations present in the saline i.e. Na^+, Ca^{++}, or Mg^{++}. Na-free saline and Tetrodotoxin (a selective Na Channel blocker) did not prevent DTX action, but removal of divalent cations or addition to the saline of 0.25 mM Cadmium Chloride (a selective Ca Channel blocker) prevented DTX depolarization.

The data is discussed in the light of the hypothesis that DTX directly or indirectly acts to open endogenous Ca Channels in the muscle membrane.

Abbreviations

AP	action potential
E_k	equilibrium potential for K ions
E_m	membrane potential
gK	conductance due to K ions
$[K^+]_i$	concentration of intracellular K ions
P_K	permeability of the membrane for K^+ ions
°C	degree celsius
ca	about
CF	culture filtrate
DIW	Deionised water
DMSO	Dimethyl Sulfoxide
DTX	Destruxin
ED_{50}	50% Effective Dose
FAB MS	Fast Atom Bombardment Mass Spectrometry
g	grams
hr	hour
λ	wavelength
LD_{50}	50% lethal dose
mm	millimetre
mV	milli Volts
min.	minute
mol. wt.	molecular weight
RPHPLC	Reverse Phase High Performance Liquid Chromatography
r.p.m.	revolutions per minute
SDA	Sabaroud's Dextrose Agar
TLC	Thin Layer Chromatography
µl	microlitre
%	percentage

CONTENTS

CHAPTER 1: GENERAL INTRODUCTION

Although the use of entomopathogenic fungi as a means of biological pest control has received sporadic interest since 1879 when *Metarhizium anisopliae* was first mass produced and used against the cockchafer *Anisoplea austericaca* by Metchnikoff (cited in Bell, 1974), it has only been over the last decade that commercial preparations of entomopathogenic fungi have become available as insecticides.

The deleterious effects of synthetic insecticides on the environment, the presence of pesticide residues in food, problems with resistance in target species and the current popularity of Integrated Control Programs have all led to the resurgence of interest in fungi as a natural means of control. One of the few successful examples is "Metaquino" a preparation of *M. anisopliae* used in Brazil to control leaf hoppers (Hemiptera: Cercopidae) on sugar cane (Wilding, 1983).

A greater understanding of the factors conferring pathogenicity would be advantageous in the selection of more virulent isolates of entomopathogenic fungi, thus providing more effective biological control agents.

Many factors are thought to be determinants of virulence, but quantitative evidence for their roles *in vivo* is often lacking (Charnley, 1984). Enhanced virulence may be conferred during the first stage of an infection, i.e. at the time of attachment of the conidia to the insect cuticle. The normal incidence of conidial attachment to *Culex pipiens* was found to be greatly reduced in a hypovirulent mutant of *M. anisopliae*, when compared to the virulent wild type (Al-Aidroos and Roberts, 1978).

Speed of conidial germination is also an important factor, fast germinating conidia being more pathogenic (Hassan, 1983).

Penetration of the host cuticle may be enhanced in isolates which produce greater amounts of cuticle-degrading enzymes (CDE). Evidence in support of this hypothesis was provided by Pavlyushin (1978) who found that low *in vitro* proteolytic and lipolytic activity in certain isolates of *Beauveria bassiana* correlated with a low virulence to *Galleria mellonella*. On the other hand, Yanagita (1980) found no correlation between *in vitro* levels of lipase and cellulase activity to virulence in *Aspergillus flavus-oryzae* against *Bombyx mori* larvae. However, infection of *M. sexta* larvae by *M. anisopliae* strain ME1 is delayed by a specific inhibitor of the pathogen's chymoelastase, which indicates that this enzyme is a virulence factor (R. St. Leger, R.M. Cooper and A.K. Charnley, unpublished result, Bath University).

Fungal enzymes could also act as insecticidal toxins. Both *M. anisopliae* and *B. bassiana* have been shown to produce proteases *in vitro* which are toxic by injection to *G. mellonella* (Kucera and Samsinakova, 1968; Kucera, 1980). It is not clear whether these enzymes are produced in sufficient amounts to cause toxic effects during the course of normal infections. Their main relevance to the *in vivo* situation may be to facilitate cuticle penetration by hydrolysing cuticle proteins, or in the saprophytic digestion of protein following host death and not in causing toxicosis (Roberts, 1980). Low molecular weight toxins have, however, been implicated in pathogenesis for certain host/pathogen interactions where there is limited mycelial invasion prior to death. Fungal toxins are normally classified as being non enzymatic, low molecular weight non antigenic compounds. Many of these low molecular weight insecticidal compounds

have been isolated from the culture filtrates of entomopathogenic fungi and their chemical structures determined (see Table 1). However, the relevance of most of these toxins to mycosis has yet to be established.

The Aflatoxins have been extensively studied as they are potent carcinogens secreted by the saprophytic activities of *Aspergillus flavus* on human food products. Entomopathogenic strains of this fungus also produce Aflatoxins *in vitro* and in the bodies of mycosed *Bombyx mori* larvae, although probably not in sufficient quantities to cause death (Murakoshi *et al.*, 1977).

Aspochracin is produced by *Aspergillus ochraceus* in liquid culture. This compound was found to be a novel cyclotripeptide, showing toxicity by injection, *per-os* and contact to Lepidopteran larvae although very high concentrations were required to cause 100% kill (Myokei *et al.*, 1969).

Beauveria bassiana, responsible for the white muscardine disease of insects, produces two cyclodepsipeptides *in vitro*, Bassianolide and Beauvericin (Suzuki *et al.*, 1977; Hamill *et al.*, 1978). Beauvericin is toxic to brine shrimps but less so to insects, whereas Bassianolide, although similar in structure to Beauvericin, is highly toxic to insects (Roberts, 1980). Bassianolide is also produced by entomopathogenic strains of *Verticillium lecanii* (Murakoshi *et al.*, 1978).

An isolate of *Fusarium solani* pathogenic to the beetle *Scolytus scolytus* produces a number of insecticidal compounds. Claydon *et al.* (1977) accounted for the insecticidal activity by isolating the naphthazarin pigments Fusarubin and Javanicin and Fusaric acid (5-n-butyl-pyridine-2-carboxylic acid). Javanicin was found to be highly toxic by

References

(1) Gudauskas *et al.*, 1967.

(2) Matsumura and Knight, 1967.

(3) Kirk *et al.* 1971.

(4) Murakoshi *et al.* 1977.

(5) Al Adil *et al.*, 1972.

(6) Myokei *et al.*, 1969.

(7) Suzuki *et al.*, 1977.

(8) Kanaoka *et al.*, 1978.

(9) Murakoshi *et al.*, 1978.

(10) Hamill *et al.*, 1969.

(11) Kodaira, 1961b

(12) Kodaira, 1962.

(13) Tamura *et al.*

(14) Suzuki *et al.*

(15) Kuyama and Tamura, 1966.

(16) Roberts, 1969.

(17) Suzuki *et al.*, 1970a.

(18) Suzuki and Tamura, 1972.

(19) Lee *et al.*, 1975.

(20) Païs *et al.*, 1981.

(21) Claydon *et al.*, 1977.

Table 1. Low Molecular Weight Toxins Produced by Entomopathogenic Fungi.

Species	Toxin	Reference
Aspergillus flavus	Aflatoxins B_1, B_2, B_3, M_2, G_1, G_2	1, 2, 3, 4, 5, 6.
Aspergillus ochraceus	Aspochracin	6
Beauveria bassiana	Cyclodepsipeptides Bassianolide	7, 8, 9
	Beauvericin	10
Verticillium lecanii	Cyclodepsipeptide Bassianolide	7, 8, 9
Fusarium solani	Fusaric Acid, Javanacin, Fusarubin	21
Metarhizium anisopliae	Cyclodepsipeptides Destruxin A, B, C, D	11, 12, 13, 14, 15, 16, 17
	Desmethyldestruxin B	17, 18
	Protodestruxin	19
	Destruxin E, A_1, A_2, B_1, B_2 C_2, D_1, D_2, E_1	20

injection to *Calliphoria erythrocephala* (LD_{50} 0.7 μg/fly), the other compounds showed lower activities. Good evidence for the involvement of any of the above "toxins" in pathogenesis has yet to be produced.

The Destruxins, highly active insecticidal cyclodepsipeptides, which are produced by *Metarhizium anisopliae*, were discovered by Kodaira (1961a). Significantly, the Destruxins are the only fungal toxins to have been detected in moribund insects in sufficient quantities to cause death (Suzuki *et al.*, 1971). Zacharuk (1971) noted ultrastructural changes prior to death and mycelial invasion when Elaterid larvae were infected with *M. anisopliae*, indicating the action of a toxin. Symptoms of sluggishness and uncoordinated movement were also seen during mycoses suggesting that toxins may act on the nervous system (Charnley, 1984).

Many naturally occurring poisons have been found to be active against the nervous system. The potency of these toxins is often a result of a specific block of key processes in nerve and muscle cell membranes and some toxins have thus become exceedingly useful experimental tools in neurobiology (see Leake and Walker, 1980; Chapter 10).

Toxic compounds used as defences against predation usually occur singly or in simple mixtures. For example, Tetrodotoxin (TTX), isolated from the ovaries of the Puffer Fish *(Sphaeroides)* selectively blocks the voltage-dependent activation of membrane permeability to Na (P_{Na}) without affecting K permeability (P_K) or resting P_{Na} (Kao, 1966).

Saxitoxin (STX) isolated from the dinoflagellate *Gonyaulax*, is chemically related to TTX and has a very similar mode of action (Leake and Walker, 1980).

Gryanotoxins extracted from the leaves of various plants of the family *Ericaceae*, cause a reversible depolarization of the squid axon membrane by eliciting a specific increase in resting P_{Na} (Seyama and Narahashi, 1973).

Batrachotoxin (BTX) is a steroid obtained from the skin of the Columbian poison arrow frog *Phylobates aurotaenia*, which causes paralysis when injected into mammals. It induces a specific increase in P_{Na} of both pre and post synaptic membranes. Depolarization of the presynaptic terminals causes massive transmitter release. Post-synaptic depolarization is thus caused both directly and indirectly (Warnick *et al.*, 1971). BTX acts by blocking sodium inactivation (i.e. activated sodium channels remain open). The effects of BTX are of course antagonised by TTX which prevents the channels opening in the first place.

It is very often found that venoms used to disable prey contain a mixture of toxic chemicals, not all of which may be neuroactive. For example, snake neurotoxins which act postsynaptically causing non-depolarizing neuromuscular block, are a mixture of several different substances, most of which are neurotoxins, but others have enzymic, cardiotoxic, haemorrhagic coagulant and anticoagulant properties. One of the most studied Elapid toxins is the long chain polypeptide α bungarotoxin (α BUTX) utilised by *Bungarus multicinctus* (Lee *et al.*, 1967). α BuTX has been used to identify and isolate nicotinic cholinoreceptor proteins in vertebrates.

The poisons produced by certain Hymenoptera, are mixtures of amines and proteins, the small peptides acting as neurotoxins. The venom of the wasp *Philanthus triangulum* contains two agonists, acetylcholine and glutamate and three low mol. wt. antagonists beta, gamma and

delta-philanthotoxin. The efficacy of this mixture is thought to be due to the synergistic action of its components (Piek *et al.*, 1985).

The rationale of possessing multiple toxins may be to ensure rapid paralysis and/or killing of the prey animal, thus facilitating its capture (Olivera *et al.*, 1985).

Toxins are sometimes produced by pathogens, e.g. botulinum toxin from *Clostridium botulinum* which is highly active against vertebrates, inducing paralysis followed by muscle atrophy due to presynaptic block at skeletal nerve-muscle junctions (Das Gupta *et al.*, 1966). However, specific neurotoxins are perhaps the exception rather than the rule in this category. Most bacterial pathogens that make toxins, produce more generally acting chemicals, e.g. Diphtheria toxin which irreversibly inhibits protein synthesis and thus has deleterious effects on many cell types (Collier, 1975) and *Bacillus thuringiensis* δ-endotoxin which interacts with specific plasma membrane lipids causing a detergent-like rearrangement of these lipids, disrupting membrane integrity eventually resulting in cytolysis (Thomas and Ellar, 1983).

Avermectins, a group of natural insecticidal and helminthicidal compounds produced by the Actinomycete *Streptomyces avermitis*, have received much attention recently as they have been claimed to display a highly selective mode of action inhibiting post-synaptic transmission of GABAergic synapses (Mellin *et al.*, 1983).

Fungal toxins are not known to act specifically on the nervous system but are more well known as antibiotics. Many of these are active principally against prokaryotes (e.g. penicillin and other medically useful antibacterial drugs) but some are toxic to eukaryotes. Most of the studied fungal toxins are not specialist

neurotoxins, but are cytotoxins, e.g. Valinomycin, an ionophore which interfers with ionic gradients in mitochondria and thus inhibits or uncouples oxidative phosphorylation.

Javanicin, Fusarubin and Fusaric Acid are thought to act by inhibiting a variety of enzyme reactions by virtue of their metal-chelating properties (Claydon *et al.*, 1977).

Beauvericin and Bassianolide (BASS) produced by *B. bassiana* do not appear to be specifically neurotoxic, acting as neutral ionophores (Pressman, 1976; Abalis, 1981). Lepidopteran larvae injected with BASS are not immediately paralysed but die a few hours later (Abalis, 1981).

The Destruxins are thought to be more specific neurotoxins as Lepidopteran larvae injected with these compounds immediately become paralysed (Roberts, 1980). However, very little is known concerning the mode of action of the Destruxins and one aim of this study was to rectify this situation (see Chapter 4).

It is not immediately obvious how neurotoxin production by a parasitic fungus would aid in the establishment of a mycosis. However, it may be that rapid host death would promote an epizootic by

(i) killing the insect rapidly and allowing transmission of the fungus to other insects in a possibly transient population;

(ii) killing the insect whilst it is still present on the host plant and therefore close to other susceptible insects (Lepidopteran larvae leave their hosts in order to pupate);

(iii) halting growth before the cuticle gets too thick. This might pose an appreciable problem to the rapid exit of hyphae from the mycosed insect, slowing the subsequent spread of the disease.

On the other hand,Hall and Papierok (1982) state that fungi which kill their hosts rapidly often fail to invade the host effectively, perhaps because of strong competition from saprophytes and consequently they sporulate poorly.

At all events, it was of interest to test the hypothesis that production of DTX by *M. anisopliae* is a determinant of pathogenicity. This is the subject of Chapters 2 and 3.

The results obtained were not conclusive but do suggest that DTX's are worthy of study. They appear to have a novel mode of action on Lepidopteran muscle membranes, causing depolarization by opening Ca Channels. Among the range of isolates tested, virulence toward the caterpillar *Manduca sexta* was well correlated with DTX production *in vivo*.

Since *M. anisopliae* may have considerable potential as an agent of biological control, it is important that the role of DTX in virulence be more fully evaluated.

CHAPTER 2: PATHOGENICITY OF *METARHIZIUM ANISOPLIAE* TO *MANDUCA SEXTA* LARVAE

Introduction

The biology of entomopathogenic fungi has been reviewed many times (see Madelin, 1963; Roberts and Humber, 1981; Ferron, 1985). Entomopathogenic fungi do not occupy a particular systematic position, but are distributed among four major groups, viz. Deuteromycetes, Phycomycetes, Ascomycetes and Basidiomycetes. The majority of entomopathogens are found to be Deuteromycetes. Infection by Deuteromycete fungi, e.g. *Metarhizium anisopliae* is effected by conidiospores which have four possible modes of entry, i.e.directly through the external cuticle, via the digestive tract, through other natural openings (i.e. spiracles) or through wounds. It is well documented that the major route of entry is directly through the outer integument (Veen, 1966; McCauley *et al.*, 1968; Schabel, 1978)

Conidia attaching to the cuticle, germinate and proceed to penetrate the cuticle by a combination of mechanical pressure and enzymatic degradation (Ferron, 1978; Roberts and Humber, 1981). Once in the haemocoel penetrant hyphae sooner or later bud off hyphal bodies which then circulate in the haemolymph (Prasertphon and Tanada, 1968). The extent of the fungal invasion of the haemolymph and tissues prior to death of the host is an indicator of the mode of pathogenicity of the fungus in question. Extensive invasion leads to lethal physical disruption of the tissues (Madelin, 1963), whilst limited invasion implies that fungal toxins play a role in mortality (Roberts, 1980; Ferron, 1985).

The symptoms of sluggishness, increase in irritability, uncoordinated movements, inability of the insect to right itself and partial or complete paralysis seen during the later stages of some host/pathogen relationships indicate the action of a neurotoxin (Charnley, 1984). Diagnosis of the disease during incubation has been mainly through observation of symptoms such as changes in host appearance and behaviour, although certain physiological indicators may express more quantifiable modifications in the condition of the host. The effects of mycoses on host physiology has received little attention (Charnley, 1984). Until there is a greater understanding of the physiological basis of pathogenicity, fungal infections can only be expressed in terms of mortality (Ferron, 1985).

The pathogenicity of *M. anisopliae* to *M. sexta* had previously been established (Hassan, 1983) using the isolate ME1. Further detailed studies of this host/pathogen relationship have been carried out using a range of *M. anisopliae* isolates (pathotypes) in order to gain a greater understanding of the factors contributing to death of the host, with particular reference to fungal toxins.

Materials and Methods

Four isolates of *Metarhizium anisopliae* were used, the details of which are given in Table 2. Hassan (1983) established the pathogenicity of ME1 to *Manduca sexta* and this strain had thus been 'passaged' periodically through the insect to maintain its virulence.

1. Preparation of Conidia

ME1 was isolated from cadavers of *Manduca sexta* on Sabourand's Dextrose Agar (SDA) + Antibiotics (Appendix 1), to suppress the growth of bacteria and saprophytic fungi. Subsequent subcultures were made on SDA alone. Cultures were incubated for 5-6 days at 27°C prior to sporulation, then stored at 4°C for two weeks before use. RS549 and RS1094 were cultured on SDA as above but RS324 required a different medium (Appendix 1) to induce sporulation.

2. Preparation of the Conidial Suspension

All apparatus involved in the preparation of the suspensions was autoclaved at 121°C for 15 min. Conidia were harvested by pouring a sterile 0.04% Tween 80 (BDH) solution onto the sporulating plate and dislodging the conidia with a glass rod. The suspension was agitated in a Waring blender for two minutes at high speed to reduce clumping of conidia, then sieved through a double layer of muslin and centrifuged at 3,120× g for 4 minutes. The supernatant was decanted and the pellet resuspended in fresh 0.04% Tween 80 by vortex mixing. Centrifugation and resuspension as above was repeated twice. The concentration of conidia in the final suspension was measured using a haemocytometer and appropriate serial dilutions made.

Table 2. Details of the isolates of *Metarhizium anisopliae* used in this study.

Isolate	Collector	Date	Site	Host
ME1	D.W. Roberts	Unknown	Mexico	*Curculio caryae**(Coleoptera)
RS549	D.W. Roberts	Nov. 1980	Brazil	Comm. Prep. of Metabiol B+
RS1094	G. Teetor	Unknown	Unknown	RS23■ passed through a Lepidopteran+
(RS23■	R. Rabb	1961	North Carolina	Elatinidae (Coleopteran))
RS324	R.S. Soper	Feb. 1979	Queensland, Australia	Austraeris sp. (Coleopteran)+

* - obtained from Microbial Resources Ltd. (= Tate and Lyle Ltd.)

\+ - obtained from USDA-IPRU

3. Experimental Insects

Newly emerged 5th instar larvae of *Manduca sexta* weighing between 1.3 and 1.8 g were used. These larvae were raised on artificial diet (Bell and Joachim, 1978; Appendix 2) at 25°C, 17 hr light and 7 hr dark ca. 50% R.H.

4. Methods of Inoculation

(i) Dipping into Conidial Suspensions

Larvae held by the 'horn' with a pair of forceps were lowered into the conidial suspensions until completely submerged and then transferred to individual sterile 50 mm plastic petri dishes (Sterilin). The larvae were held at 25°C; 100% RH; 17L : 17 D in a hydrator without diet for 24 hrs, to allow germination of the conidia, then the insects were transferred to containers with diet at 25°C and 50% RH to follow the course of infection. This procedure prevented inhibition of germination by antibiotics in the diet.

(ii) Injection of Conidial Suspensions

Larvae were injected dorsoventrally through the intersegmental membrane between the 7th and 8th abdominal segments with 10 µl of the conidial suspensions using a Hamilton Microsyringe (25 µl) and a 27 gauge sterile disposable needle. The larvae were anaesthetised before injection by placing on ice until they became flaccid. This enabled injection with little loss of blood and closure of the wound by blood coagulation occurred before the insect recovered.

The larvae were kept on diet at 25°C and 50% RH whilst the course of infection was followed. For both methods of inoculation, dead

insects were placed in petri dishes on moistened filter paper and kept at 27°C to promote sporulation of the fungus on the cadaver.

Controls for both methods of inoculation were either dipped into or injected with autoclaved conidial suspensions.

5. Parameters of Infection

Inoculated insects were examined regularly for changes in both behaviour and appearance of the cuticle, and for the presence of hyphal bodies in the haemolymph.

Insects were weighed and faeces collected at intervals during the incubation period of the disease. Faeces were dried at 70°C for 48 hrs to remove all water before weighing.

6. Determination of the LC_{50} of *Metarhizium* for *Manduca* using the dip-inoculation method

The concentration of conidiospores required to give 50% kill of 5th instar larvae within 7 days of inoculation was calculated for the isolates ME1, RS549 and RS1094 by Probit Analysis (Finney, 1952) using the maximum-likelihood programme (MLP) (Ross, 1977) from Rothamsted Research Station.

Results

1. The Pathogenicity of four isolates of *Metarhizium anisopliae* to *Manduca sexta*

Three of the isolates tested were pathogenic while the fourth RS324 did not kill *Manduca* up to a concentration of 5×10^6 conidia ml^{-1}. The LC_{50} values and fiducial limits for the pathogenic isolates are given in Table 3. ME1 had a significantly lower LC_{50} than the other two strains (no overlap of 95% fiducial limits, $P < 0.05$) and the greatest value for the slope of the probit line. All three probit lines show no heterogeneity with good fit of the points about the lines at the 5% level.

The symptoms typically observed during mycosis initiated by dip-inoculation are recorded in Table 4. The appearance of localized brown spots on the cuticle (Plate 1) was the first visible symptom of mycosis, although reduced weight gain was the first recordable symptom (see page 22). The extent of the melanic deposits appeared to be positively correlated with the concentration of the inoculum (Plate 2). Localized melanization appeared during the mycosis of all three pathogenic isolates but a generalized melanization of the tissues and haemolymph was only recorded during ME1 infections. This observation indicates that the mode of pathogenicity of ME1 may be fundamentally different to that of RS1094 and RS549.

In addition, only ME1 infected larvae became paralysed. Paralysis was determined as the inability of an insect to right itself when placed on its dorsum.

Haemolymph samples taken 24 hours prior to death from larvae infected with the 3 pathogenic isolates were examined by light microscopy for the presence of hyphal bodies. It was noted at this

Table 3. The LC_{50} values for 3 isolates of *M. anisopliae* pathogenic to *Manduca* (inoculum applied by dipping)

Isolate	LC_{50} conidia $ml^{-1}g^{-1}$ (fiducial limits)	Slope ± S.E.	Chi^2	Degrees of freedom
ME1	1.68×10^5 ($1.076 - 2.252 \times 10^5$)	2.42 ± 0.50	0.602	3
RS1094	3.51×10^5 ($2.391 - 6.73 \times 10^5$)	1.83 ± 0.41	2.90	3
RS549	4.42×10^5 ($3.182 - 6.678 \times 10^5$)	1.79 ± 0.28	1.38	3
RS324	non pathogenic	-	-	-

Table 4. Symptoms observed during Infections, insects inoculated by dipping

Isolate	Dose (conidia/ml)	Localized brown spots$^{\Delta}$	Gen. melanization$^{\Delta}$	"Haemolymph" colour	"Presence of hyphal bodies"	Paralysis$^{\Delta}$	Death$^{\Delta}$
ME1	5×10^6*	74 hrs	95 - 118 hrs	darkened	few	95 hrs	118 hrs
RS1094	5×10^6*	80 hrs	-	normal	numerous	-	144 hrs
RS549	$6,5 \times 10^6$*	89 hrs	-	normal	numerous	-	162 hrs
RS324	5×10^6	-	-	-	-	-	non-pathogenic

* > LC_{100} dose

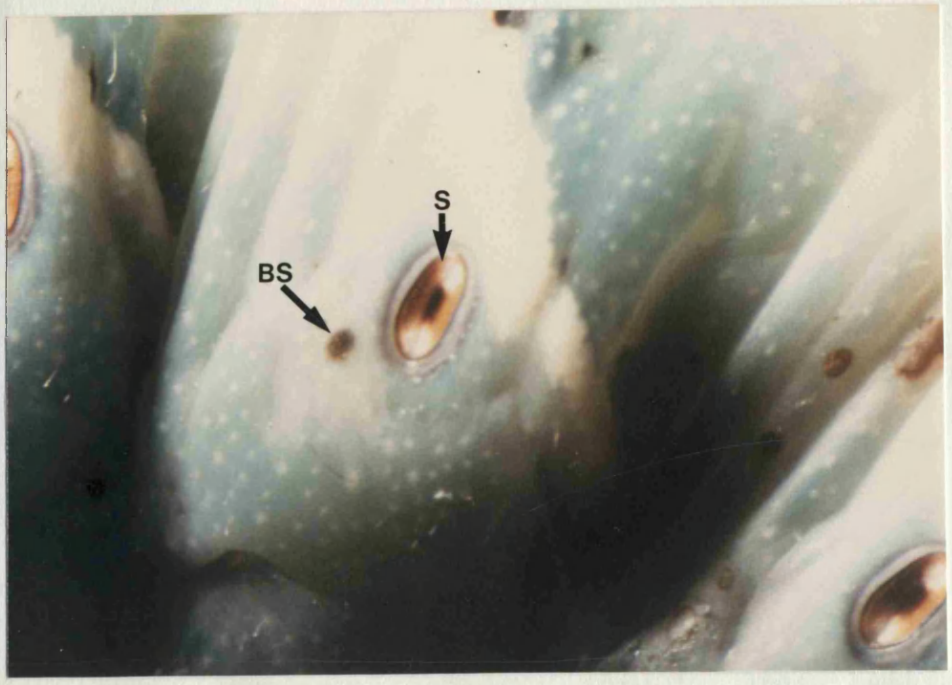
Δ time first noted

Plate 1. Localized brown spots on the cuticle are the first visible symptom of mycosis (low dose of inoculum)

BS - Brown spot (melanization)

S - Spiracle

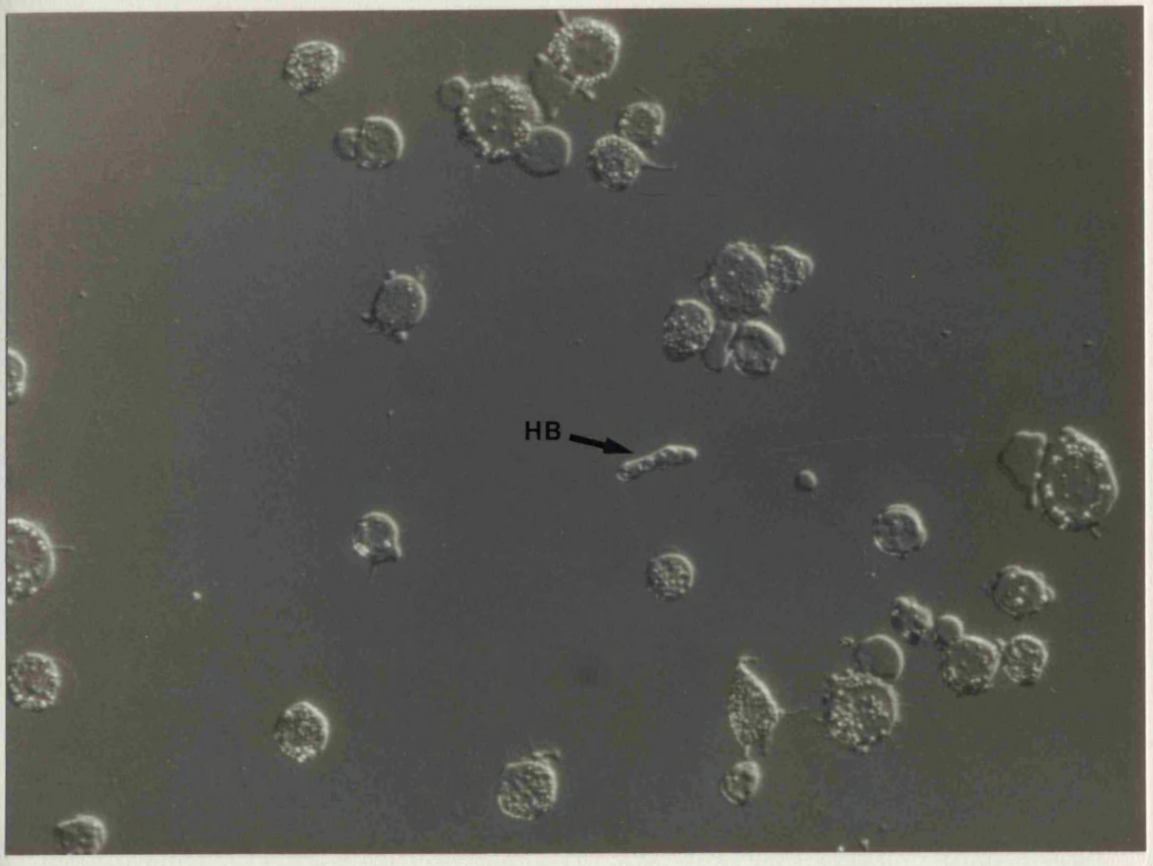
Plate 2. Melanic deposits increase with increasing concentration of inoculum


S
BS
1mm


1mm

Plate 3. Haemolymph of ME1 infected larvae, very few hyphal bodies (HB) could be found (X 1,500)

Plate 4. Haemolymph of RS549 infected larvae packed with hyphal bodies (X 1,500)


HB

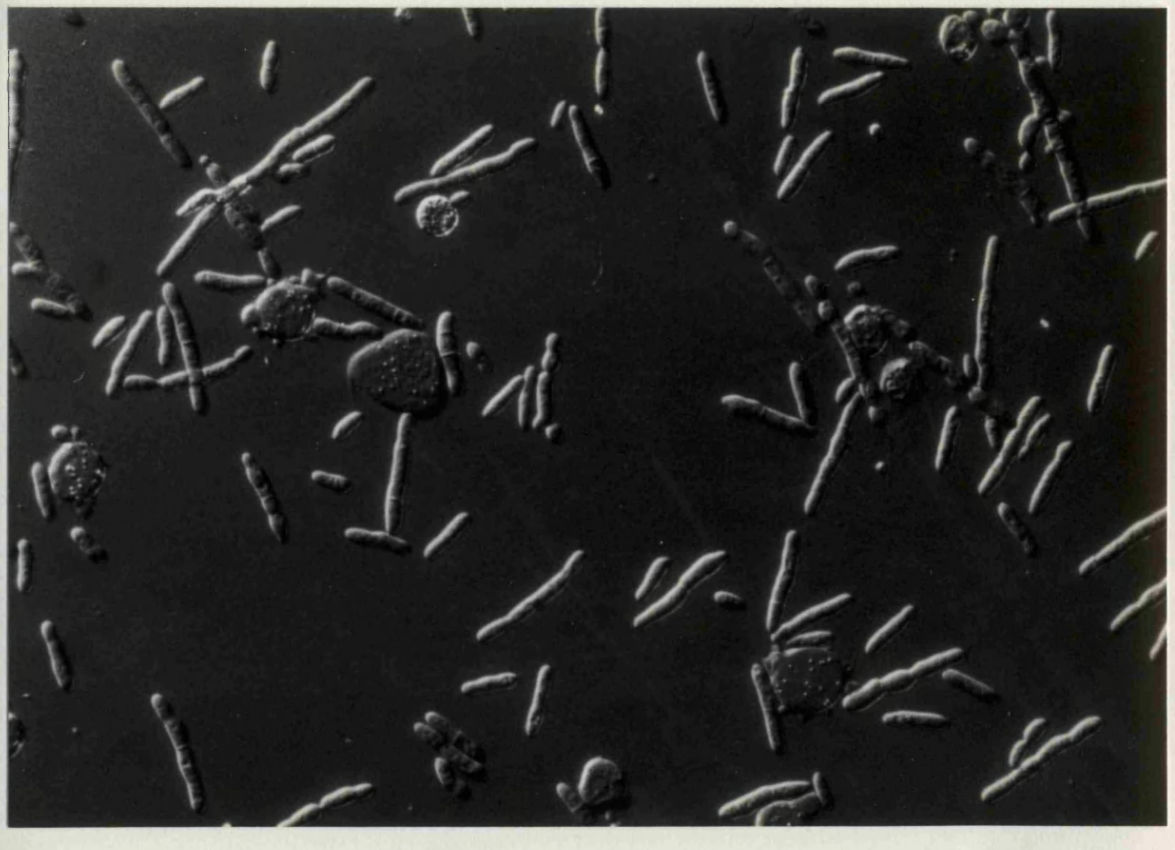


Plate 5. Cadavers of *Metarhizium* infected larvae covered in white mycelia which in certain areas are sporulating green conidiospores.

