Studies of the Action of Venom and Venom Constituents on *Escherichia Coli*.

BY

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CONTENTS

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Special thanks go to my sister, Mrs. Therese K. Wilkinson for the drawing and to Mrs. Betty Wright for the typing.

Dedication

To the memory of my father.

Synopsis

Studies of the action of venom and venom constituents on *Escherichia coli*.

The antibacterial activity of honeybee venom (*Apis mellifera*), three snake venoms (*Naja naja sputatrix, Vipera russellii* and *Crotalus adamanteus*) and the polypeptide melittin (a component of honeybee venom) was investigated against the gram-negative organism *Escherichia coli*.

Minimum inhibitory concentration (MIC) values were determined and action against proliferating and non-proliferating cells was in the order: *Apis mellifera* venom > melittin > *Naja naja sputatrix* > *Vipera russellii* venom > *Crotalus adamanteus* venom.

Cell lysis was determined by absorbancy changes and was caused by the more active venoms (*Apis mellifera* and *Naja naja sputatrix*) and melittin.

Alteration of the permeability of the cell envelope of *Escherichia coli* cells harvested in mid-log phase was followed principally by measuring β-galactosidase release from cells. Venom activity decreased in the same order as MIC above.

Phospholipases A_2 from *Apis mellifera* and *Naja naja sputatrix* venoms, melittin and polymixin B (polypeptide antibiotic) increased β-galactosidase release. No synergism between the phospholipases A_2 and melittin was seen under the conditions employed.

Separation of *Apis mellifera* and *Naja naja sputatrix* venoms was carried out by gel filtration and electrophoresis. The action of venom components implies that the antibacterial activity of whole

venom is not totally accounted for by that of the venom polypeptide toxins melittin or direct lytic factor (DLF).

That the antibacterial activity of Apis mellifera and Naja naja sputatrix venoms and melittin is due at least in part to membrane disruption is supported by electron microscopy studies.

ABBREVIATIONS

DLF	Direct lytic factor
EDTA	Ethylene diamine tetraacetic acid, disodium salt
EM	Electron microscopy
ITGP	Isopropyl β-D-thiogalactopyranoside
ML agar	Minimal lactose agar
MLM	Minimal lactose medium
MBC	Minimum bactericidal concentration
MIC	Minimum inhibitory concentration
OD	Optical density
ONP	ortho-Nitrophenol
ONPG	ortho-Nitrophenylgalactopyranoside
PMSH	Phosphate-mercaptoethanol
PL A_2	Phospholipase A_2
RBC	Red blood cell
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy

Apis mellifera	*A. mellifera*
Crotalus adamanteus	*C. adamanteus*
Escherichia coli	*E. coli*
Naja naja sputatrix	*N. naja sputatrix*
Staphylococcus aureus	*S. aureus*
Vipera russellii	*V. russellii*

TABLES

FIGURES

Section 1

INTRODUCTION

Many venoms from stinging insects and reptiles possess membrane active properties (Bloom and Haegermark 1967). The exact nature of the membrane active effects vary between animal orders such as bees and snakes and also between species, e.g. cobra and rattlesnake (Bhargava et al 1970).

These venoms are used as a defense against dangers and have been the subject of much research by man (O'Connor and Peck 1978; Lee 1979), whose reaction when stung or bitten ranges from slightly painful to fatal (Barnard 1967).

Venoms are complex mixtures containing for example many enzymes and peptides making accurate analysis of the action of venoms and their constituents difficult (Habermann 1972).

HONEY BEE (*Apis mellifera*) VENOM

The honey bee is typical of one of the main categories of the social Apidae, the Apini. The other two main categories are the Bombini (bumble bees) and the Meliponini ("stingless" bees). The venom of the stinging honey bee has been one of the most studied subjects in insect biochemistry. The first chemical investigation reported the main component to be formic acid (Langer 1897). This claim was repudiated by later more careful studies e.g. O'Connor et al. (1965), which showed the venom to be a mixture of enzymes, peptides and smaller molecules, many of which exhibit physiological activity (Peck and O'Connor 1974).

The quantity and composition of honey bee venom varies with the age of the bee and the season of the year. To overcome this, venom composition is often described as that of a "typical adult worker bee" rather than "pure honey bee venom" (O'Connor and Peck 1978). The general character of *Apis mellifera (A. mellifera)* venom is summarised in Table 1.

SNAKE VENOMS

Of the 2,500 or so species of snakes found around the world only about 250 are venomous. Most of these are classified within three families: Colubridae, Viperidae (vipers, rattlesnakes) and Elapidae (cobras, sea snakes) (Underwood 1979). The advantages to snakes of venom and the means with which to inject the venom into their prey are those of immobilization and increased speed of digestion (Pearce 1973).

Like bee venom, snake venoms are complex mixtures of proteins, peptides and smaller molecules with enzymic and toxic properties (Iwanaga and Suzuki 1979). The biological effects of snake venoms and their components vary with the snake involved, marked differences being found at a family level. These differences are less at genus and species level while at the subspecies level they are mainly quantitative. However, again like bee, comparison of venoms between closely related snake species is difficult because composition can vary with the condition of the snake e.g. nutrition, age (Bonilla et al. 1973) and venom treatment (vacuum-drying, lyophilization, storage methods) (Iwanaga and Suzuki 1979).

TABLE 1 - General Character of *A. mellifera* Venom (O'Connor and Peck 1978).

Type of Component	% of venom[a]	Physiological Properties and Comments
WHOLE VENOM	100 (ℓ)	Toxic, directly haemolytic, inhibits mitochondrial activity, antibacterial and antifungal activity.
WATER	88 (ℓ)	From venom sac contents.
VOLATILE COMPOUNDS	4-8 (ℓ)	Possibly pheromones, possibly 13 compounds
Isoamyl acetate	≃ 0.1 (ℓ)	From ether extract of sac and glands.
ENZYMES	13-15 (s)	Prior to phospholipase A multiplicity.
Hyaluronidase (β-hexosaminidase)	1-3 (s)	Mol.wt. 38,000, pH optimum 4-5, enzyme primarily responsible for spreading of venom through tissue. Minor allergen.[b]
Phospholipase A system	12 (s)	Two possibly three fractions, pH optimum 7.0, toxic, antigenicity and anaphylactogenicity.
"Esterases and Phosphatases"	?	Suggested α esterase, β esterase, 2 alkaline phosphatases, 3 acid phosphatases in natural venom.
LARGE PEPTIDES	50-60 (s)	Mol.wt. range 2000-6000, strongly basic, potent physiological activities.
Melittin family	≃ 50 (s)	Toxic, directly haemolytic, anti-acetylcholinesterase activity, inhibits mitochondrial activity, antibacterial and antifungal activity. Separation of original melittin into three components.

continued.....

(Table 1 contd....)

Type of Component	% of venom	Physiological Properties and Comments
Mast cell degranulating (MCD) peptide	≃ 2 (s)	Structure determined, activity characterized - plays a role in release of histamine from mast cells.
Apamin	≃ 2 (s)	Powerful neurotoxin, structure partially characterized: basic peptide, 18 amino acid residues, 2 disulphide bridges, mol.wt. 2038. No surfactant properties[c].
Minimine	≃ 3 (s)	Structure partially characterised. Acts on Drosophila larvae to reduce adult size.
Protease inhibitor	?	Structure partially characterized.
SMALL MOLECULES	≃ 24 (s)	Mostly small peptides.
Histamine terminal peptide	≃ 1 (s)	First such compound definitely characterized from natural source.
Procamine	≃ 1 (s)	
7 small peptides	≃ 13 (s)	Some may contain histamine.
19 free amino acids	≃ 1 (s)	Amino acid content determined from individual bees.
Histamine	≃ 1 (s)	Age and seasonal variation studied.
Simple sugars	≃ 2 (s)	Glucose and fructose by chromatographic study.
6 phospholipids	≃ 5 (s)	Determined by colourimetric reagent.

a - (ℓ) = % of natural liquid venom; (s) = % of dried venom solids

b - Kemeny et al. (1984); c - Callewaert et al. (1968).

The snake venoms used in this study are from *Naja naja sputatrix (N. naja)*, *Vipera russelli (V. russelli)* and *Crotalus adamanteus (C. adamanteus)*: see Figure 1.

VENOM TOXINS

(i) Bee

Melittin is the principal toxic component of honey bee venom (Habermann and Zeuner 1971). It has an effect on all biological systems tested. For example melittin damages erythrocytes and leucocytes and their lysosomes (Hegner 1968). The melittins form approximately 50% of the dry weight of *A. mellifera* venom (Habermann 1972). They consist of the related peptides melittin, an N_{α}-formulated derivative and a precursor, promelittin (Kreil 1973).

The melittin system is strongly basic and the 26 amino acid sequence (Habermann 1972) of the principal component melittin (Figure 2) is consistent with its surfactant properties, the important areas being a basic hydrophilic region (positions 1-20) next to a generally hydrophobic region (positions 21-26). The surfactant properties contribute to the potent direct lytic activity of melittin (Shipman and Cole 1969) although there is no strict parallelism between the two.

In aqueous solutions of low ionic strength melittin exists as a monomer (molecular weight 2840) and in aqueous solutions of high ionic strength as a tetramer (Brown et al 1980). The four chains of the tetramer are composed so that the interior is almost entirely of apolar side chains and the surface coated with polar residues. The balance between hydrophobic adhesion and electrostatic

FIGURE 1 - Classification of snake species.

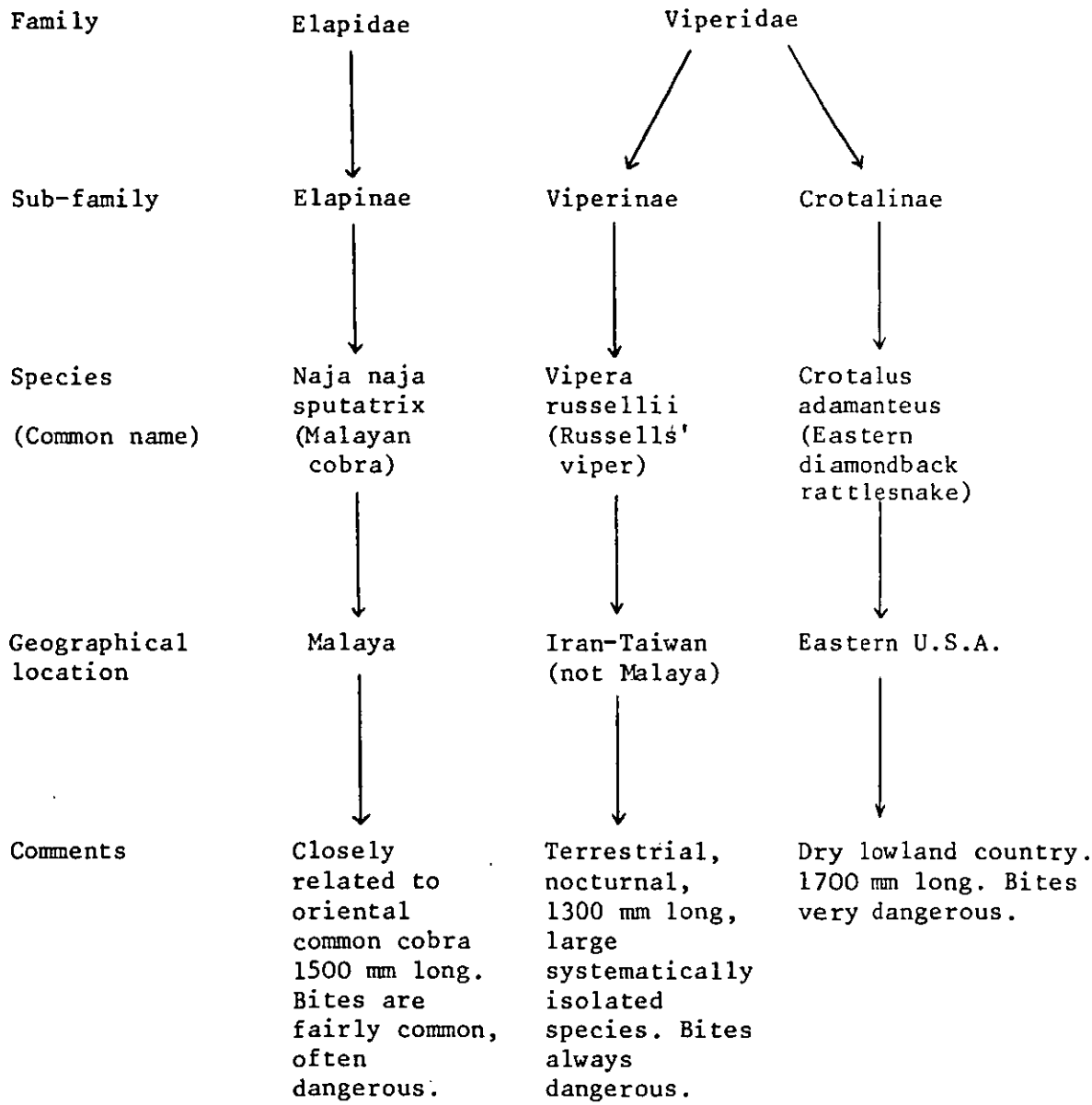


FIGURE 2 - Primary Amino Acid Sequence of Melittin (Yunes et al 1977)

Gly - Ile - Gly - Ala - Val - Leu - Lys - Val - Leu - Thr - Thr -

Gly - Leu - Pro - Ala - Leu - Ile - Ser - Trp - Ile - Lys - Arg -

Lys - Arg - Gln - Gln (NH_2).

repulsion of the subunits is such that melittin is tetrameric at concentrations prevailing in the bee venom sac and monomeric at the minimum concentrations required for cell lysis (Terwilliger and Eisenberg 1982a,b). Tetramer formation may be by subunit aggregation after structural modification rather than by conformational changes (Salerno et al. 1984). Like other detergents melittin damages enzyme systems bound to cell membranes such as cation activated adenosine triphosphatases (Repke and Portius 1963).

Another important polypeptide toxin present in honey bee venom is apamin which has no surfactant properties.

(ii) Snake

Like bee venom, snake venoms contain many mammalian toxins (see Table 2) and extensive reviews on the subject are available (Lee 1972; Howard and Gundersen 1980; Tu 1973; Karlsson 1979). The majority of toxins are to be found in elapid venoms (cobra) rather than viperid or crotalid venoms (Iwanaga and Suzuki 1979). They are mainly looked upon as neurotoxins such as cobrotoxin and crotoxin or as cardiotoxins e.g. direct lytic factor (D.L.F.).

The neurotoxins fall into two groups, those which act post-synaptically e.g. cobrotoxin and those which act presynaptically e.g. notexin. All the toxins of this latter type exhibit phospholipase A_2 activity (Chang et al. 1977), which may be linked with their neurotoxicity whereby the phospholipids at the neuromuscular junction are preferentially hydrolysed.

Cardiotoxins have been found only in the venoms of elapid snakes particularly cobra. Various toxins from many species have been isolated e.g. DLF from *N. naja* (Lee et al. 1968) and

TABLE 2 - Major toxins of snake venoms and some of their properties.

SNAKE (family) Common name	TOXIN, SUBUNIT	MOLECULAR WEIGHT	No. OF AMINO ACID RESIDUES	NEUROTOXIC SITE OF ACTION	ISOELECTRIC pH	TOXICITY LD50 I.V. µg/g mouse	PLA_2 Activity	ACTIVATORS	INHIBITORS	CHARACTERISTICS
REFERENCES	1,5	1,3,10	1,2,4,10, 16	1,3,8,13	1,4,10,15	3,5,7,15	3,9,11, 14,15,17	7,10,12,15	2,7	1,2,3,4,5,6,7,10,12,15,16
Crotalus durissus terrificus (Crotalid) Brazilian rattlesnake	Crotamine	5,500	42/46		10.3				Ca^{2+}, Mg^{2+}, K^+	Induces contracture of skeletal muscle Veratrine-like action
C. durissus terrificus (Crotalid)	Crotoxin	22,000	186	Presynaptic	4.7	0.05	Active	$0.5mM<Ca^{2+}<9mM$	$9mM>Mg^{2+}, Ca^{2+}<0.5mM$	2 noncovalently linked proteins Haemolytic
				Postsynaptic		1.1		Ca^{2+} dependent. Most active at phase transition temp.	Sr^{2+}	no cardiotoxic activity
	PhA_2	14,000	110		8.6	5.4	Active			Haemolytic, sequence homology with notexin
	Crotapotin (crotactin)	8,500	76		3.7	7500	None			
Bungaris multicinctus (Elapid) Formosan Krait	β-Bungarotoxin	20,500		Presynaptic		0.025/0.01	Active	$0.5mM<Ca^{2+}<9mM$ Ca^{2+} dependent. Most active at phase transition temp. (p-t temps.)	$9mM>Mg^{2+}, Ca^{2+}<0.5mM$ Sr^{2+}	2 subunits linked by at least one S-S bond. PhA activity and neurotoxicity lost on S-S reduction.
	1	13,500					Active	Ca^{2+} dependent. Most active at p-t temps.		sequence homology with notexin
	2	7,000					None			
Oxyuranus scutellus Scutellus (Elapid) taipan	Taipoxin	46,000		Presynaptic	basic	0.002/ 0.0021	Active	$0.5mM<Ca^{2+}<9mM$ Ca^{2+} dependent. Most active at p-t temps.	$9mM>Mg^{2+}, Ca^{2+}<0.5mM$ Sr^{2+}	noncovalently linked. 3 subunits, all homologous with notexin.
	α	13,000			basic	0.3	Higher			PhA_2 activity suppressed when in complex.
	β	14,000			neutral	> 2	None			
	γ	18,000			acidic	> 2	Lower	Ca^{2+}		contains carbohydrate moiety
Notechis scutatus scutatus (Elapid) Australian tiger snake	Notexin	13,600	119	Presynaptic	basic	0.025/0.01	Active	Ca^{2+} dependent. Most active at p-t temps. $0.5mM<Ca^{2+}<9mM$	$9mM>Mg^{2+}, Ca^{2+}<0.5mM$ Sr^{2+}	single chain myonecrotic specific targets in nerve terminals & muscle membranes. Amino acid sequence homologous to porcine & venom PhA_2 (N.melanoleuca). Modification of one His residue causes 99% reduction in PhA_2 activity & neurotoxicity

(Table 2 contd.....)

SNAKE (family) Common name	TOXIN, SUBUNIT	MOLECULAR WEIGHT	No. OF AMINO ACID RESIDUES	NEUROTOXIC SITE OF ACTION	ISOELECTRIC pH	TOXICITY LD50 I.V. ug/g mouse	PLA_2 Activity	ACTIVATORS	INHIBITORS	CHARACTERISTICS
REFERENCES	1,5	1,3,10	1,2,4,10, 16	1,3,8,13	1,4,10,15	3,5,7,15	3,9,11, 14,15,17	7,10,12,15	2,7	1,2,3,4,5,6,7,10,12,15,16
Naja naja atra (Elapid) Formosan cobra	Cobrotoxin	6,959	62	Postsynaptic	> 9.0					Typical Type I neurotoxin, 4 S-S bonds. Fully active in 8M urea. Left handed α helix. Reduction of S-S bonds causes reduction in toxicity.
Naja naja simiensis (Elapid)	Neurotoxin	8,000	71	Postsynaptic	9.4					Typical Type II neurotoxin, 5 S-S bonds. Fractions producing a neuromuscular block also depolarize skeletal muscle.
Bungarus multicinctus (as above)	α-Bungarotoxin		74	Postsynaptic						5 S-S bonds. Very similar to Type II cobra neurotoxins.
Vipera palestinae (Viperid)	Viperotoxin	11,600	108		basic					Non dialyzable protein. 3 S-S bonds. Causes circulatory not respiratory failure.

References for Table 2

1. Lee 1972
2. Chang 1979
3. Howard and Gundersen 1980
4. Meldrum 1965
5. Rosenberg 1979
6. Breithaupt 1976
7. Karlsson 1979
8. Chang and Lee 1977
9. Breithaupt et al 1977
10. Tu 1973
11. Strong 1976
12. Kelly 1977
13. Harris et al 1973
14. Fohlman et al 1976
15. Fohlman et al 1979
16. Harris and Johnson 1978
17. Halpert et al 1976
18. Moroz et al 1966

Haemachatus haemachatus (Condrea et al 1964), cobramines (Larsen and Wolff 1968) and cytotoxin (Braganca et al 1967) from *N. naja*, but they are virtually identical with each other and can be classified as a family of homologous peptides. Molecular weights are in the range 6-7000 (Larsen and Wolff 1968; Aloof-Hirsch et al 1968), isoelectric pH above 12 and in crude venom they are often found in stable binding with proteins of larger molecular size (Lee 1972). Cardiotoxin is the most basic and abundant constituent of cobra venoms amounting to 25-55% dry weight and is heat stable at acid pH but not at alkaline pH. The activity of the peptides is not related to phospholipase, phosphodiesterase or alkaline phosphatase activity and the number of amino acid residues varies slightly, cardiotoxin having 60, DLF 57 and cobramine B 52 (Tu 1973). The single polypeptide chains are cross-linked by four disulphide bonds and although the amino acid composition is quite different from neurotoxins there is some sequence homology.

VENOM ENZYMES

In addition to the toxins, venoms contain many enzymes. The enzymes and their properties of bee venom are summarised in Table 1 and of snake venoms in Table 3. Snake venom enzymes are thought to act in the following ways: (a) local capillary damage and tissue necrosis by proteases, phospholipases, arginine ester hydrolases and hyaluronidase; (b) coagulant and anticoagulant actions by proteases, phospholipase A_2 and (c) acute hypotension and pain due to release of vasoactive peptides by kininogenase (Meldrum 1965).

TABLE 3 - Some Properties of Enzymes found in Snake Venoms (Iwanaga and Suzuki 1979)

TRIVIAL NAME	EC NO	TYPICAL SUBSTRATE	MOLECULAR WEIGHT	TYPICAL VENOM	SPECIFIC CHARACTERISTICS	CHARACTERISTICS - General
Phospholipase A_2	3.1.1.4	Phosphatidylcholine	11,000-15,000	Elapid Viperid Crotalid	several isoenzymes several isoenzymes I, II, forms dimers	Simple protein, heat stable, histidine active site. Basic PLA_2 appear to be more toxic (LD_{50} 500µg/kg mouse) than acidic (LD_{50} >2000µg/kg mouse). Limited sequential homology of elapid PhA_2 when compared with viperid and crotalid PhA_2.
L-amino acid oxidase	1.4.3.2	L-amino acid	100,000-130,000	Elapid Viperid Crotalid		Glycoprotein, 2 moles FAD per mole enzyme (confers yellow colour onto venom eg. *C. adamanteus*), heat unstable.
Phosphodiesterase	3.1.4.1	Oligonucleotides	115,000	E,V,C		Heat labile, EDTA sensitve, acid unstable, pH9 optimum.
5'Nucleotidase	3.1.3.5	5'Mononucleotides	100,000	E,V,C		Heat labile, Zn^{2+} sensitive, EDTA sensitive, acid unstable, optimum at pH8.5.
Phosphomonoesterase	3.1.3.2	p-Nitrophenylphosphate	100,000	E,V,C		Heat labile, Zn^{2+} sensitive, EDTA sensitive, acid unstable, pH8.5-9 optimum.
Deoxyribonuclease	3.1.4.6	DNA,RNA		E,V,C		pH5.0 optimum.
Ribonuclease	2.7.7.16	RNA	15,900	E,V,C		pH7-9 optimum, specific towards pyrimidine nucleotides.
Adenosine triphosphatase	3.6.1.8	ATP		E,V,C		
Hyaluronidase	4.2.99.1	Hyaluronic acid		E,V,C		Heat labile, pH4.6 optimum, resembles testicular enzyme.
NAD-nucleosidase	3.2.2.5	NAD	100,000	E,V,C		Heat labile, pH7.5 optimum, nicotinamide sensitive
Arylamidase		L-leucine naphthylamide	100,000	E,V,C		Heat labile, SH-enzyme, p-chloromercuribenzoate + Hg^{2+} sensitive, pH8.5 optimum.
Peptidase				E,V,C		
Acetylcholinesterase	3.1.1.7	Acetylcholine	126,000	Elapid only		Heat labile, Diisopropylphosphorofluoridate (DFP) sensitive, pH8-8.5 optimum.
Phospholipase A_1	3.1.1.5	Lysolecithin		Elapid only		Heat stable pH10 optimum.
Glycerophosphatase		Glycerophosphate		Elapid only		
Endopeptidase		Casein, haemoglobin, insulin, bradykinin	21,400-95,000	Viperid, Crotalid	V.russellii pH3.6 optimum	Glycoprotein, metal (Ca^{2+},Zn^{2+}) protease, generally pH8.5 optimum. Some inactivated by 10^{-2}M EDTA. Differ from mammallian endopeptidases.
Arginine ester hydrolase		Benzoyl-L-arginine ethyl ester (BAEE); Tosyl-L-arg. Methyl ester (TAME)	27,000-30,000	V,C		Glycoprotein, heat stable, DFP sensitive, pH8-9 optimum. Activity doubled by 1mM Mn^{2+},Zn^{2+},Co^{2+}. No effect by Mg^{2+}. Possibly involved in causing hypotension and increasing capillary permeability
Kininogenase	3.4.4.21	Plasma Kininogen, BAEE	33,500	V,C		Heat stable, DFP sensitive, specific towards kininogen.
Thrombin-like enzyme		Fibrogen, BAEE	28,000-33,000	V,C		Glycoprotein, heat stable, DFP sensitive. Large difference between V and C enzymes.
Factor X activator		Factor X	78,000			Glycoprotein, heat liable, DFP insensitive, EDTA sensitive. Activates also Factor IX.
Prothrombin activator		Prothrombin	56,000	V,C		Glycoprotein, heat liable, DFP insensitive, EDTA sensitive

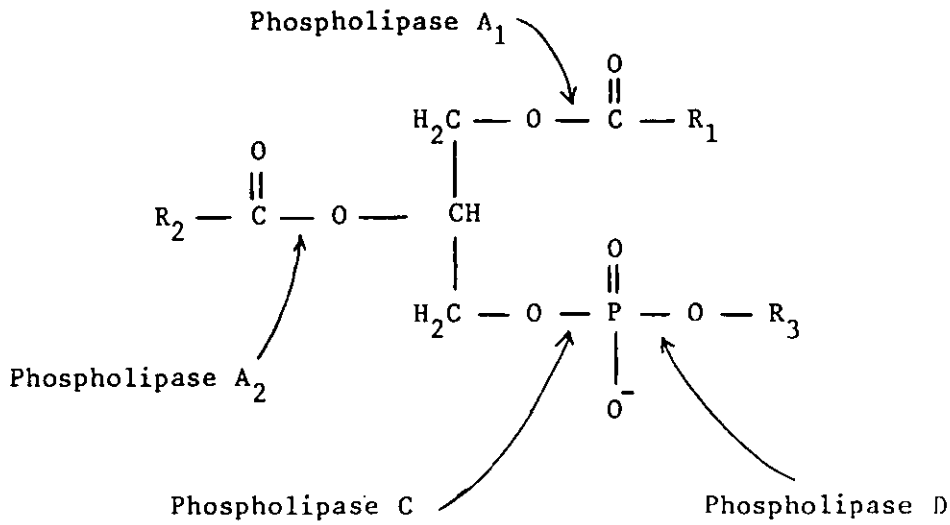
Immunological studies show an absence of common antigens between remotely related snakes e.g. cobras and rattlesnakes and the widely distributed enzymes are believed to differ in molecular properties. Certain enzymes characterize certain families e.g. acetylcholinesterase is typical of elapid venoms but is never found in viperid or crotalid venoms (Mebs 1970).

Particularly relevant to toxic action in the context of the present work are both bee and snake venom phospholipases, since these are reported to cause the partial disruption of *Escherichia coli (E. coli)* cell envelopes pretreated with EDTA or lysozyme (Slein and Logan 1967; Op den Kamp et al. 1972).

Phospholipases are lipolytic enzymes which work at lipid/water interfaces. They have a narrow substrate specificity and hydrolyse the phospholipid molecule as shown in Figure 3. Phospholipase A_2 (PLA_2) will hydrolyse natural and synthetic sn-3 phosphoglycerides (De Haas and Van Deenen 1966) irrespective of the nature of the fatty acids to give the lysophospholipid but will not attack sn-1 phosphoglycerides of D-isomers (Van Deenen and De Haas 1963), Figure 4.

PLA_2 are universally distributed and can be divided into three classes: (1) enzymes of venom; (2) digestive enzymes (mammalian, similar to class 1); (3) intracellular enzymes occurring in animal tissues e.g. pancreas and in micro-organisms e.g. *E. coli*. The venoms of the honey bee and of snakes of the sub-families crotalinae, viperinae, elapinae and hydrophiinae represent the richest sources of PLA_2 although the content varies from venom to venom.

FIGURE 3 - Substrate specificity of phospholipases.



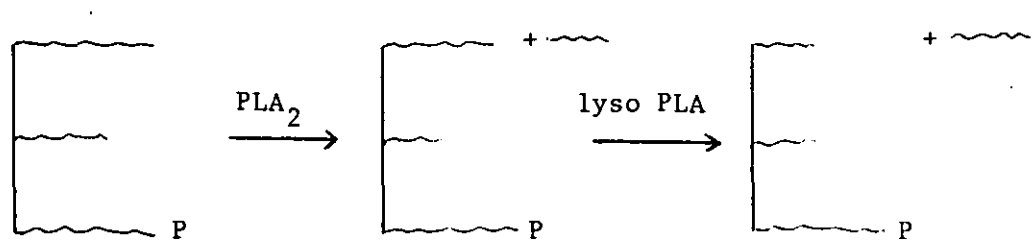
R_1, R_2 = long chain fatty acids

R_3 = polar head group eg. CH_2CH_2N (phosphatidyl choline)

$CH_2CH_2NH_2$ (phosphatidyl ethanolamine)

$CH_2CHOHCH_2OH$ (phosphatidyl glycerol)

FIGURE 4 - Action of phospholipases and lysophospholipases.



The PLA_2 of venoms have been generally reviewed by Condrea and de Vries (1965), Iwanaga and Suzuki (1979) and Rosenberg (1979). They have an absolute requirement for Ca^{2+} (Magee and Thompson 1960) and their catalytic activity is also enhanced by many other substances such as Na^+, Mg^{2+}, Mn^{2+}, deoxycholate and ether. It is inhibited by Fe^{2+}, Cu^{2+}, Na_2EDTA, phosphate, oxalate, citrate, lysophospholipids and long chain fatty acids (Smith et al. 1972) amongst others (Iwanaga and Suzuki 1979; Rosenberg 1979; Long and Penny 1957).

Inhibition is effected by two processes, the first preventing enzyme-substrate association or orientation e.g. Triton, Tween, sodium dodecyl sulphate (Rohalt and Schlamowitz 1961) and depend on the overall charge requirement, thus an inhibitor of one enzyme may be an activator of another one. Other inhibitors react at the active site and compete with the substrate.

Other general properties of PLA_2 are their stability when heated, frozen, thawed repeatedly, lyophilized and treated with acid or urea (6 M). The optimum temperature varies with the enzyme e.g. 65°C for *N. naja* enzymes while *C. adamanteus* enzymes lose their activity above 45°C, PLA_2 are usually most active in the pH range 7.0 to 8.5.

PLA_2 have small molecular weights, usually between 8,500 and 24,000 for the monomers: c.f. proteinases 25,000, lipases 50,000. Most are active as monomers e.g. *N. naja*, *V. russelli*, some as dimers e.g. *C. adamanteus* (Wells and Hanahan 1969), *A. mellifera* (Shipolini et at. 1971a) and some such as *A. mellifera* have large carbohydrate moieties. There appears to be a dynamic equilibrium

between dimer and monomer which alters with respect to ionic strength and Ca^{2+} concentration. In some e.g. *N. naja* the important factor is probably the charge difference due to the balance of isoenzymes.

PLA_2 have been purified from many venoms and the primary amino acid sequences determined for some e.g. *A. mellifera*, *N. naja* and *C. adamanteus*, see Table 4 (Shipolini et al, 1971b; Tsai et al, 1981; Heinrikson et al, 1977). Most have 120-140 amino acid residues and 6-8 disulphide bridges. While there is a high degree of homology within snake enzymes, elapid PLA_2 differ from viperid PLA_2 in some basic constituents such as disulphide bridges. Various properties of the enzymes such as the lack of free sulphydryl groups make the molecule compact and resistant to unfolding which could be an adaptive device to prevent denaturation at the phospholipid/water interface.

The mode of action of venom PLA_2 has been studied by several workers e.g. Wells (1972, 1974a, 1974b), Roberts et al (1977a). Phospholipid hydrolysis occurs at the phospholipid/water interface and is dependent on the physical state of the substrate. Substrate and supersubstrate (micelles formed by phospholipids grouping with hydrophobic fatty acids inwards and hydrophilic headgroups outwards) binding is important. Dennis (1973) has shown that synthetic substrates must be as micelles rather than mono- or bi- layer. Lipids in cell membranes are thought to be as lipid-protein complexes resulting in their being held more loosely than in micelles. Differences in hydrolysis rates of different substrates are probably due to many factors important among which are interface charge, polarity matching and substrate size. A possible mode of action of PLA_2 on mixed

TABLE 4. Comparison of primary amino acid sequences of phospholipases A from various sources.

	1									10										20										30				
1	A	L	W	Q	F	R	S	M	I	K	C	A	I	P	G	S	H	P	L	M	D	F	N	N	Y	G	C	Y	C	G	L	G	G	S
2	N	*	Y	*	*	K	N	*	*	Q	*	T	V	*	S	-	R	S	W	W	*	*	A	D	*	*	*	*	*	*	R	*	*	*
3	S	*	V	*	*	E	T	L	*	M	-	K	V	A	K	R	S	G	*	L	W	Y	S	A	*	*	*	*	*	*	W	*	*	H
4																					I	I	Y	P	G	T	L	W	*	*	H	*	N	K

	40													50										60										
1	G	T	P	V	D	E	L	D	R	C	C	E	T	H	D	N	C	Y	R	D	A	-	K	N	L	D	S	C	K	F	L	V	D	N
2	*	*	*	*	*	D	*	*	*	*	*	Q	V	*	*	*	*	*	N	E	*	E	*	-		S	G	*	-	W	-	-	-	N
3	*	R	*	Q	*	A	T	*	*	*	*	F	V	*	*	C	*	*	G	K	*	T	N	-	-	-	-	*	-	-	-	-	-	*
4	S	S	G	P	N	*	*	G	*	F	K	H	*	D	A	C	*	R	T	H	D	M	C	P	N	V	M	S	A	G	E	S	K	H

	70											80									90										100
1	P	Y	T	E	S	Y	S	Y	S	-	C	S	N	T	E	I	T	C	N	S	K	N	N	A	C	E	A	F	I	C	N	C	D	R
2	*	*	F	K	T	*	*	*	E	C	S	*	Q	G	T	L	*	*	K	G	G	*	*	-	*	A	*	A	V	*	D	*	*	*
3	*	K	*	V	*	*	T	*	S	-	E	E	*	G	*	*	V	*	G	G	D	D	-	P	*	G	T	Q	*	*	E	*	*	K
4	G	L	*	D	T	A	*	R	L	S	*	N	D	N	D	L	F	Y	K	D	S	A	*	T	I	S	S	Y	F	V	G	K	M	Y

										110										120										130				
1	N	A	A	I	C	F	S	K	A	-	-	P	Y	N	K	E	H	K	N	L	D	T	K	-	K	Y	C							
2	L	*	*	*	*	*	G	G	*	-	-	*	*	*	D	N	N	N	Y	I	*	L	*	-	A	R	*	Q							
3	A	*	*	*	*	*	R	D	N	I	P	S	*	D	-	N	K	Y	-	W	L	F	P	P	*	D	*	P	Q	E	P	E	P	C
4	F	D	L	I	D	T	K	C	Y	K	L	E	H	P	V	T	G	C	G	E	R	*	E	G	R	C	L	H	Y	T	V	D	K	S

											140																							
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The sequences compared are 1) porcine pancreatic (Puijk et al, 1977); 2) *Naja naja atra* (Tsai et al, 1981); 3) *Crotalus adamanteus* (Heinrikson et al, 1977) and 4) *Apis mellifera* (Shipolini et al, 1971b). Gaps (-) have been introduced in order to obtain alignments of half cysteines and maximal homology. Residues identical to the corresponding residue in porcine pancreas PLA are indicated with an asterisk. The numbering has been based on porcine pancreas PLA and does not necessarily correspond to the numbering used in the original publication. The IUPAC one-letter notation for amino acids has been used (IUPAC-IUB Commission, 1968).

micelles: the "Dual Phospholipid" model, has been proposed by Roberts et al (1977a, 1977b), see Figure 5.

INTERACTION BETWEEN VENOMS AND INDIVIDUAL CELLS

(a) Bacterial cells

Some venoms and venom components have been shown to have antibacterial properties.

That honey bee venom has antibacterial properties was discovered by Schmidt-Lange in 1941 and confirmed by Ortel and Markwaldt (1955) who found that gram positive bacteria were more sensitive than gram negative bacteria. Melittin was put forward as the anti-bacterial component of honey bee venom by Fennell et al (1968). This study showed the antibacterial activity of melettin to be of the same order as that of whole honey bee venom. Fennell et al also reported that neither whole bee venom nor melittin showed an antibacterial effect on *E. coli*. However, later work by Dorman and Markley (1971) reported that melittin was effective against *E. coli* and other bacteria at a similar concentration to that used by Fennell et al. Dorman and Markley (1971) synthesized N-terminal peptide sequences homologous with natural melittin, none of which exhibited antibacterial activity.

Elapid (cobra) venoms have been designated as direct lytic venoms due to the combined action of PLA_2 and DLF on mammalian membranes. Since micro-organisms contain phospholipids as an integral part of their cell envelopes this could render them susceptible to attack by PLA_2.