Plate 6. The end result of *Metarhizium* mycosis, the cadaver is converted to conidiospores


mm


10mm

time that the haemolymph of ME1 infected larvae had become melanized but very few hyphal bodies could be found (Plate 3).

The haemolymph of RS1094 and RS549 infected insects was normal in colouration although when examined microscopically it was found to be packed with hyphal bodies (Plate 4).

The time to death is dependent on the virulence of the isolate and concentration of the inoculum. ME1 caused the most rapid deaths, which at high doses (20×10^6 conidia $ml^{-1}g^{-1}$) occurred within 74 hours of inoculation. Within hours of death, under suitable conditions of temperature and humidity, the insect became covered in mycelia (Plate 5). It was noted that initial emergence from the cadaver occurred through the spiracles, although Roberts and Humber (1981) found that emergence occurred primarily through the intersegmental membranes. Either route affords the least resistance to penetrant hyphae. The infection cycle was complete when the cadaver became coated with conidiospores (Plate 6).

2. The Effect of Mycosis on Larval Body Weight and Faeces Production

Insects inoculated with pathogenic isolates of *Metarhizium* rapidly showed a loss of appetite indicated by a reduced consumption of diet and a failure to produce normal amounts of faecal pellets. These symptoms were noticed previously by Hassan (1983) for *Manduca sexta* 2nd instar larvae infected with ME1 although no detailed investigation was carried out.

The body weight gain of 5th instar larvae inoculated by dipping was negatively correlated with the size of the inoculum (Fig. 1). Larvae dipped into 22×10^6 conidia $ml^{-1}g^{-1}$ increased in weight only marginally before death, whereas larvae inoculated with a dose of

0.49×10^6 conidia $ml^{-1}g^{-1}$ initially showed near normal increases in body weight. The dose response of body weight analysed by probit transformation showed that the concentration of conidia (EC_{50}) required to reduce the weight gain to 50% of normal 74 hours after inoculation was 9.954×10^5 conidia $ml^{-1}g^{-1}$ (Fd. Lmts. 14.22 - 6.18×10^5)

Normal symptoms of ME1 infections were recorded throughout the experiment i.e. brown spots, general melanization and paralysis, the time of appearance of which was directly related to the dose of inoculum.

In order to compare the two methods of inoculation (dipping and injection) larval body weight changes during an infection initiated by intrahaemocoelic injection were recorded (Fig. 2). As with dip-inoculated insects the normal pattern of weight gain is disrupted in a dose dependent manner. The dose response of body weight analysed by probit transformation showed that the concentration of conidia (EC_{50}) required to reduce the normal weight gain by 50%, 68 hours after inoculation was 1513.88 conidia g^{-1} (Fd. Lmts. 6190-751.2).

The dry weights of faeces produced during infection were recorded (Fig. 3). Faecal pellet production by diseased insects did not increase at the same rate as the controls and by ca. day 2 post inoculation, started to decline, which reflected a loss of appetite.

3. Comparison of Body Weight Changes during Infection by ME1 and RS549

The whole body wet weights of control insects increased rapidly during the course of the experiment (Fig. 4). In contrast body weight of both groups of infected larvae were less than those of the controls

from as early as 25 hr after inoculation. The isolate, ME1, suppressed weight gain to a greater extent than RS549. Mortality with ME1 occurred as early as 96 hr after inoculation compared to 116 hr for RS549 infections. The difference between the effects of ME1 and RS549 on body weight became significant 51 hr after inoculation and remained so until the end of the experiment (51 hr; $t = 2.718$ df5; $P < 0.05$, 96 hr; $t = 2.809$ df5 $P < 0.05$).

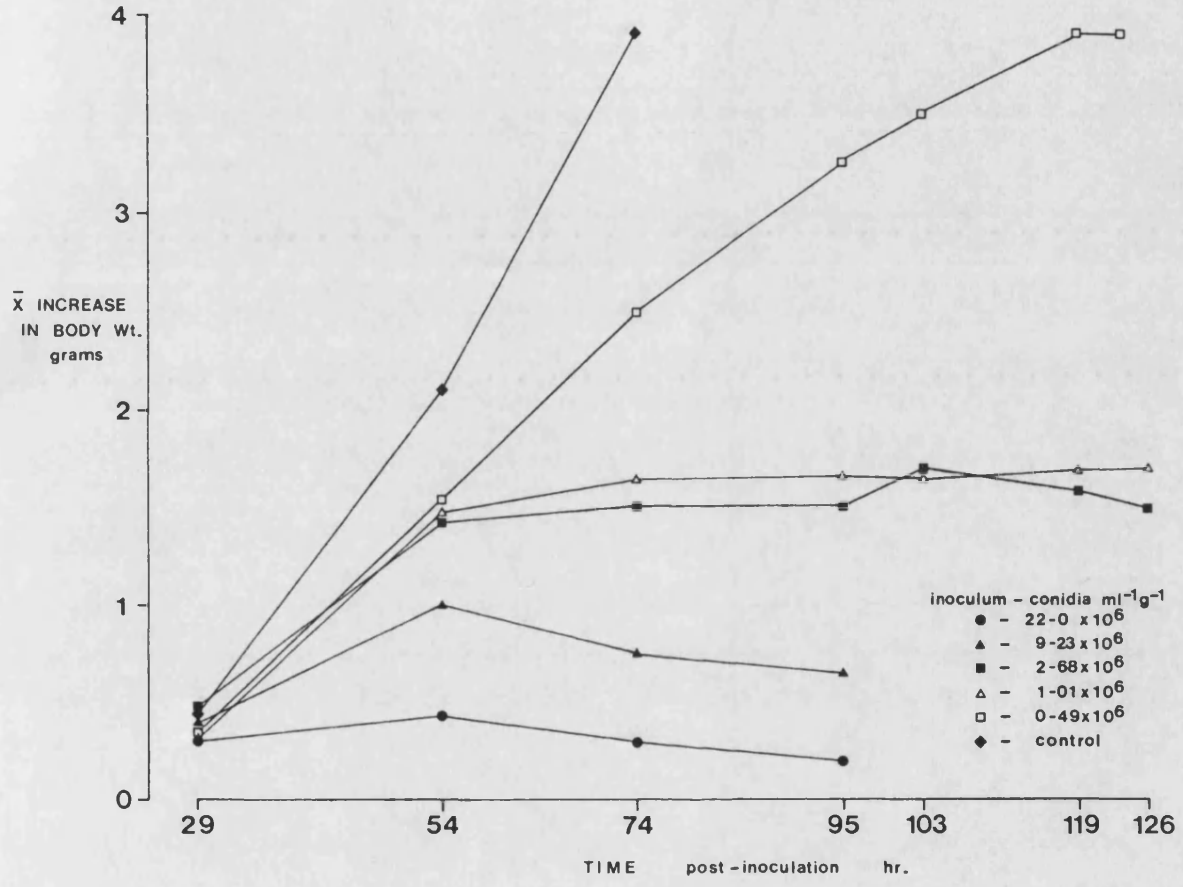


Fig. 1. Changes in larval body weight during ME1 mycosis

Note : dip-inoculation

$\bar{x}$ body wt. increase from initial wt. (n = 10/point)

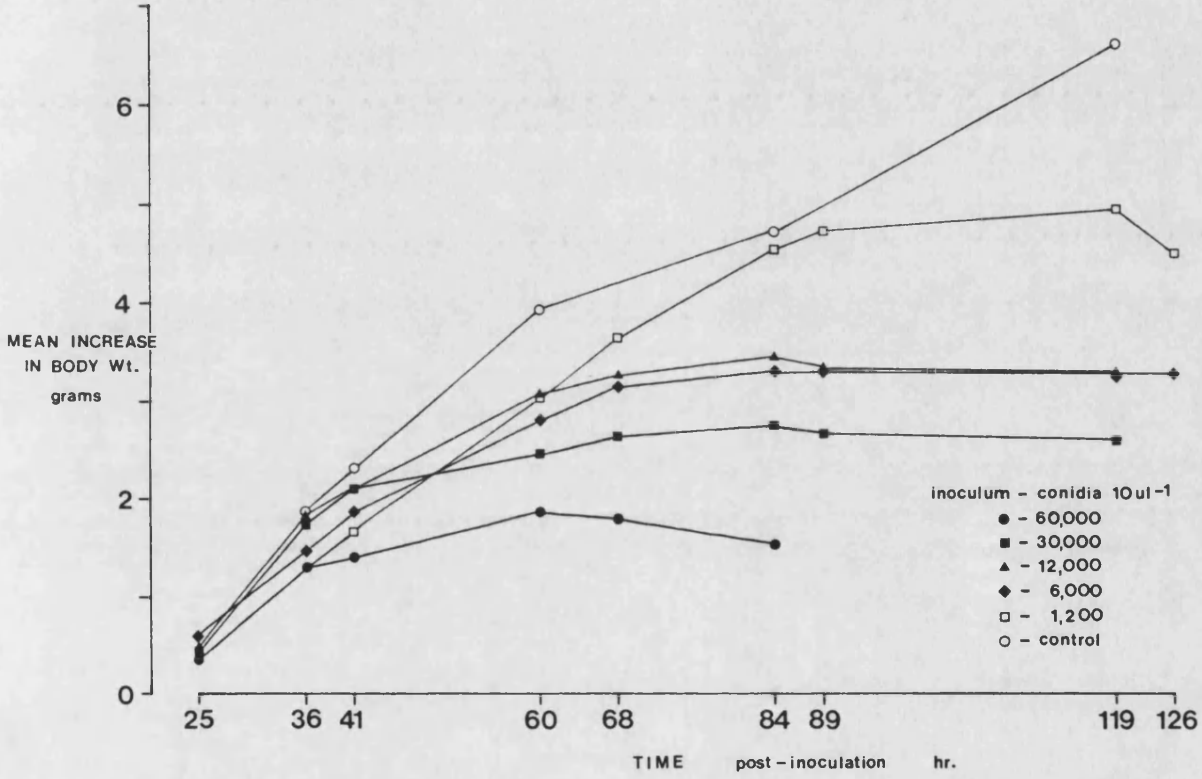


Fig. 2. Changes in larval body weight during ME1 mycosis

Note : injection-inoculation

$\bar{x}$ body wt. increase from initial wt. (n = 10/point)

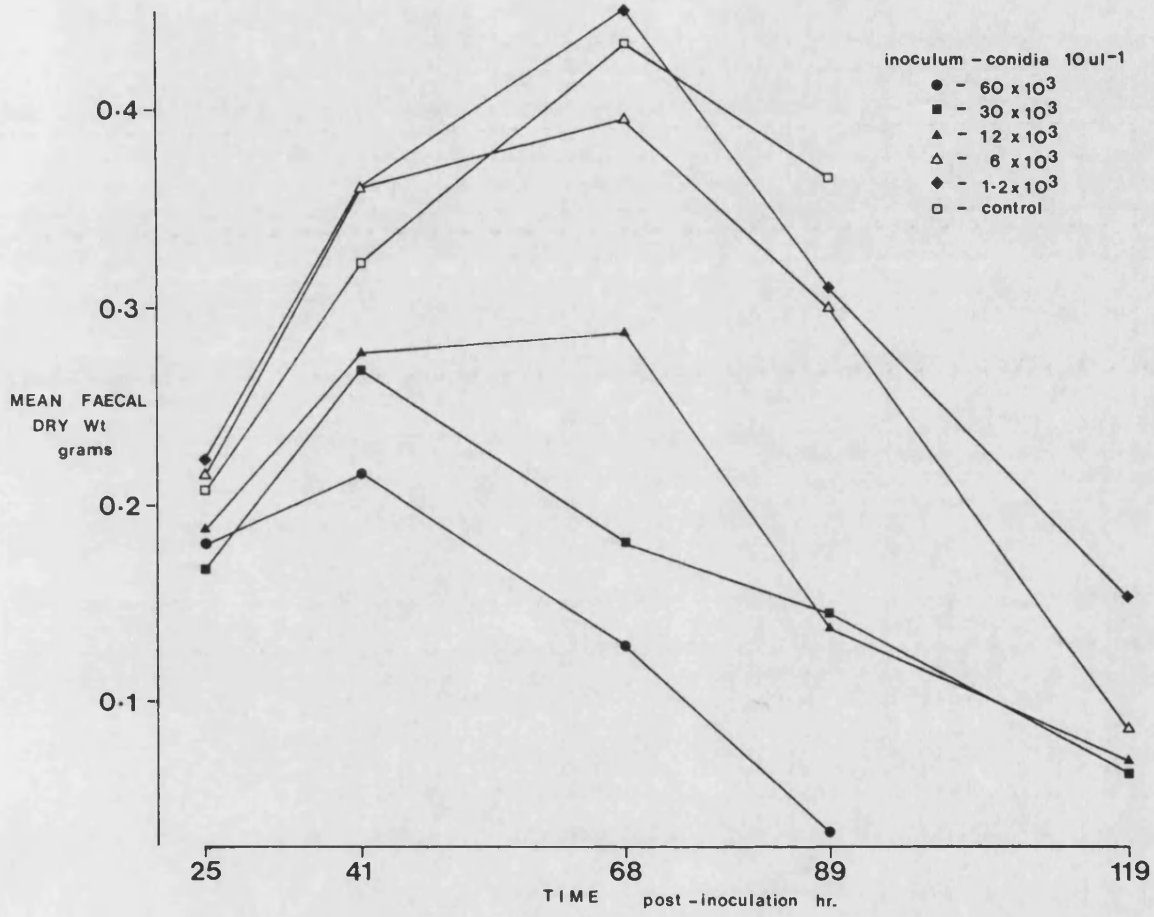


Fig. 3. Changes in faeces dry weight during ME1 mycosis

Note : injection inoculation

$\bar{x}$ faeces dry wt. (n = 10/point)

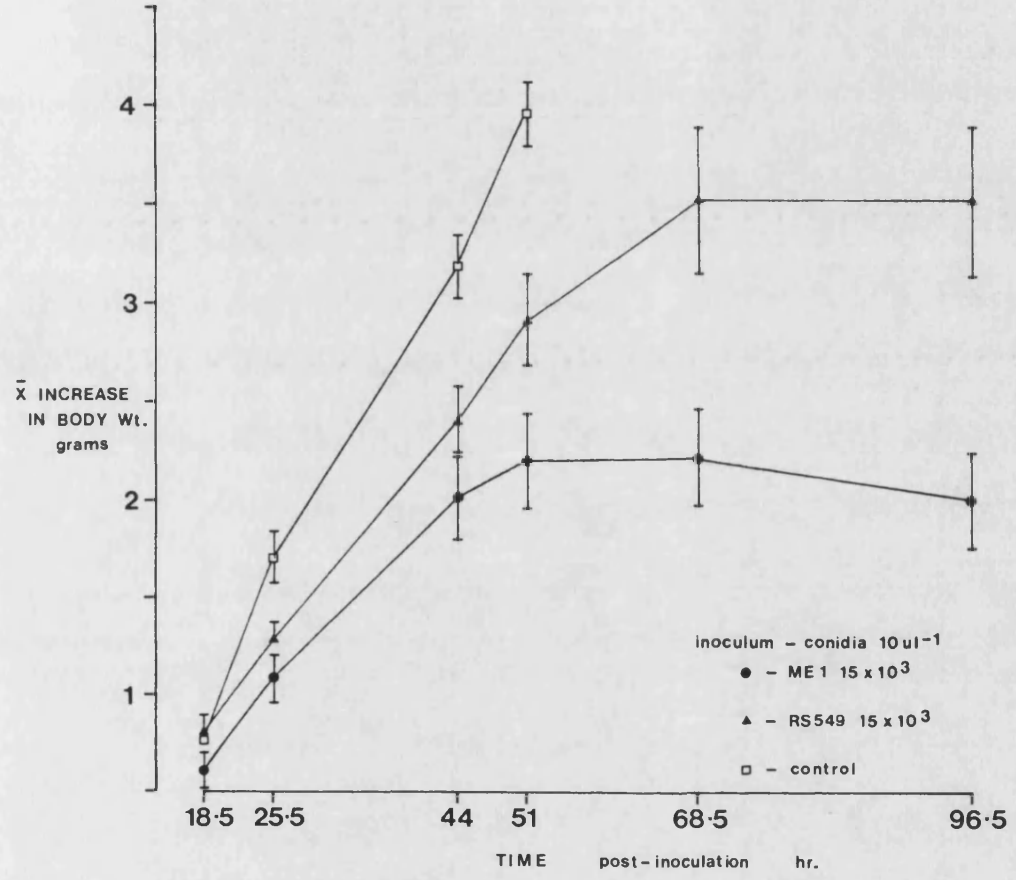


Fig. 4. Changes in larval body weight during ME1 and RS549 mycoses

Note : injection inoculation

> LD_{100} ME1 and RS549 used

$\bar{x}$ body wt. increase from initial wt (±SE, n=10)

Discussion

The first priority of an investigation of the interaction between *Metarhizium anisopliae* and *Manduca sexta* was to achieve reproducible host responses to infection (bioassay). Hall (1976) is critical of many workers for their imprecise methods of inoculation, e.g. allowing insects to crawl over culture plates or applying conidia with a brush.

Intrahaemocoelic infection of a known number of conidia should be the most reproducible method of inoculation, but this method has a number of disadvantages. Firstly if used to study pathogenicity, it circumvents two important stages of infection, attachment and penetration. Secondly the conidia may initiate a host immune response, which would not normally occur and most importantly injection of conidia bears no relevance to the practice of biological control.

Dipping larvae into a uniform conidial suspension is a compromise between the desire for a method of inoculation relevant to pest control practices (viz. spraying) and the need for a quantitative and reliable bioassay. Dip-inoculation has previously been shown to be a reproducible method of bioassaying the pathogenicity of *M. anisopliae* against *Manduca* larvae (Hassan, 1983).

Hassan (1983) stated that ME1 was a very effective pathogen of *M. sexta* with an LC_{50} of 1×10^5 conidia ml^{-1} for 2nd instar larvae. This value compares favourably with that of a virulent strain of *Verticilium lecanii* against *Macrosiphoniella sanborni* ($LD_{50} = 10^5$ conidia ml^{-1}; Hall, 1976).

The LC_{50} of ME1 for 5th instar *Manduca* larvae was found to be 1.68×10^5 conidia $ml^{-1}g^{-1}$, the values for RS1094 and RS549 were higher, indicating a lower virulence. This is also reflected in the

slower kill of these two isolates. It is difficult to compare Hassan's (1983) results to those found here due to differences in technique.

All three of the pathogenic isolates screened evoked melanization of the cuticle. Localized melanization has been recorded as a symptom of mycosis in other fungus - Lepidopteran interactions e.g. *Beauveria* spp. and *Peris brassicae* (Tanada, 1955) and *Beauveria bassiana* and *Bombyx mori* (Aoki and Yanase, 1970). This melanization is commonly thought to contribute to the host defences but it often appears to be ineffective (Charnley, 1984).

Once through the cuticular layers and into the haemocoel, the isolates appeared to behave differently. Hyphal bodies of RS549 and RS1094 proliferated within the haemolymph but very few hyphal bodies were found in the haemolymph of ME1 infected insects prior to death.

Determinants of hyphal circulation within the haemolymph are thought to be size of hyphal bodies, optimal size for growth of the fungus and behaviour of a specific fungal isolate in a specific host (Prasertphon and Tanada, 1968).

Limited hyphal growth prior to death has been reported previously as circumstantial evidence for toxin production by a fungus (McCauley *et al.*, 1968; Roberts, 1980; Ferron, 1985). Pathotypes which grow extensively within the haemocoel (prior to death), probably cause death indirectly by physical disruption of host tissues and host nutrient depletion.

Disruption of normal locomotor activity and eventual complete paralysis is only seen when *Manduca* are infected with ME1. Infections by RS549 and RS1094 are characterised by normal behaviour until death, which indicates another important difference in pathogenicity between ME1 and the RS isolates. Paralysis of mycosed insects may indicate

neurotoxin production. Similar paralysis has also been reported when a *Cephalosporium* sp. infected larvae of European corn borer (Baird, 1954) and when termites were infected by *B. bassiana* (Bao and Yendol, 1971).

General melanization is seen only during the later stages of ME1 infections. The cuticle appears darker than normal, but it is in fact the underlying tissues and haemolymph that have become melanized. This melanization may be a consequence of the cytotoxic action of fungal metabolites (Quiot *et al.*, 1985) causing breakdown of haemocytes which would then release tyrosinase activating factors (e.g. proteases) into the blood and tissues (Ratcliffe, 1986).

Loss of appetite during mycoses has been noted by many workers (Bell, 1974; Cheung and Grula, 1982; Hassan, 1983). Reduced weight gain seen during ME1 and RS549 infections was probably mainly due to reduced food intake, as faecal production was concurrently reduced. Increased oxygen consumption, transpiration and metabolism of the host tissues caused by the activities of the invading fungus may also contribute to reduced weight gain, but these factors were not examined here.

The pathogenicity of ME1 and RS549 appears to differ in many respects i.e. general melanization, presence of hyphae in the blood and paralysis. Both caused reduced weight gain, although for ME1, the effect was significantly greater than that of RS549. However, > LD_{100} doses were used in the comparative weight gain experiments. This does not discount the possibility that an LD_{50} dose for each isolate may have equal effects on body weight. Alternatively, the greater effects on weight gain caused by ME1 may reflect a different mode of pathogenicity to that of RS549.

If ME1 kills by toxicosis, the toxin produced by the fungus may account for the characteristic effects on host physiology seen here. Water loss (transpiration) may be enhanced by toxin production, however, preliminary investigations showed no difference in transpiration rates of ME1 and RS549 infected insects (D. Youle: Bath University).

Sussman (1952) found that pupae of *Platysamia cecropia* infected with *Aspergillus flavus* lost weight, probably via water loss through the spiracles, which remained continuously open possibly due to the action of toxins on the nervous system or musculature controlling the spiracles.

The virulence of an isolate is a function of many aspects of pathogenicity and therefore it is very difficult to assess individually, their contributions to host mortality unless single lesion mutants are employed. However, Meynell and Meynell (1965) suggest that if a pathogen produces a toxin (e.g. *B. thuringiensis)* the slope of the LC_{50} probit line should exceed 2▼. Considering the LC_{50}'s of the three pathogenic isolates of *Metarhizium*, only the slope for ME1 exceeds 2 which together with the symptomology of this isolate provides evidence in support of the hypothesis that ME1 produces toxins during the course of pathogenesis.

The virulence of a pathogen may be dependent on the number of times an isolate has been 'passaged' through the host in question (Schaerffenberg, 1964). The virulence of certain strains of *Metarhizium anisopliae* increased 1.63 - 2.45 times with one passage through mosquito larvae (Daoust and Roberts, 1982) although less significant increases in virulence are recorded after subsequent passage (Ferron, 1985). This phenomenon may contribute to the difference in

▼ **slope < 2 no toxin**

virulence of the 3 pathogenic isolates, the highly virulent ME1 has been passed through *Manduca* numerous times, RS549 only a few times and RS1094 having never been 'passaged' through *Manduca* (originally isolated from a Coleopteran and passed through a Lepidopteran by USDA-IPRU).

Highly virulent isolates which cause a rapid loss of appetite may be advantageous in the control of pest species where crop damage could be curtailed. In addition, reduced food intake would cause considerable stress to the host, increasing its susceptibility to chemical insecticides. The dual application of a fungus and a conventional insecticide in an integrated pest control program would thus require reduced levels of application of both agents to that needed if they were used independently (Roberts and Humber, 1981).

The results so far presented display the diversity of action of different isolates of one species of entomopathogenic fungus when infecting Lepidopteran larvae.

The pathogenicity of these isolates ranges from the avirulent RS324 to the seemingly highly virulent ME1, which kills *Manduca* by a very different mode of action to RS549 and RS1094.

These findings are relevant to those of the next chapter where toxin production *in vitro* and *in vivo* of the different isolates was investigated.

CHAPTER 3. ISOLATION AND PURIFICATION, TOXICITY AND DETECTION *'IN VITRO'* AND *'IN VIVO'* OF THE DESTRUXINS

Introduction

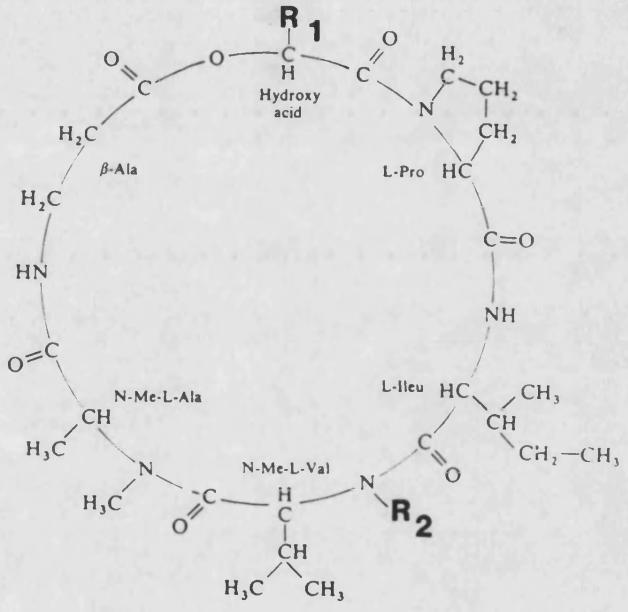
In 1961 Kodaira carried out an extensive study of the interaction between Muscardine fungi and *Bombyx mori*. He discovered and isolated two low molecular weight substances from the culture filtrates of *M. anisopliae* Destruxin A (DTXA) and Destruxin B (DTXB), both of which were highly toxic on injection to *B. mori* larvae. Further analytical studies of the properties of these compounds showed DTXA to be $C_{29}H_{47}O_7N_5$ mol. wt. 577, within a m.p. of 188 - 188.5°C and DTXB to be $C_{30}H_{51}O_7N_5$ mol. wt. 593 with a m.p. of 234°C (Kodaira, 1961b; 1962).

On the basis of degradation studies Tamura *et al.*(1964 a,b) proposed that DTXB was a depsipeptide, cyclo-D-αhydroxy-γ-methyl-valeryl-L-prolyl-L-isoleucyl-N-methyl-L-valyl-N methyl-Lalanyl-β-alanyl (Fig. 5). The structure was confirmed by total synthesis (Kuyama and Tamura, 1965). The structure of DTXA was subsequently elucidated by IR and NMR spectra (Suzuki *et al.*, 1966). Amino acid analysis of an acid hydrolysate confirmed the structure as cyclo-D-2 hydroxy-4-pentenoyl-L-proplyl-L-isoleucyl-N-methyl-L-valyl-N methyl-L-alanyl-β alanyl (Fig. 5).

DTXA is different from DTXB only in the hydroxyl acid constituent and is considered to have been derived from it by elimination of elements corresponding to a methane molecule CH_4, in the hydroxy acid.

Three new insecticidal Destruxins were isolated from *M. anisopliae* culture filtrates by Suzuki *et al.* (1970a)and their structures

The Chemical Structure of Destruxins



			mol. wt.
DESTRUXIN A	R_1 =	$CH_2{=}CH{-}CH_2{-}$	577
	R_2 =	$CH_3{-}$	
DESTRUXIN B	R_1 =	$(CH_3)_2CH{-}CH_2{-}$	593
	R_2 =	$CH_3{-}$	
DESMETHYL-DESTRUXIN B	R_1 =	$(CH_3)_2CH{-}CH_2{-}$	579
	R_2 =	$H{-}$	

Fig. 5. The Chemical Structure of 3 Destruxins.

elucidated. These were named DTXC, DTXD and Desmethyl-DTXB. The most complete isolation of Destruxins to date separated 14 depsipeptides; the previously characterised DTXA, B, C, D and Desmethyl-DTXB and the newly discovered E, A_1, A_2, B_1, B_2, C_2, D_1, D_2 and E_1 (Pais *et al.*, 1981). The structures of all 14 DTX's were established from the mass spectral analysis of their corresponding open chain derivatives.