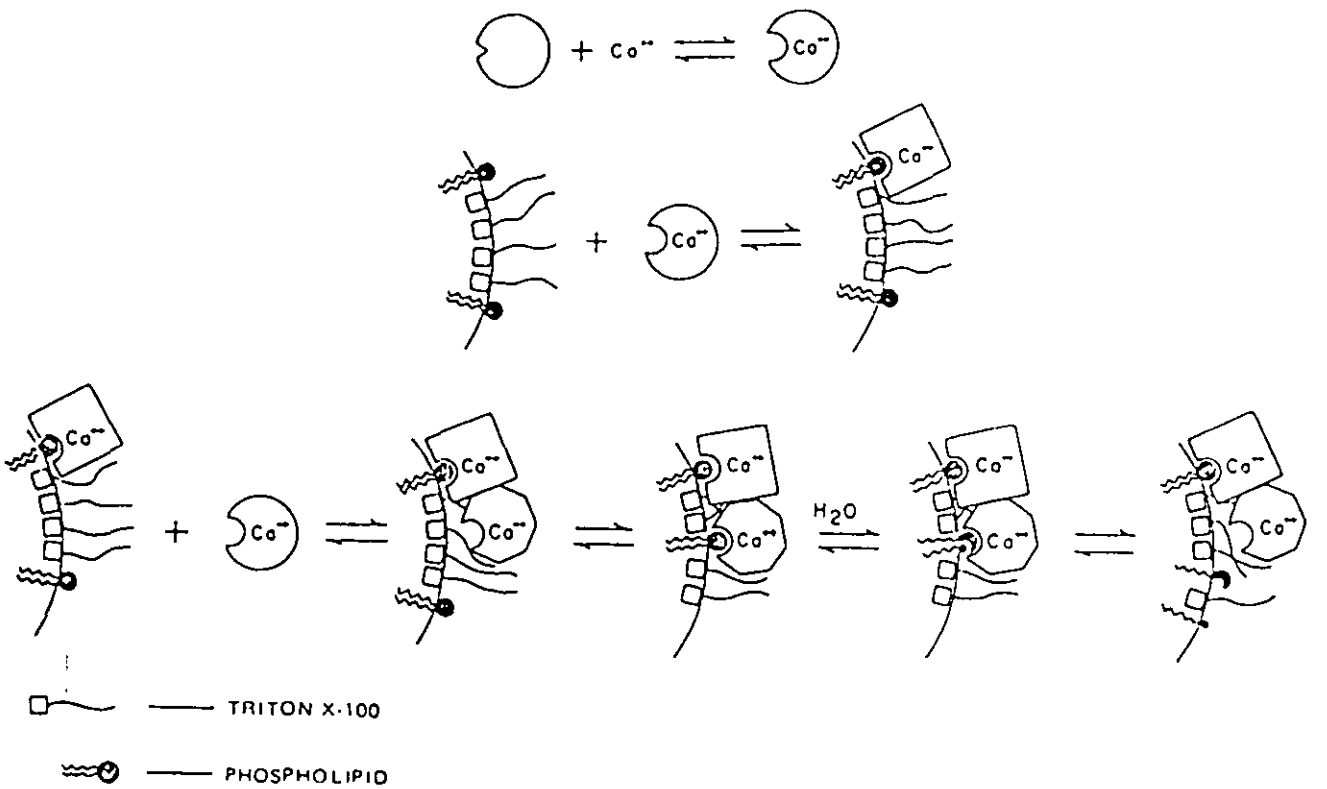


FIGURE 5 - Schematic diagram of the "dual-phospholipid" model for the action of phospholipase A_2 toward phospholipids contained in mixed micelles. Enzyme first binds Ca^{2+} and undergoes a a conformational change that allows it to bind to phospholipid in the mixed micelle. The presence of interfacial phospholipid causes the enzyme to form an asymmetric dimer. One subunit of this dimer is responsible for binding to the interface via phospholipid, while the other hydrolyzes an accessible phospholipid. Lateral diffusion of phospholipid in the mixed micelle may be involved before the catalytic subunit binds phospholipid. Alternatively, two enzyme molecules bound to interfacial phospholipid may associate to form the asymmetric dimer directly. Once catalysis occurs, the products may diffuse away from the enzyme and either be retained in the mixed micelle or released into the solution.

The cell envelope of *E. coli* is typical of gram negative bacteria. It consists of three distinct layers: the inner or cytoplasmic membrane, the peptidoglycan layer and the outer membrane.

Peptidoglycan is very rigid, it gives the cell stability and shape and prevents lysis during growth. Between the peptidoglycan layer and the inner membrane is the periplasmic space containing hydrolytic enzymes and soluble binding proteins involved in transport and chemotoxin.

The membrane structure is thought to conform to the fluid mosaic model proposed by Singer and Nicholson (1972) as shown in Figures 6 and 7. This model is supported by freeze-etching studies (Branton 1966). Peripheral proteins can be removed by mild treatment, for example chelating agents, and integral ones by stronger treatment such as detergents.

The bulk of the phospholipids is in the bilayers forming matrix-containing proteins. Distribution of both proteins and phospholipids between inner and outer membranes is asymmetric (Rothman and Lenard 1977) and while the phospholipid composition between the two is similar, approximately 60% of the total is recovered in the outer membrane (Osborn et al 1974). The predominant species of phospholipid is phosphatidylethanolamine with some phosphatidylcholine and cardiolipin also present (Figure 3).

The inner membrane acts as the primary permeability barrier for the cell containing specific permeases and transport proteins and is the site of biosynthesis of major membrane constituents.

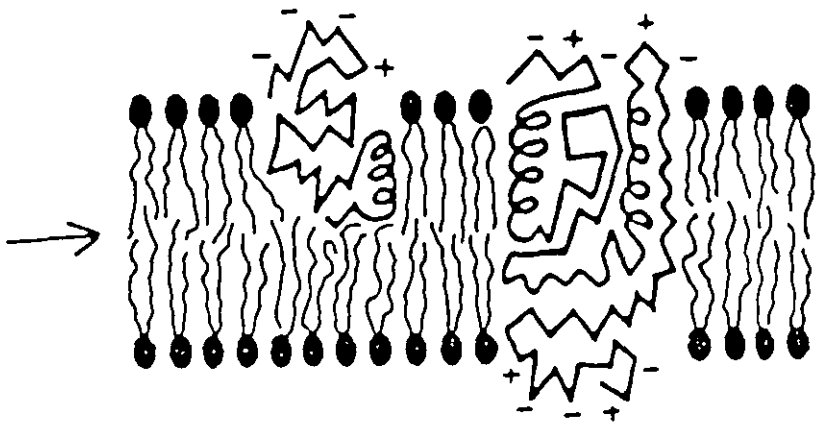


FIGURE 6 - The lipid-globular protein mosaic model of membrane structure: schematic cross-sectional view. The phospholipids are arranged as a discontinuous bilayer with their ionic and polar heads in contact with water. The integral proteins, with the heavy lines representing the folded polypeptide chains, are shown as globular molecules partially embedded in, and partially protruding from, the membrane. The protruding parts have on their surfaces the ionic residues (- and +) of the protein, while the nonpolar residues are largely in the embedded parts; accordingly, the protein molecules are amphipathic. The degree to which the integral proteins are embedded and, in particular, whether they span the entire membrane thickness depend on the size and structure of the molecules. The arrow marks the plane of cleavage to be expected in freeze-etching experiments.

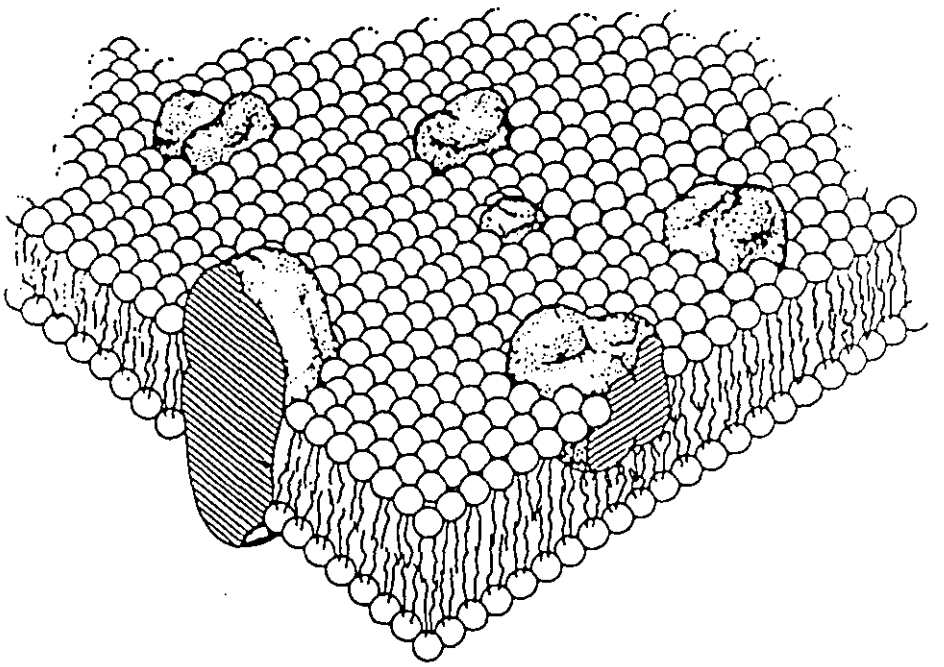


FIGURE 7 - The lipid-globular protein mosaic model with a lipid matrix (the fluid mosaic model); schematic three-dimensional and cross-sectional views. The solid bodies with stippled surfaces represent the globular integral proteins, which at long range are randomly distributed in the plane of the membrane. At short range, some may form specific aggregates, as shown.

The outer membrane has been extensively reviewed by Nikaido and Nakae (1979). It is exposed to the environment providing a passive barrier to substances with molecular weight greater than 600-700. It contains lipopolysaccharide and protein in the outer half of the bilayer leaflet and lipoprotein, phospholipid and protein in the inner half. The fluidity and permeability is very different from the inner membrane and hydrophobic molecules do not penetrate easily. The entry of some hydrophilic molecules is determined by specific proteins e.g. Omp A (Osborn and Wu 1980) but the majority of small hydrophilic molecules enter through channels provided by porin proteins. This is not selective other than by relative molecular mass and to an extent on charge characteristics.

The only enzyme known to be present in the outer membrane of *E. coli* is a phospholipase A. This is thought to be of the PLA_2 type but PLA_1 and lysophospholipase activities have also been claimed to be exhibited (Raetz 1978). Lysophospholipases are enzymes which hydrolyse lysophospholipid molecules (Figure 4).

The cell envelope of bacteria is a prime target for antibacterial action. Many chemical agents cause cell lysis thought to be due to metabolic disturbance followed by uncontrolled action of the lytic enzymes normally functioning during cell envelope growth and cell division. One of the first effects of such action is the leakage of cell constituents into the surrounding medium and this can be followed by a variety of methods e.g. measurement of 260 nm - absorbing cell constituents. Slein and Logan (1967) studied the effect of phospholipases from *Bacillus cereus* on *E. coli* by

determining changes in β-galactosidase activity and cell absorbancy and *E. coli* cell envelopes were shown to be disrupted by the action of porcine PLA_2 and bacterial phospholipase C provided they were pretreated with EDTA (Slein and Logan 1967) or lysozyme (Op den Kamp 1972). Aloof-Hirsch et al.(1968) reported that DLF from Ringhals (Haemachatus haemachatus) venom inhibited growth of *E. coli* and S. aureus at concentrations of 50 $\mu g/cm^3$ and higher.

(b) Mammalian cells.

Venoms have many effects on mammalian cells, most of which are ascribed to the components PLA_2 and the membrane-active polypeptides e.g. DLF and melittin.

Cytotoxicity has been demonstrated against Yoshida sarcoma cells by cytotoxin (*N. naja*) (Braganca et al 1967) and by cardiotoxin (*N. naja atra*) (Lee 1972) and against HeLa and KB cells by cardiotoxin (*N. naja atra*) (Lee 1972). Against Ehrlich ascites tumour cells effects were shown to be time and dose dependent, not affected by cell concentration and inhibited by Ca^{2+} but not Mg^{2+} (Leung et al 1976).

Haemolysis of red blood cell (R.B.C.) membranes is produced by melittin which renders the erythrocyte ghosts shrunken and empty unlike other detergents e.g. lysolecithin which dissolves them totally (Hegner 1968). Cardiotoxins have a direct though weak haemolytic activity (Slotta and Vick, 1969; Condrea et al. 1964; Aloof-Hirsch et al 1968) and DLF is thought to induce perturbations of RBC membrane permeability leading to impairment of the active transport system.

PLA_2 from varying sources have different effects on RBC membranes. Those from *N. naja* and bee cause 70% hydrolysis of membrane lecithin (Zwaal et al 1975) while those from *C. adamanteus* (Zwaal et al 1975) and *V. palestinae* (Condrea et al 1964) cause none. This action alone does not usually lead to cell lysis.

Synergism between PLA_2 and melittin or DLF has been reported. Thus synergism between PLA_2 and melittin was observed by Yunes (1977) and Vogt (1970). Mollay and Kreil (1974) suggested that the binding of melittin could be an intermediate step in the mechanism of venom action on cell membranes. Vogt (1970) showed that both melittin and DLF potentiated haemolysis of RBC at concentrations which were not lytic for the polypeptides alone and which was enhanced by Ca^{2+} and inhibited by EDTA.

The synergistic action of PLA_2 and DLF has been demonstrated on various membranes e.g. axonal membranes (Leung et al 1976). In the presence of DLF, PLA_2 continues its hydrolytic action through increased access to the lipid matrix provided by structural changes caused by DLF.

The mode of action of DLF has not been elucidated but two theories have been proposed. One suggests that the toxin attaches to the cell membrane via its basic residues and hydrophobic amino acids. The membrane structure is then disturbed by insertion of lipophilic residues. Condrea et al. (1965) showed that the initial binding of DLF is weak and Vincent et al (1976) suggested a two step binding of cardiotoxin (*N. mossambica*) with axonal membranes. This mechanism would resemble that of melittin (Mollay and Kreil 1973). The continual presence of PLA_2 and DLF is required for synergism implying a role for DLF as both membrane modifier and enzyme-binding mediator.

An alternative mechanism is proposed by Vogt et al (1970) whereby DLF potentiates PLA_2 haemolysis by interacting with SH groups in the membrane proteins. Reduction of DLF causes loss of lytic but not detergent activities and synthetic SH reagents e.g. N-ethylmaleimide can mimic DLF action while having no detergent properties. However, melittin is devoid of S-S bridges yet potentiates PLA_2.

There are several ways in which lysis and phospholipid cleavage can be enhanced, the basic requirements being membrane modification and facilitation of enzyme access to substrate. This has been achieved by using hypotonic media which induce membrane stretching, SH agents which interfere with membrane proteins and detergents which interact with membrane lipid, the direct action of venom polypeptides putting them into the last group (Mollay and Kreil 1974).

Most of the determination of venom action is based on studies of the whole venom, single major components or pairs of major components. In many cases the studies may have used incompletely purified components. The net effects of venom action on an organism depends on the interaction between the components and while a specific effect may be linked with a specific component, the end reaction may well be modified by the other components present.

OBJECTIVES OF THIS STUDY

The object of this present work was to compare the antibacterial effects of different venoms on *E. coli* particularly with reference to the cell envelope. Some venoms such as *A. mellifera* and *N. naja sputatrix* contain membrane-active polypeptides in contrast to venoms which have had no such polypeptides identified: *V. russelli* and *C. adamanteus*.

Slein and Logan (1967) showed that leakage of intracellular material from *E. coli* by phospholipase C (*B. cereus*) could be measured by determining β-galactosidase changes. This study was aimed at determining whether the action of crude venoms and some of their components such as phospholipase A_2 or polypeptide toxins on *E. coli* cells could be followed by this method. It was also intended to investigate whether or not venom action can be accounted for by the action of these specific components.

Section 2

MATERIALS

1. ORGANISMS

Escherichia coli NCIB 9552 (lac^+ $perm^-$) and *Staphylococcus aureus* NCIB 8625 were used in this study and will be referred to as *E. coli* and *S. aureus* respectively.

Organisms were maintained on nutrient agar slopes and subcultured in minimal lactose medium (see below 3.2) when required.

2. REAGENTS

2.1 Chemicals

All laboratory reagents used were of analytical grade supplied by Fisons Scientific Apparatus p.l.c., Loughborough or British Drug Houses Ltd., Atherstone.

Chemicals used for electron microscopy were of electron microscopy purity and obtained from Taab Laboratories Ltd., Reading or Polaron Equipment Ltd., Watford.

Sephadex G75 (fine and superfine) was supplied by Pharmacia Ltd., Hounslow.

2.2 Biochemicals

Crude venoms: *Apis mellifera (A. mellifera), Naja naja sputatrix (N. naja sputatrix), Vipera russellii (V. russellii)* and *Crotalus adamanteus (C. adamanteus).*

Phospholipases A_2: partially purified enzymes from *A. mellifera* and *N. naja sputatrix,* obtained as lyophilized powder with activities of 1500 and 200-600 units/mg respectively. Both were reconstituted in distilled water at a concentration of 0.8 mg/cm^3 before use.

Peptides: melittin from *A. mellifera* and cardiotoxin from *N. naja siamensis*. Both were reconstituted in distilled water prior to use.

The above and all biochemical reagents were obtained from the Sigma (London) Chemical Company Ltd., Poole, Dorset.

3. MEDIA

All media used for culturing micro-organisms were obtained from Oxoid Ltd., Basingstoke.

3.1 Nutrient agar

This was used for the maintenance of both *E. coli* and *S. aureus*, and for experiments involving *S. aureus* and contained: (% w/v) Lab-lemco, 1; neutralised bacteriological peptone, 1; sodium chloride, 0.5; agar No3 1.5. The pH was adjusted to 7.5 before use. Sterilization was carried out by autoclaving at 121°C for 20 minutes before use.

3.2 Minimal lactose medium (M.L. medium)

This medium was used for all experimental work involving *E. coli* where a liquid medium was required unless otherwise stated. It contained (% w/v) $Na_2HPO_4 . 12H_2O$, 0.6; KH_2PO_4, 0.3; NH_4Cl, 0.1; $MgSO_4 . 7H_2O$, 0.05; NaCl, 0.02; lactose, 0.4. Sterilization was carried out by autoclaving at 117°C for 10 minutes, lactose being autoclaved separately and added to the medium before use. The pH was adjusted to 7.5 prior to use.

3.3 Minimal lactose agar (M.L. agar)

Agar No3 was added to minimal lactose medium as above to a final concentration of 1.5% w/v to give a solid minimal medium.

4. BUFFERS

4.1 Tris-HCℓ buffer

Tris-HCℓ buffer was prepared by adjusting the pH of a 0.6% w/v solution of tris [hydroxymethyl] methylamine (0.1 M) to 7.5 with dilute HCℓ.

4.2 Mercaptoethanol buffer (PMSH)

This was used to dilute cells prior to β-galactosidase determination and was composed of 0.01 M potassium phosphate buffer containing (% w/v) NaCℓ, 0.9; $MnCℓ_2$, 0.002; mercaptoethanol, 0.348; adjusted to pH 7.2.