The techniques used to isolate and purify the Destruxins have varied enormously. Pure samples of DTX are a pre-requisite for studying their natural product chemistry and more importantly the mode of action of DTX which up until now have received virtually no attention.

The procedure originally used by Kodaira (1961a) to obtain pure samples of DTXA and DTXB was laborious and inefficient involving silicic acid and partition chromatography, alumina chromatography and fractional crystallization. The technique was improved by treating the culture filtrate with 0.7% charcoal (Tamura *et al.*, 1965). The insecticidal principles taken up by the charcoal were eluted with butanol-water (1:1 vol/vol). The butanol phase was separated and concentrated under reduced pressure to a syrup which was then extracted with benzene. The benzene extract was applied to an alumina column which was developed successively with Benzene +5% and 10% ethyl acetate to give DTXB and DTXA respectively. Higher yields were obtained by further modifications involving CCl_4 extraction of culture filtrates, ion exchange and alumina chromatography, although this technique was unable to separate DTXA and DTXB (Roberts, 1969).

The biogenesis of the Destruxins *in vitro* and *in vivo* has not been properly investigated due to the lack of a direct means of qualitative and quantitative detection. The Destruxins have no

characteristic UV spectra and can only be identified by Mass Spectroscopy after extensive purification.

Destruxin yields from liquid cultures have been investigated by extraction and purification (Suzuki and Tamura, 1971). This has shown that DTXA may be formed from DTXB (pathway as stated earlier) as DTXA levels increase with time of culture, to become the predominant Destruxin. DTXC and D are considered to be intermediates in the transformation of DTXB to DTXA. An apparent biosynthetic precursor Desmethyl- DTXB, Protodestruxin, has been isolated (Suzuki and Tamura, 1972) which is a compound without N-methylation of any amino acid and it is found to be harmless to *Bombyx mori* larvae (Lee *et al.*, 1975). N-methylation of alanine converts Protodestruxin to Desmethyl-DTXB and fixes the molecules in a specific configuration, which may be related to its mode of action as Desmethyl-DTXB is toxic to *Bombyx mori* (Naganawa *et al.*, 1976).

The relationship between the toxicity of the different Destruxins and their structures has received little attention. From a range of eight Destruxins, DTXA and DTXB were found to be particularly toxic to *Bombyx mori* by injection (Suzuki and Tamura, 1971). Certain aspects of structure thus appear to confer differences in toxicity.

The toxicity spectrum of the Destruxins to insects has also received little attention, only the susceptibility of Lepidopteran larvae to injected DTX has been fairly well documented (Roberts, 1980). A prior understanding of Destruxin toxicity to *Metarhizium* mycosis susceptible insects is necessary before evaluating the possible role of DTX in pathogenicity.

The quantitative detection of fungal toxins in the bodies of moribund insects is required to evaluate their role in pathogenicity.

To date, all of the host/pathogen interactions where toxins have been implicated in pathogenesis by circumstantial evidence from symptomology, only the Destruxins have actually been found *in vivo* when *Bombyx mori* larvae were infected with *M. anisopliae* (Suzuki *et al.*, 1971).

Here, the relationship between DTX production *in vitro* by different isolates of *M. anisopliae* and the pathogenicity of these isolates to *Manduca sexta* larvae have been investigated. The toxicity of DTX to a range of insect species and *in vivo* detoxification of DTX were studied. Finally levels of DTX in the blood of diseased *Manduca sexta* larvae were investigated in order to gain an insight into the possible role of DTX as a causative agent of host death during mycosis.

Materials and Methods

1. Isolation and Purification of Destruxins

Preliminary studies were performed to find the optimal culture conditions for DTX production and the most efficient method for purification of Destruxins. The effect of changes in culture nutrients, pH, culture period, shake versus stationary and fungal strain were assessed by effects on culture growth (dry wt. of fungus), crude toxin yield (wt.), bioactive components and TLC estimation of levels of DTX and impurities.

Fungi were cultured on Czapek-Dox liquid Modified Medium (Oxoid) + 0.5% Bacteriological Peptone (Oxoid) (Appendix 1). Culturing was carried out in 2 litre conical flasks with 1 litre of medium, adjusted to pH 7.0 before autoclaving at 115°C for 20 minutes. The flasks were inoculated with a spore suspension containing 30 x 10^6 conidia per flask. The flasks were then placed in an orbital shaking incubator (Gallenkamp) at 27°C and 150 r.p.m. for approximately 14 days. At this time the culture filtrate (CF) was obtained by sieving through double muslin. The CF was then poured into a separating flask and extracted five times with 70 - 80 mls of Analar CCl_4 (Fisons) (ratio of 10:1 CF:CCl_4). Emulsions which sometimes formed, were broken by centrifugation. The CCl_4 extract was then filtered through Whatman 1 P.S. filter paper to remove any aqueous residues and thence through a 0.22µm47 mm Millipore membrane filter to remove any conidia that had passed into the CCl_4 layer (the CCl_4 extract was stored if necessary in dark bottles at 4°C). The CCl_4 was removed by evaporation, using a Büchi Rotavapor-R at 50 - 60°C and rotating at maximum speed, under appropriate vacuum. The residue, a yellow oil, contained the Destruxins and impurities.

Ion exchange chromatography was used for purification. The first column contained Dowex 50 W - X8 20 - 50 US mesh (hydrogen ion form), a cation exchanger which retains all negative charged impurities. As cyclodepsipeptides are neutral compounds they pass unaffected through ion exchange resins. The 200 ml bed volume of Dowex 50 was prepared by washing the resin in 4 - 5 bed volumes of deionised water (DIW) before degassing. The resin was then carefully poured into an LKB 2137 chromatography column of 26 mm internal diameter and filled to a height of approx. 400 mm. The resin was allowed to settle before being charged with the appropriate ionic form. Two bed volumes of 1N HCl (diluted from 10N with DIW) were passed through the resin at a rate of 2 ml min^{-1}. The resin was then rinsed with DIW at 2 ml min^{-1} until the effluent pH returned to normal.

A colloidal suspension of the crude toxin extract was prepared by first dissolving it in 10 ml of Acetone (Analar BDH) and then adding this to 100 ml of DIW. The acetone was removed by evaporation at 70°C. The colloidal suspension was then pumped onto the top of the column via a 3-way valve, at a flow rate of 1 ml min^{-1}. Once the colloidal suspension was on column the value was switched over to DIW. The flow rate was controlled throughout by an LKB Varioperpex II pump at the outlet of the column. Detection of compounds not retained on the resin was by an LKB Uvicord S at 206 nm and recorded by an LKB chart recorder. The major toxin containing fraction, determined by bioassay and TLC, was collected and stored at 4°C until ready for use.

The second resin used was Biorad Ag 1-X8 20-50 US Mesh (Acetate form), an anion exchanger which will retain all positive charged

impurities. This resin was obtained in the chloride ion or hydroxide ion forms and converted to the acetate form. The resin was expanded and washed in 4 bed volumes of DIW before pouring into a column. Conversion was most efficiently carried out in the column mode. The resin was washed with 20 bed volumes of 1N NaOH. On completion of conversion the resin was rinsed with DIW (approx 4 bed vol.), until the effluent pH was <9.0. The second stage of the conversion could then be performed. Two bed volumes of 1N HAc▲ was passed through the resin until the effluent pH < 2.5. Then the resin was rinsed with DIW until the effluent pH > 4.8. The resin was then ready for use and packed into a 16 mm internal diameter LKB column to a height of approx. 400 mm. The toxin fraction from the previous column was pumped onto the anion exchange column, followed by DIW at a flow rate of 1 ml min^{-1}. Fractions were again collected with absorbance in the 206 nm range and then freeze dried. The freeze dried extracts from ion exchange chromatography were dissolved in acetone and approx. 5 mg streaked onto a TLC plate (Merck Kieselgel 60 F254 10 x 20 cm). The plate was run in 19:1 chloroform:MeOH△ for about 2 hr. Analytical TLC was used to assess the efficiency of the purification stages, non-destructive visualization of spots was achieved using iodine vapour. For semi-preparative work the DTX band was identified under UV light at 260 nm, although DTX does not fluoresce at this wavelength, a shadow appears with large amounts of DTX (∿ 5 mg). This band was scraped off and suspended in DIW, whirlimixed for 2 min, then centrifuged at 3000 x g for 3 min. The supernatant was carefully removed and freeze-dried ready for Semi-preparative Reversed Phase HPLC.

▲HAc – ACETIC ACID △ MeOH – METHANOL

(i) Reverse Phase High Performance Liquid Chromatography (RPHPLC)

Apparatus: Gilson Chromatograph which comprised two Gilson Model 302 pumps with 5S heads, Rheodyne injection valve fitted with a 25µl or 100µl sample loop. A Gilson Holochrome Model HM detector (λ variable) set to 210 nm, with a 8µl flow cell. The chromatograph was controlled via an Apple Microcomputer with Gilson Gradient Manager program. The output from the UV dectector was recorded on a Rikadenki Chart Recorder.

HPLC Packing: i. Semi-preparative HLC - Support material was Spherisorb C_{18} 5µm (HPLC Technology) prepacked in 30 cm x 8 mm i.d. stainless steel column and guard column.

ii. Analytical HPLC - support material was Spherisorb C_{18} 5µm (Anachem) prepacked in a 25 cm x 4.6 cm i.d. stainless steel column and guard column.

Other chemicals and Equipment: HPLC grade Acetonitrile (CH_3CN) (Koch Light Ltd.) and Ultrapure Milli-Q-quantity water (Millipore equipment) were used.

Method: Prior to use the mobile phases were degassed with helium. Samples were dissolved in the initial mobile phase before injection of amounts not exceeding 5 mg per run for semi-prep. but for the analytical column, 20 - 40 µg was the maximum permissible loading. Preliminary experiemnts using the analytical column established that a mobile phase of 45 - 50% CH_3CN in the isocratic mode with a flow rate of 1 ml min^{-1} produced optimum separation of Destruxins.

Fractions were collected every 30 seconds for bioassay on the *Manduca* heart preparation or the body wall muscle preparation (see Chapter 4). These fractions were freeze-dried in an Edwards Freeze Dryer Modueyo and Savant Speed-Vac Concentrator. The residues were redissolved in 5µl ethanol, whirlimixed before adding 200µl of *Manduca* saline (Appendix 3), then whirlimixed again before application to the heart or muscle preparations.

The fractions displaying bioactivity corresponded to the four major peaks of UV absorbance (Fig. 6) now to be designated as P_1, P_2, P_3 and P_4. Samples of these peaks were subjected to fast-atom bombardment mass spectrometry (FAB-MS) (see below).

The purification technique was then extended to large scale separation of the four peaks by using the semi-prep column, 50% CH_3CN isocratic at a flow rate of 3.25 ml min^{-1}. The four absorbance peaks were collected and freeze dried ready for use in physiological studies.

(ii) Fast Atom Bombardment Mass Spectromety

A VG Analytical 70 70E Mass Spectrometer was used with a DS2000 Data system. Xenon gas was used in the atom gun running at 6 KV and Glycerol was used as the matrix.

2. Assay of Toxicity by Injection of Pure Destruxins to *Manduca* larvae

Toxic extracts of CF with known purity (HPLC) were assayed for biological activity by conventional ED_{50} and LD_{50} techniques (see Busvine, 1971). The ED_{50} was based on immediate knockdown following intrahaemocoelic injection of Destruxins. Knockdown was classified as the inability of the insect to right itself when placed on its

dorsum one minute after injection. The LD_{50} was based on mortality of the insect after injection of Destruxins. Death was defined as a lack of response to tactile stimulation 48 hours after administration of the toxin. The values for the ED_{50} and LD_{50} were calculated from the data by Probit Analysis (see p. 15).

Destruxins were weighed out on a balance and then dissolved in a small volume of Dimethyl Sulfoxide (DMSO) before adding E & B saline (Appendix 3). Appropriate serial dilutions were then carried out in E & B saline. Injection of Destruxins was by the method stated previously (see p. 14) but without anæthetisation. Controls were injected with E & B + 1% DMSO. A minimum of ten larvae weighing between 1.3 and 1.8 g were used per dose of Destruxin.

3. Toxicity of Injection of Destruxins to a Range of Insect Species

Due to the lack of published data on the susceptibility of different insects to Destruxins a range of insect species was screened for immediate knockdown after intrahaemocoelic injection. In these experiments, three species of Lepidopterans, a Dipteran, an Orthopteran, a Dictyopteran, a Coleopteran and a Phasmid were screened (see Appendix 4 for details of insect cultures). Three arbitrary doses were chosen, 10 µg g^{-1}, 20µg g^{-1} and 100µg g^{-1}. The appropriate dilutions for all the tests were made from a stock solution of Destruxins. For certain species, symptoms seen after the 1 min knockdown period were recorded. The method of injection of DTX for all insects was as described previously (p. 14). All injections were performed on unanaesthetised animals although *Periplaneta americana* and *Calliphoria vomitoria* adults were cooled on ice before injection. Injection was normally dorsoventrally through the intersegmental

membranes of the abdomen.

4. Detection of Destruxins *'in vitro'*

A technique was developed to enable the rapid assessment of the levels of Destruxin in culture filtrates, without requiring extensive extraction procedures. This new direct detection technique utilized the quantitative analytical capabilities of RPHPLC.

(i) Preparation of Crude Culture Filtrates for Destruxin Assessment

Cultures were set up in 250 ml conical flasks containing 100 ml of Czapek Dox medium inoculated with 3×10^6 conidia. After incubation at 27°C and 150 r.p.m. for various lengths of time the cultures were filtered through Whatman 1 to remove the mycelial mat, which was subsequently dried at 60°C, and dry weight recorded. The culture filtrate was filtered through a second Whatman 1, then through a glass fibre filter (Whatman GFF 0.7µm) before the pH was recorded. The samples were then frozen at -20°C until required.

'Sep-Pak' C_{18} cartridges (Waters) were prepared by the passage of 5 ml of HPLC grade MeOH, followed by 10 ml of HPLC grade water. Two ml of a CF sample was then passed through the 'Sep-Pak' cartridge and the eluent discarded. Finally 2 ml each of 40%, 50% and 60% CH_3CN were passed successively through the cartridge and the eluents collected.

20µl samples were taken from each of the 3 fractions and injected onto an analytical RPHPLC column. It was found that 50% CH_3CN eluted DTXs off the 'Sep-Pak' with least impurities and was adopted for subsequent experiments.

(ii) Identification and Quantification of DTX

RPHPLC fractions corresponding to the major absorbance peak that had a retention time very similar to that of pure Destruxin A (DTXA) standard , were subjected to FAB-MS for identification. The level of DTXA in the culture was quantified by using an internal standard. Each sample of CF was divided into two 2 ml aliquots and to one of these aliquots was added a known amount of DTXA standard (40µg in 10µl of HPLC H_2O). The pairs of samples were passed through a 'Sep-Pak' then run on HPLC and the peak absorbance recorded at 210 nm. The peak areas were measured using a Hayashi Denko Automatic Area Meter (Type AAM-5). The DTXA concentration in the CF was calculated from the difference in peak areas between CF and DTXA and CF alone.

5. Detection of Destruxins *'in vivo'*

300 µl of haemolymph taken from *Manduca sexta* or *Bombyx mori* larvae (see Appendix for *Bombyx* culture details) which had previously been injected with Destruxin (25µg g^{-1}), was heat fixed at 80°C for 3 minutes to deactivate enzymes and precipitate blood proteins. 1 ml of E & B saline was added to each sample which was thoroughly whirlimixed and then centrifuged at **11600 x g** (Micro-Centaur) for 5 min. The supernatant was passed through a Sepak Cartridge which was eluted with 40% and 50% CH_3CN successively. The 50% fraction was freeze dried and the subsequent residue was redissolved in 100µl of 50% CH_3CH. 20µl of this sample was injected onto an analytical RPHPLC column. Spiked blood samples with known amounts of DTX and unspiked blood were used as positive controls and blank controls respectively. The results were quantified with respect to the positive controls using peak area measurements (see previous section).

6. Detection of Destruxins in Diseased *Manduca* Larvae

Insects were infected by dipping larvae into spore suspension (technique, see p. 14). Two isolates of *M. anisopliae* were used in these experiments, ME1 and RS549.

Blood samples were taken from insects at different stages of mycoses and pooled to give a total volume of 2 ml for each stage of infection. Samples were processed in the normal manner before injection onto an analytical RPHPLC column. Fractions were collected and bio-assayed on *Manduca* heart (technique, see p. 83).

Results

1. Isolation and Purification of Destruxin from the Culture Filtrates of *Metarhizium anisopliae*

Destruxins are known to be produced in liquid cultures of *M. anisopliae* and many techniques for their isolation and purification have been documented (Tamura and Takahashi, 1971).

Experiments were carried out in order to determine the most suitable conditions for Destruxin production. The choice of culture media proved to be important, in particular, the yield of toxin was much greater when Bacto-peptone was used as opposed to Myco-peptone. The advantages of shake culture over stationary culture were that similar amounts of toxin were produced in 5-6 days of shaking to that produced by 3-4 weeks of still culture. The amounts of toxin in CFs were compared to those in whole culture extracts. Liquidized cultures and whole cultures gave the highest yields of crude toxin but the levels of impuriti es rose concurrently when compared to CFs. The time period of culture was also an important consideration with the maximum amounts of crude toxin found after 2-3 weeks, although this was somewhat dependent on temperature and amount of shaking. Loss of culture toxicity occurred with age concomitant with the degeneration of the mycelia and may be due to the utilization of the toxin out-stripping production.**(see Appendix 6)**

Estimates of Destruxins based on the sizes of identified spots on TLC plates suggested an order for toxin production by different isolates of *Metarhizium* as being ME1 > RS549 > RS324. A detailed study of Destruxin A production by these three isolates was carried out (see p. 58).

Thus conditions of large scale culturing to give the highest yields of Destruxins were defined. TLC was used to check the efficiency of the isolation and purification technique at each stage i.e. CCl_4 extraction, Ion Exchange freeze dried extracts and HPLC (Fig. 7).

In addition to the Destruxins, up to 10 impurities were visualized by iodine staining of TLC chromatograms of CCl_4 extracts. One of the major impurities has been identified as a diketopiperazine (Suzuki and Tamura, 1971), the source of which is the Bacto-peptone from the culture media. TLC of ion-exchange fractions showed that the two resins used are highly efficient at removing the impurities of CCl_4 extraction. TLC of RPHPLC purified Destruxin A, as expected, showed no impurities and this spot was used as a standard. Tamura and Takahashi (1971) stated that it was not possible to resolve different Destruxins by TLC and the Rf value of the DTX group was 0.6. Using an identical solvent system,the Rf value obtained here was 0.8. The difference was probably due to recent advances in TLC plate technology, giving increased resolution but still failing to separate the Destruxins.

The purity of the Ion Exchange extract DTX spot was established by RPHPLC and the resulting chromatogram is shown in Fig. 6. Fractions corresponding to the four major peaks (P_1, P_2, P_3 and P_4) were collected and bioassayed on *Manduca* heart. All four caused increased rate and amplitude of the heart (Fig. 8).

P_2 and P_4 were initially identified as Destruxin A and Destruxin B respectively, by their retention times in comparison to pure standards (generously supplied by M. Païs CNRS, France). The structures and identity of all four peaks was determined by Fast Atom Bombardment Mass Spectrometry. The positive ion Mass Spectra are shown in Fig. 9

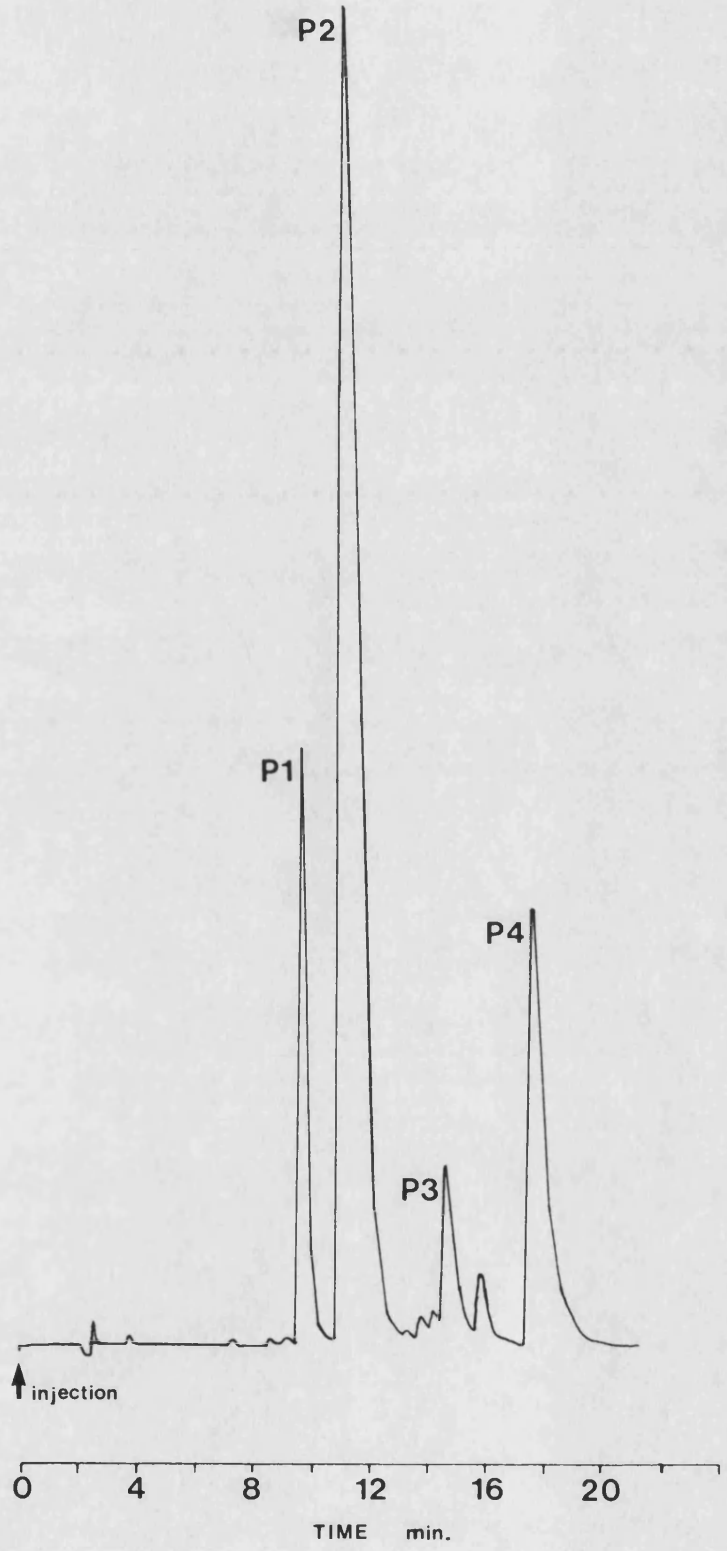


Fig. 6. HPLC Chromatogram of Semi-Preparative Separation of the Destruxins

P_1 - Destruxin A_2

P_2 - Destruxin A

P_3 - Desmethyl-destruxin B

P_4 - Destruxin B

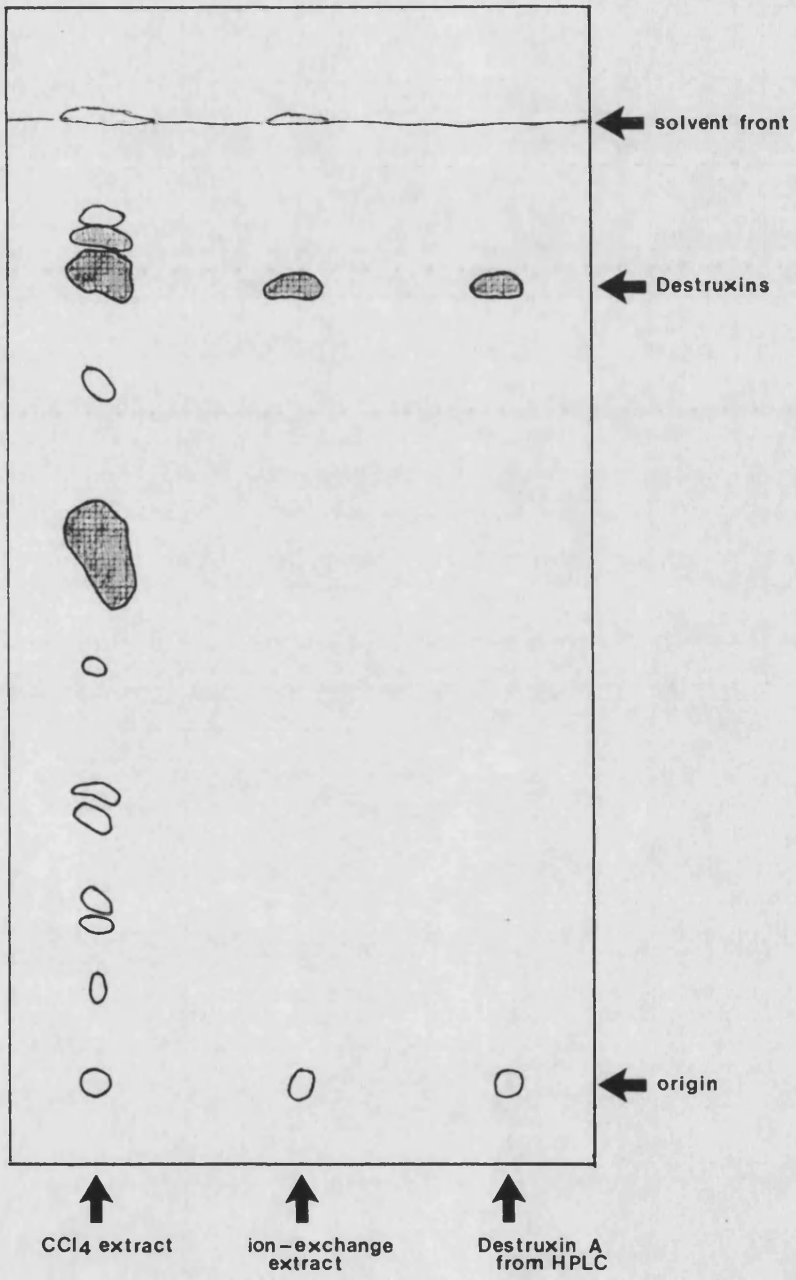


Fig. 7. TLC Chromatogram showing the 3 Stages of Purification

technique: Chloroform : Methanol 19:1

Kieselgel $60F_{254}$

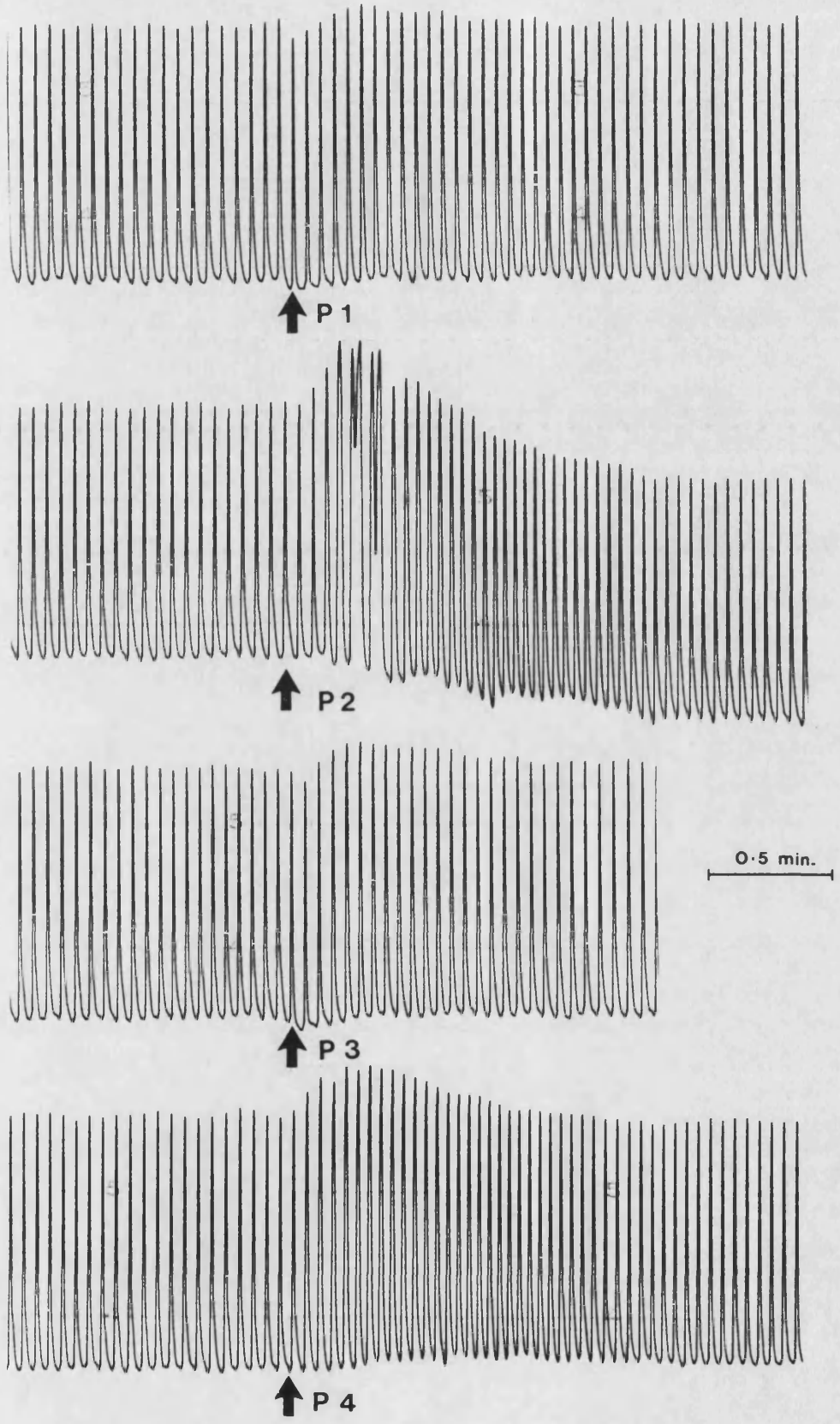


Fig. 8. Bioassay of HPLC fractions on *Manduca* larval heart

↑ -50µl pulse of test solution

HPLC SEPARATION OF DESTRUXINS

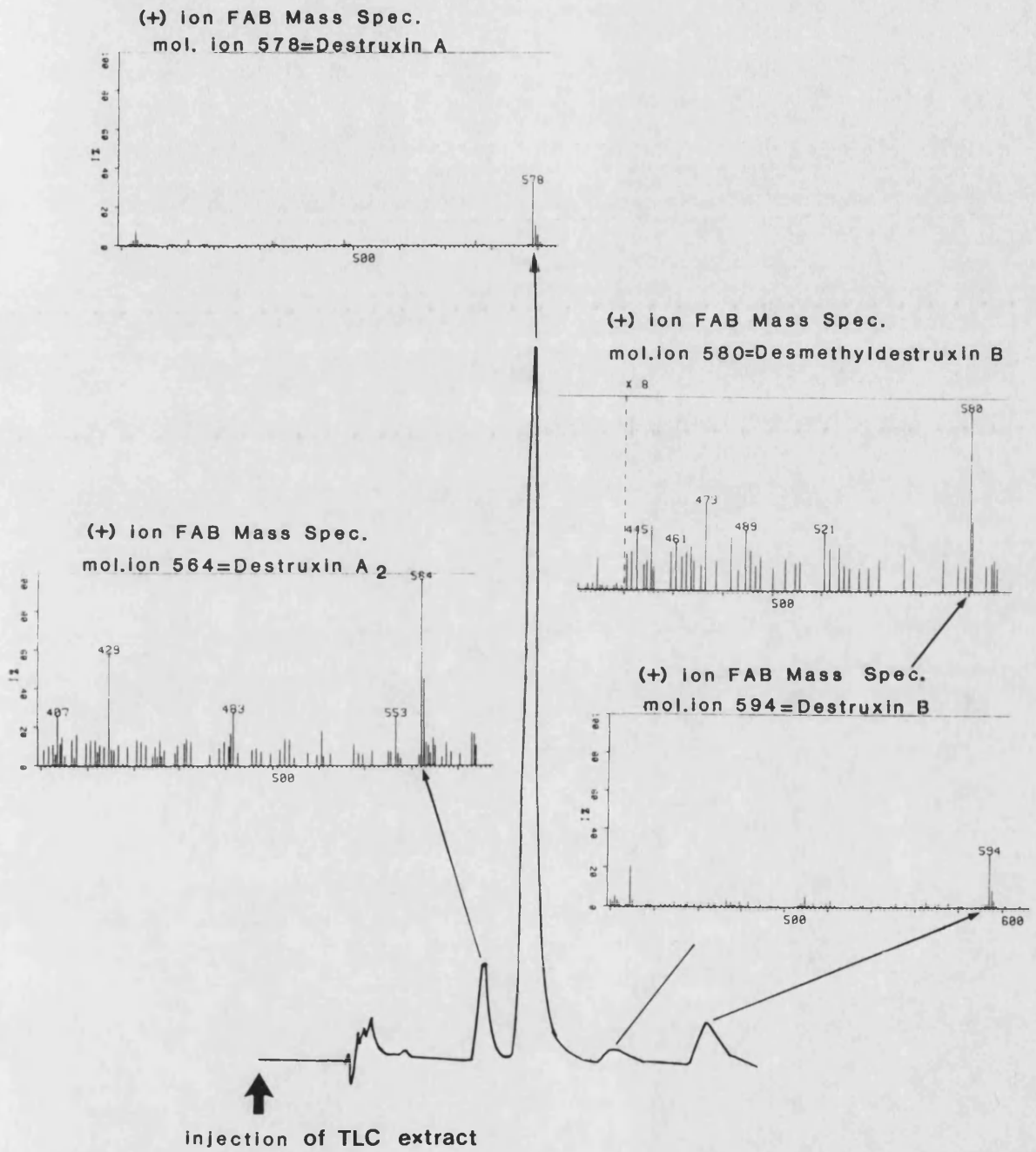


Fig. 9. HPLC Separation of the Destruxins and their corresponding (+) ion FAB.MS.