4.3 Ortho-nitrophenylgalactopyranoside (ONPG) solution

This was prepared as a 0.4% w/v solution of ONPG in 0.25 M potassium phosphate buffer, final pH 7.2.

4.4 ¼ Strength Ringers' solution

Treated cells were diluted in ¼ strength Ringers' solution prior to cell viability measurements and it contained (% w/v) NaCℓ, 0.225; KCℓ, 0.0105; $CaCℓ_2$, 0.012; $NaHCO_3$, 0.005; the pH being adjusted to 7.0.

5. CHROMATOGRAPHY AND ELECTROPHORESIS SOLUTIONS

5.1 Sephadex G75

Gel chromatography supports, Sephadex G75 fine and superfine, were prepared for use by being left to swell in distilled water overnight before packing into glass columns.

5.2 Barbitone buffer

This was composed of: (% w/v) barbitone, 0.085; barbitone sodium, 0.665; sodium acetate, 0.4875; with an ionic strength of 0.075 and a pH of 8.6.

5.3 KOH/acetic acid buffer

2.69% w/v KOH and 17.2% v/v glacial acetic acid were mixed and the pH adjusted to 4.3 if necessary.

5.4 Ponceau S

Protein staining of electrophoresis separation of venoms was by Ponceau S which consisted of 0.5% w/v Ponceau S in 5% w/v trichloroacetic acid.

6. ELECTRON MICROSCOPY SOLUTIONS

6.1 Veronal acetate buffer

The basic solution was composed of: (% w/v) sodium acetate, 1.943; barbitone sodium, 2.94; NaCℓ, 3.4. To 5.0 cm^3 of this solution was added 7.0 cm^3 HCℓ (0.1 M), 13.0 cm^3 distilled water, 0.25 cm^3 $CaCℓ_2$ (14.7% w/v) and 0.25 cm^3 MgCℓ (20.3% w/v), the pH being adjusted to 6.1.

6.2 Purified agar

Cells for transmission electron microscopy were embedded in agar prepared by dissolving by boiling 0.4 g agar (Oxoid Ionagar No2) in 10 cm^3 veronal acetate buffer.

6.3 Osmium tetroxide

4% w/v OsO_4 was diluted to 1% w/v by addition of veronal acetate buffer.

6.4 Uranyl acetate

This was prepared by dissolving 0.2 g uranyl acetate in 10 cm^3 veronal acetate buffer.

6.5 Araldite resin

27 cm^3 araldite (CY212), 23 cm^3 dodecenyl succinic anhydride (DDSA; HY964) and 1 cm^3 benzyldimethylamine (BDMA) were carefully and thoroughly mixed to provide the araldite resin.

6.6 Lead citrate

This was prepared by dissolving 2.66 g lead nitrate and 3.52 g sodium citrate in 60 cm^3 distilled water which had been freshly boiled and cooled. To this was added 16 cm^3 sodium hydroxide (1M, 4% w/v) and the solution was then made up to 100 cm^3 and stored being careful not to shake it.

7. APPARATUS

7.1 Spectrophotometer

All optical density measurements were made using a CE202 ultra-violet spectrophotometer (Cecil Instruments Ltd., Milton, Cambridge) with the exception of ultra-violet scans for Job plots for which a Pye Unicam SP800 was used.

7.2 Sonication

Cells were disrupted for determination of total β-galactosidase activity by sonication using a 150 watt ultrasonic disintegrator with a titanium exponential microprobe (end diameter 3 mm) supplied by MSE Ltd., Crawley.

7.3 Centrifugation

Routine harvesting of *E. coli* to prepare cell suspensions was carried out by centrifuging cultures at 3000 rpm for 30 minutes using a Mistral 6L centrifuge. Centrifugation of treated cells for electron

microscopy was carried out for 15 minutes at 3000 rpm using the Mistral 6L centrifuge. Other centrifugation was performed for 10 minutes at 3,100 rpm using an MSE Minor centrifuge. Both centrifuges were supplied by MSE Ltd., Crawley.

7.4 Electrophoresis

This was carried out using Cellogel cellulose acetate strips obtained from Gelman Hawksley Ltd., Lancing, in a Reeve-Angel electrophoresis tank with a Vokam SAE 2761 power pack supplied by Shandon Southern Products Ltd., Runcorn.

7.5 Microscopy

(a) Light microscopy

Cells were viewed using a Carl Zeiss Jena student microscope.

(b) Scanning electron microscopy

Cell preparations were coated in gold (10 nm) using an SEM coating unit (Polaron Equipment Ltd., Watford) and scanned on an Alpha 9 scanning electron microscope (International Scientific Instruments (U.K.) Ltd., Newmarket).

(c) Transmission electron microscopy

Cell preparations were embedded in araldite resin using Beem capsules (Taab Laboratories Ltd., Reading). Ultrathin sections were cut using an Ultracut microtome (Reichert-Jung, Vienna) and examined under an acceleration voltage of 60 kV in an AEI EM6B transmission electron microscope.

METHODS

1. DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION (MIC) VALUES AND MINIMUM BACTERICIDAL CONCENTRATION (MBC) VALUES

MIC values were determined in M.L. medium. A range of concentrations of each test substance was prepared in the medium (5 cm^3) and inoculated with one drop of an overnight culture of *E. coli*. After incubation at 37°C for 48 hours the presence or absence of growth was noted.

To obtain approximate MBC values, a loopful of medium from all tubes showing no visible growth was subcultured onto an M.L. agar plate, the presence or absence of growth being noted after 48 hours incubation at 37°C.

2. EFFECT ON GROWING CULTURES

E. coli was grown at 37°C in shaken flasks containing M.L. medium. An inoculum was prepared by incubating a loopful of cells from an overnight slope culture in 5 cm^3 of M.L. medium for 24 hours. This culture was used to inoculate 20 cm^3 of the medium. The OD_{420} was measured at various intervals and the cells also examined by light microscopy. A growth curve was obtained in this manner to determine the duration of log phase.

Growth curves for *E. coli* were also determined by shake culture in the presence of test substances added in water. Concentrations of the test substances used were determined by the MIC values obtained previously.

3. AGAR DIFFUSION ASSAY

Wells 5 mm in diameter were cut out of M.L. agar plates seeded with *E. coli* using a number 3 cork borer. A solution in water of the material under test (0.05 cm^3) was placed in each well each solution being tested at four different concentrations. The plates were kept at 4°C for 2 hours to allow diffusion before incubation at 37°C overnight. The zones of diffusion and inhibition of growth were measured.

A similar procedure using nutrient agar plates was used to determine the effect of *A. mellifera* venom on *S. aureus*.

4. PREPARATION OF BACTERIAL CELL SUSPENSIONS

E. coli was grown by shake cultures using the method described for the effect on growing cultures (Section 2) with a total volume of 125 cm^3. The cells were harvested by centrifugation after 4½ hours. They were then suspended in water at a concentration such that a small sample when diluted by 1 in 100 had an optical density at 420 nm (OD_{420}) of 0.50 to 0.55 and stored at 4°C.

5. CELL TREATMENT

Immediately before use cell suspensions were diluted two fold with 0.1 M Tris-HCℓ buffer. Samples (0.2 cm^3) were added to pre-warmed 0.004 M EDTA (0.1 cm^3) and incubated at 37°C for 3 minutes. 0.02 M $MnCℓ_2$ (0.05 cm^3) was added to stop the reaction. After a further 1 minute 0.05 cm^3 of appropriate venom solution was added and the mixture incubated for 20 minutes before quenching the reaction by addition of cold PMSH (3.6 cm^3). In some circumstances water replaced

EDTA and $MnCl_2$. Other solutions were added before, after or instead of the venom solution as required. The final volume of the reaction mixture was always 0.4 cm^3.

6. β-GALACTOSIDASE ASSAY

After treatment as above cells were diluted 1 to 50 with PMSH. 2 cm^3 aliquots of this mixture were assayed for β-galactosidase activity:-

The samples were warmed to 37°C for 2 minutes, ortho-nitrophenylgalactopyranoside (ONPG)(0.2 cm^3) added and the reaction stopped after 10 minutes incubation by addition of 1M Na_3CO_3 (1 cm^3). The OD_{420} was read against a blank of distilled water. A calibration curve of ortho-nitrophenol (ONP) was constructed (Figure 1) and β-galactosidase activity was calculated in terms of the μg ONP produced / cm^3/min.

Total β-galactosidase activity was measured by sonication of the cells following the method of Slein and Logan (1967), the probe being immersed as far as possible and four 30 second treatments at 12 μ each followed by a minute in ice were used.

7. CELL VIABILITY MEASUREMENTS

For cell viability measurements 1 cm^3 samples of the cells were diluted in series (1+9) with $\frac{1}{4}$ strength Ringers' solution. Using the method of Miles and Misra (1938) one drop ($\frac{1}{30}$ cm^3) of the diluted suspension was applied to each quarter of a poured M.L. agar plate. Two plates were used for each dilution of each suspension to give eight samples. After 48 hours incubation at 37°C, colonies of viable cells were counted.

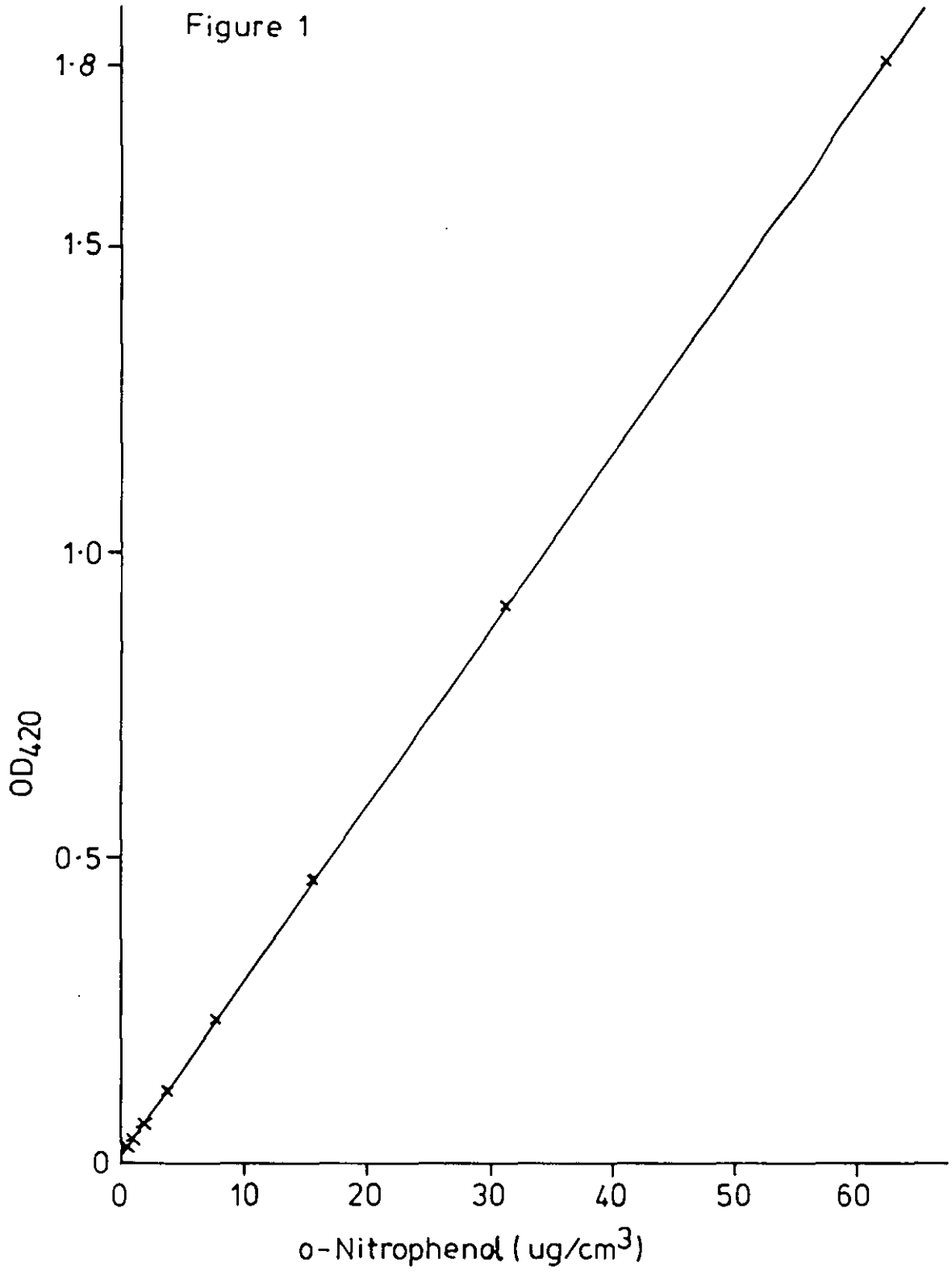


FIGURE 1

Calibration curve for ortho-nitrophenol

8. DETERMINATION OF CELL LYSIS

The degree of cell lysis was estimated by measuring the OD_{420} of treated cells after addition of PMSH (3.6 cm^3) (Section 5 above). Lysis was considered to have occurred when the OD_{420} of the treated cell suspension was less than that of the control cells.

9. LOSS OF CELL PROTEIN CONSTITUENTS

After determination of cell lysis, cell suspensions were centrifuged (bench centrifuge) and the OD_{260} of the supernatant solutions were measured to determine loss of the 260 nm-absorbing material (protein) from the cells.

10. SEPARATION OF VENOM COMPONENTS

10.1 Gel Filtration

Separation by gel filtration was carried out using a column (14 cm high with an internal diameter of 1.2 cm) packed with Sephadex G75 (fine or superfine). Venom samples (25 mg/2 cm^3) were eluted with distilled water, 4 cm^3 fractions being collected. The OD_{235} of the fractions was measured. Fractions (pooled where applicable) were freeze-dried and redissolved in distilled water to give concentrations approximating to the original sample. These were then tested on *E. coli* suspensions and compared with whole venom.

10.2 Electrophoresis

This was carried out for 30 minutes with a constant voltage of 200 using a barbitone buffer with methyl green as a marker unless otherwise specified. Two cellulose acetate strips were run

simultaneously, one being stained with Ponceau S while the other was laid face down onto an M.L. agar plate either seeded or flooded with *E. coli* cells. The plates were incubated at 4°C. After 2 hours, the strips were removed and incubation of the plates continued at 37°C overnight. Zones of inhibition of growth were noted and measured.

11. ELECTRON MICROSCOPY

11.1 Scanning Electron Microscopy

Glutaraldehyde (25% w/v) was added to an *E. coli* culture (25 cm^3 M.L. medium) to give a final concentration of 1.5% w/v. After two minutes contact, cells were removed by centrifugation at 3000 rpm for 15 minutes at 4°C. The cells were resuspended in 2 cm^3 glutaraldehyde (5% w/v) in veronal acetate buffer and stored at 4°C for 16 hours. The cells were collected by centrifugation as before, washed three times with distilled water and resuspended to the required cell density. One drop of this suspension was allowed to air dry on a 13 mm coverslip and was then dehydrated over calcium chloride under partial pressure (Bulman and Stretton 1974). They were then gold coated (10 nm) and examined in an Alpha 9 scanning electron microscope.

11.2 Transmission Electron Microscopy

Cells were initially prepared as above but washed three times with veronal acetate buffer. The final cell pellet was embedded into 4% w/v purified agar which was then cut into 1 mm cubes, stained with 1% w/v osmium tetroxide for 30 minutes followed by 2% w/v uranyl acetate. The samples were dehydrated using the

following alcohol series: (% v/v) 25, 50, 75, 100. They were then taken through alcohol/propylene oxide (50/50), propylene oxide (100%), propylene oxide/araldite resin (50/50) into araldite resin (100%). Ultrathin sections were supported on copper grids and stained with Reynolds' lead citrate to increase contrast. One drop of lead citrate solution and two drops of water were placed separately on a wax strip. The grid was placed onto the lead citrate for 2-5 minutes, then onto one water drop for 2 minutes and finally onto the second water drop before being dried. They were then examined in an EM6B transmission electron microscope.

12. INTERACTION OF Ca^{2+} WITH TEST SUBSTANCES: JOB PLOTS

A series of solutions was set up whereby the concentration of the test solution increased as that of Ca^{2+} ions decreased. The solutions were scanned over the ultraviolet range over a period of time. Job plots were obtained by plotting the initial and final optical densities at a wavelength where absorption occurred (Job 1928).

Section 3

RESULTS

1. GROWTH CURVE FOR *E. coli*

Figure 1 shows the growth curve obtained for *E. coli* in M.L. medium with vigorous shaking at 37°C. Log phase was from 0-5 hours.

2. ACTION OF VENOMS AND POLYPEPTIDES AGAINST *E. coli*

2.1 Minimum Inhibitory Concentration (MIC) Values

The MIC values determined for the venoms and melittin against *E. coli* are given in Table 1. Greatest activity is shown by *A. mellifera* venom ≅ melittin > *N. naja sputatrix* venom > *V. russellii* venom > *C. adamanteus* venom.

TABLE 1

Minimum Inhibitory Concentration Values against *E. coli* (using one drop of culture containing *ca* 4×10^6 cells/cm^3 as inoculum).

Test Substance	MIC values (mg/cm^3)
C. adamanteus venom	>24
V. russellii venom	24
N. naja sputatrix venom	1.6
A. mellifera venom	0.05
Melittin	0.07

2.2 Minimum Bactericidal Concentration (MBC) Values

Approximate MBC values (Table 2) for the more active substances were generally found to be double the MIC values.