4 peaks resolved by HPLC identified by MS

(complete spectra - Appendix 5) and confirm P_2 as Destruxin A (mol. wt. 577), P_4 as Destruxin B (mol. wt. 593), P_1 as Destruxin A_2 (mol. wt. 563) and P_3 as Desmethyldestruxin B (mol. wt. 579). The results for FAB Mass Spectrometry confirm the previously postulated structure of Destruxins (Suzuki *et al.*, 1970b).

Peak area measurements established that P_2 constituted 80% of the Destruxins on column, P_4 was 10% with P_1 and P_3 making up the remainder of the total.

2. The Toxicity by Injection of Pure Destruxins to *Manduca sexta* Larvae

The ED_{50} of a mixture of 4 Destruxins (propns. stated above), the ED_{50} of DTXA and the LD_{50} of the DTX mix were calculated by Probit Analysis and the results are shown in Table 5. The ED_{50} values for the DTXA and the DTX mix were not significantly different (overlap of 95% Fiducial Limits $\equiv P > 0.05$). This was expected as the DTX mix contained 80% DTXA and confirmed the reproducibility of this bioassay.

3. The Toxicity by Injection of Destruxins to a Range of Insect Species

A range of readily available laboratory insects were screened for knockdown by Destruxin and the results are summarized in Table 6. It was immediately obvious from the results that insect species vary considerably in their susceptibility to Destruxins. The only coherent group appeared to be the Lepidopterans, all with similar knockdown susceptibility. The only insects other than the Lepidopterans to display knockdown at 10 $\mu g\ g^{-1}$ are *C. vomitaria* adults. Interestingly the larvae of this species are less sensitive. All other species screened

Table 5. The Toxicity by Injection of Pure Destruxins to 5th Instar *Manduca sexta* larvae.

Destruxin	ED_{50} $\mu g\ g^{-1}$ (fiducial limits)	Slope ± SE	Chi^2	Degrees of Freedom
DTX mix	3.643 (0.446-5.670)	1.913 ± 0.845	0.501	3
DTX A	3.681 (2.585-4.787)	3.611 ± 1.084	0.660	3
Destruxin	**LD_{50} $\mu g\ g^{-1}$ (fiducial limits)**	**Slope ± S.E.**	**Chi^2**	**Degrees of Freedom**
DTX mix	125.358 (96.237 - 311.851)	3.242 ± 1.128	0.356	2

Table 6. Distribution of Destruxin Toxicity Among the Insecta

Species	Dose 10 μg^{-1}	20 μg^{-1}	100 μg^{-1}
Lepidoptera			
Manduca sexta larvae	KD/REC	-	ca. LD_{50}
adults	UNCOORDINATED	KD/REC	-
Pieris brassicae			
larvae	KD/REC	-	-
Bombyx mori			
larvae	KD/REC	KD/NO REC	-
Diptera			
Calliphora vomitoria			
larvae	NE	NE	PAR-2 min/NO REC
adults	KD/REC	KD/REC	KD/NO REC
Coleoptera			
Tenebrio molitor			
larvae	NE	-	KD /REC
Dictyoptera			
Periplaneta americana			
adults	NE	-	PAR-5 min/REC
Orthoptera			
Schistocerca gregaria			
adults	NE	-	NE
Phasmida			
Carausius morosus			
adults	NE	-	PAR-1 h /NO REC

Key: KD = 100% knockdown
REC = Recovery
NO REC = No recovery
NE = No effect
PAR = Paralysis min/h after injection
- = Not recorded

Table 7. The Sensitivity of Lepidopteran Larvae to Injected Destruxin A

Species	Dose ED_{50} $\mu g\ g^{-1}$	Dose LD_{50} $\mu g\ g^{-1}$	Source
Bombyx mori	0.015-0.030	0.15-0.30	Kodaria (1961a)
Bombyx mori	∿0.15	∿0.30	Tamura *et al.* (1964)
Bombyx mori	∿1.5	∿5.0	Suzuki and Tamura (1971)
Galleria mellonella	-	40-60 (80-150 DTXB)	Roberts (1980)
Galleria mellonella	-	41.8-59.5 (130 DTXB)	Fargues and Roberts (1984)
Manduca sexta	3.681 (3.643 DTX mix)	(125.36 DTX mix)	Present work
Bombyx mori	∿5.0	∿8.0	Present work

show responses to DTX at the 100 μg g^{-1} level (or not at all) which must be considered to be a non-physiological dose.

4. Destruxin A Production '*in vitro*'

This study investigated the extent of Destruxin A production in liquid culture by three isolates of *M. anisopliae* ME1, RS549, and RS324. These isolates were chosen as they display significant differences in pathogenicity. ME1 was found to be highly virulent causing symptoms which were indicative of death by toxicosis. RS549 was less virulent and appeared to kill by physical disruption of host tissues. RS324 was not pathogenic to *Manduca* larvae by dipping (for details, see Chapter 2).

The techniques used here enabled rapid detection and quantification of Destruxins *'in vitro'*. Previously DTXs could only be quantitatively detected after extensive purification of culture filtrates, involving unknown losses at each stage. Minimal losses using the present technique were compensated for by use of controls and internal standards.

In a preliminary study, ME1 CFs were used to find the most suitable solvent system for 'Sep-Pak' purification and RPHPLC. A typical chromatogram (Fig. 10) displayed a peak of absorbance with the same retention time as that of DTXA as standard. The identity of this peak was confirmed by FAB MS (Fig. 10). The spectrum shows a molecular ion peak at 578, the molecular weight of DTXA + 1 unit (+ ion FAB).Interference peaks are seen as a shadow to the molecular ion peak around 600 units , which corresponds to unavoidable Na^+ and K^+ contamination.

Additional peaks seen on RPHPLC may possibly be other Destruxins, but only DTXA was identified and quantified in the present study.

Fig. 11 shows the changes in dry weight of fungus, pH and DTXA

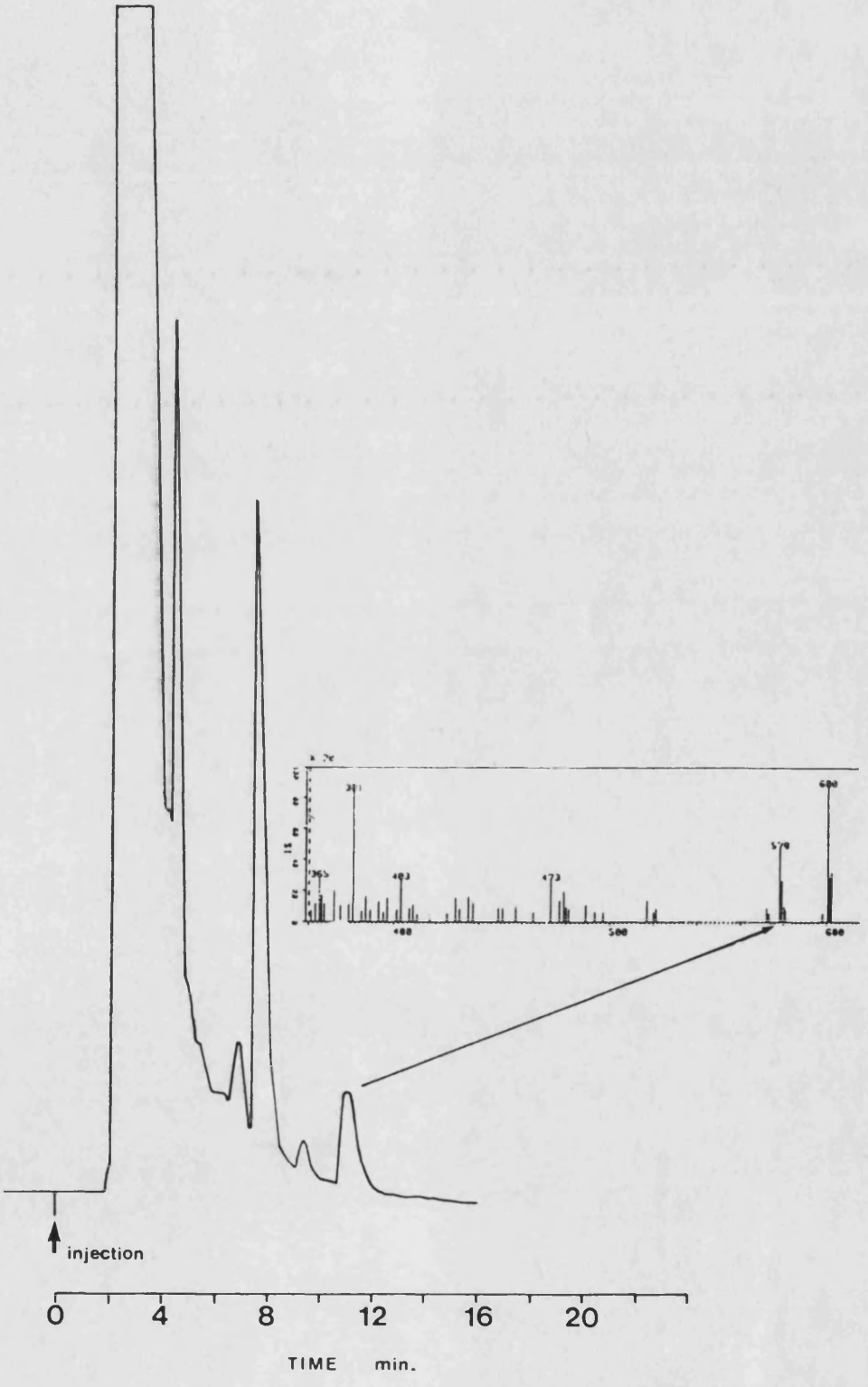


Fig. 10. HPLC Chromatogram of Fungal Culture Filtrate
note: presumptive DTXA peak identified by FAB.MS (insert) mol. ion peak 578 (interference at 600 from Na^+ contamination

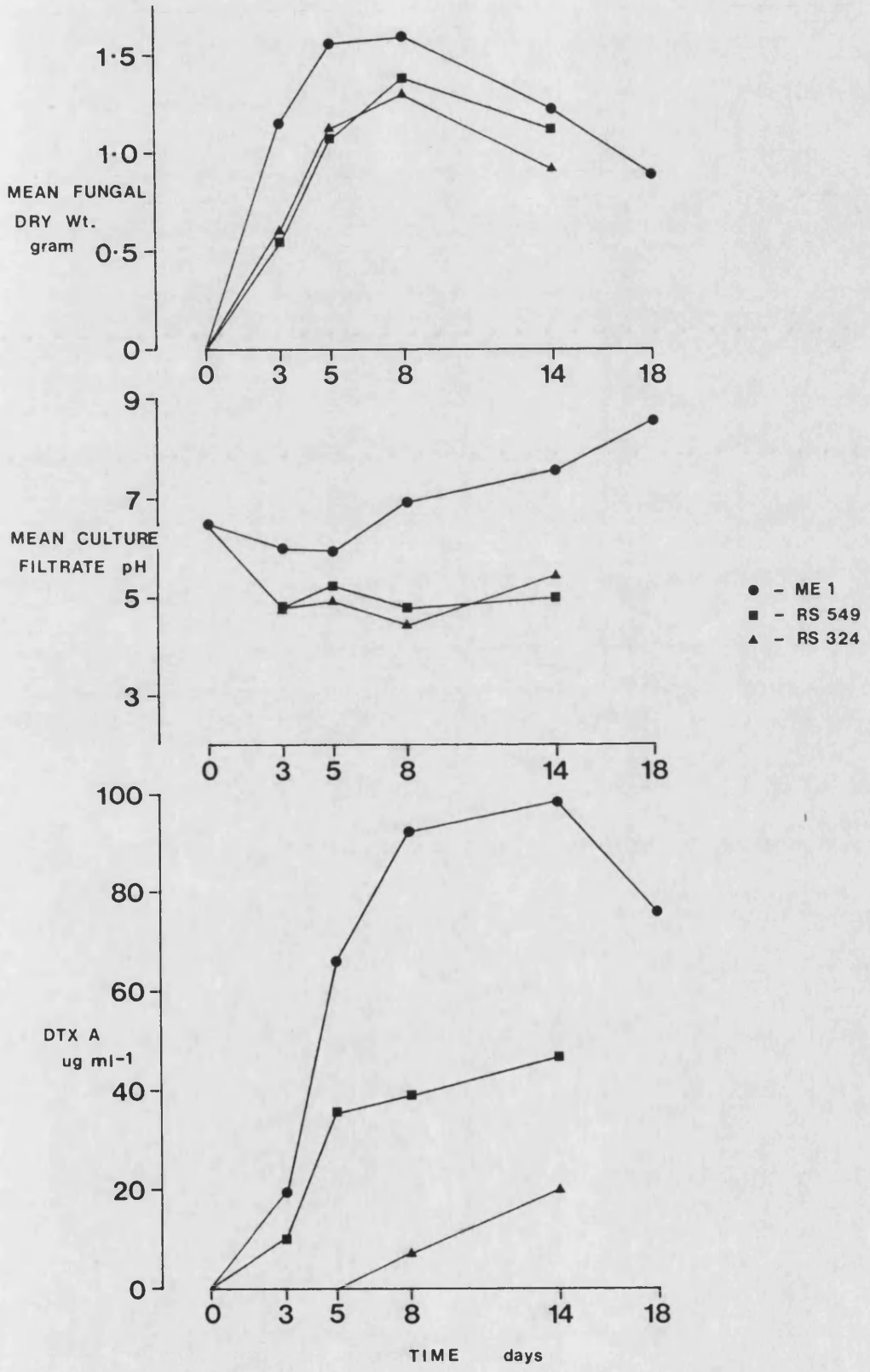


Fig. 11. Changes in Mycelial Dry Wt., Culture Filtrate pH and DTXA titre of *M. anisopliae* liquid cultures using isolates ME1, RS549, and RS324.

production during culture of ME1, RS549 and RS324. DTXA was detectable in RS549 and ME1 cultures after only 3 days although DTXA could only be detected after 8 days in RS324 cultures. ME1 appeared to produce twice as much DTXA as RS549 throughout the experiment. Production of DTXA by ME1 rises exponentially over the first 8 days of culture with the peak of mycelial growth also reached after 8 days. There is a small increase in production after this point and a sharp decline in production after 14 days, coinciding with a decline in mycelial growth.

RS324 and RS549 show very similar growth rates and pH changes during liquid shake culture, whereas ME1 shows more rapid and greater growth with higher CF pH throughout the experiment. Further experiments are required to understand the significance, if any, of culture pH to DTX production. When compensating for the differences in mycelial growth, ME1 and RS549 would have produced similar amounts of DTXA after 3 days but from then on ME1 outstrips RS549.

5. Determination of the Rate of Removal of an Injected Dose of Destruxin from the Haemolymph of *Manduca* and *Bombyx*

Manduca larvae injected with 25 µg g^{-1} of DTX eventually recovered from the poisoning but as seen in Tables 6 and 7 *Bombyx mori* larvae do not recover from this dose. Both species of larvae are equally susceptible to knockdown doses but *Bombyx* are significantly more sensitive when considering mortality. These results indicated a fundamental difference in the ability of the larvae to detoxify Destruxins.

The rate of removal of DTX from the blood of previously injected insects was investigated. 5th instar *Manduca* were injected (25 µg g^{-1}) and bled at 5 min, 1 hr, 2 hr, 5 hr, 6 hr and 24 hr. 5th instar *Bombyx*

were injected (25 μg g^{-1}) and bled at 5 min, 2 hr, 5 hr and 24 hr (limited availability of insects). The results are shown in Table 8.

The Destruxin titre of *Manduca* blood declined steadily from the time of injection. Six hours after injection, the larvae started to recover from complete flaccid paralysis with some movement of the appendages. This coincided with low levels of DTX ca. < 0.1 μg μl^{-1}, whereas immediately after injection the blood contained >1.5 μg μl^{-1}. A rapid change in the DTX titre was seen over the first hour after injection and this was probably due to DTX dispersal throughout the body. At this stage the larvae were completely flaccid, showing no response to tactile stimulation but 10 hours later they had totally recovered.

Bombyx larvae injected with the same dose as *Manduca* larvae were also paralysed immediately but did not recover and eventually died (48 hr). Twenty-four hours after injection no DTX could be detected in *Manduca* blood but ca. 0.3 μg μl^{-1} was found in the blood of *Bombyx* and consequently these insects remained paralysed.

6. Detection of Destruxins in the Blood of Mycosed *Manduca*

From the previous experiment it can be seen that very small amounts of DTXA can be detected by HPLC in the blood of individual insects. However, no DTX's were found in blood samples taken from individual insects paralysed by ME1 mycosis.

Pooled blood samples from five flaccid paralysed insects were processed and produced a peak on HPLC with the same retention time as pure DTXA (Fig. 13). The identification of the presumptive DTXA peak was supported by bioassy of peak fractions using the *Manduca* heart preparation. Attempts to obtain FAB MS spectra on this material proved unsuccessful due to the limitations of sensitivity of this

technique. Peak area measurements with respect to DTXA standards indicated that ca. 0.018 $\mu g\ \mu l^{-1}$ (± 0.03) of presumptive DTXA was present in the blood of flaccid paralysed ME1 infected *Manduca* larvae. A very small, non quantifiable peak corresponding to DTXA was found in the blood of insects in the 'uncoordinated' stage of infection. No peaks at the correct retention time were obtained from RS549 infected blood and bioassay of fractions produced no response on the *Manduca* heart preparation

Table 8. Rate of Removal of an Injected Dose of Destruxin from the Haemolymph of *Bombyx* and *Manduca* larvae.

Time of Sample Hr after Injection	Mean titre of Destruxin µg/µl ± SE (N=5)	
	Manduca	*Bombyx*
0.08	1.53 ± 0.1	1.37 ± 0.22
1	0.77 ± 0.07	-
2	0.57 ± 0.03	0.65 ± 0.2
5	0.17 ± 0.06	0.45 ± 0.15
6	0.09 ± 0.02	-
24	0	0.21 ± 0.11

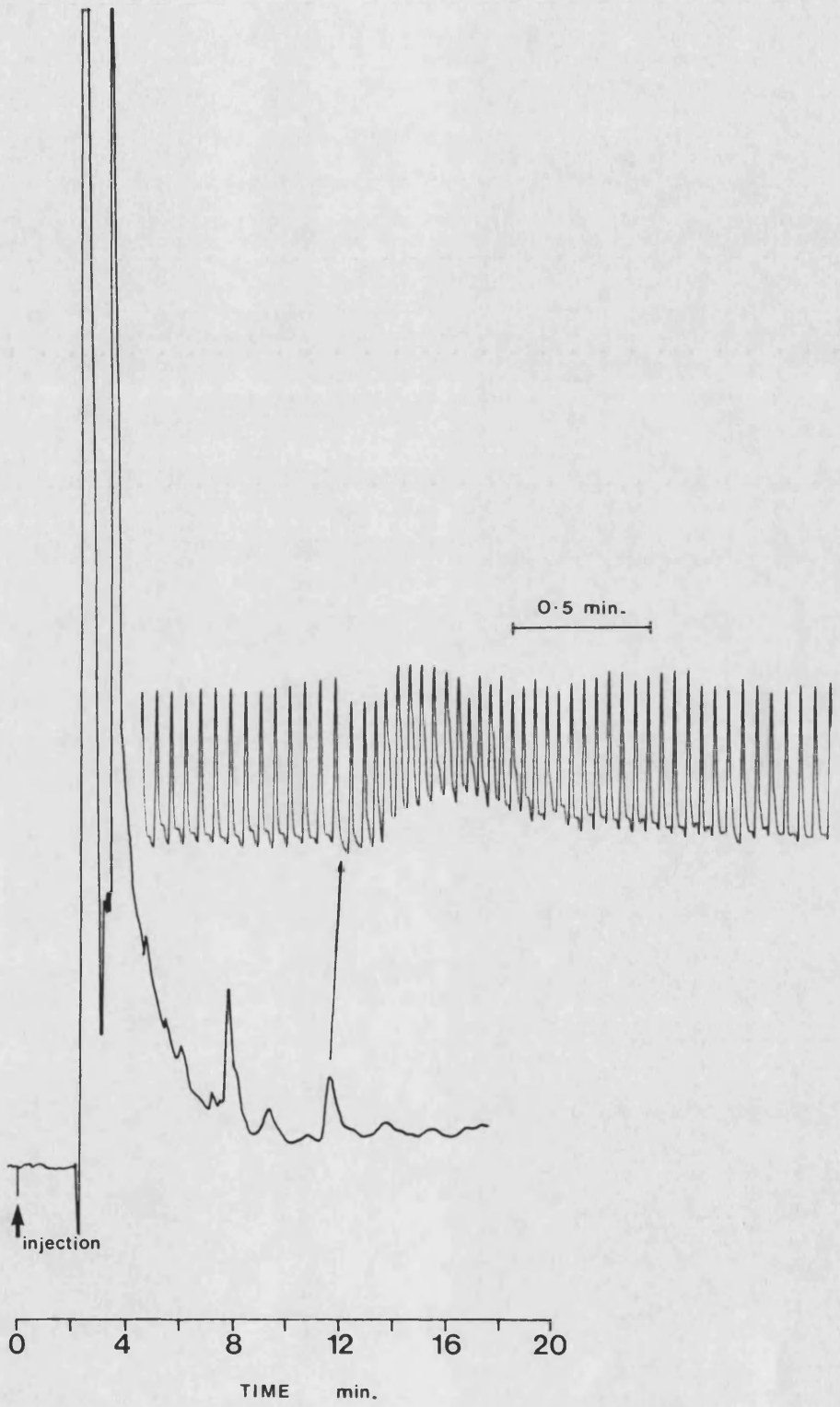


Fig. 13. HPLC Chromatogram of Mycosed Insect Blood
insert: *Manduca* heart bioassay of presumptive DTXA peak (x4)

Discussion

Destruxins of known purity were required for toxicity and mode of action studies. Unfortunately Destruxins are not available commercially and synthesis was financially prohibitive. The logical approach was to extract these fungal metabolites from the liquid cultures of *M. anisopliae*. The primary source of host-specific toxins was the culture filtrate. Some toxins may be mycelial bound and although the largest amount of crude toxin was obtained by whole culture extraction, a large percentage of the available Destruxin was extracted from the culture filtrates with least impurities.

The separation of the Destruxin from the CF was carried out by CCl_4 extraction. This method also extracts many unwanted compounds from the CF which can only be removed efficiently by preparative ion-exchange chromatography. Ion-exchange resins completely remove most of the contaminants but the toxicity of the extract is concurrently reduced. There are two possible reasons for the loss in toxicity (a) the contaminants show toxic activity, and (b) some of the DTXs are not recovered from the ion-exchange resins. Metabolites other than the DTX are known to be insecticidal (Roberts, 1966b) and losses of DTX 'on column' are unavoidable.

Separation of the purified cyclodepsipeptides to give constituent Destruxins has previously required laborious fractionation techniques. In order to obtain 14 DTXs, Païs *et al.* (1981) processed 300 litres of CF, extracted with CH_2Cl_2 followed by silica gel chromatography, fractionation, rechromatography, refractionation etc. etc. The end result was 2.5 grams of Destruxin, 47% of which was DTXA and 30% DTXB.

In this study pure samples of Destruxin A, B, Desmethyl-DTXB and DTX A_2 were obtained by semi-preparation RP HPLC (verified by

FAB Mass Spec.) which proved to be a highly efficient technique. This technique was further developed as an analytical method for the detection of DTX *in vitro* and *in vivo*, which did not require extractions (with inherent losses) before quantification.

The chemistry of the Destruxins has been fully investigated, e.g. Tamura and Takahashi (1971), therefore no further studies were carried out here. However, the results obtained here from FAB MS provide more accurate details of the structure of Destruxins than those previously published (A.J. Floyd, University of Bath, pers. comm.).

The toxicity by intrahaemocoelic injection of the Destruxins to *Manduca sexta* was investigated. The HPLC defined mixture of 4 DTXs and HPLC separated DTXA were used in standard ED_{50} and LD_{50} tests. *Manduca* 5th instar larvae responded to injected DTX by immediate tetanic paralysis followed by flaccid paralysis which occurred with a time-course which was dose dependent. *Manduca* are highly sensitive to DTX when considering knockdown but not in terms of mortality. The high dose required to kill 50% of the larvae 48 hours after injection indicated rapid detoxification of this compound. Most available data on toxicity of fungal toxins towards insects is poor due to a lack of standardization and failure to use purified toxins. It is therefore difficult to make direct comparisons between the present work and most published results.

The knockdown susceptibility to injected Destruxins of a range of insect species was investigated. All Lepidopterans tested were equally sensitive to a knockdown dose of DTX but varied in their ability to recover. *Bombyx mori* larvae were killed by an injection of 20 $\mu g\ g^{-1}$, a dose from which *Manduca* larvae recovered within 12 hours. A comparison of the results seen here and those of other workers

is shown in Table 7. There appears to be some discrepancy over the published values for ED_{50} and LD_{50} of DTXA to *Bombyx* larvae, with a 10 fold difference between each set of data. The results obtained here for *B. mori* larvae were similar to those obtained by Suzuki and Tamura (1971). Differences in the doses required for knockdown and mortality may be a reflection of Destruxin purity and/or the physiological state of the test insects.

Of the other insects screened, only *C. vomitoria* (adults) were as susceptible to DTX as the Lepidopteran larvae. A similar finding has also been noted by Abalis (1981) for the closely related dipteran, *Musca domestica* (adults). The physiological basis of differences in toxicity can only be the subject of conjecture as the mode of action of the Destruxin remains uncertain (see Chapter 4). The uniformity of the response to injected DTX by the Lepidopteran larave suggests that DTX acts at a site peculiar to this group of insects. The susceptibility of *C. vomitoria* adults (but not the larvae) is interesting but provides few insights.

Toxicity of the DTXs by contact and ingestion to *Manduca* larvae has not been investigated here, however, DTX has been found to be a phagodepressant to *Spodoptera littoralis* and *Spodoptera exempta* larvae (M.S.J. Simmonds, Birkbeck College, University of London, pers. comm.).

Certain fungal toxins have been shown to display toxicity by contact and ingestion. Bassianolide from the cultures of *B. bassiana* or *Verticilium lecanii* is toxic to *Bombyx* larvae *per os* and causes paralysis at 4 ppm with mortality at 8 ppm (Kanaoka *et al.*, 1978). Aspochracin from *Aspergillus ochraceus* cultures shows some contact toxicity but only at high concentrations, i.e. 800 µg/ml kills 50%

of dipped 1st instar *bombyx* larvae (Myokei *et al.*, 1969).

The consistent use of *Bombyx mori* larvae as test animals for the study of fungal toxins is questionable as this is not a pest species but in fact a commercially valuable insect which is known to be highly susceptible to stress! The potential of fungal metabolites for contact, *'per os'* and phagodepressant activity against pest species has therefore yet to be realised.