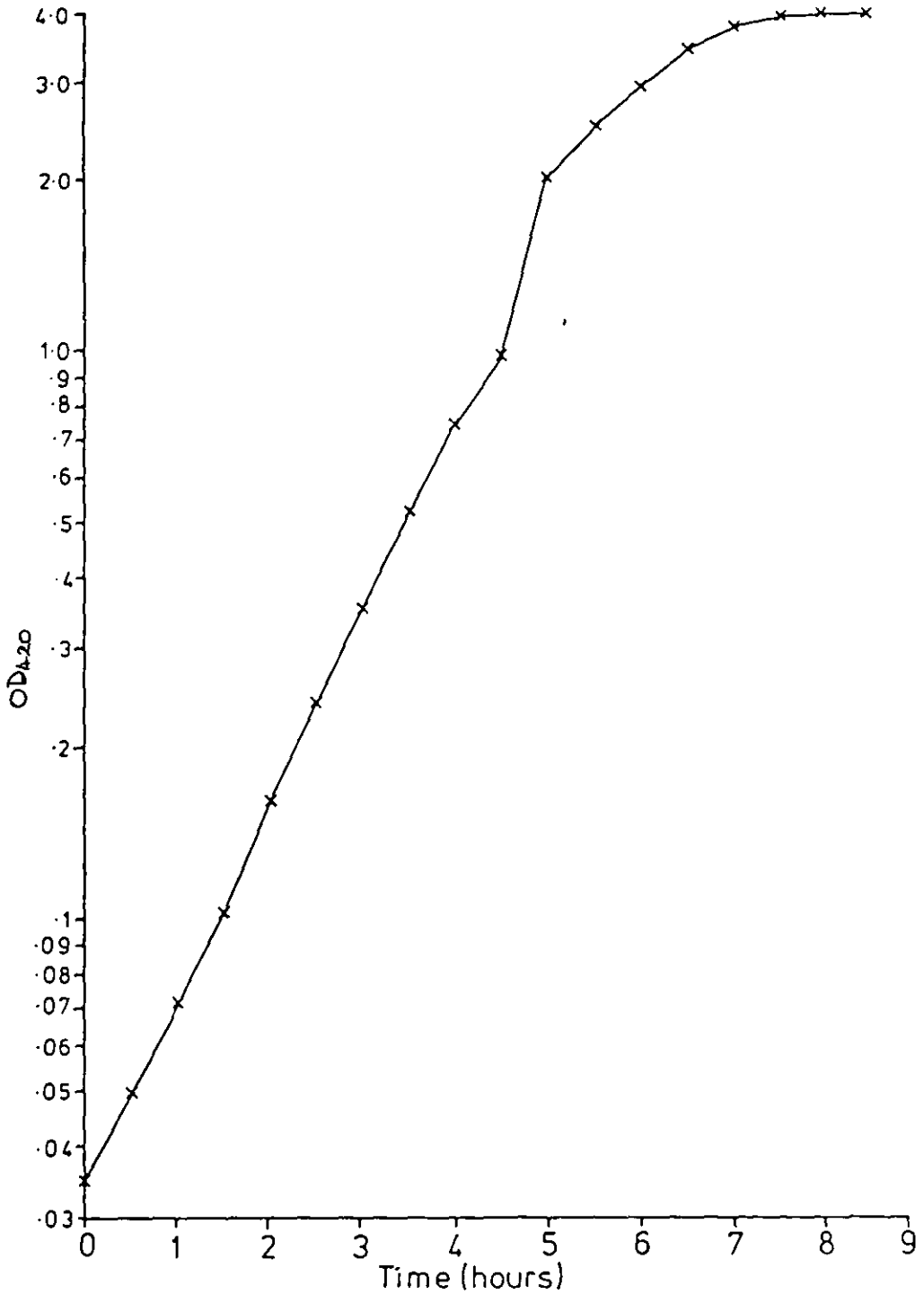


FIGURE 1

Growth curve for *E. coli* incubated at 37°C utilising minimal lactose medium (M.L.medium).

TABLE 2

Minimum Bactericidal Concentration Values against *E. coli* (inoculum as in Table 1)

Test Substance	MBC Values (mg/cm^3)
C. adamanteus venom	>24
V. russellii venom	24
N. naja sputatrix venom	>16
A. mellifera venom	0.1
Melittin	0.14

2.3 Effect on Growing Cultures

Addition of *N. naja sputatrix* and *A. mellifera* venoms and melittin to log phase shake cultures of *E. coli* caused inhibition of growth (Figures 2-4). In the presence of levels of >0.5 × MIC for *N. naja sputatrix* or >0.25 × MIC for *A. mellifera* venoms (MIC values from Table 1) growth continued for 45 minutes after addition before stopping completely. With lower concentrations the growth rate was reduced, but with time approached that of the control system.

Examination of the cells by light microscopy showed no apparent changes in cell morphology but the cells became fewer and less motile as the concentrations increased.

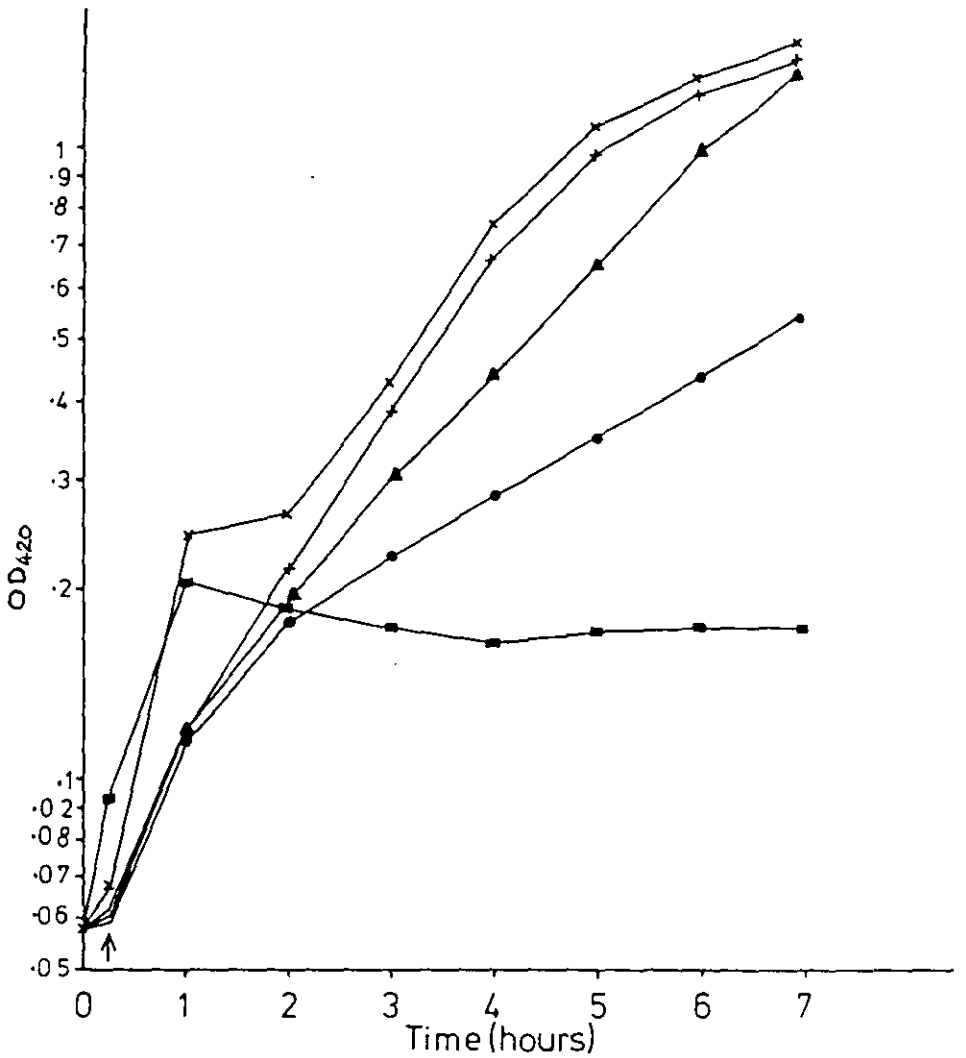


FIGURE 2

The effect of *N.naja sputatrix* venom on a log. phase shake culture of *E. coli* incubated at 37°C grown on M.L.medium. Addition of the venom is shown by ↑ .

× Control culture

\+ 100 μg/cm^3 *N. naja sputatrix* venom

▲ 200 μg/cm^3 *N. naja sputatrix* venom

● 400 μg/cm^3 *N. naja sputatrix* venom

■ 800 μg/cm^3 *N. naja sputatrix* venom

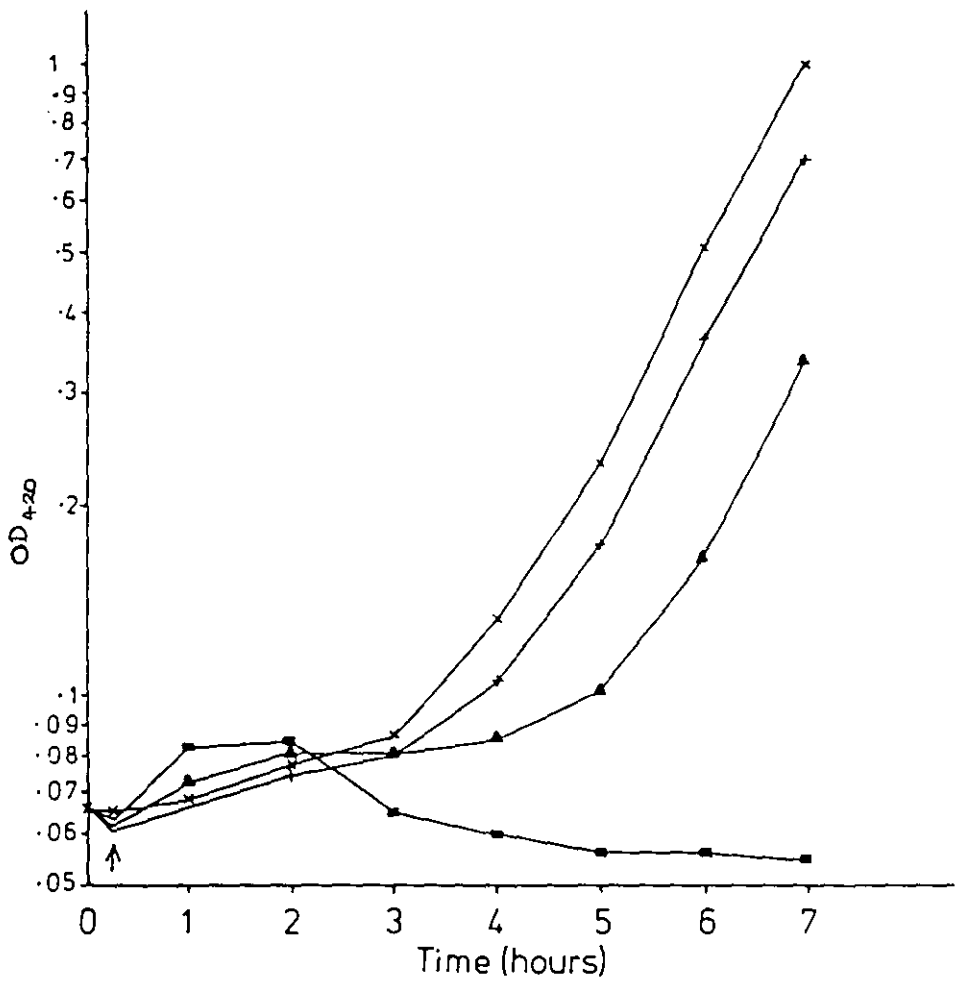


FIGURE 3

The effect of *A. mellifera* venom on a log. phase shake culture of *E. coli* incubated at 37°C grown on M.L. medium. Addition of the venom is shown by ↑.

× Control culture

\+ 3.2 μg/cm^3 *A. mellifera* venom

▲ 6.4 μg/cm^3 *A. mellifera* venom

■ 12.8 μg/cm^3 *A. mellifera* venom

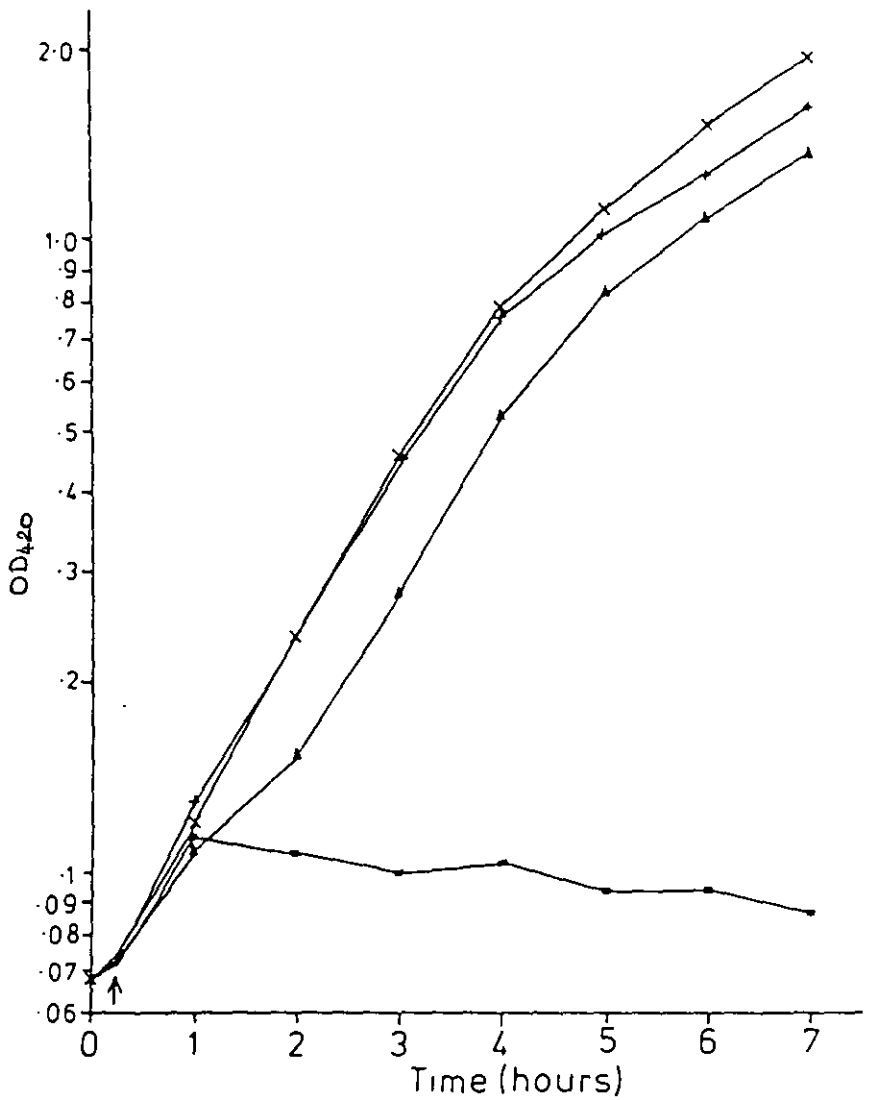


FIGURE 4

The effect of melittin on a log. phase shake culture of *E. coli* incubated at 37°C grown on M.L.medium. Addition of melittin is shown by ↑ .

× Control culture

\+ 4.8 μg/cm^3 melittin

▲ 9.2 μg/cm^3 melittin

■ 18.4 μg/cm^3 melittin

2.4 Agar Diffusion Assay

Neither *V. russellii* (5-40 mg/cm^3) nor *C. adamanteus* (5-40 mg/cm^3) venoms could be seen to have diffused into the agar (Figure 5a,b). *N. naja sputatrix* venom (5-40 mg/cm^3) and cardiotoxin *(N. naja siamensis)* (2.5-20 mg/cm^3) both diffused over large areas (Figure 6a,b) without showing any inhibition of *E. coli* growth. On the other hand inhibition of growth of *E. coli* over the whole of the well-defined diffusion zones could be seen with both *A. mellifera* venom (5-40 mg/cm^3) and melittin (1.25-10 mg/cm^3) (Figure 7a,b).

For comparative purposes the action of *A. mellifera* venom against *S. aureus* was investigated. *A. mellifera* venom (5-40 mg/cm^3) was added to wells in nutrient agar plates seeded with *S. aureus*. Growth was inhibited by all concentrations within the whole diffusion zone.

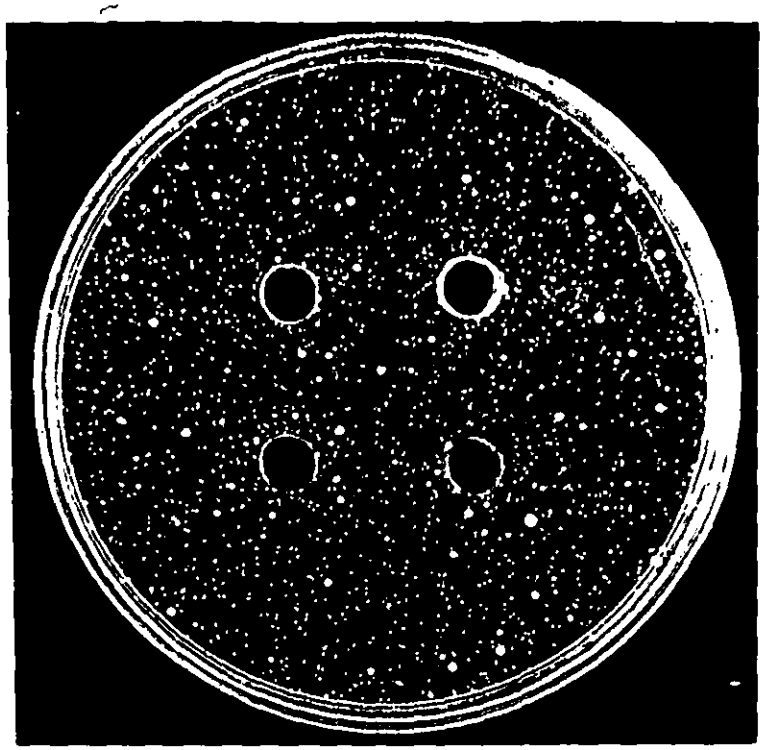
3. RELEASE OF β-GALACTOSIDASE FROM NON-PROLIFERATING CELLS.

Having ascertained that some venoms exhibit antibacterial activity, studies were carried out to investigate the nature of this activity and to determine whether certain components such as polypeptide toxins may be the cause. The principal method was by measuring leakage of the enzyme β-galactosidase from non-proliferating cells which would result from physical damage to the cell envelope.

3.1 Assay Conditions for β-galactosidase.

(a) Enzyme optimum parameters.