Destruxin production *in vitro* by *M. anisopliae* was affected by nutrients, temperature, agitation, culture time period and most importantly the fungal isolate used. The DTXA titre was followed by RPHPLC and it was found that this compound was very rapidly produced in growing cultures of ME1, but production declined with degeneration of the mycelia. The toxin titre of *Beauveria bassiana* liquid cultures also increased during the growing phase which indicated that toxins were produced by living fungal cells and not elaborated upon lysis during senescence of the culture (West and Briggs, 1968).

The maximum amount of DTXA found in the culture filtrates of ME1 was ca. 100 mg litre^{-1}. An isolate of *M. anisopliae* (F-84) used by Roberts (1969) and Abalis (1981) yielded 86 mg l^{-1} and 101 mg l^{-1} respectively, after extensive purification, which would entail some loss. If a 50% loss is assumed, then F84 would appear to produce substantially more DTXA than ME1 (minimal loss by HPLC technique). Tamura and Takahashi (1971) found 40 - 50 mg litre^{-1} of DTXA after purification, a yield which accounting for losses would be similar to that produced by ME1. The Destruxin titre of culture filtrates may be influenced by the isolate used. From the results it can be seen that the isolates examined here vary greatly in DTX production. The extent of '*in vitro*' Destruxin production appeared to correlate

with pathogenicity. ME1, which produced the greatest amounts of DTX was also found to be highly virulent to *Manduca*, apparently killing by toxicosis (see Chapter 2). RS549 was found to be less virulent to *Manduca* than ME1 and coincidentally produced less Destruxin. The avirulent isolate RS324, produced very small quantities of Destruxin but this was thought not to be related to its lack of pathogenicity as this isolate appears to be unable to penetrate the cuticle[Δ]. DTX production may not be the only factor determining pathogenicity. Other factors may also be related to pathogenicity, e.g. numbers of conidia attaching to the cuticle, speed of germination and levels of enzyme production influencing penetration. Therefore the relationship between DTX production and pathogenicity can only be confirmed by using single lesion mutants.

Destruxin production may be enhanced by 'passaging' an isolate repeatedly through the host insect. Abalis (1981) claimed enhanced toxin production for F-84 (over that quoted by Roberts, 1969) by repeated 'passage' through *Galleria melonella* before *in vitro* culturing. However, examining the figures more closely it is evident that the DTX yield in either case was exactly the same . West and Briggs (1968) noted increased toxin production by *B. bassiana* following 'passage'. Of the isolates used here, ME1 has been 'passaged' through *Manduca* many more times than RS549, which may account for some of the differences in DTX production seen. However direct comparisons of DTX production by different isolates *in vitro* may be questionable as culturing was carried out on commercial media, not defined media for optimal growth of a particular isolate. ME1 growth was the greatest on Czapek Dox media when compared to RS549 and RS324, however this could not account for the large differences in

Δ see table 4

DTX titre. Isolates differ in their form of growth on similar media, the size of the mycelial pellets may also influence toxin production (Roberts, 196 6a).

No studies have previously investigated the fate of intrahaemocoelically injected Destruxins, probably as these compounds are difficult to detect at very low concentrations. Differences in the toxicity of the Destruxins to different species of Lepidopteran larvae are consistent with the hypothesis that increased susceptibility may be correlated with a reduced ability to detoxify Destruxins. *Bombyx mori* larvae do not recover from injected doses of DTX from which *Manduca* larvae recover after a period of paralysis. Using RPHPLC it was possible to follow the DTXA titre of *Manduca* and *Bombyx* blood. Incubation of DTX in the blood of these insects *in vitro* had no effect on the DTX titre over a 24 hour period. It is unlikely that the serum would contain enzymes capable of breaking down DTX and it is known that insect blood serum possesses strong protease inhibitors (R. St. Leger, University of Bath, pers. comm.).

The titre of DTXA in the blood of injected *Manduca* falls rapidly with time, the rate of removal and concurrent recovery from paralysis would require a highly efficient detoxification or sequestration system. *Bombyx* larvae appear to be much less able to detoxify DTXA as it can still be detected in the haemolymph 24 hours after injection. Krieger *et al.* (1971) found that monophagous Lepidopteron larvae (e.g. *Bombyx*)have low mono-oxygenase activity when compared to polyphagous larvae (e.g. *Manduca*). These characteristics may account for the low LD_{50} of *Bombyx* to DTX when compared to *Manduca*. *Manduca* 5th instars are known to possess mono-oxygenase activity in the midgut and fat body (Tate *et al.*, 1982). Therefore a possible site of

injected DTX detoxification in *Manduca* is the fat body.

The interaction between toxin producing isolates of *Metarhizium* and Lepidopteran larvae may be influenced by the insects ability to detoxify fungal metabolites produced during mycosis. As seen in Chapter 1, fungi produce many low molecular weight toxins *in vitro*, however, only the Destruxins have been detected in mycosed insects. Suzuki *et al.* (1971) were the first to find Destruxins in the bodies of mycosed Lepidopteran larvae. They extracted DTX from the whole body homogenates of 300 5th instar *Bombyx mori* larvae infected with *Metarhizium anisopliae*. The presence of Destruxin B was established by Mass Spectrometry and quantified on the basis of the β-alanine present in the purified extract (amino acid analyser). This technique assumed that all β-alanine extracted was derived from DTX. However, no control extractions were apparently performed and it is known that many insect cuticles contain β-alanine (reviewed in Neville, 1975; p. 135). 0.0004 µmol/larvae was found on day 4 and 0.0006 µmol/larvae on day 5 post inoculation. These figures are equivalent to 0.238 and 0.356 µg/larvae of DTXB respectively.

Metarhizium anisopliae isolate ME1 does not appear to invade the tissues of *Manduca* prior to death and the DTXs must therefore circulate in the blood, which as a consequence may be expected to have the highest titre of DTX. 0.018 µg/ml of DTXA was found in the blood of paralysed 5th instar *Manduca* larvae. Assuming a blood volume in diseased 5ths of ∿200 µl (w.r.t. ∿ 1000 µl in healthy controls), there is a ∿3.6 µg of DTXA present in the blood. This is 10 times more than that found by Suzuki *et al.* (1971) in whole larvae of the comparably sized *Bombyx mori* larvae. However, their extraction procedure would have led to extensive losses. Accounting for the fact that *Bombyx*

larvae are highly sensitive to DTX, the concentrations found by Suzuki *et al.* (1971) could have been responsible for host death.

A comparison of the symptoms seen during the rate of removal experiment (p. 61) and those noted during mycosis, indicated that the blood of paralysed mycosed insects should contain approximately 0.1 µg/ml of DTX. Although only 0.018 µg/ml was found, it is possible that continued localized secretion of DTX by the fungus growing beneath the cuticle could exert deleterious effects on the muscles while detoxification keeps the overall blood titre relatively low. It is significant here that the circulation of the haemolymph in caterpillars is extremely sluggish. Injected [^{14}C]-insulin takes more than 2 h to be uniformly distributed within the haemocoel in mature fifth instar *Manduca* larvae (S.E. Reynolds, University of Bath, pers. comm.). These results support the hypothesis that fungal toxins are key determinants of pathogenicity during certain host/pathogen interactions.

Champlin and Grula (1979) failed to detect the presence of methanol soluble toxins, e.g. Beauvericin, in the haemolymph of *B. bassiana* infected *Heliothis zea* larvae. They also failed to detect Beauvericin in the culture filtrates of *B. bassiana*. Beauvericin was not found to be toxic to *H. zea* larvae on injection but another toxin known to be produced by *B. bassiana*, Basianolide (Kanoaka *et al.*, 1978), was found to be highly toxic. From this evidence it was concluded that Beauvericin plays no role in the pathogenicity of *B. bassiana* to *H. zea* larvae, which is hardly surprising when considering the low toxicity of Beauvericin to this species of Lepidopteran.

The role of fungal toxins as key determinants of pathogenicity requires further investigations which could be aided by some of the newly devised techniques described here.

CHAPTER 4: THE MODE OF ACTION OF DESTRUXINS

Introduction

The chemistry of the Destruxins has been extensively investigated but their mode of action has recieved virtually no attention. The Destruxins, first discovered over 20 years ago (Kodaira, 1961a), cause very striking symptoms of poisoning when injected into Lepidopteran larvae (Roberts, 1980). These symptoms are immediate tetanic paralysis followed by flaccidity, from which it can be concluded that DTX acts as either a neurotoxin or a myotoxin.

DTX is rather selective in its toxicity to insects, being far more poisonous to Lepidoptera than most other insects (see Chapter 3). Significantly, Lepidoptera are also one of the groups of insects most susceptible to *Metarhizium* mycosis. It therefore seemed desirable to investigate the mode of action of DTX in a Lepidopteran (i.e. *Manduca*).

Ionic basis of resting and action potentials in Lepidoptera

The use of *Manduca* raises the problem that the ionic bases of resting and action potentials in Lepidopteran nerve and muscle are only imperfectly understood. Partly, this problem arises from the unusual ionic composition of the extracellular fluid (haemolymph) in Lepidoptera, as compared to most insects and other animals. Lepidopteran caterpillars in particular have very low extracellular Na ion activity, and moderately high extracellular K^+ activity, a situation which is opposite to the normal state of affairs in most animals. Additionally $[Mg^{2+}]_o$ may reach concentrations which would severely compromise synaptic transmission in most animals.

These ionic problems are the consequence of an exclusive diet of leaves, which are high in K^+ and Mg^{++}, but low in Na^+. A similar state of affairs is also found in another phytophagous insect, the stick insect *Carausius morosus* (Wood, 1963).

The way in which nervous transmission is maintained despite this unpromising ionic environment has been elucidated largely by Treherne and his co-workers (see Treherne and Pichon, 1972; Lane and Treherne, 1980). Essentially, neuronal function depends on the active maintenance by perineurial cells of a privileged ionic micro-environment in the extracellular space within the nervous system. Conduction processes in the nerve cord of *Manduca sexta* were investigated by Pichon *et al.* (1972), who found that there was a peripheral barrier to the movements of sodium and potassium. It was suggested that the tight junctions between adjacent perineurial cells were the possible sites for generating the privileged ionic micro-environment (Lane and Skaer, 1980).

However muscle cells are not so protected from the ionic milieu of the haemolymph, so that a different explanation must be found for the ionic basis of resting and action potentials in muscle. Since it will be shown in this chapter that DTX acts to depolarize E_m in *Manduca* body wall muscle, it is necessary to review briefly what is known of the membrane biophysics of Lepidopteran muscle.

The resting potential (E_m) of Lepidopteran muscle membranes that would be predicted from measured concentrations of $[K^+]_i$ and $[K^+]_o$ using the Nernst Equation range from -8.0 to -44 mV (Duncan *et al.*, 1976), but directly measured values range from -42 to -75 mV (Wood, 1963). These inconsistencies are not present in the majority of insect orders. The E_m of cockroaches, locusts, grasshopppers and stick

insect muscle calculated from the Nernst equation much more closely approach those recorded directly (Hoyle, 1955; Duncan *et al.*, 1976).

The E_m of Lepidopteran muscle may be better described by the Goldman Equation which takes into account the different permeabilities of the membrane to different ion species. Changes in E_m of Lepidopteran muscles are most readily evoked by changes in $[K^+]_o$, but E_m is also affected (though to a much lesser extent) by changes in $[Ca^{2+}]_o$, $[Mg^{2+}]_o$ and $[Na^+]_o$ (Belton, 1960). The Lepidopteran muscle membrane may thus behave as a multi-ion electrode where E_m is determined in a complex way, depending not only on the passive permeability of the membrane to K , but also on its permeability to other ions (Huddart, 1967). It has been suggested that divalent cations play a role in the maintenance of E_m in the muscles of the caterpillar *Antheraea pernyi* (Weevers, 1966), but in the closely related *Antheraea polyphemus* Rheuben (1972) found no significant resting permeability to Mg or Ca ions.

The multi-ion electrode model still fails to account for a large proportion of the recorded E_m. The difference between calculated and recorded values may be due to active processes. Metabolic inhibition has a very significant effect on Lepidopteran E_m. In *Sphinx lugustri*, E_m declines over a period of 2 hours from -50 mV to -20 mV when exposed to DNP (Huddart and Wood,1966). Similar effects on E_m are produced by lowering the temperature of the muscles to 5°C (Duncan *et al.*, 1976) and during anoxia (Rheuben, 1972), when the value for E_m approaches E_k. The muscles become inexcitable at this lowered E_m (Wareham *et al.*, 1975), which is presumably due to inactivation of the voltage sensitive Ca Channels thought to be responsible for the action potential (see below). These observations have been taken to be

an indication of the importance of active transport to the normal functioning of Lepidopteran muscles. The identities of the ions transported by the electrogenic pump have not been established with certainty in Lepidopteran muscles. In cockroaches, the much smaller metabolic contribution to E_m is dependent on HCO_3^- ions and may be a consequence of an active HCO_3^- pump (Duncan *et al.*, 1976).

Calcium Channels and the Ionic Basis of Insect Muscle Action Potentials

Action potentials of most excitable tissues are a result of a transient increase in **Na** conductance. The original and classic example is of course the squid axon (Hodgkin and Katz, 1949), other well known Arthropod examples are the lobster giant axon (Narahashi *et al.*, 1964) and the cockroach giant axon (Pichon and Boistel, 1967).

Fatt and Ginsborg (1958) were the first to investigate the novel ionic requirements for the production of APs in an Arthropod muscle. They found that Na^+ was not involved in production of APs and suggested that an increase in Ca ion conductance was responsible for the inward transfer of charge across the membrane and balancing the outward current carried by K and Cl ions.

Following this discovery, many workers have investigated the 'Ca Spike' and the membrane channels responsible for it. The Ca Channel is not a unique molecular structure, unlike the Na Channel which shows almost identical selectivity in tissues as diverse as squid axon, frog nerve and tunicate egg (Hagiwara and Byerly, 1981). The different permeability properties of Ca **Channels** that have been documented are evidence for the presence of more than one type of Ca Channel.

Six criteria are available to identify 'Ca Spikes' (Hagiwara and Byerly, 1981), although it is rare that all are given as evidence for voltage dependent Ca permeability of membranes. One of the most important criteria is that Calcium Spikes are blocked by polyvalent cations such as Co^{2+}, La^{3+}, Mn^{2+}, Cd^{2+} and Ni^{2+} at concentrations less than 10 mM. The AP of Barnacle muscle fibres was inhibited by Mn^{2+}, probably as it competes with Ca for occupancy of the sites on the membrane necessary for spike initiation (Hagiwara and Nukajima, 1966). Calcium APs were found by McCann (1971) in Lepidopteran Myocardial fibres which were blocked by $MnCl_2$ and zero $[Ca^{2+}]_0$. A similar situation is found in *Manduca* where the heartbeat is independent of the presence of external Na but dependent on the presence of external Ca, and is blocked by a number of divalent cations. In order of potency, these are Cd^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+} (S.M. Luckman and S.E. Reynolds, University of Bath, pers. comm.).

The TTX independence of locust muscle AP shows that Na plays little, if any, role in the spike configuration (Washio, 1972). Na does appear to make a small contribution to the AP of *Sarcophaga bullata* flight muscle, although Na will not support spikes in the absence of Ca (Patlak, 1976).

It has also been suggested that Mg may carry charge across the muscle membrane during depolarization (Usherwood, 1969), however, this was not found to be the case in *Xlyotrupes dichtomus* larvae (Fukuda *et al.*, 1977).

The most exacting study to date of Calcium Spikes in insect muscle was carried out on *Carausius morosus* muscle under Voltage Clamp Conditions (Ashcroft and Stanfield, 1982). The voltage-activated inward current of this membrane was found to be carried solely by Ca

ions. In this case, Na^+ and Mg^{++} appeared to be unable to pass through the Ca Channel.

Conflicting data from different membrane systems is not surprising, as each has its own unique adaptations. Ca currents with very different properties can even be found in the same membrane (Hagiwara and Byerly, 1981).

Materials and Methods

1. Dissection

For preliminary experiments 5th instar *Manduca* were injected with a paralytic dose of DTX (5 μg g^{-1}) and when flaccid the larvae were dissected. Dissections of all *Manduca* preparations either anesthetised by chilling on ice (30 min) or after injection of DTX, was by cutting along the dorsal midline from the 'tail' to the head capsule. Larvae were then pinned out on a Sylgard dish (Dow-Corning) over a cavity which allowed the spiracles access to air. The gut, fat body and CNS were then carefully removed. The dish was mounted at a slight angle (25°) and the body wall preparation perfused from the caudal region using either an LKB peristaltic pump or a gravity feed system at a flow rate of 2 ml min^{-1}.

2. Recording Body Wall Muscle Contractions (Spontaneous and Evoked)

A Grass FTO3C transducer was attached to the 'tail' of a dissected larva via a miniature suture clamp. Signals from the transducer were amplified by a Devices Sub 1C amp. and output recorded on a Houston Omniscribe Chart recorder.

Muscles were field stimulated via gross electrodes (dissection pins) in the body wall connected to a Harvard Research Stimulator. 15 volt,0.15 msec pulses were delivered every 1.33 min.

3. Intracellular Recording, Direct and Indirect Muscle Fibre Stimulation

Intracellular recordings were made from the great ventro-recti muscles of the A_3 and A_4 segements. These fibres were stimulated directly by current injection and indirectly by stimulation of the appropriate ventral motor neuron.

Membrane potentials were recorded with glass micro-electrodes (Clark Electromedical) filled with 3 M KCl, having a resistance of 5 - 20 mΩ and connected to a NL-102DC Preamp (Digitimer). The reference silver/silver chloride electrode was placed in 3 M KCl and connected to the preparation via an agar bridge (0.1 M KCl in 2% Agar). The output from the DC Preamp was displayed on a Telequipment DM 64 Storage Oscilloscope. E_m's were read directly from the storage oscilloscope screen, but APs and injected current were recorded using a Medelec (FOR-4) fibre-optic recording oscilloscope.

Current was injected via a glass microelectrode filled with 1.5 M K-Acetate having a resistance of 10 - 15 MΩ and connected to a NL-800 stimulus isolator (Digitmer) or its home-made equivalent driven by a Harvard Stimulator. Motor neurons were stimulated via a suction electrode isolated and driven in the same way.

4. Experimental Solutions

Standard saline consisted of Na 5.5 mM, K 40 mM, Mg 18 mM, Ca 3 mM, Cl 65 mM, Sucrose 193 mM and MOPS (Sigma) 10 mM, pH adjusted to 6.5 using KOH.

In Na -free solutions isotonic Choline Chloride was substituted for Na . In Ca free solutions Mg was substituted for Ca and 1 mM EDTA added. For Ca and Mg free solutions Choline Chloride was substituted for Ca and Mg and 1 mM EDTA added.

Hypertonic saline contained 400 mM Sucrose (BDH) but was otherwise identical to the normal saline. Where required, 0.25 mM Cadmium chloride (Sigma), 0.05 mM 2,4-Dinitrophenol (BDH) and 1 mM L-Glutamic Acid Sodium Salt (BDH) were simply added to normal buffered salines and pH checked.

Tetrodotoxin (CalbioChem) in citrate buffer was injected intra-haemocoelically using a 100 µl Unimetrics fixed needle syringe.

All experiments were carried out using a mixture of Destruxins (defined by HPLC) consisting mainly of DTXA (80%) as described in Chapter 2. This was initially dissolved in Dimethyl-sulfoxide (Sigma) and appropriately diluted in experimental solutions to give a maximum DMSO concentration of 1%, Control experimental solutions contained 1% DMSO.

All reagents were Analar grade.

5. Semi-isolated *Manduca* Larval Heart Preparation

Fifth instar larvae (8 - 10 g) were anæsthetised in water for 15 min. The body wall was then cut laterally, ventral to the spiracles so that the entire dorsal surface could be removed and pinned out in a sylgard dish. The gut was completely removed and the preparation washed well in standard saline.

Movements of the dorsal vessel were monitored by the deflection of a hook made from a IAA gauge entomological pin inserted underneath the heart between abdominal segments 5 and 6. This was connected by a light cotton thread to the lever of a Palmer isotonic movement transducer. Permanent records of the output from the transducer were made on a chart recorder (Houston Omniscribe).

The semi-isolated preparation was held at an angle of approx. 25° to the horizontal and saline perfused continuously onto the caudal end of the heart, at a rate of about 10 ml/min. All test substances were applied into the perfusion drip using a Hamilton microsyringe.

6. Ionophore Experiments

Pyrex U-tubes were filled with 10 ml of CCl_4 containing 40 µg ml^{-1} DTX mix or 40 µg ml^{-1} Valinomycin (Sigma). Plastic coated magnetic fleas were placed in the bottom of the U tubes to stir the CCl_4. 5 ml of Deionised water (DIW) was placed in one arm of the U tube and 5 ml of standard Ion solution in the other arm. The standard solution consisted of 100 mM NaCl, 100 mM KCl, 100 mM $CaCl_2$ and 100 mM $MgCl_2$ (all Analar BDH).

The tubes were then placed in the dark at 20°C and the CC14 barrier slowly stirred for 4 days. Samples were removed from the DIW arm and ionic content measured using a Pye Unicam SP9 Atomic Absorption Spectrophotometer. The photometer was calibrated using a range of standard solutions of Na, K, Ca and Mg.

Results

1. The Effect of Destruxins on Body Wall Muscles

Intrahaemocoelic injection of DTX (5 μg g^{-1}) causes immediate paralysis. An initial tetanic phase which lasted approx. 30 min was followed by a phase of flaccid paralysis (> 6 hr). With larger doses of DTX the tetanic phase was shorter, while the flaccid phase was prolonged. Insects in tetany are firm to touch due to the muscles being constantly contracted, increasing the haemocoelic turgor pressure. At this stage of paralysis *Manduca* larvae regurgitate their gut contents (Plate 7), a symptom also noted for DTX poisoned *Bombyx* but not *Galleria* larvae (Roberts, 1966). Complete muscle relaxation occurs when the insect reaches the flaccid paralysis stage (Plate 8). Normal muscle tension is restored if and when the insect recovers.

The effect of Destruxins on denervated (CNS removed) semi-isolated body wall muscles of *Manduca* larvae was investigated. This preparation allowed quantitative measurement of muscle contraction caused by exposure to DTX. An example of the response of body wall muscle to DTX is shown in Fig. 14. DTX 0.25 μg ml^{-1} caused contraction within one minute. The muscle tension developed was approximately 12 grams. The effects of this concentration of DTX are not as quickly reversed as lower doses on washing. The muscle tension developed by the body wall preparation is a product of the many layers of muscle fibres in the body wall, which may account for the fluctuations seen in the records. The great ventro-recti muscles were the first to be affected, followed by the underlying muscle groups.

To mimic the effects of injected DTX, i.e. muscle flaccidity , the semi-isolated muscle preparation was continuously exposed to DTX for over 30 min (Fig. 15). The muscle tension initially increased in the normal pattern but after 10 min exposure the tension began

to fall and eventually returned to normal levels.

Body wall muscle tension showed a dose response to perfusion by DTX. The maximal value of the tetanic tension evoked was recorded in response to a range of DTX doses (Fig. 16). The minimum dose required to cause contraction was approx. 0.1 μg ml^{-1} and maximal tension was exerted when the muscles were perfused with 1.0 μg ml^{-1}. The upper limit may be set by the maximum sustainable tension that can be exerted when all body wall muscles are fully activated.

The body wall preparation was occasionally used as a means of detecting bioactivity of HPLC fractions but was less sensitive than the response of the semi-isolated heart (see below) and therefore was not used routinely.

2. The Effects of Destruxin on Evoked Muscle Contractions

Normal twitch contractions of *Manduca* body wall muscles were evoked by field stimulation which depolarized nerve terminals and the effects of DTX on this contraction investigated. The threshold for eliciting a response directly from the muscle fibres by such an external stimulus is considerably higher than that necessary to depolarize the presynaptic membrane (Miller, 1979).

An example of the response to evoked contractions is shown in Fig. 17. Normal twitch contractions developed 8 - 10 g of tension. This pattern was immediately disrupted by perfusion of DTX. The toxin caused an immediate increase in the basal (i.e. unstimulated) tension of the body wall muscles, reaching a plateau after about 20 min. During this period, the amplitude of the evoked contraction also declines, so that the twitch size falls to a fraction of its normal force during the plateau of the DTX-induced change in base tension.

Plate 7. *Manduca* larvae in tetanic paralysis immediately after injection of DTX.

Plate 8. *Manduca* larvae, following a period of tetany (above) become completely flaccid.

5mm

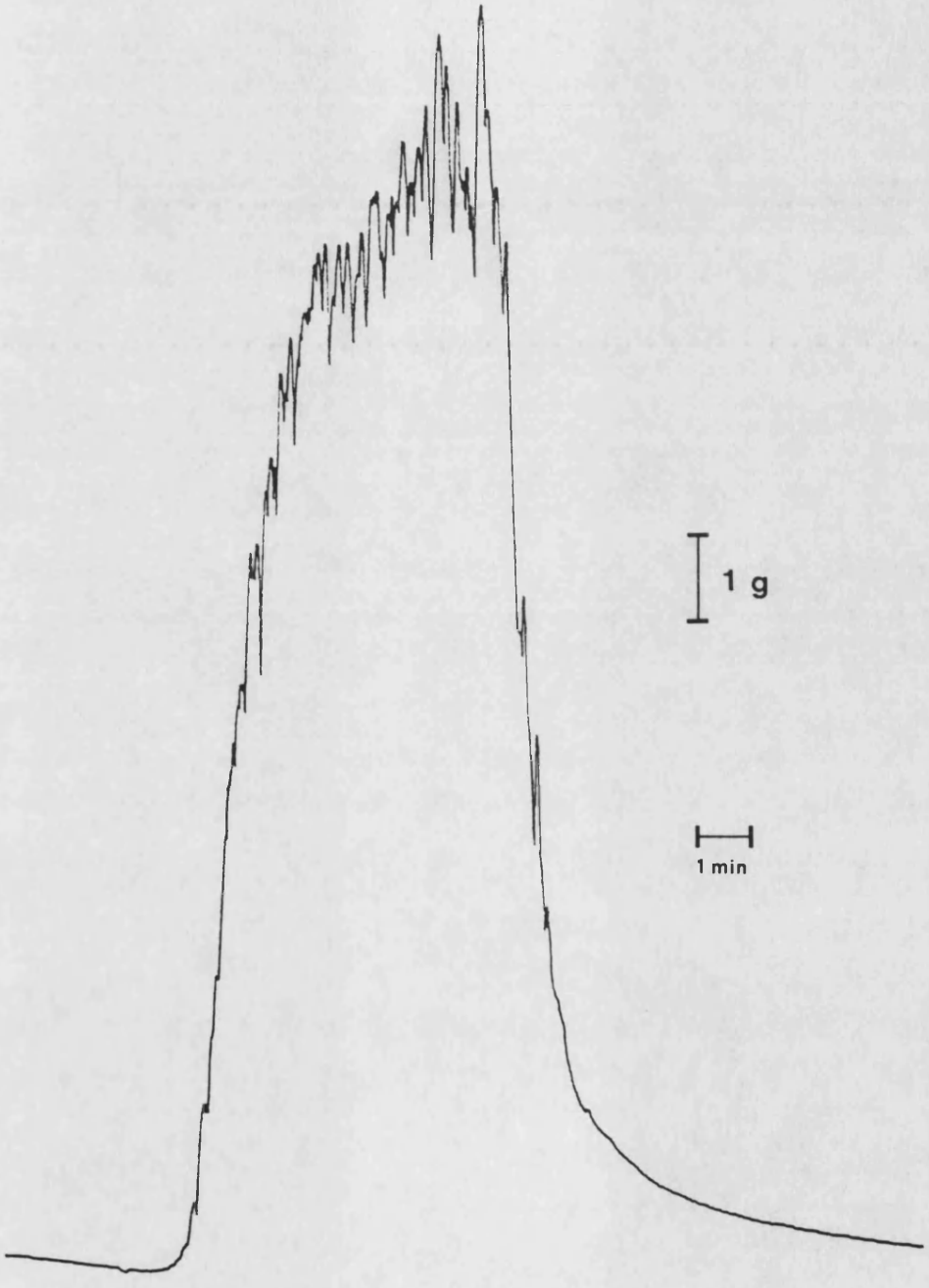


Fig. 14. A typical response of *Manduca* body wall muscle to perfused Destruxin
note: Perfusion: 2 ml.min^{-1}

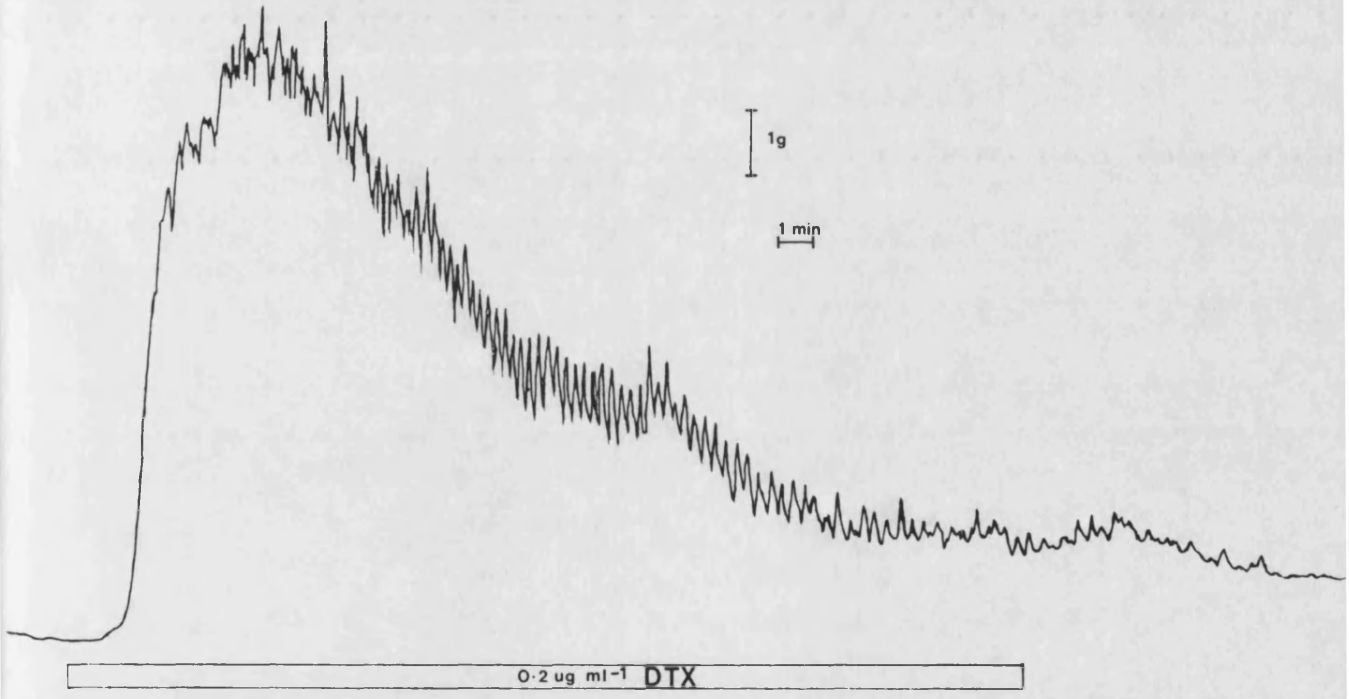


Fig. 15. The Response of *Manduca* body wall muscle to continuous exposure to Destruxin

note: initial tetanic paralysis is followed by muscle relaxation (flaccidity)

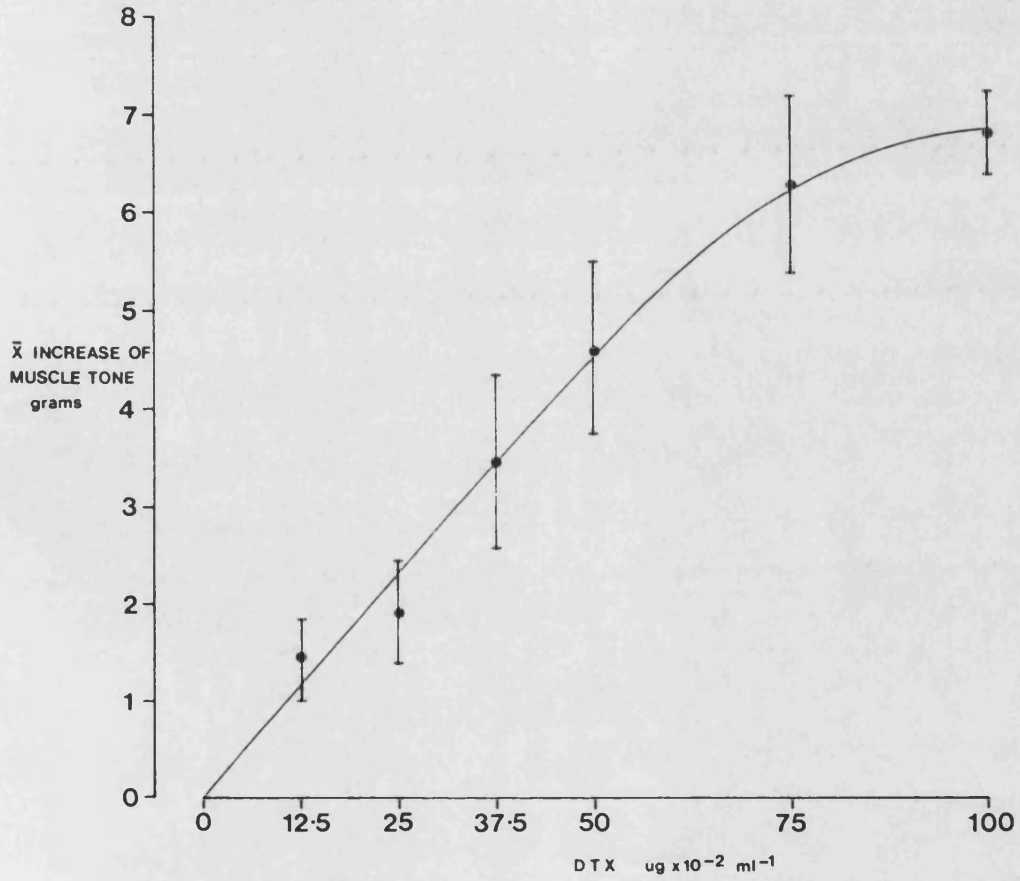


Fig. 16. Dose Response of *Manduca* body wall muscle tension to perfused Destruxins

note: $\bar{x}$ Peak tetanic contraction recorded

(± 95% con. lmts, n=3)

Perfusion: 2 ml min^{-1}, 5 min.