Cell suspensions were treated with water and assayed for β-galactosidase activity. Optimum conditions were found to be an initial concentration of ONPG of 4 mg/cm^3 (Figure 8) and a pH of 7.2 (Figure 9). Activity increased with temperature up to 45°C



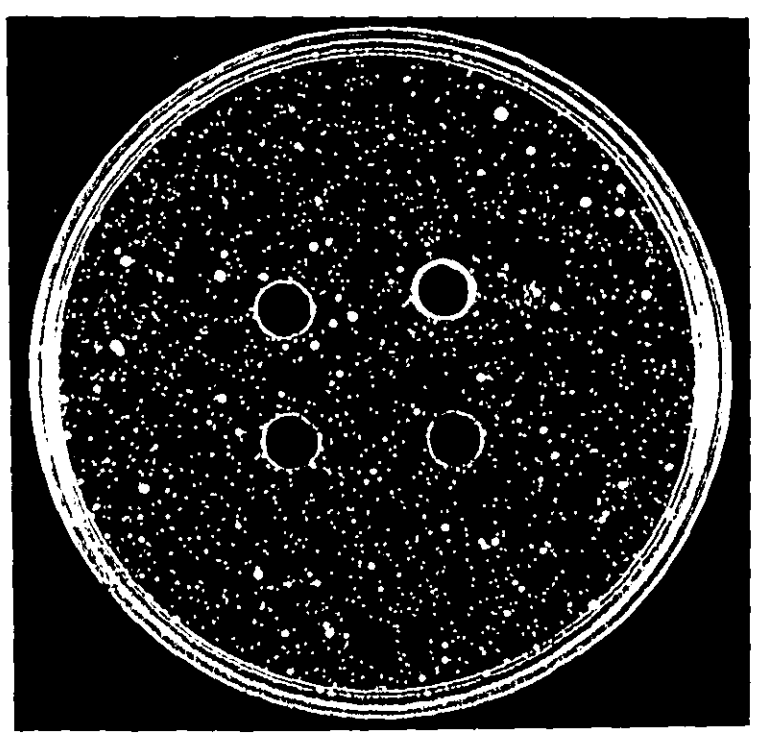


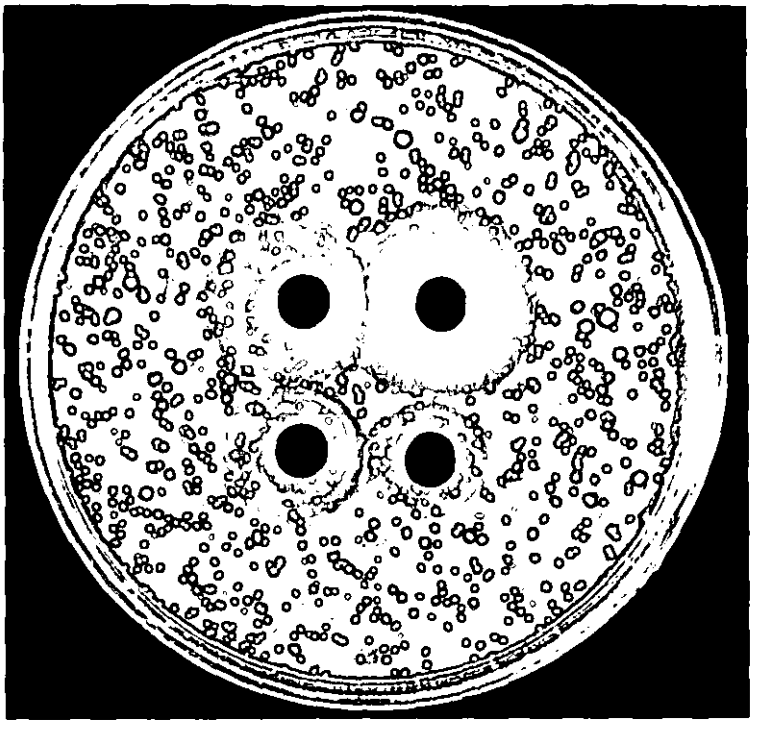
FIGURE 5

The effect of venoms after diffusion in minimal lactose agar on *E. coli* incubated at 37°C.

a) *V. russellii* venom

b) *C. adamanteus* venom

a)



b)

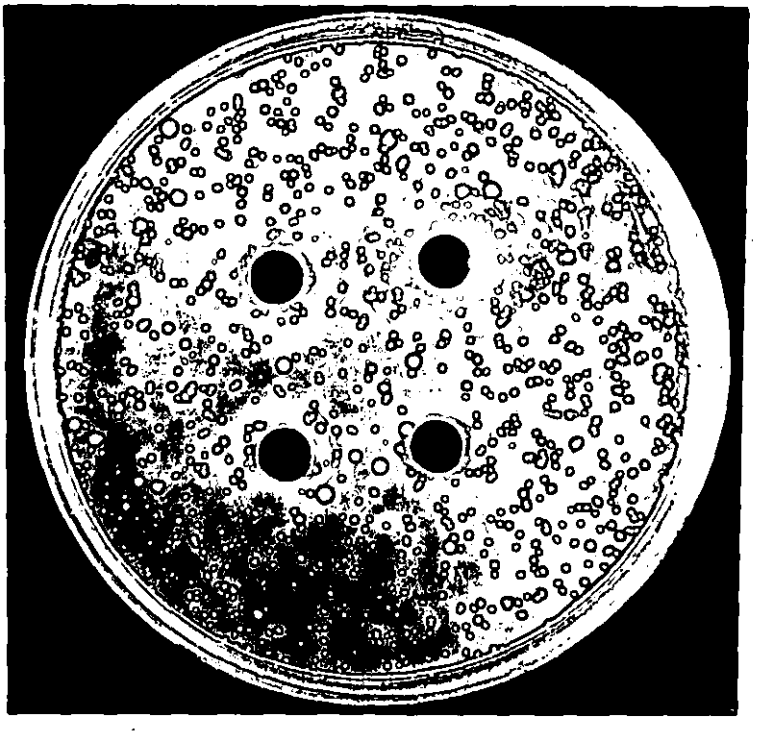
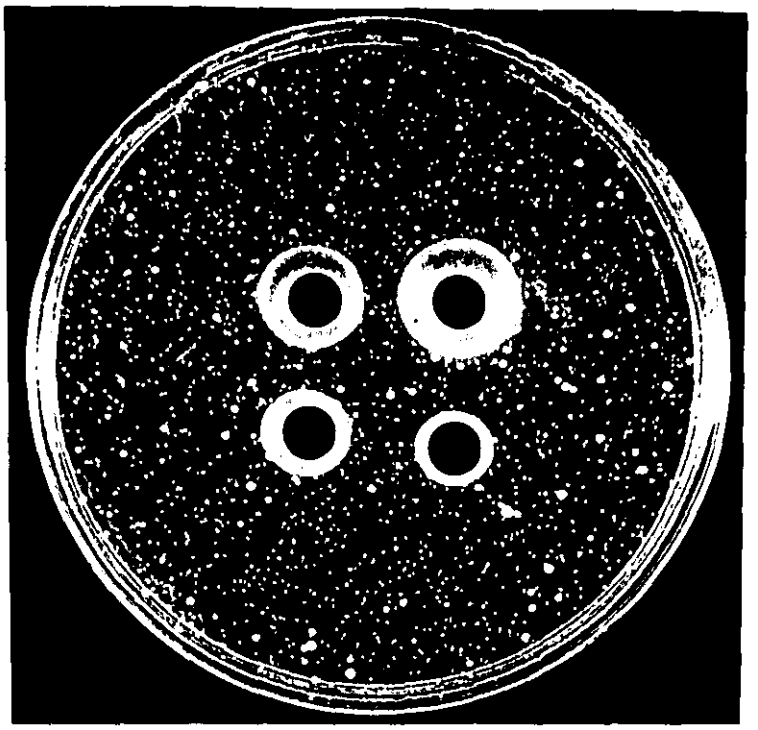


FIGURE 6

The effect of venoms and toxin after diffusion in minimal lactose agar on *E. coli* incubated at 37°C.

a) *N. naja sputatrix* venom

b) Cardiotoxin



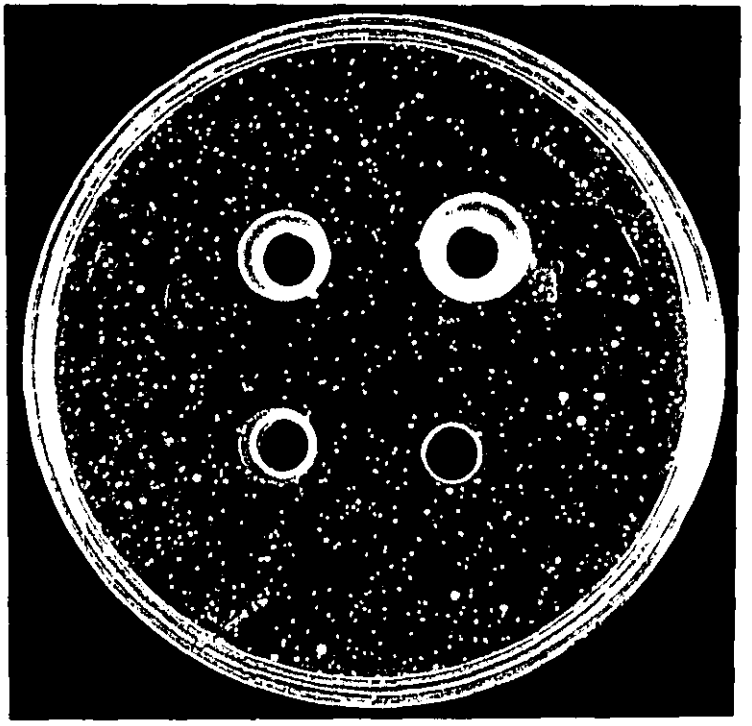


FIGURE 7

The effect of venom and melittin after diffusion in minimal lactose agar on *E. coli* incubated at 37°C.

a) *A. mellifera* venom

b) Melittin

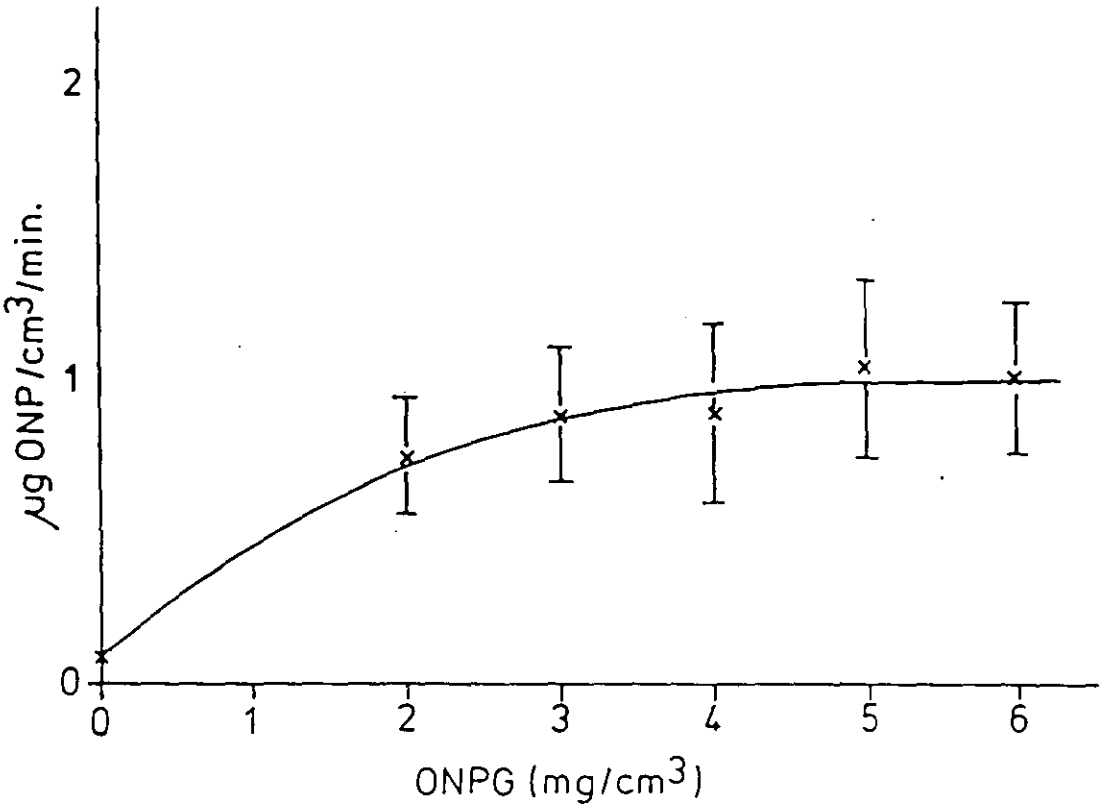


FIGURE 8

The effect of ONPG concentration on the assay of ß-galactosidase activity in *E. coli* cell suspensions incubated for 10 minutes at 37°C, pH 7.2.

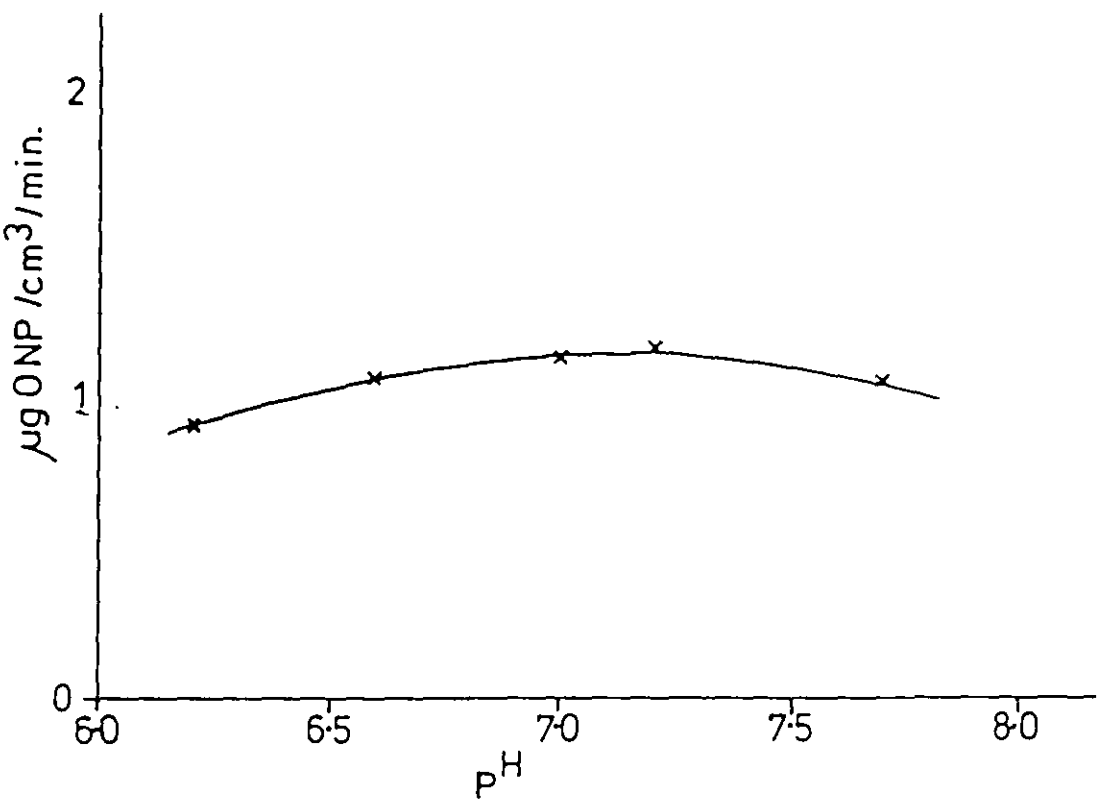


FIGURE 9

The effect of pH on the assay of ß-galactosidase activity in *E. coli* cell suspensions incubated with 4 mg ONPG/cm^3 for 10 minutes at 37°C.

(Figure 10) and had not reached maximum after 120 minutes incubation (Figure 11).

As the cell concentration was increased the β-galactosidase release also increased but this was complicated by increased turbidity of the solution. A concentration was chosen where the light scattering caused by the cells did not affect the absorbance of the ortho-nitrophenol (ONP) at OD_{420}.

Conditions chosen for future work were:-

Time	:	10 mins to give a suitable OD reading
pH	:	7.2
ONPG	:	4 mg/cm^3
Cell number	:	approx. 2×10^4 $cells/cm^3$

(b) *E. coli* growth medium.

E. coli was grown on a variety of media utilising either glucose or lactose as carbon source with or without isopropyl-β-D-thiogalactopyranoside (ITGP) and assayed for β-galactosidase. An increase of β-galactosidase in lactose-grown cells compared with glucose-grown cells was observed in both basal, unstimulated release (Figure 12 and Table 3) and in total levels as adjudged by cell sonication (Table 3). Addition of ITGP to the growth medium caused an increase in β-galactosidase (Figure 12).

Unless otherwise stated the minimal medium containing only lactose as inducer (M.L. medium) was used in future experiments.

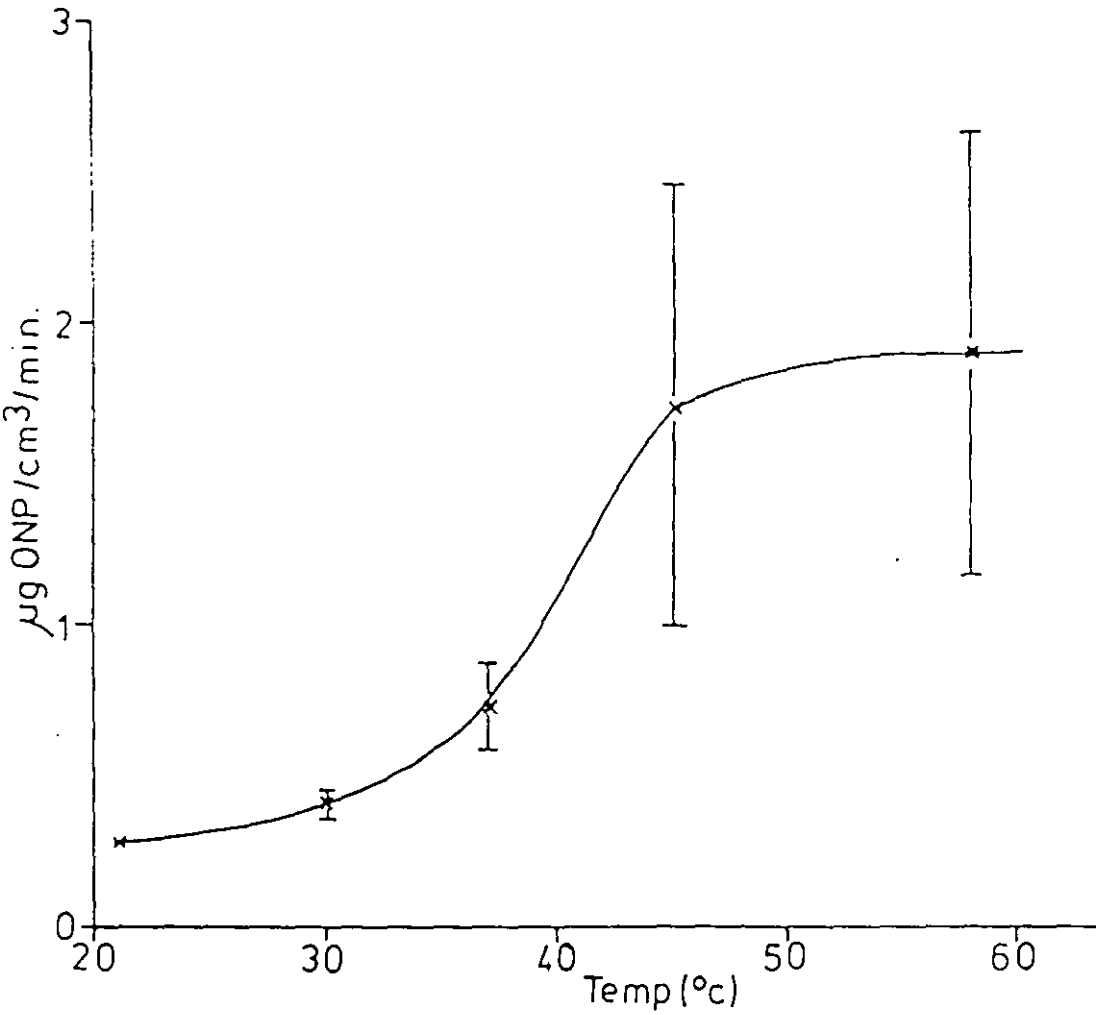


FIGURE 10

The effect of temperature on the assay of β-galactosidase activity in *E. coli* cell suspensions treated with 4 mg ONPG/cm^3 for 10 minutes at pH 7.2.