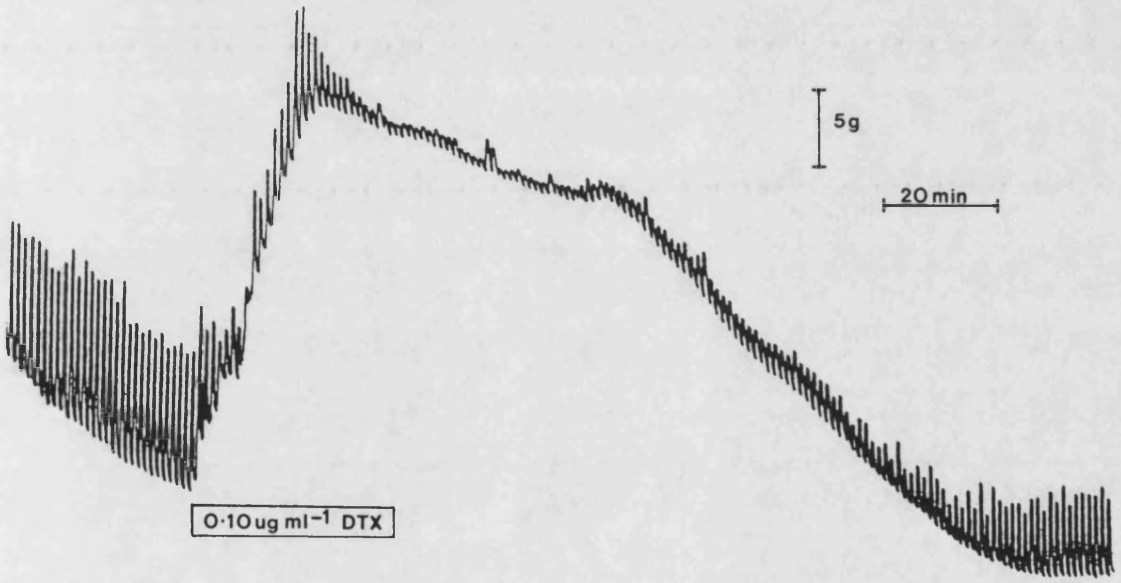


Fig. 17. The Effects of Destruxin on evoked contractions of *Manduca* larval body wall muscles

note: baseline of contractions prior to DTX perfusion is unstable due to body wall muscle stretching. Stimulus: 15V, 0.15 msec. pulse every 1.33 min.

The evoked contraction recovered to some extent, along with base tension, after washing with control saline.

3. The Effects of Destruxins on *Manduca* Larval Muscle Membrane Potentials

(i) The Effect of DTX injection on the Membrane Resting Potential

Larvae which had received DTX injections (5 μg g^{-1}) and had become flaccid, were dissected and the resting potential (E_m) of the ventral longitudinal body wall muscles determined.

The muscle membranes of DTX-paralysed insects were found to be depolarized with an E_m of -21.06 ± 0.67 mV (n = 15) compared to control E_m of -65.26 ± 1.42 mV (n = 15) [mean ± SE]. The depolarizing effect of Destruxins could be reversed by continuous perfusion of the muscle fibres with DTX-free saline. This eventually (75 min) restored E_m to normal levels (Fig. 18).

Control fibres contracted normally when stimulated by a depolarizing 100 na pulse administered through the recording electrode, but fibres from DTX-treated insects failed to contract.

(ii) The Effects of DTX Perfusion on E_m of Semi-isolated Muscle

A similar depolarization of the ventral longitudinal muscles to that found in injected larvae was produced by perfusion of DTX (40 μg ml^{-1}) over the denervated body wall preparation. Within 5 min of exposure to DTX, E_m fell from ca. -60 mV to ca. -20 mV (Fig. 19A).

It seems likely that E_m actually fell more rapidly than this, but it was not found to be possible to maintain stable intracellular recordings during the period 0 - 5 min after DTX addition, owing

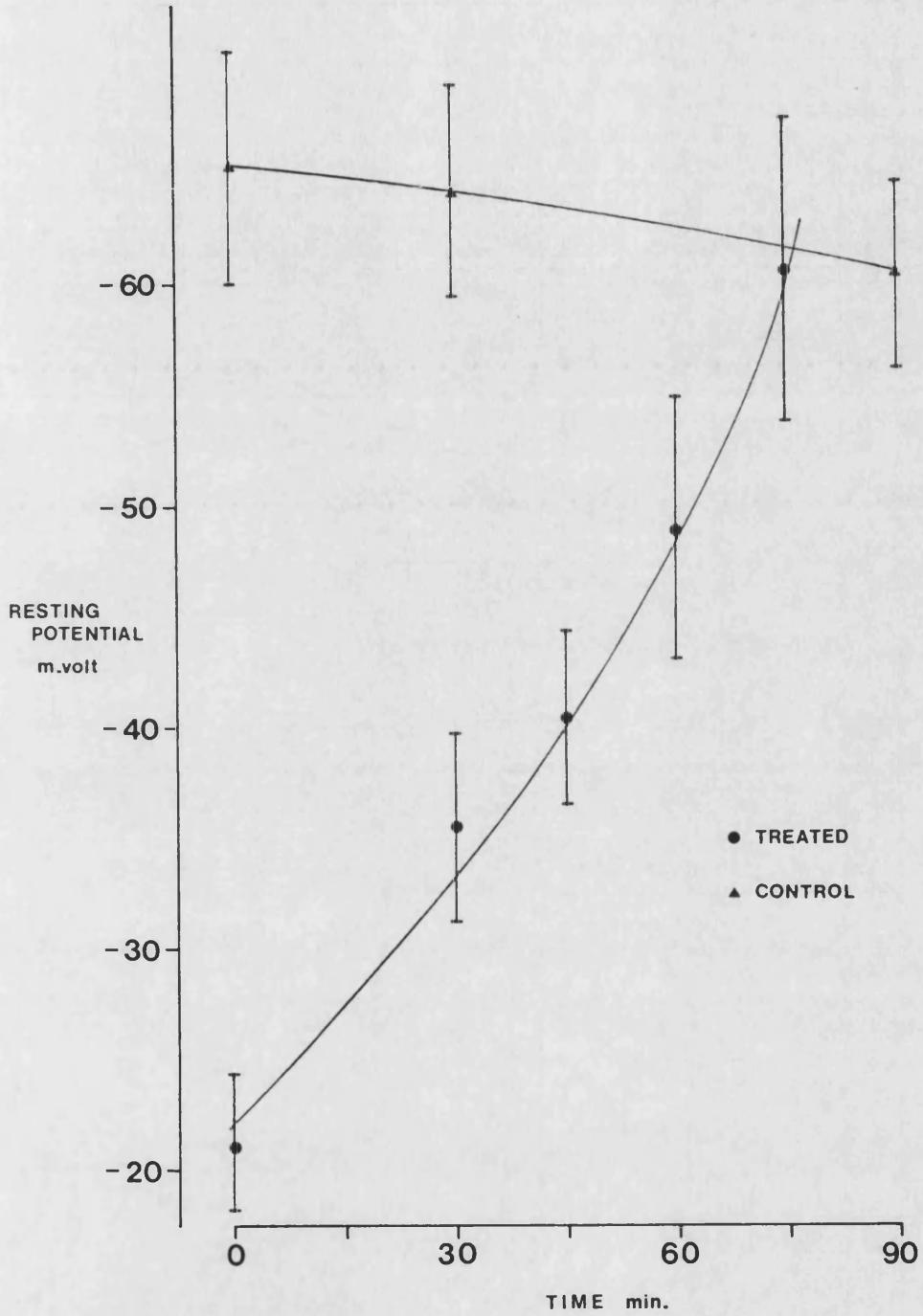


Fig. 18. Recovery of the Muscle Membrane Resting Potential from the effects of intrahaemocoelically injected Destruxins ($\bar{x}$ E_m ± SD, n = 15)

note: Perfused after dissection (from time 0) at 2 ml.min^{-1}

to movement of the previously quiescent muscles. After 5 min, however, the body wall muscles appeared to be in a state of tetany, with movement sufficiently reduced to allow intracellular penetration with the recording electrode. Extensive washing (2 hr) with DTX-free saline restored E_m to control values, with muscle relaxation occurring concurrently.

(iii) Ion Substitution and Ion Channel Blocking Experiments

The DTX-induced depolarization of the body wall muscles is likely to result from an increase in permeability to one or more of the three positive ions, Na^+, Ca^{++} and Mg^{++}, that are present in higher concentrations outside than inside the cell. The identity of the ionic permeability responsible was sought by means of ion-substitution experiments and by use of specific ion channel blockers.

(a) The Effects of Tetrodotoxin on DTX action. TTX is a highly potent and selective blocker of voltage dependent Na permeability, which at a concentration of 3×10^{-8} μM completely prevents AP conduction in crayfish neurons (Leake and Walker, 1980).

The effect of this Na channel blocker on DTX action was investigated by intrahaemocoelically injecting TTX (10 μg g^{-1}) into *Manduca sexta* larvae. When flaccid (> 1 hr), due to neuronal conduction block, the larvae were then injected with DTX (10 μg g^{-1}) which caused immediate tetany. This showed that TTX and DTX have different modes of action. Blocking Na Channels did not prevent DTX induced tetany or (presumably) depolarization of the muscle membrane.

Unfortunately, it was not possible to perfuse TTX saline over the denervated body wall preparation in order to investigate this

point directly, as TTX was not available in sufficient quantities.

(b) Na Free Saline. Pre-equilibration (3 hr) of the body wall preparation to Na -free saline failed to prevent either depolarization or contraction in response to DTX (Fig. 19B). The absence of Na did not affect either the resting potential or the muscle's ability to contract in response to indirect stimulation *via* the motor nerve (suction electrode).

(c) Ca -Free + EGTA Saline. 1 mM EGTA was used to selectively chelate Ca ions and thus reduce extracellular Ca activity to a very low level. Perfusion of Ca -free saline for 3 hours was required to stop muscle contractions evoked by field stimulation. This presumably indicates that the reduction of free $[Ca]_o$ at the muscle membrane takes a long time, suggesting that privileged microenvironments may exist near to the muscle membrane. Other authors have made similar observations. Piek (1975) has suggested that the t-system of insect skeletal muscle may constitute such a privileged microenvironment. Interestingly, in *Manduca* heart muscle, perfusion of Ca-free saline stops the heart beat within a few seconds (S.E. Reynolds, University of Bath, pers. comm.).

The Ca-free treatment did not prevent the normal pattern of depolarization in response to the subsequent application of DTX in the Ca -free/EGTA-saline (Fig. 19C), even after nerve evoked contractions had been completely abolished. Interestingly, no muscle contraction in response to DTX was seen. The absence of contraction reinforces the supposition that Ca entry to the muscle fibres is abolished under these conditions. The ability of DTX to depolarize

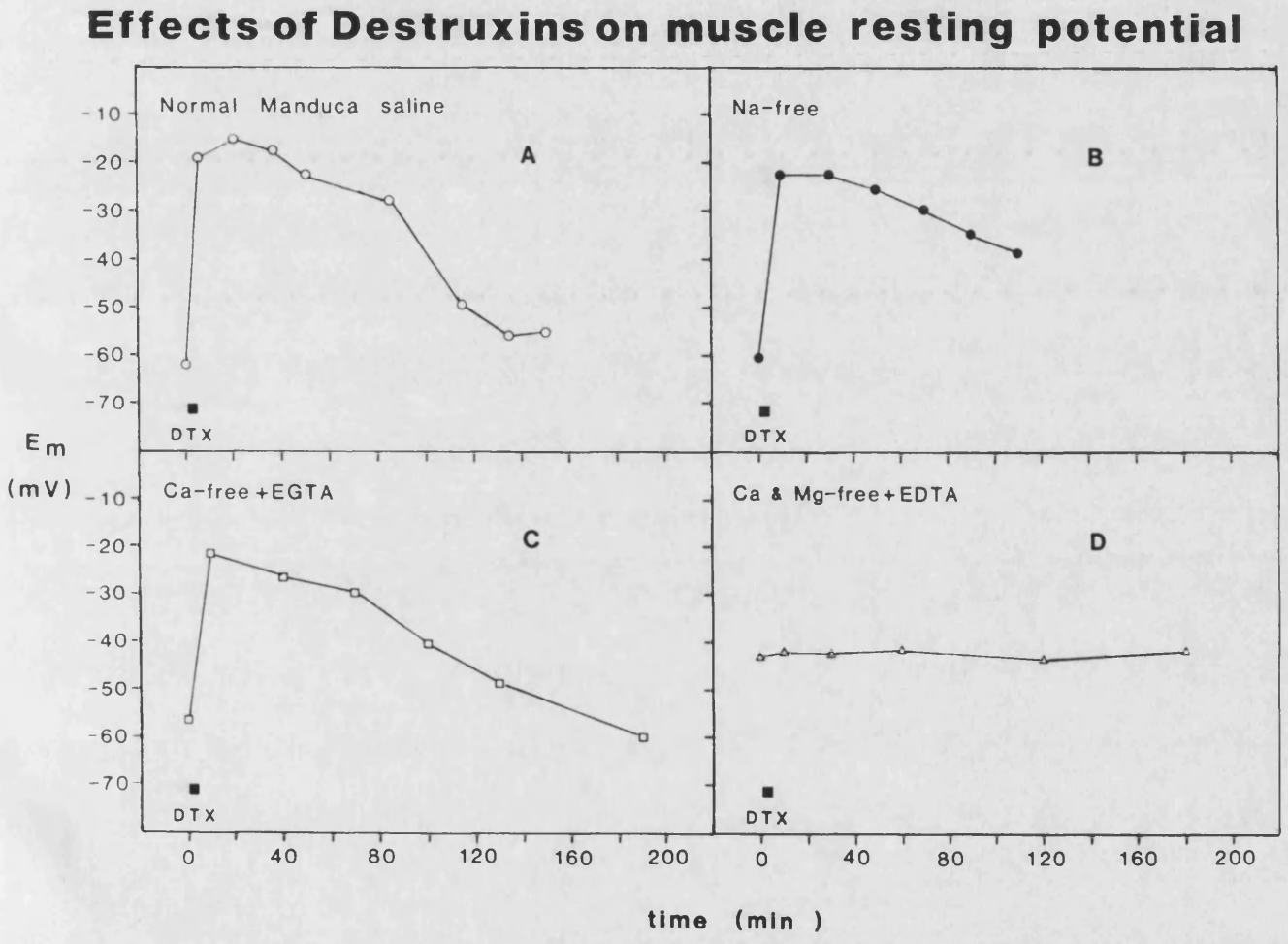


Fig. 19. The Effects of DTX on the Muscle Membrane Resting Potential in Normal and Experimental Salines
A: Normal Saline
B: Na-free Saline (Choline substituted for Na)
C: Ca-free + EGTA (Mg substituted for Ca, EGTA to chelate Ca)
D: Ca and Mg-free + EDTA (Choline and Na substituted for Ca and Mg, EDTA to chelate Ca and Mg)

note: Perfusion: 2 $ml.min^{-1}$
DTX: 40 $\mu g\ ml^{-1}$ for 5 min
All preps. pre-equilibrated in Normal Saline for 2 hr. then Experimental Salines for 3 hr.

the membrane therefore implies either that Ca Channels are not normally responsible for DTX-induced depolarization; or that these channels can accept other ions.

(d) Ca and Mg free and EDTA-Saline, It was not possible to remove Mg alone from the saline as Mg is evidently required for normal muscle function. Although reduction of Mg to 1 mM (normal = 18 mM) had no adverse effects on E_m or excitability of *Manduca* muscle, complete removal of Mg immediately caused muscle spasms. Therefore Mg and Ca were removed together after pre-equilibration in Ca -free saline to prevent muscle contraction.

1 mM EDTA was used to chelate both Ca and Mg , ensuring low extracellular activities of both ions. Removal of both Mg and Ca caused a slight fall in E_m to ca. -45 mV but completely prevented depolarization in response to DTX (Fig. 19D).

(e) The Effcts of Ca Channel Blockers on DTX action. Cadmium chloride is known to act as a Calcium Channel Blocker (Hagiwara and Byerly, 1981). The interaction between $CdCl_2$ and DTX was investigated in the denervated body-wall preparation. Perfusion with 0.25 mM $CdCl_2$ completely blocked evoked muscle contraction by direct or neuronal stimulation within 10 min. Further exposure for 6 min. was found to completely prevent DTX depolarization (Fig. 20). Perfusion with DTX after washing off the $CdCl_2$ resulted in immediate depolarization and tetanic contraction of the muscles.

E_m of DTX exposed muscles were somewhat restored by perfusion with $CdCl_2$ in the continued presence of DTX (Fig. 21).

(iv) The Effect of DTX on Muscle Membrane Resistance

The resistance of the muscle membrane was assessed as the input resistance (R_i) of the cell to an injected pulse of hyper-polarizing current. Because the cells were large it was necessary to pass a relatively large current (100 nA) from a second intracellular electrode. The membrane's contribution to the total resistance in the circuit was assessed by comparing the sizes of recorded voltage pulses when measured inside and outside the cell.

The normal membrane resistance was found to be 28.4 ± 0.52 kΩ (mean ± SE), but 10 min after exposure to DTX the resistance had fallen to 5.42 ± 0.85 kΩ. This result indicates that a substantial increase in membrane permeability occurred in the presence of DTX.

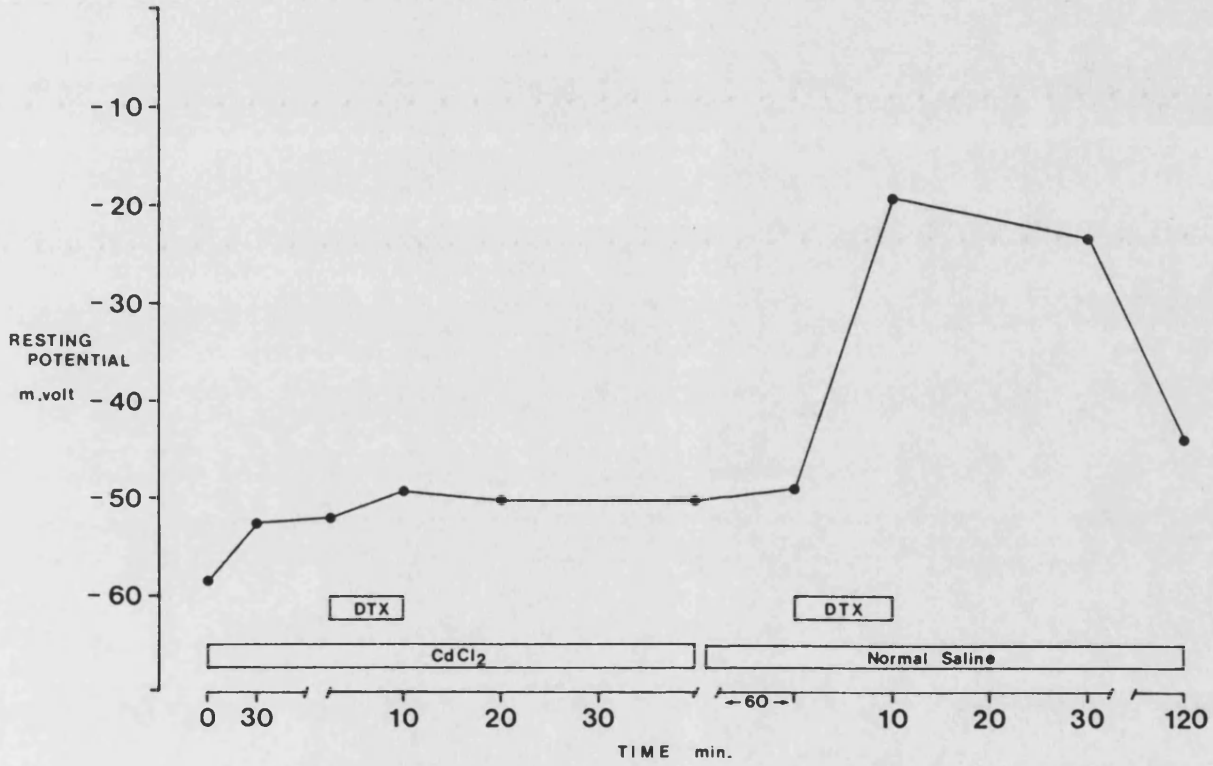


Fig. 20. The Effects of a Calcium Channel Blocker ($CdCl_2$) on Destruxin Action

note: Perfusion: 2 $ml.min^{-1}$

$CdCl_2$: 0.25 mM

DTX : 20 $\mu g\ g^{-1}$

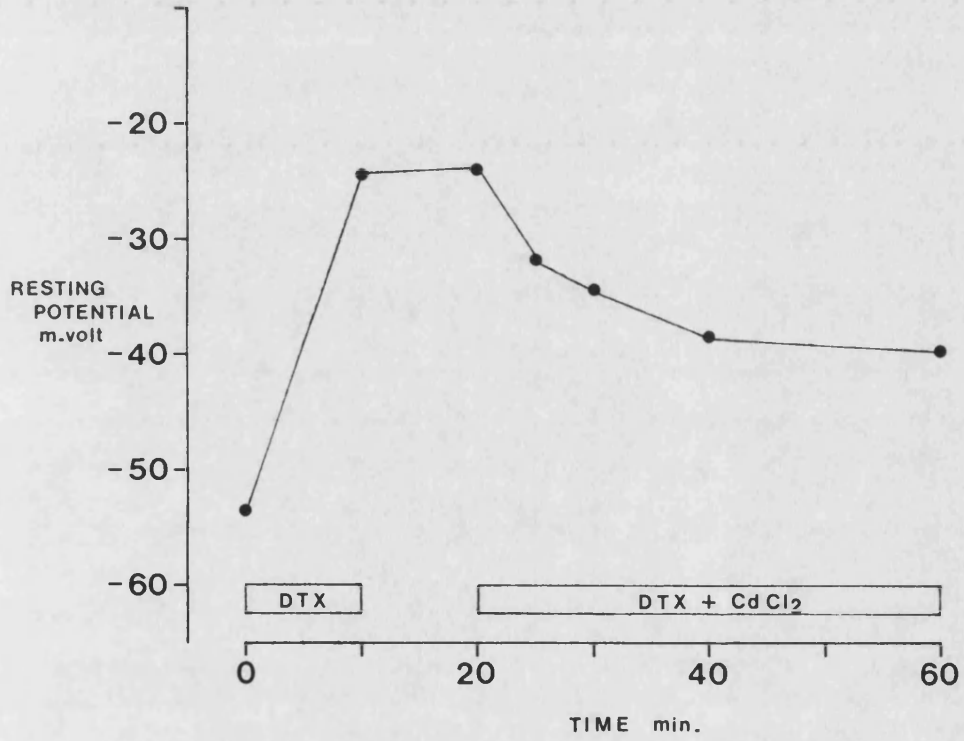


Fig. 21. The Recovery of the Muscle Membrane Resting Potential on Exposure to $CdCl_2$ in the Continued Presence of Destruxin

note: Perfusion: 2 $ml.min^{-1}$

$CdCl_2$: 0.25 mM

DTX : 20 $\mu g\ g^{-1}$

(v) The Effects of Metabolic Inhibition on Muscle Fibre E_m and DTX Action

A large percentage of the Lepidopteran muscle resting potential is probably generated by active processes. As DTX caused a decline in the membrane E_m, its effects were compared to those of metabolic inhibition.

When *Manduca sexta* larval body wall muscles were exposed to the metabolic inhibitor 2:4-Dinitro-phenol (which uncouples the electron transport chain from phosphorylation of ATP) there was a gradual decline in E_m (Fig. 22). The rate of decline was dependent on the concentration of the inhibitor. It was found that 0.5 mM DNP caused immediate and irreversible depolarization (with some muscle contraction), whereas the effects of 0.05 mM DNP were less dramatic and reversible if washed off within 30 min. Continued exposure reduced the E_m to a value of ca. -25 mV, after which no further depolarization occurred. The introduction of DTX at this stage caused an additional small but significant ($t = 12.12$ df 16 $P < 0.01$), decline in the E_m to ca. -15 mV, which was reversed on washing with DTX-free DNP saline. Interestingly, the membrane E_m then recovered to a higher value than that seen prior to DTX exposure.

4. The Effects of DTX on Muscle Action Potentials

Preliminary experiments showed that Destruxins had no effect on the conduction of nerve impulses,but acted specifically on the muscle membrane. Depolarization of the membrane affected the excitability of the muscle which was examined by recording the muscle action potential evoked by stimulation of the ventral nerve branches with a suction electrode. Unfortunately, the large twitch contraction,

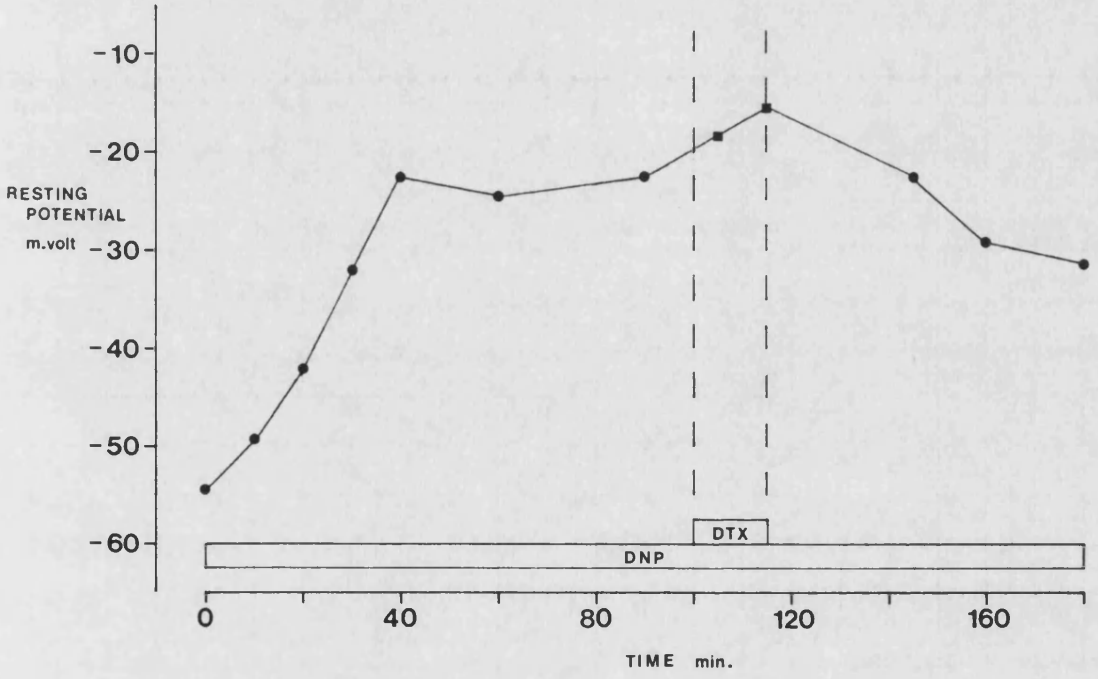


Fig. 22. The Effects of 2:4-Dinitrophenol on the Muscle Membrane Resting Potential and Destruxin Action

note: Perfusion: 2 ml.min^{-1}

DNP : 0.05 mM

DTX : 20 µg g^{-1} for 15 min.

evoked by neuronal stimulation, and the tetany caused by DTX, prevent the microelectrode from remaining within the cell. Therefore hypertonic sucrose salines were used to abolish contraction.