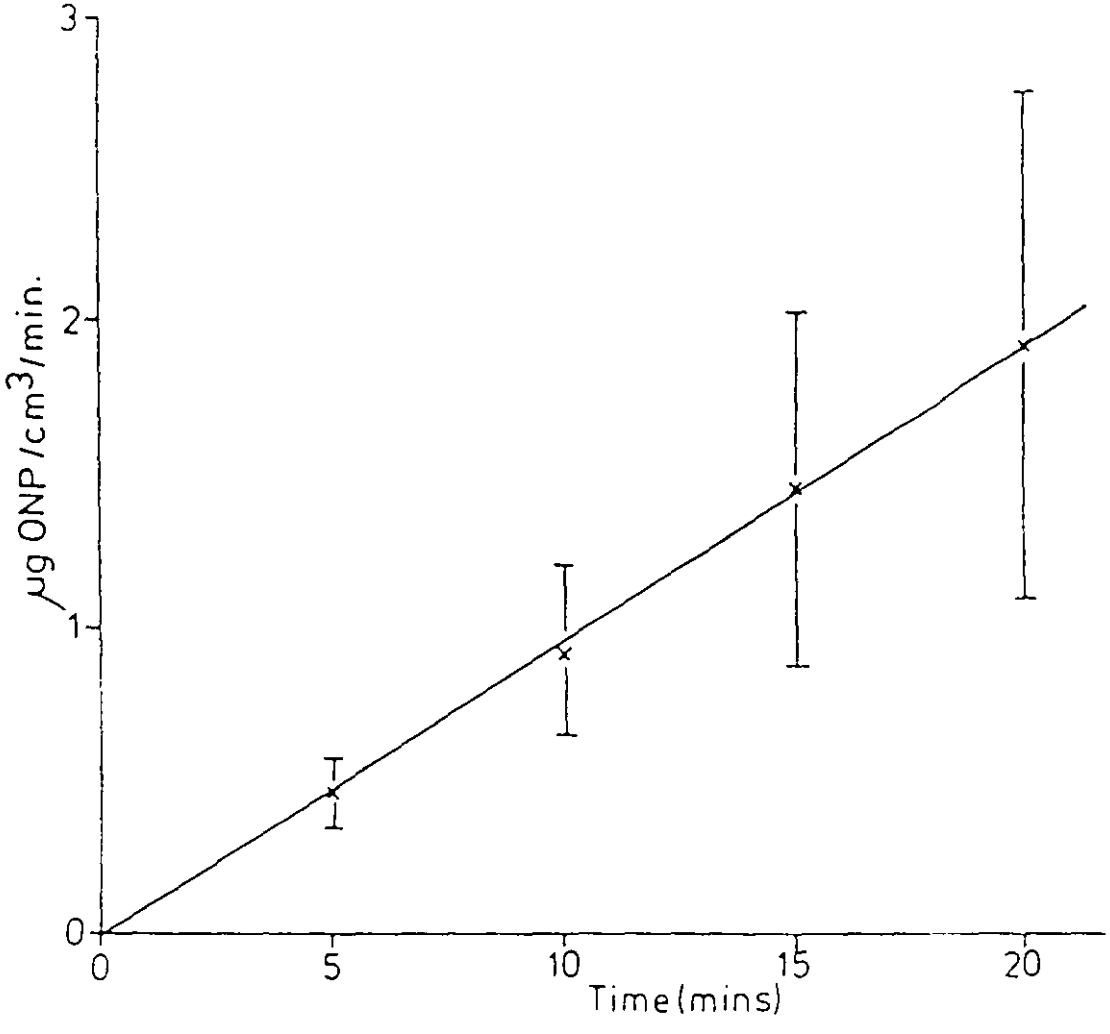


FIGURE 11

The effect of time on the assay of β-galactosidase activity in *E. coli* cell suspensions treated with 4 mg ONPG/cm^3 at 37°C, pH 7.2.

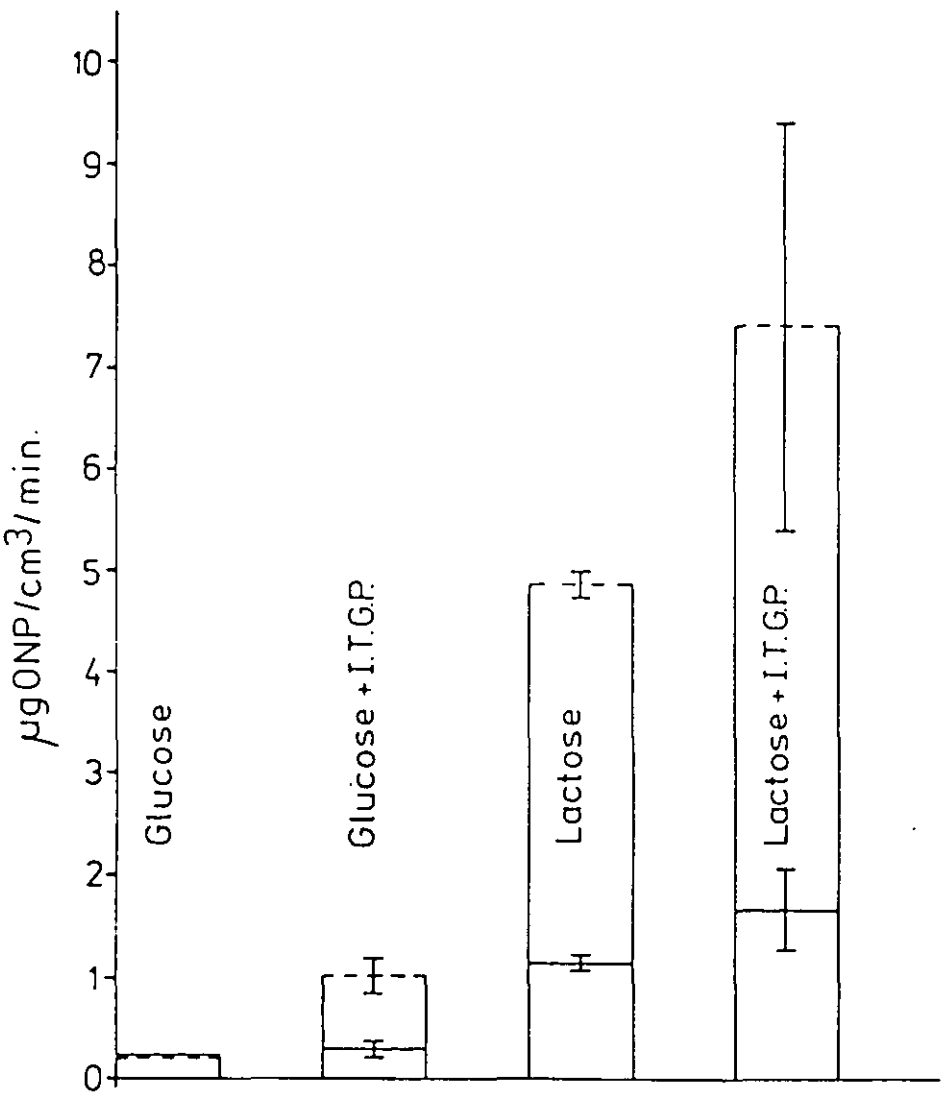


FIGURE 12

The effect of growth medium composition on the β-galactosidase activity of *E. coli* cell suspensions. Conditions of β-galactosidase assay were 4 mg ONPG/cm^3 for 10 minutes at 37°C, pH 7.2.

——— Control suspensions

----- Suspensions treated with *A. mellifera* venom, 625 μg/cm^3

TABLE 3

β-Galactosidase Activity of *E. coli* Cells Grown on Different Carbon Sources Enzyme conditions as given in 3.1a

Carbon source	β-galactosidase activity (μg/cm^3/min)	
	Un-sonicated cells	Sonicated cells
Lactose	1.35	6.50
Glucose	0.38	0.80

(c) Cell Storage.

Cell suspensions were stored at 4°C prior to use and samples over a period of 17 days were assayed to measure the effect of storage time. Untreated cell samples showed little overall difference in released enzyme activity. After 17 days storage β-galactosidase was 0.85 μg ONP produced /cm^3/min compared with 0.7 μg ONP produced /cm^3/min prior to storage. Stored cell suspensions were always used within 14 days.

3.2 β-Galactosidase Release Induced by Whole Venom.

(a) Dose-response curves.

None of the venoms were shown to have inherent β-galactosidase activity when incubated with the substrate ONPG alone.

Treatment of *E. coli* with crude venoms in the range 0-1250 μg/cm^3 caused increasing β-galactosidase activity in the supernatant with increasing concentration. The venoms varied in effectiveness in the order *A. mellifera* > *N. naja sputatrix* > *V. russellii* > *C. adamanteus* (Figure 13).

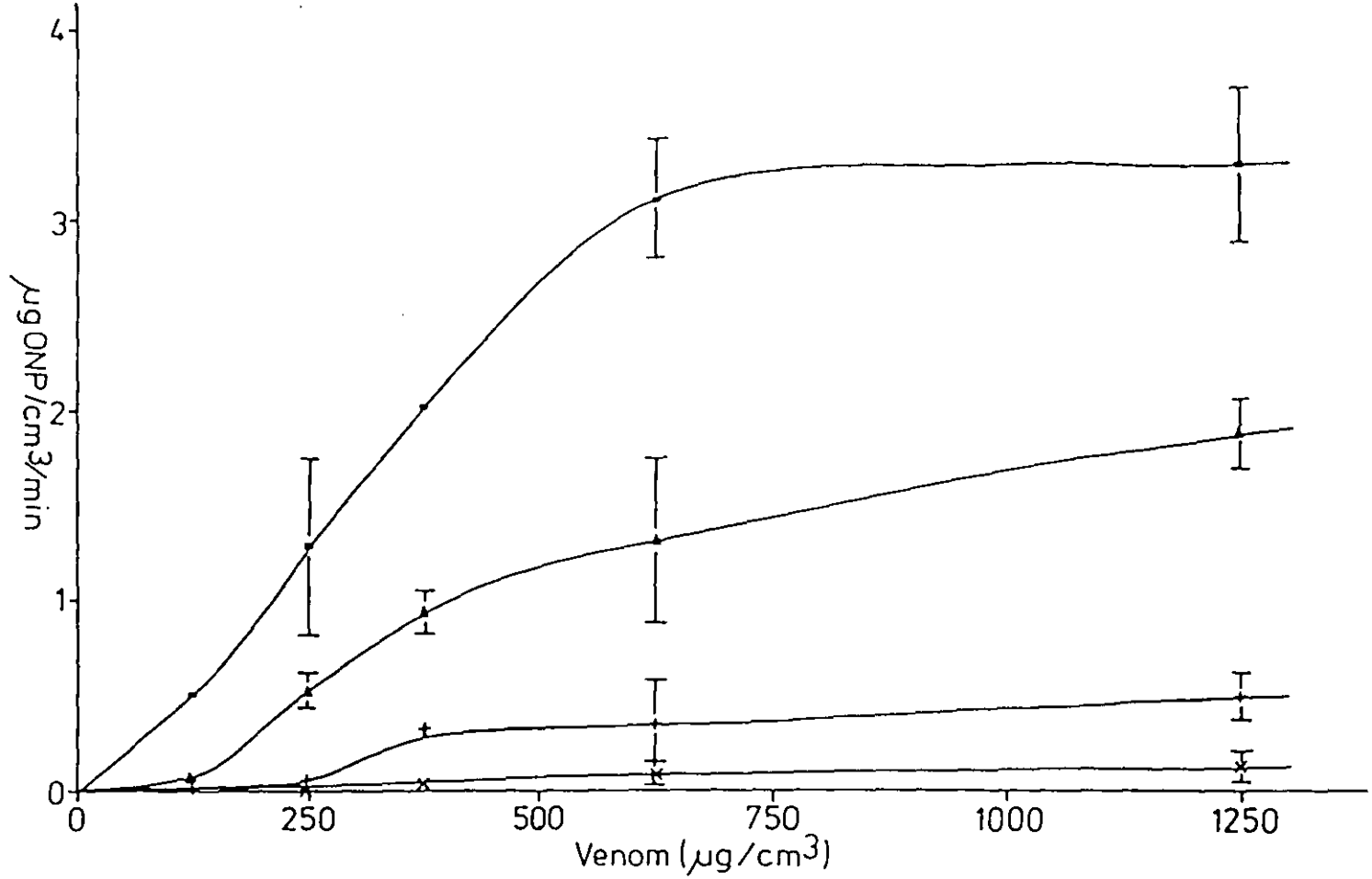


FIGURE 13

Release of β-galactosidase from *E. coli* by venom treatment. Cell suspensions were incubated at 37°C with or without venom for 20 minutes before being diluted 1 in 50 with PMSH buffer and assayed for β-galactosidase.

×—— × *C. adamanteus* venom.

+—— + *V. russellii* venom.

▲—— ▲ *N. naja sputatrix* venom.

■—— ■ *A. mellifera* venom.

Since it was intended to store the cell suspensions, venom action was investigated after varying times of storage. Little difference was observed when compared with controls (Figure 14).

(b) Assay conditions.

Experiments in which the β-galactosidase assay conditions were evaluated (Section 3.1a above) were repeated in the case of *A. mellifera* treated cells. The enzyme responses were seen to be the same as control responses (Figures 15-18).

(c) Effect of Ions and other materials.

(i) EDTA

When cells were treated with EDTA (1 mM), β-galactosidase release increased by 0.41 ± 0.31 μg ONP/cm^3/min (10 experiments) from a control value of 1.0 ± 0.29 μg ONP/cm^3/min.

Increasing the concentration of EDTA in the presence of *A. mellifera* venom results in an increase in β-galactosidase release (Figure 19). This was not observed with the other venoms though there was a small initial rise. With increased venom (625 μg/cm^3) and 1 mM EDTA a stimulation was found with *V. russellii* and *N. naja sputatrix* venoms but not with *A. mellifera* or *C. adamanteus* venoms under the conditionsemployed (Figure 20).

(ii) $MnCl_2$

A preliminary survey showed that $MnCl_2$ (2.5 mM) did not cause any alteration in β-galactosidase release from *E. coli* cells alone or in the presence of crude venom (250 μg/cm^3). $MnCl_2$ added after 3 minutes incubation with EDTA in the absence of venom, blocked

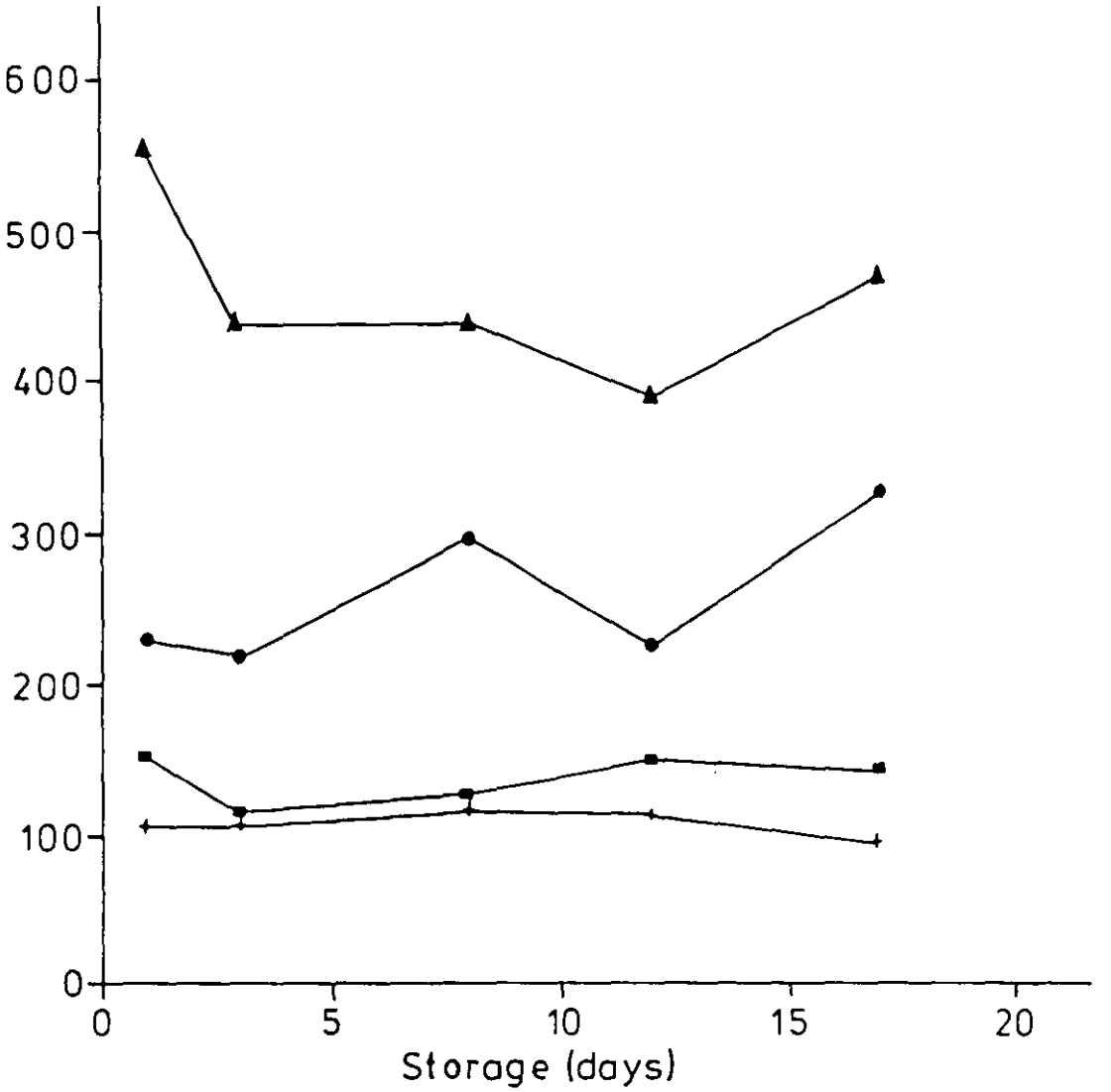


FIGURE 14

The effect of storage on *E. coli* cell suspensions. Release of β-galactosidase from venom-treated cells was compared with control cells (= 100%). All venom concentrations were 625 $\mu g/cm^3$. Treatment was for 20 minutes at 37°C.
Release by control cells ($\mu g/cm^3$/min) after 1 day : 0.7; after 3 days : 0.95; after 8 days : 0.8; after 12 days : 1.1; after 17 days : 0.85.