Hypertonic saline is thought to interfere with excitation-contraction coupling without affecting the excitability of the membrane (Hodgkin and Horowicz, 1957).

Fig. 23i shows the normal response of the muscle membrane to a single suprathreshold motor nerve stimulus. The depolarizing response includes a contribution from the excitatory junction potential and a regenerative membrane response. This fast AP of ca.+ 33 mV in amplitude and ca. 20 msec. in duration did not overshoot as E_m was ca. -60 mV. The APs of a variety of Lepidopteran muscles have been found to be ca. +40 mV (Huddart, 1966a).

The changes in magnitude of the AP during exposure to DTX are shown in Fig. 23 i & ii.The size of the AP declined very rapidly over the first 5 min.in conjunction with E_m. E_m stabilized at -25 mV after 4 min but the AP continued to decline progressively in size until it was almost impossible to measure at 15 min. On washing the AP recovered, but at a slower rate than the resting potential.

The AP changed in magnitude and conformation during exposure to DTX. The falling phase of the ATP immediately (1 min) became more rapid and after 4 min showed a very distinctive undershoot below the "resting" E_m. Oscillations of the baseline seen between 1 and 4 min after DTX addition were probably artifactual, caused by small contractions of the muscles not completely paralysed by the hypertonic saline.

Despite this noise, an additional slight depolarization following the undershoot could consistently be seen in the AP's recorded 4-5 min after DTX exposure.

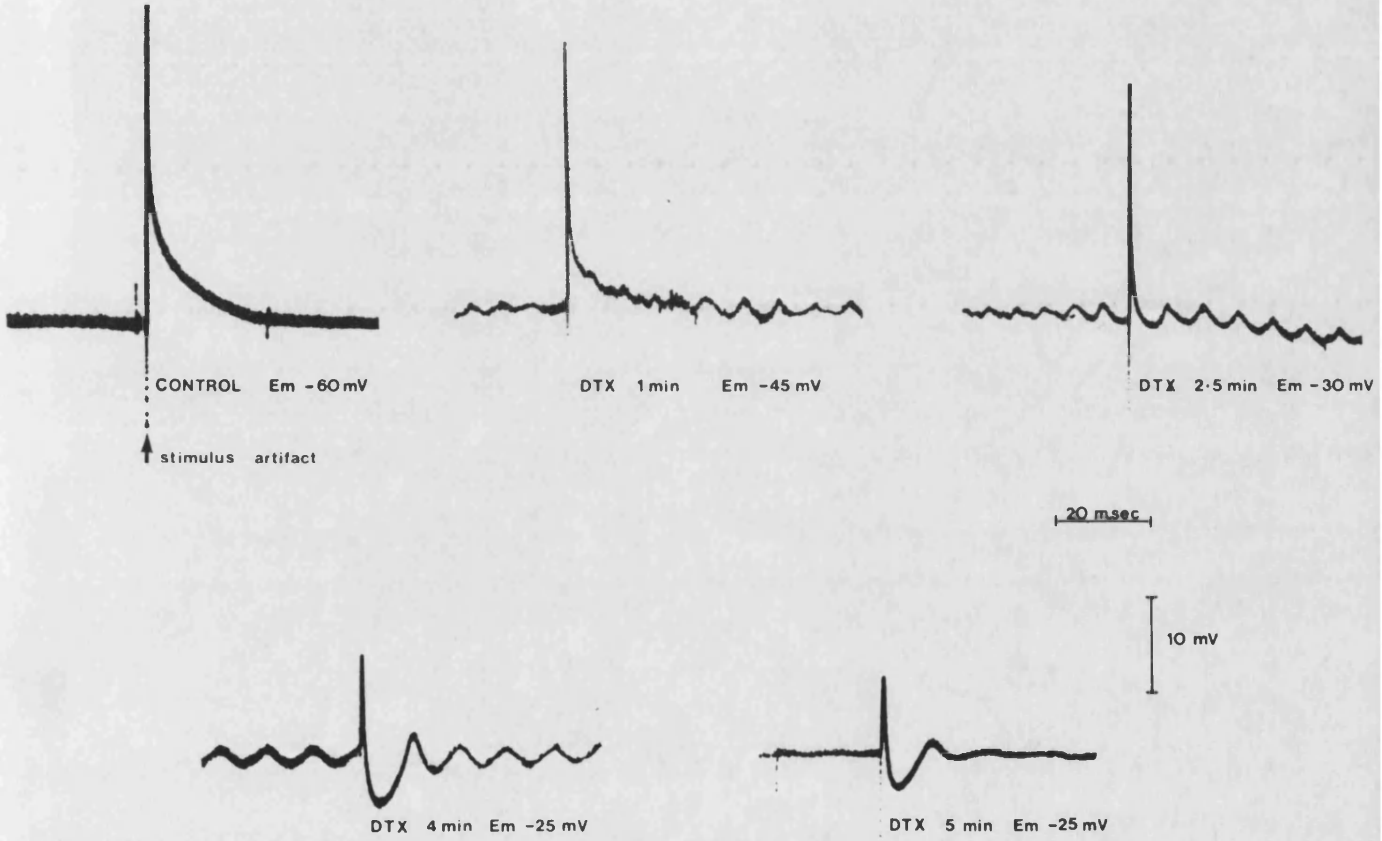


Fig. 23 i. The Effects of Destruxin on the Muscle Membrane Action Potential

note: Perfusion: 0.5 ml.min^{-1}

DTX : 2 µg g^{-1}

Stimulus : 0.01 m.sec.

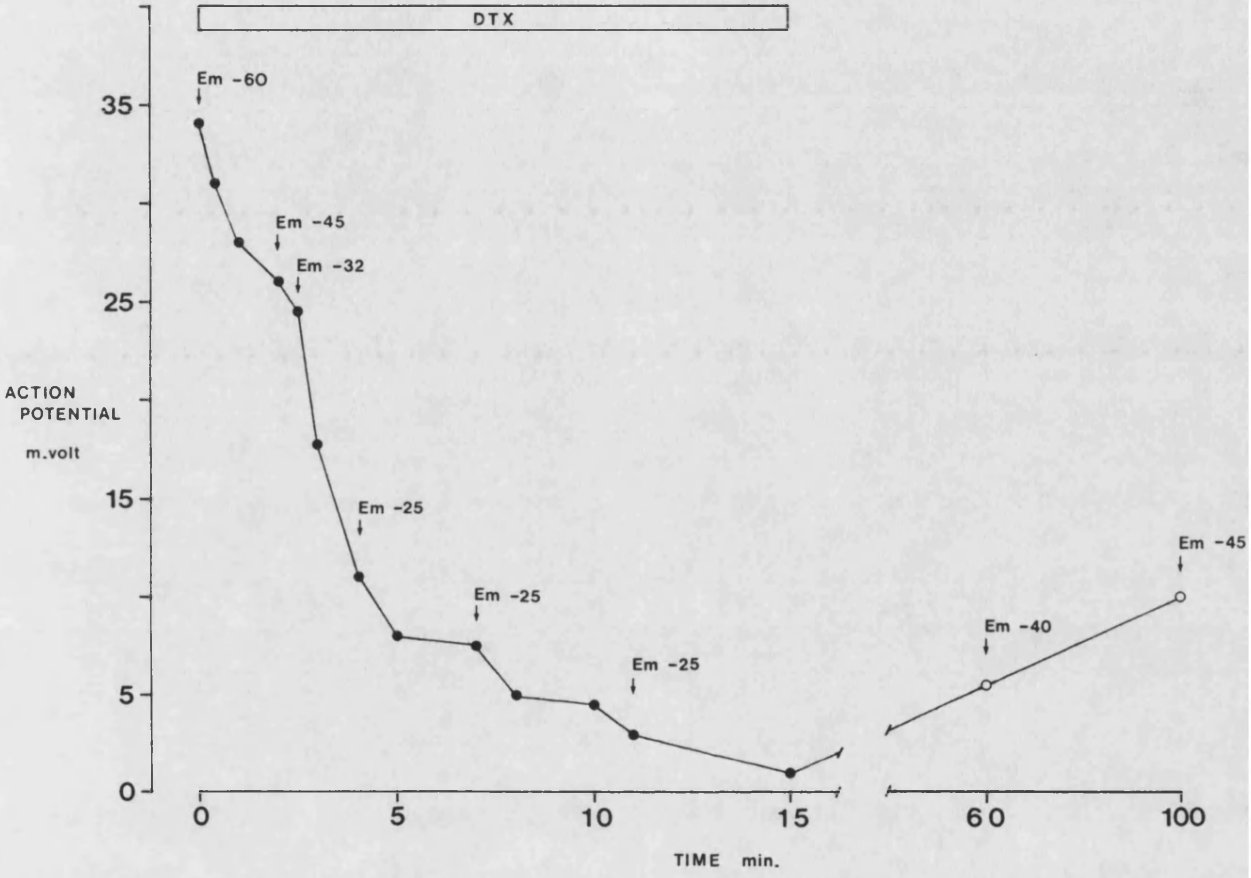


Fig. 23 ii. The Effects of Destruxin on the Muscle Membrane Action Potential

Resting Potential (E_m) recorded simultaneously

note: Perfusion: 0.5 ml.min^{-1}

DTX : 2 µg g^{-1}

Stimulus : 0.01 m.sec.

5. The Effects of Glutamate on the Muscle Action Potential and DTX Action

Perfusion of the denervated body wall muscle preparation with L-glutamate (1 mM) eventually desensitised the post-synaptic receptor sites on the muscle membrane. This concentration of L-glutamate abolishes the fast EPSP within 10 seconds in *Musca domestica* larvae (Irving and Miller, 1980), but in *Manduca* the synaptic potential was only abolished after 1½ hours perfusion (Fig. 24). The delayed effects of L-glutamate may be due to the inacessibility of the Lepidopteran neuromuscular junction (Piek, 1975).

Densensitization of the post-synaptic membrane did not affect the depolarization caused by subsequent application of DTX in the continued presence of L-glutamate.

6. The Effects of DTX on *Manduca* Larval Heart and its use as a Bioassay for HPLC Fractions

DTX increased the frequency and amplitude of heart beat (Fig.25i and 25iiA. Above a certain dose the heart became paralysed in systole. The *Manduca* heart preparation was extremely sensitive to DTX, showing accelerated beat when exposed to a pulse application of only 20 ng (∿ 35 pmol) in a volume of 50 µl. This sensitivity was useful in allowing detection of small quantities of DTX in HPLC fractions (Chapter 2).

The effects of DTX on the heart beat were compared to the effects of 5HT and Carioactive Peptides (CNS extracts) (Fig. 25ii B and C). Perfusion of 2-bromolysergic acid diethylamide (BOL) only slightly affects heart rate but completely abolishes the response to 5HT, i.e. blocks 5HT receptors (Platt and Reynolds, 1986).

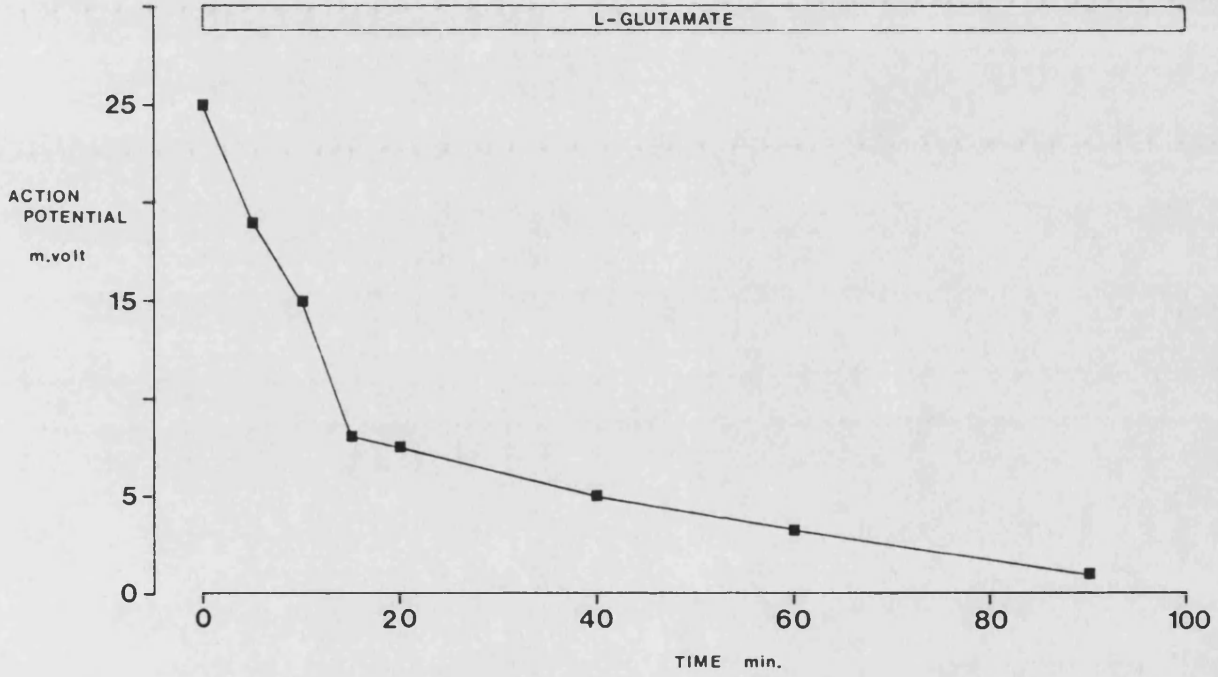


Fig. 24. The Effects of L-Glutamate on the amplitude of the Muscle Membrane Action Potential

note: L-Glutamate: 1 mM

Stimulus : 0.01 m.sec.

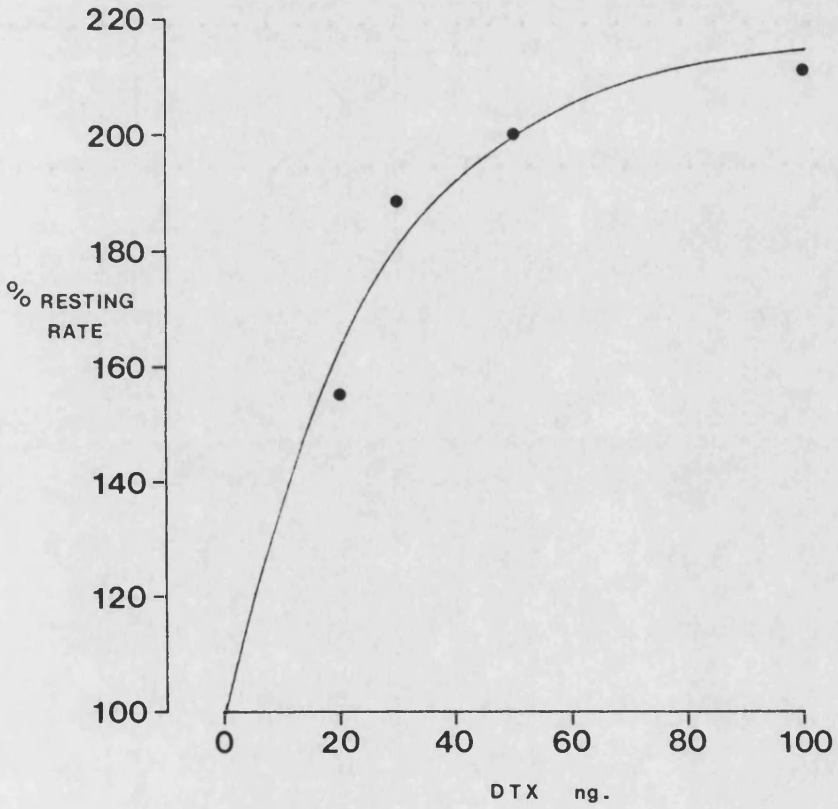


Fig. 25 i. Increase in the frequency of *Manduca* heart beat on exposure to Destruxin.

Note: DTX pulsed on in 50 µl of saline

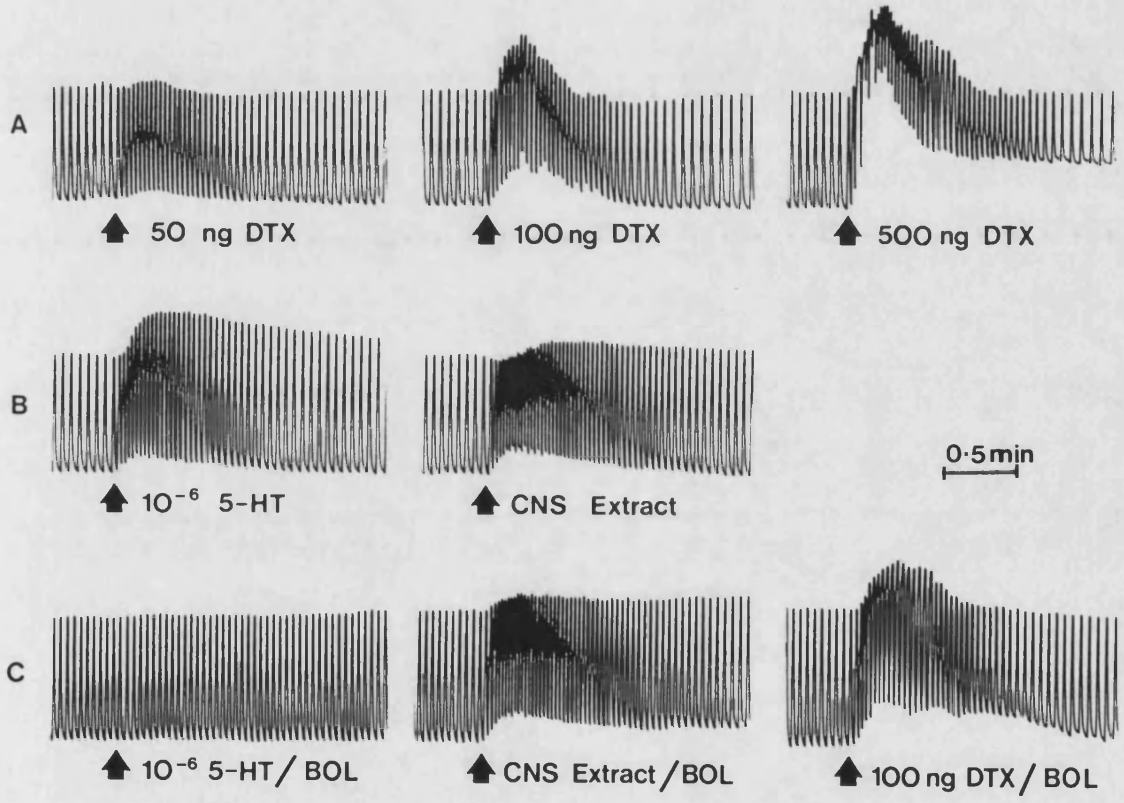


Fig. 25 ii. The Effects of Destruxin on *Manduca* larval heart beat.

A. the effects of increasing concentration of DTX

B. cardioaccelerator properties of 5HT and CNS extract

C. blocking of 5HT receptors with Bromolysergic Acid

BOL did not abolish the response to CNS extract or DTX. Therefore DTX must act at sites other than 5HT receptors. Although it seems unlikely that DTX acts at the same receptors as the cardioactive peptides (CAPs), this possibility cannot be ruled out at present.

7. Destruxins as Ionophores?

The effects of DTX on muscle membrane potentials and on the heart might be explained if they acted as neutral ionophores. Although Abalis (1981) had previously concluded that DTX lacked ionophoric properties he did not test their ability to transport Mg ions.

Several experiments with Pressmann Cells (see methods) confirmed Abalis' results, and failed to show any evidence of ionophoric activity. Transport of Na , K , Ca and Mg ion was not increased over control levels (i.e. when no DTX was present) even when the experiment was continued over 4 days. Under these conditions Valinomycin transported K ions as would be expected (Table 9).

It must be concluded, in concurrence with Abalis (1981), that Destruxins are not neutral ionophores.

Table 9. The Ionophoric Activity of Destruxins and Valinomycin in Pressman Cells over 4 Days. Na detected was probably due to use of Soda Glass Sample Tubes.

	Control	Valinomycin	Destruxin
Potassium	0	0.113 mM	0
Sodium	0.0153 mM	0.0231 mM	0.0169 mM
Calcium	0	0	0
Magnesium	0	0	0

The lower limits of detection for these ions were:

K : 0.281×10^{-3} mM

Na : 0.20×10^{-3} mM

Ca : 1.99×10^{-3} mM

Mg : 0.123×10^{-3} mM

Discussion

Injection of DTX causes immediate tetanic contraction of body wall muscles followed by flaccid paralysis. Tetanic contraction in response to DTX occurs whether or not the CNS is present, and thus must be independent of any central effects of DTX. The extent of muscle contraction is dose dependent, reversible and can be elicited repeatedly on exposure to DTX.

One hypothesis is that DTX may act presynaptically. A massive release of neurotransmitter from the presynaptic junction could result in a short term tetanic contraction of the muscles, which would then relax after desensitisation. For example, the venom of the black widow spider *Latrodectus mactans* causes a massive transmitter release from locust neuromuscular synapses, subsequently blocking evoked and spontaneous transmitter release without affecting the electrical excitability of the muscle (Usherwood and Cull-Candy, 1975). Such a mode of action is unlikely for DTX. Desensitisation of the post-synaptic receptors by continuous perfusion of *Manduca* muscle with the putative neurotransmitter L-glutamate did not prevent muscle contraction on exposure to DTX. Therefore DTX does not act by causing transmitter release nor does it act as an agonist of the excitatory transmitter. Tetany of *Galleria* larvae also appears to result from the direct action of DTX on the muscles without intervention of the nerves, since *Bracon hebetor* venom, a known presynaptic blocker (Walther and Rathmayer, 1974), fails to prevent tetanic paralysis (unpub. result cited in Roberts, 1980).

In this study DTX was found to act directly on the muscle fibres of *Manduca* larvae, causing a 32% decline in the membrane resting potential. This immediate depolarization was accompanied by tetanic

contraction of the muscles. These two events are intimately linked. The effects of DTX are fully reversible, at physiological doses, although extensive washing is required. Depolarization of the muscle membrane is probably the result of an increase in membrane permeability to one or more of the major cations in the saline i.e. Na^+, Ca^{++} and Mg^{++} (a selective increase in K^+ permeability would result in hyperpolarization).

Substitution of Na^+ in the saline by an impermeant cation (choline) fails to prevent depolarization. Additionally, TTX failed to prevent DTX action. These results were expected as voltage-sensitive Na channels are thought to play little or no role in Lepidopteran muscle physiology (Huddart, 1967).

Removal of Ca^{++} does not prevent depolarization but completely prevents contraction in response to DTX. Ca^{++}-free saline also blocks directly evoked contractions of *Manduca* body wall muscle. This has been stated as evidence for the presence of voltage dependent calcium channels (Hagiwara and Byerly, 1981). Removal of divalent cations from the saline i.e. Mg^{++} and Ca^{++}, completely prevent depolarization in response to DTX. Therefore it would appear that Destruxins cause an increase in permeability of the membrane to divalent cations. This is consistent with the observed decline in input resistance of body wall muscle in the presence of DTX.

It was found that Cd^{++} completely prevents DTX depolarization. Furthermore, muscles that were exposed to Cd^{++}, after DTX depolarization, repolarized to a considerable extent despite the continued presence of DTX. Cd^{++} thus appears to compete with DTX for a site on the muscle membrane and that site may well be a Calcium Channel. Cd^{++} is known to block voltage dependent calcium permeability (Hagiwara

and Byerly,1981). Cd^{2+} is the most potent of the divalent metal cations in blocking the Ca-dependent action potential of the *Manduca* heart, completely blocking the heart beat at 0.1 mM (S.M. Luckman and S.E.Reynolds, University of Bath, pers. comm.). Removal of Ca^{2+} alone does not prevent DTX-induced depolarization, therefore the proposed Calcium Channel may not exclude the passage of other cations. The selectivity of Cd^{2+} as a blocker of Calcium Channels is uncertain, Cd^{2+} may also inhibit the permeability of less selective ion channels. Although other authors (e.g. Rheuben, 1972; Ashcroft and Stanfield, 1982) have found that voltage-activated insect muscle Ca Channels did not accept Mg^{2+}, this does not mean to say that such Ca Channels do not exist in *Manduca* body wall muscle. It is known that more than one type of Calcium Channel exists (Hagiwara and Byerly, 1981).

An increase in calcium permeability during normal muscle excitation and possibly DTX induced depolarization results in the initiation of a contraction. Ca^{2+}-free salines prevent contraction, as an increase in intracellular Ca is vital to the process of excitation-contaction coupling (Aidley, 1975). At present it is not clear how much of the Ca^{2+} required for the contractile response enters from outside the cell and how much is released from internal stores. Ca induced Ca release has not been ruled out for invertebrate muscle contraction (Hagiwara and Byerly, 1981). Calcium release may be triggered by the influx of cations other than calcium. BTX, which causes a specific increase in membrane P_{Na}, induces a contraction in vertebrate muscle. The contraction is probably due to Ca^{2+} release from internal stores. Warnick *et al.* (1971) suggested that the release of Ca is triggered by the increased intracellular Na concentration. A more likely suggestion is that Ca release is indirectly triggered by the depolarization

signal through a second messenger. The inositol triphosphate second messenger system has recently been implicated in Ca release during muscle contraction.

The effects of DTX on the excitability of the muscle membrane are probably directly related to the effects of the toxin on "resting" E_m. The magnitude of Lepidopteran muscle AP has been found to be directly proportional to the size of the "resting" E_m (Huddart, 1971). It is therefore not surprising to find that the magnitude of the AP declines with that of the E_m during exposure to DTX, although the size of the AP continues to fall after E_m has stabilized. This may be due to progressive inactivation of Ca Channels, or to a gradual increase in $[Ca]_i$, which might alter E_{Ca}.

The normal configuration of the AP is also affected during membrane depolarization. The rising phase of the muscle AP is caused by a regenerative increase in gCa. The falling phase results from inactivation of gCa and an increase in gK (Ashcroft and Stanfield, 1982). The late activation of gK may be recognised in DTX-poisoned muscles by the 'undershoot' that follows the AP. This is pronounced in the poisoned fibres because the "resting" membrane potential is less negative than usual (i.e. further from E_K) in the presence of DTX.

The mode of action of DNP, a metabolic inhibitor, and Destruxin, were compared. A large proportion of E_m of Lepidopteran muscle is thought to be generated by active processes as metabolic inhibition causes depolarization of the membrane (Huddart and Wood, 1966). This depolarization, although much slower than that elicted by DTX, is of a similar magnitude. DNP was also found to cause a contraction of *Manduca* muscle at high concentrations (0.5 mM), with immediate and irreversible depolarization, DNP has been found to act initially

as an excitant of the contractile activity of moth heart (McCann, 1967). DTX at extemely low doses also acts as a cardioaccelerator of *Manduca* larval heart. This evidence indicates certain similarities between the mode of action of DNP and DTX. DTX appears to exhibit greater selectivity than DNP, since it is highly active, but its effects can easily be reversed.

The active maintenance of Lepidopteran muscle E_m is not fully understood. It would appear that at least ca. -20 mV of the E_m is independent of the active processes ($E_m = E_k$ during inhibition), which generate a further ca. -30 to -40 mV by

(a) affecting the distribution of a permable ion,

(b) metabolically maintaining the membrane permeability characteristics,

or (c) by operating an electrogenic ion pump.

It may be significant that DNP causes a decline in membrane resistance, since it increases the membrane permeability of *Antheraea polyphemus* muscle (Rheuben, 1972). Ionic permeability depends on the structural and physiochemical properties of the membrane, which may be directly maintained by oxidative metabolism (Huddart and Wood, 1966). DNP is known to uncouple oxidative phosphorylation directly or indirectly and it may be that it is the loss of high energy phosphates which results in increased membrane permeability causing depolarization. However, DNP may have other effects. R.W. Meech (University of Bristol, pers. comm.) points out that DNP also causes rapid intracellular acidification.

The modes of action of most low molecular weight toxins produced by entomopathogenic fungi are unknown. Studies of their mode of action have rarely been carried out on insect systems. Bassianolide, a

cyclodepsidipeptide is superficially similar in structure to DTX, fails to effect muscle contraction or relaxation of guinea pig ileum but inhibits agonists of this muscle at selective sites (Nakajyo *et al.*, 1982). Bassianolide is thought to act as an ionophore and it is found to be highly toxic to Lepidopteran larvae. Its effects are not immediate, but larvae eventually succumb ($\sim$ 2 hours) to an LD_{100} dose (5 $\mu g\ g^{-1}$) (Kanaoka *et al.*, 1978; Champlin and Grula, 1979).

Beauvericin, another cyclic peptide, isolated from *Beauveria bassiana* cultures is also thought to be an ionophore but shows little toxicity against Lepidopteran larvae (injection of >100 $\mu g\ g^{-1}$ = no effect) (Champlin and Grula, 1979), although it is toxic to the brine shrimp *Artemia salina*(Hamill *et al.*, 1969). The differences in toxicity between the two ionophores, Bassianolide and Beauvericin,may be due to differences in their selectivity to cations (Roberts, 1980).

The selectivity of Bassianolide determined in Pressman Cells was $K^+ > Na^+ > Ca^{2+}$ (Abalis, 1981). Effects on K^+ permeability of the muscle membrane would not cause immediate paralysis, which may explain why Bassianolide acts differently to DTX. It has been suggested that Destruxin may affect membrane permeability by acting as an ionophore, promoting cation transport across the cell membrane (Rober ts, 1980). DTX at low doses ($\sim$ 5 $\mu g\ g^{-1}$) causes immediate paralysis of Lepidopteran larvae(unlike the slow acting ionophore Bassianolide) and no ionophoric activity for Na , K , Ca or Mg ions could be detected using the artificial environment of a Pressman Cell. Abalis (1981) when comparing the ionophoric activity of DTX and Bassianolide, found Bassianolide to be a good neutral ionophore, whereas DTX was not. It therefore seems unlikely that DTX acts as an ionophore, although further studies on living systems should be carried out.

A number of microbial toxins are known to act on the respiratory chain, e.g. the Piericidins (Tamura and Takahashi, 1971). It has been suggested that DTX might act by uncoupling electron transport (Roberts, 1980; Abalis, 1981) and as seen here DNP mimics certain aspects of DTX poisoning. DNP is thought to act by uncoupling oxidative phosphorylation but it is also known to have other less specific effects, e.g. intracellular acidification which may induce membrane depolarization. The comparison of DTX action to the action of more specific mitochondrial blockers, i.e. Oligomycin, should be investigated. However, A.K. Charnley (unpubl. result, Bath University) found no DTX induced uncoupling of oxidative phosphorylation using blow fly flight muscle mitochondrial preparations. The failure of DTX to promote ATP'ase activity (S. Watkins and A.K. Charnley, unpubl. result, University of Bath) also tends to discount an uncoupler-like action (see Lehninger, A.L., 1975, Chapter 19). This does not discount the possibility that DTX may act to inhibit metabolism in some other way, resulting in membrane depolarization.

Thus, DTX directly or indirectly affects Ca permeability of the muscle membrane (see Fig. 26), although more detailed studies of the toxins effects on membrane conductance are required to define more closely its site of action. Provided that the problem of muscle movement can be overcome, voltage or patch clamping would be powerful techniques with which to approach this problem.

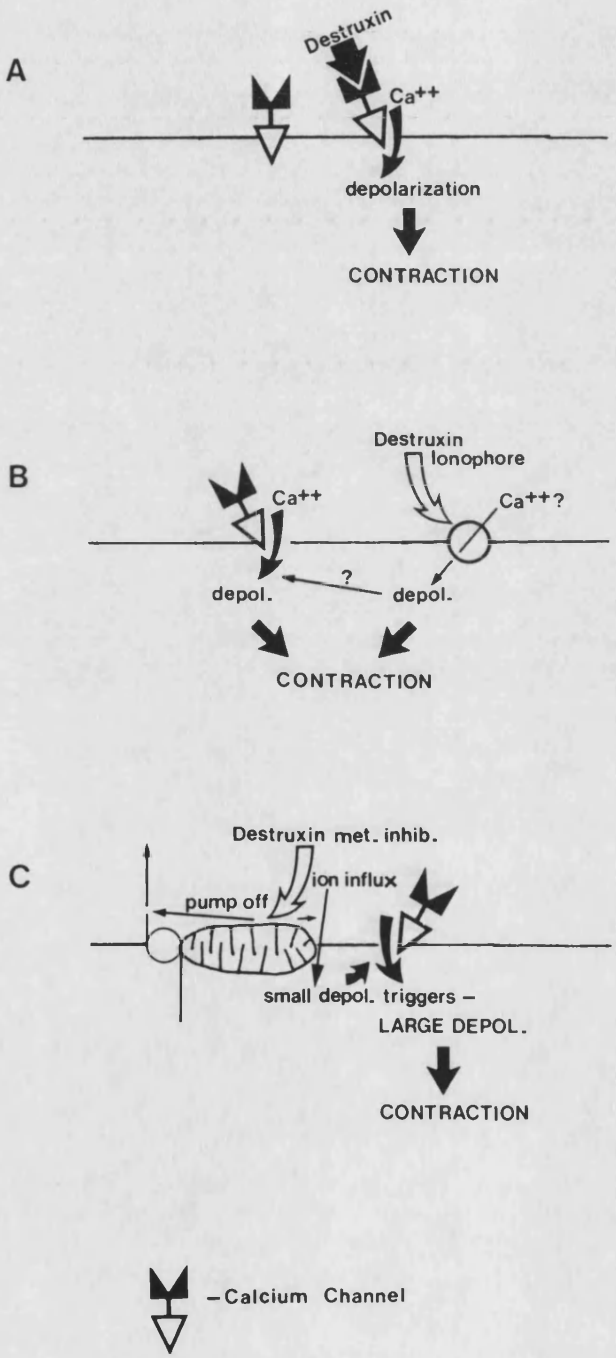


Fig. 26. Three Possible Modes of Action of Destruxins
A. by acting directly to open endogenous Ca Channels
B. by acting as an ionophore, increasing membrane permeability
C. by acting as a metabolic inhibitor, triggering depolarization indirectly

CHAPTER 5: OVERVIEW AND GENERAL DISCUSSION

The increasing incidence of resistance to chemical insecticides in economically important pests and vectors has led to increased interest in the potential of microbial pesticides. Although insect pathogenic fungi were among the first successful agents of biological control, they have yet to realise their full potential. There is an urgent need for a rational approach to strain selection and improvement, which has been hampered by ignorance of the factors that determine fungal pathogenicity.

It has been found here that production of Destruxins by a restricted range of *M. anisopliae* isolates, correlates well with virulence against *Manduca sexta* larvae. Although it has been established that there is a relationship between DTX production *in vitro* and pathogenicity and that detectable quantities of DTX are only produced *in vivo* by the highly virulent isolate ME1, it does not follow simply that DTX synthesis *in vivo* is a key determinant of pathogenicity. Comparisons between the modes of pathogenicity of natural isolates is a useful approach but it should be noted that lower isolate virulence may derive from deficiencies in several essential characteristics which may or may not include DTX production. Conversely, poor DTX production might be compensated, in naturally selected isolates, by genetic changes that affect other determinants of pathogenicity, e.g. speed of germination and cuticle degradation on penetration. A more powerful approach would be to use chemical mutagens to generate single lesion mutants of *M. anisopliae* which are hyper or hypo-productive for DTX. A comparison of the pathogenicity of such mutants against a constant genetic background should resolve this question.

Destruxin production does not appear to be essential to pathogenesis as the low producer RS549 does kill *Manduca* larvae, albeit more slowly than the high producer, ME1. Destruxin production during mycosis may cause host death, although death is more likely to be due to a combination of factors. The presence of a toxin does appear to promote early death, thus reducing the time to sporulation and possibly increasing the incidence of epizootics.

Should DTX production be found to be a key determinant of pathogenicity, then it may be possible to implement a programme of rational strain improvement using DTX production *in vitro* as a screening tool.

Fungal toxins may also be considered in their own right as prototypes for the design of chemical pest control agents At present there are no fungal metabolites under commercial development as insecticides. This approach to pest control is gaining momentum due to the staggering costs of developing new synthetic insecticides. These costs continue to escalate as the number of compounds required to be screened to yield one commercial product increases. Whereas in the decade 1950-1960, one product resulted from 2,000 bioassays, a recent estimate suggests that in the 1980's approximately 15,000 compounds must be screened for the same result (Menn, 1983).

Although the Destruxins are limited in this respect by their toxicological and/or penetration properties, they might nevertheless be capable of improvement by chemical modification. Differences in toxicity of the Destruxin to Lepidoptera are thought to be related to the insects ability to detoxify DTX but the site and mechanism of DTX detoxification remains unknown. Future studies could utilize 3H

labelled DTX as a means of localizing sequested DTX and its breakdown products.

The relationship between toxicity and mode of action indicates a specific site peculiar to Lepidopteran larvae. The proposed mode of action of DTX is the opening of Ca Channels on the muscle membrane. Whether DTX acts directly or indirectly on Calcium Channels remains uncertain, further investigation of Ca Conductances are required.

APPENDIX 1

Sabouraud's Dextrose Agar

Dextrose	40 g
Mycological Peptone	10 g
Agar (No. 3)	20 g
D. H_2O	1 litre

Mix and boil until dissolved, adjust pH to 6 - 6.5.

Sterilize 15 p.s.i. 10 min. Do not overheat

Optional 0.5% yeast extract.

Antibiotics for 1st Culture from Cadaver

0.5 g/litre Cyclohexamide (Sigma)

0.4 g/ litre Chloramphenicol (Sigma)

Add to autoclaved media when temp < 70°C

RS324 Plate Media

yeast extract	7 g
glutamic acid	2 g
soluble starch	14 g
K_2HPO_4	1 g
$MgSO_4$	0.5 g
$FeSO_4$	0.005 g
Agar (No. 3)	20 g
D. H_2O	1 litre

Czapek Dox Liquid Medium (Modified)

Dissolve 33.4 grams of Czapek Dox + 5 grams of Bacteriological Peptone in 1 litre of Distilled Water. Mix well and adjust pH to 6 - 6.5. Distribute into final containers and sterilize by autoclaving for 20 min at 10 lb/sq inch.

APPENDIX 2

Ingredients of artificial diet

Pre-mix components	weight in g
Wheat germ	750
Casein	350
Sucrose	300
Dry yeast	150
Wesson's salt mixture	100
Sorbic acid	15
Cholesterol	10
Methyl-p-hydroxybenzoate	10
Choline chloride	10

This mixture was kept in an airtight plastic container at room temperature.

A batch of diet was made up using 504 g of pre mix plus:

Other ingredients	Wt. in g or vol. in ml
Ascorbic acid	12
Aureomycin	0.30
Vanderzant's Vitamins mixture	0.30
10% Formaldehyde	12
Raw linseed oil	6
Vegetable oil	6
Agar	45

Diet Preparation

504 g of pre-mix was put into a Waring Blender, and 1250 ml of boiling distilled water was added, mixing for 5 min at low speed. The agar was heated separately in 1250 ml of distilled water

and transferred to the blender when boiling.

When the temperature of the mix had cooled to 70°C, the oils, 10% for formaldehye, ascobic acid, aureomycin and Vanderzant's vitamins mixture were added. Once thoroughly mixed the diet was poured into containers lined with aluminium foil and allowed to cool and solidify at room temperature in a flow cabinet, before being stored at 4°C.

APPENDIX 3

Ephrussi and Beadle's (E & B) Saline

10 x stock solution	/litre
NaCl	7.5 g
KCl	3.5 g
$CaCl_2$	2.1 g
Dist. H_2O	make up to desired vol.

Stock solution keeps well at room temperature - dilute stock as needed.

Composition of Final Saline

Na^+	128 mM
K^+	5 mM
Ca^{2+}	2 mM
Cl^-	135 mM

Manduca Saline (modified from Cherbas based on Weevers)

1. Making up Daily

   100 ml 10 x salt solution

   10 ml 100 x buffer solution (or 10 mM MOPS)

   66 g sucrose

   1 litre of distilled water. Adjust to pH 6.5 using KOH.

2. Recipes for Stock Solutions

   10 x salt solution

   1 litre

NaCl	2.34 g	$MgCl_2.6H_2O$	36.58 g
KCl	29.84 g	$CaCl_2$	3.33 g
		(or $CaCl_2 2H_2O$	4.41 g)

100x buffer solution

1st make up: 500 ml

150 mM Na_2HPO_4 : 11.70 g

150 mM NaH_2PO_4 : 10.65 g

2nd: mix solutions while stirring on pH meter to pH 6.9.

Composition

Na^+	5.5 mM	K^+	40 mM
Mg^{2+}	18 mM	Ca^{2+}	3 mM
Cl^-	65 mM	Sucrose	193 mM
phosphate	1.5 mM		

APPENDIX 4

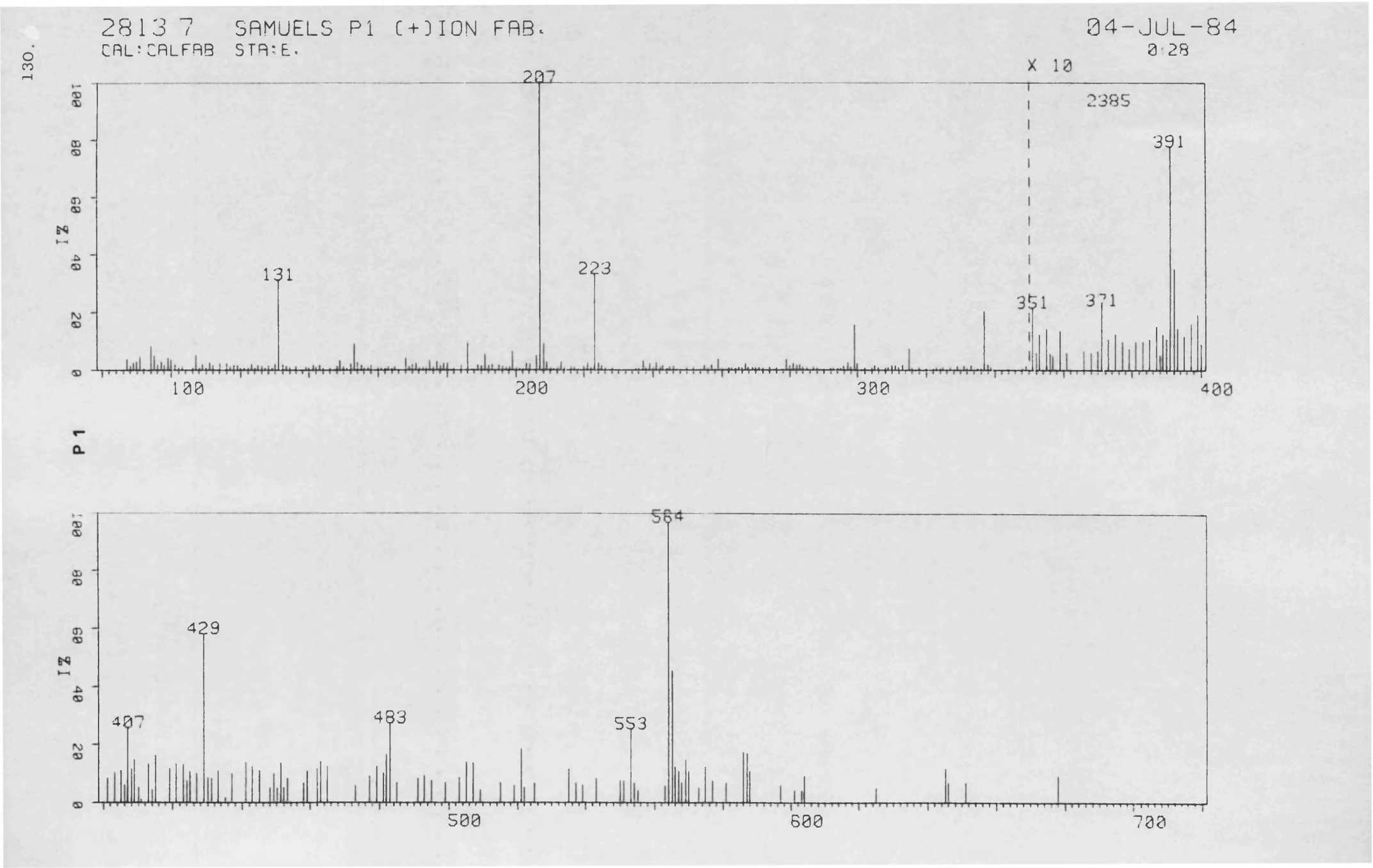
Maintenance of Insect Cultures

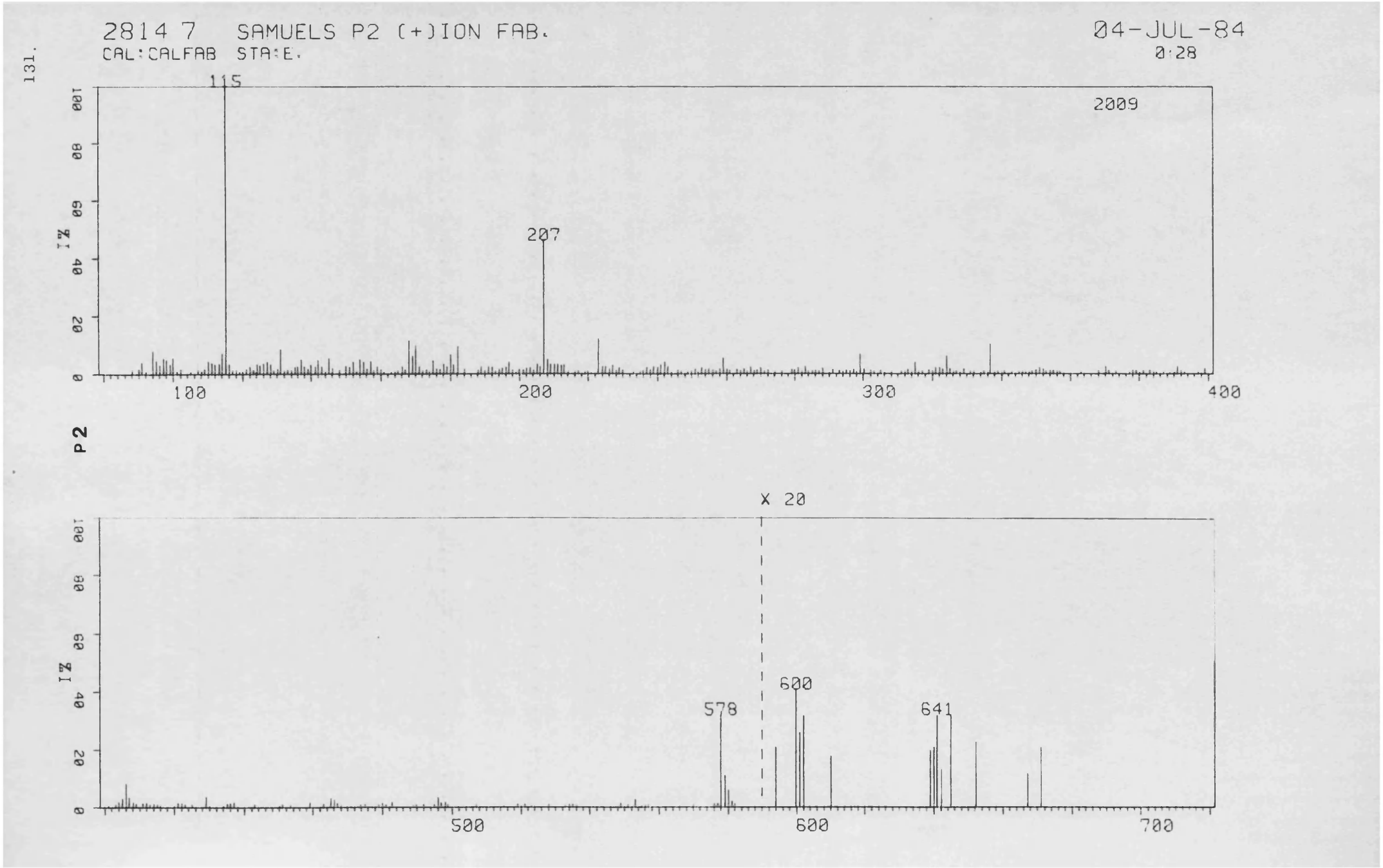
1. *Pieris brassicae* larvae were collected in the field and raised on cabbage leaves at ∿20°C.
2. *Bombyx mori* larvae were obtained as 4th instar larvae from a commercial silk farm and were raised on mulberry leaves at 25°C.
3. *Caliphora vomitoria* larvae were obtained from 'Fish and Shoot', Bath and raised on bran at 15-20°C.

   Adults were hatched from pupae and raised on sugar and water at 20°C.
4. *Tenebrio molitor* larvae were from a lab culture and raised on bran potatoes and carrot at 20°C.
5. *Periplaneta americana* adults were supplied by Bioserve and raised on bran at 25°C.
6. *Schistocerca gregaria* adults were supplied by Bioserve and raised on bran and grass at 30°C.
7. *Carausius morosus* adults were from a lab culture and raised on privet leaves at 20°C.

APPENDIX 5

(+) Ion FAB MS P_1 P_2 P_3 P_4


28137 SAMUELS P1 (+)ION FAB.
04-JUL-84
CAL:CALFAB STA:E.
0:28
130.
X 10
207
2385
391
131
223
351
371
%I
100
200
300
400
P1
564
429
407
483
553
%I
500
600
700


2814 7 SAMUELS P2 (+)ION FAB.
CAL:CALFAB STA:E.
04-JUL-84
0:28
131.
115
207
2009
I%
0
02
04
06
08
100
100
200
300
400
P2
X 20
578
600
641
500
600
700

2815 11 SAMUELS P3 (+)ION FAB. 04-JUL-84
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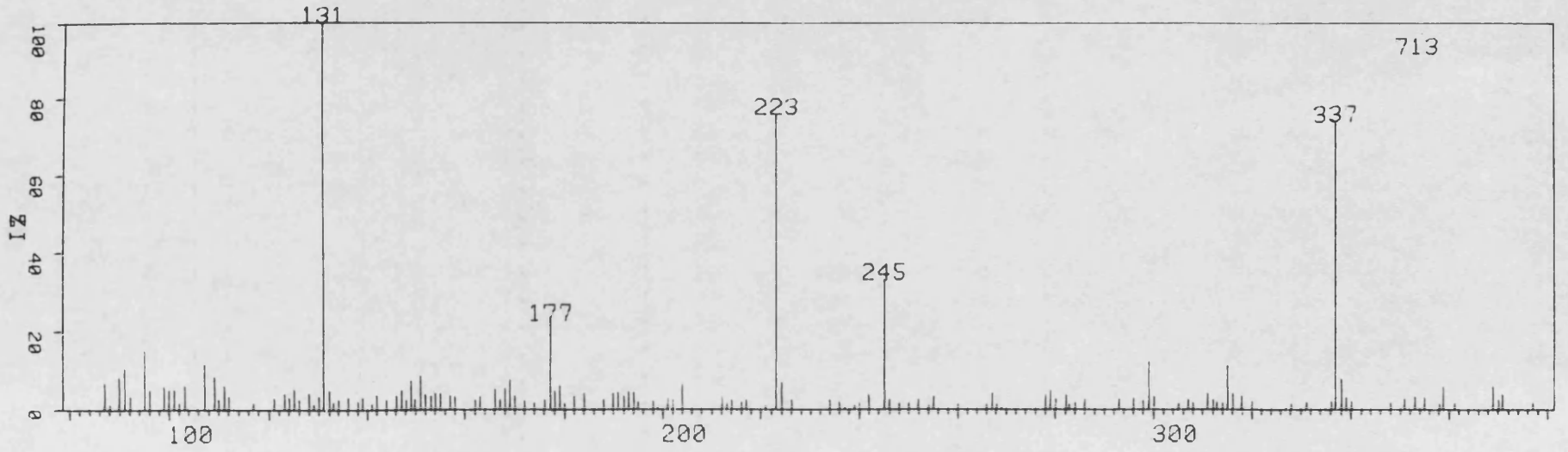
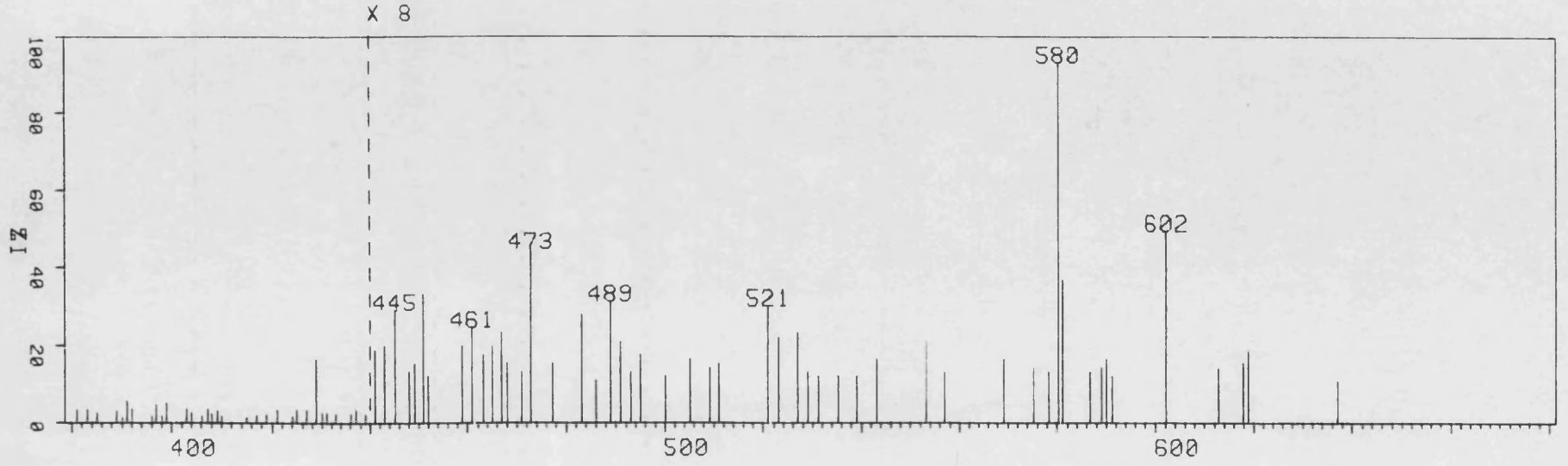


p3



2816 4 SAMUELS P4 (+)ION FAB. 04-JUL-84
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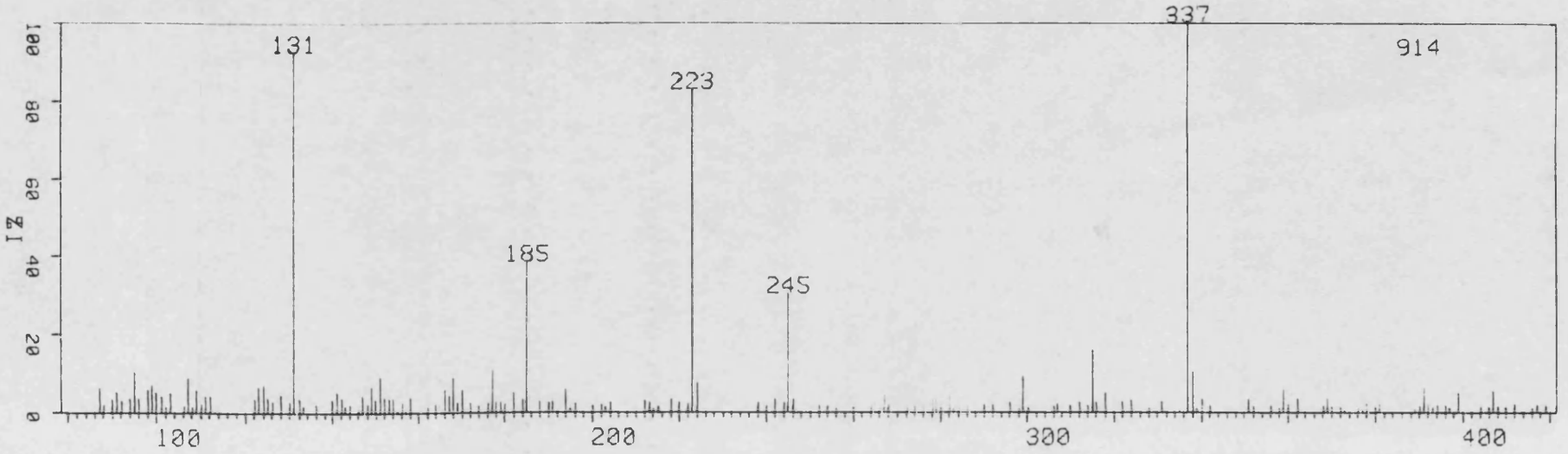
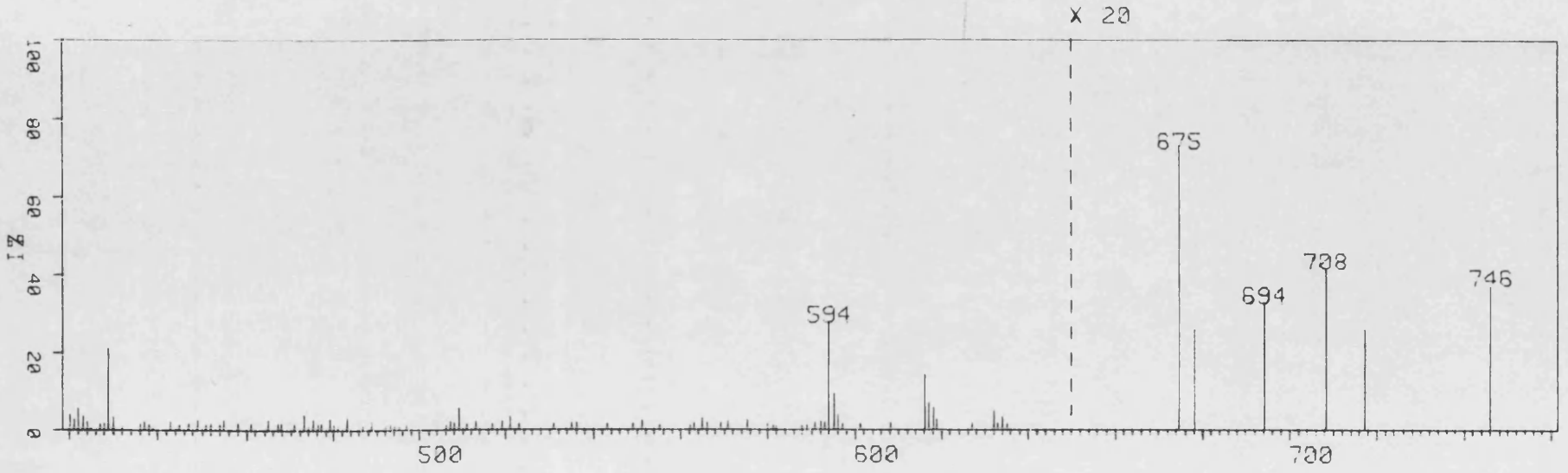


p 4



-APPENDIX 6 -

Crude toxin yields under different culture conditions

	Wt. of toxin mg L^{-1}	Fungus dry wt. g
1) Non adjusted pH	28.59	16.38
2) pH adjusted to 7.0 at start of culture	64.47	15.61
3) pH 7.0 at start and adjusted to pH 5.0 at end of culture	87.41	15.55
4) *Whole culture extracted	115.10	12.64
5) ▲Liquidized culture extracted	173.79	11.44
6) Stationary culture	22.75	7.39
7) ■Bactopeptone used in media	248.58	10.84

Culture conditions 14 days at 27^{oC} and 150 rpm (except stationary culture)

* Mycelia and culture filtrate extracted with CCl_4

▲ Mycelia and culture filtrate liquidized for 1 min in Waring Blendor

■ All other media consited of Czapek Dox + 0.5% Mycopeptone

TLC estimation of DTX in crude extract

	No. of impurities	Estimate of DTX level
1) Non adjusted pH	1	Moderate
2) pH 7.0 start	1	High
3) pH 7.0 start, pH 5.0 end	1	Moderate
4) Whole culture	3	High
5) Liquidized culture	3	High
6) Stationary culture	1	V. low
7) Bactopeptone	2	V. high

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