+ —— + *C. adamanteus* venom
■ —— ■ *V. russellii* venom
● —— ● *N. naja sputatrix* venom
▲ —— ▲ *A. mellifera* venom

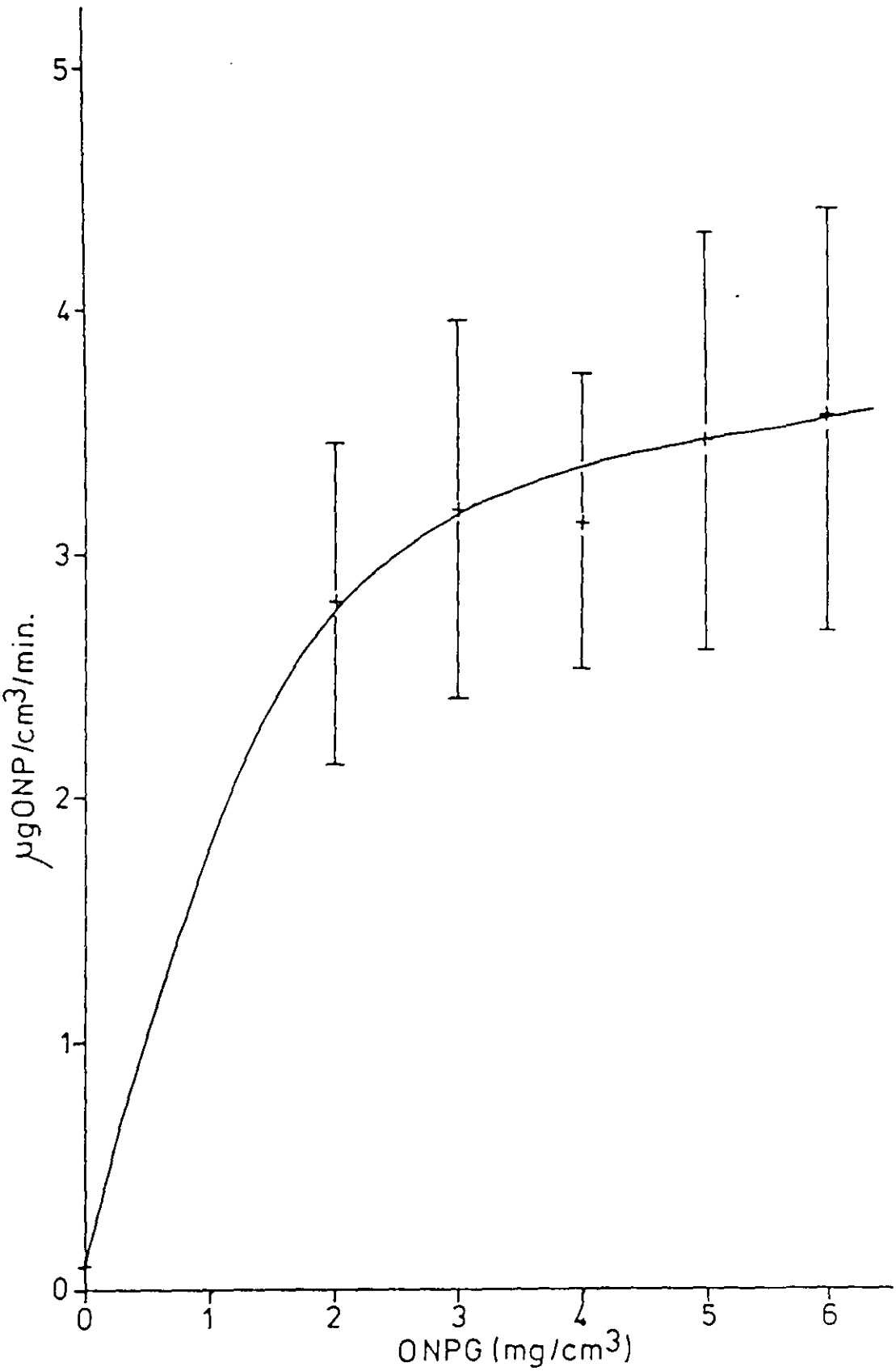


FIGURE 15

The effect of ONPG on the assay of ß-galactosidase release from *E. coli* cells treated with *A. mellifera* venom, 625 µg/cm^3. Other conditions were as figure 12.

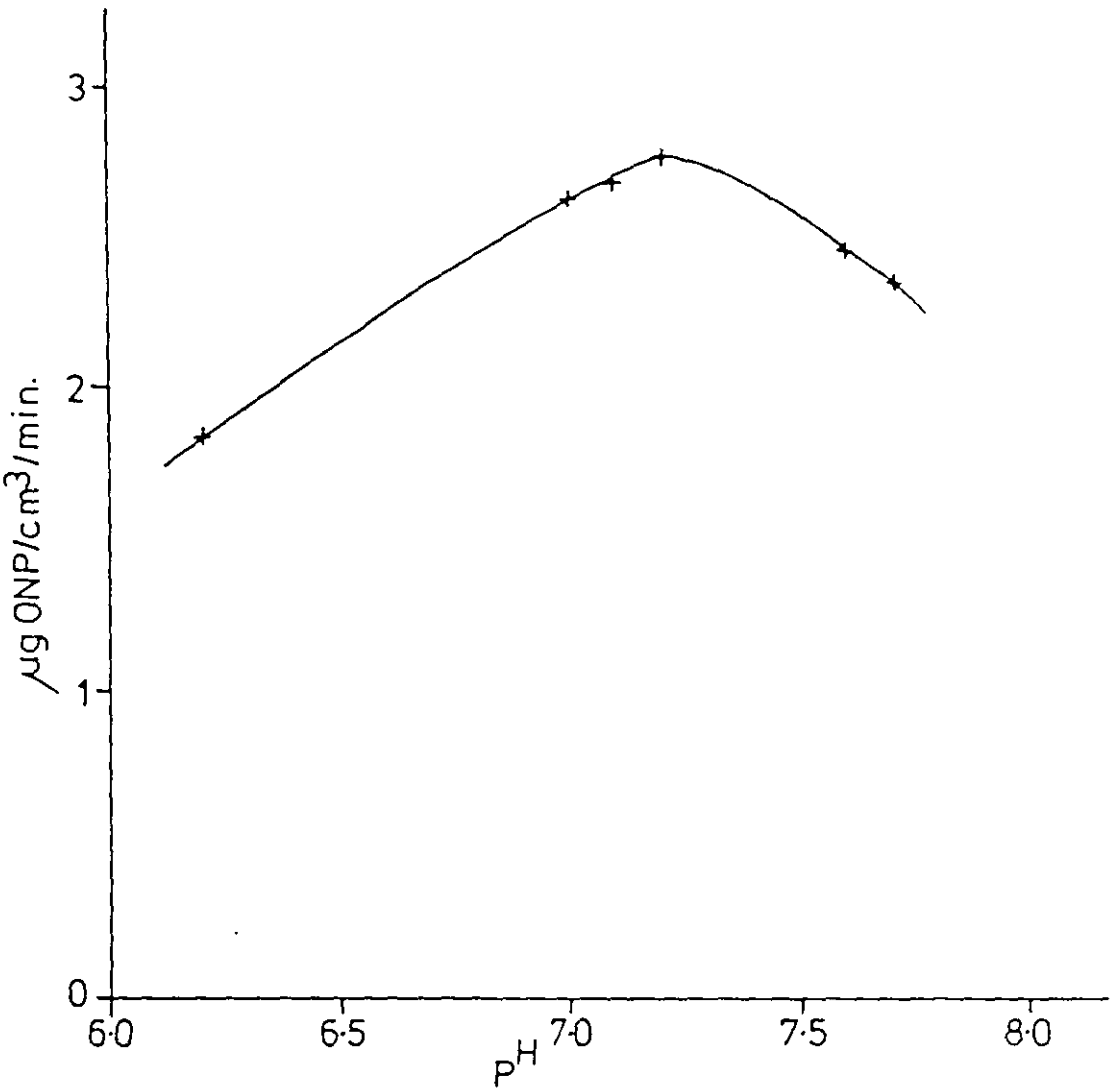


FIGURE 16

The effect of pH on the assay of β-galactosidase release from *E. coli* cells treated with *A. mellifera* venom, 625 µg/cm^3. Other conditions as figure 12.

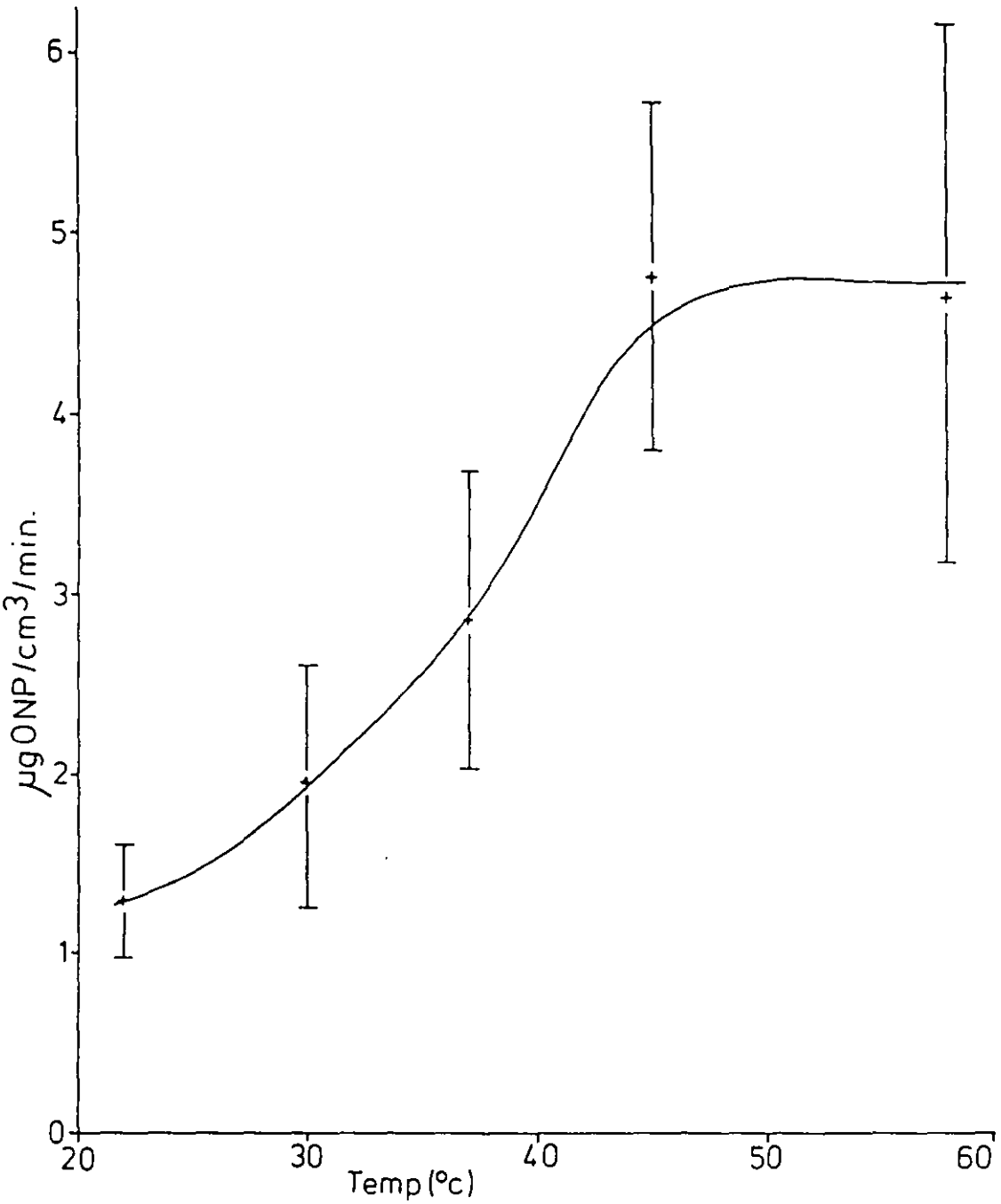


FIGURE 17

The effect of temperature on the assay of ß-galactosidase release from *E. coli* cells treated with *A. mellifera* venom, 625 µg/cm^3. Other conditions as figure 12.

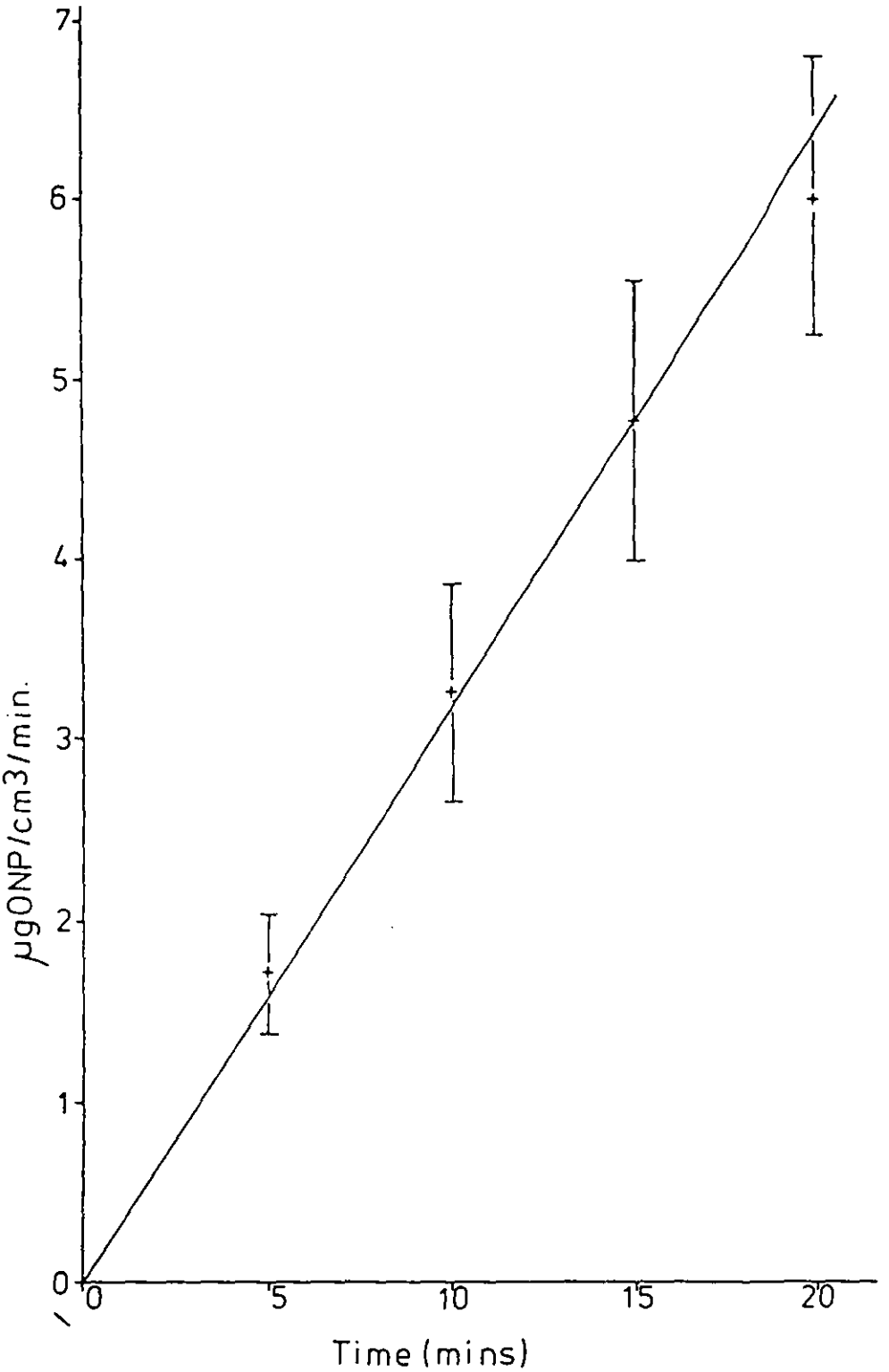


FIGURE 18

The effect of time on the assay of ß-galactosidase release from *E. coli* cells treated with *A. mellifera* venom, 625 µg/cm^3. Other conditions as figure 12.

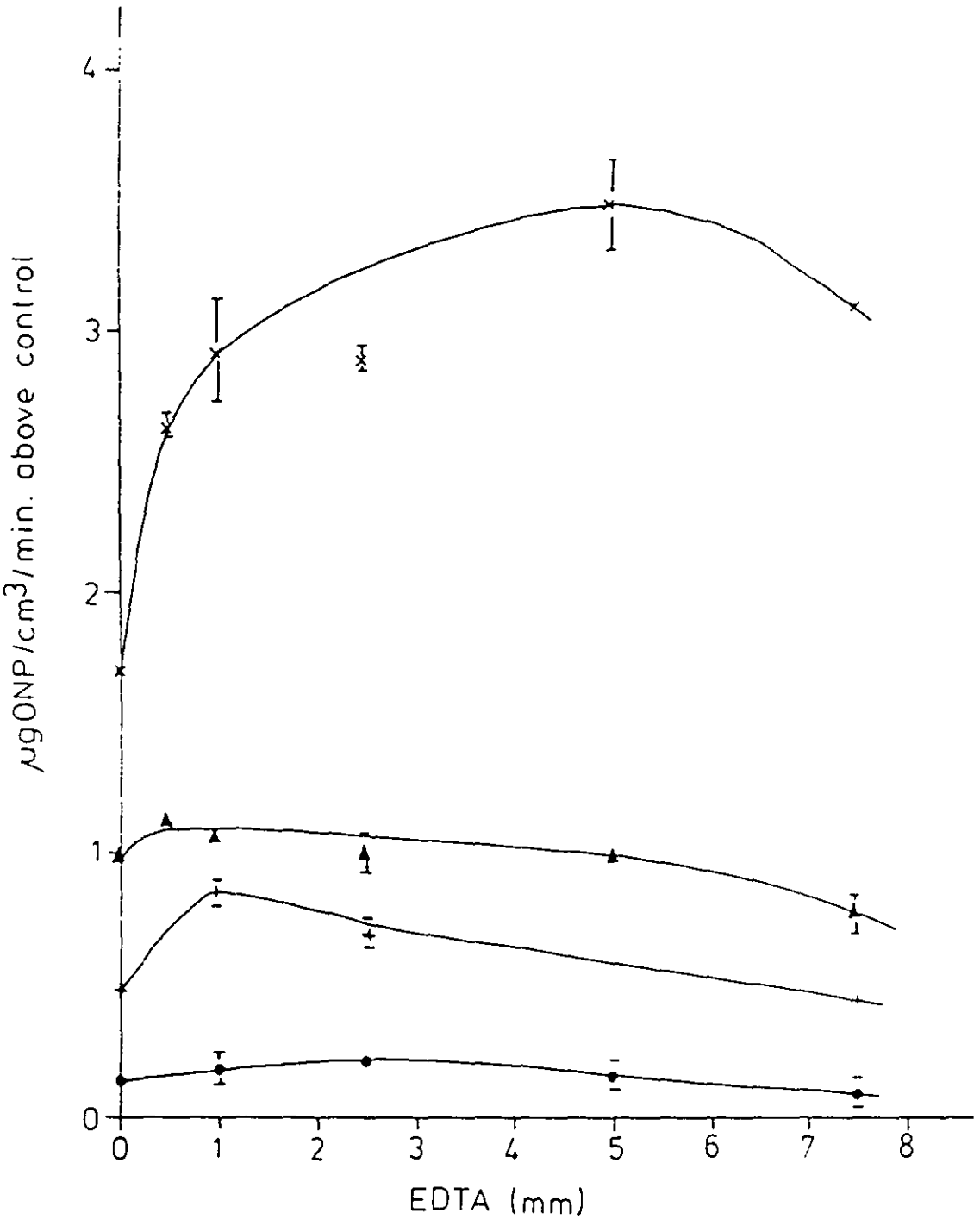


FIGURE 19

The effect of EDTA on β-galactosidase released from *E. coli* by venom treatment at 37°C. All venom concentrations were 250 μg/cm^3 and venom was added 3 minutes after EDTA addition, incubation being continued for 20 minutes.

●—● *C. adamanteus* venom

+ — + *N. naja sputatrix* venom

▲—▲ *V. russellii* venom

× — × *A. mellifera* venom

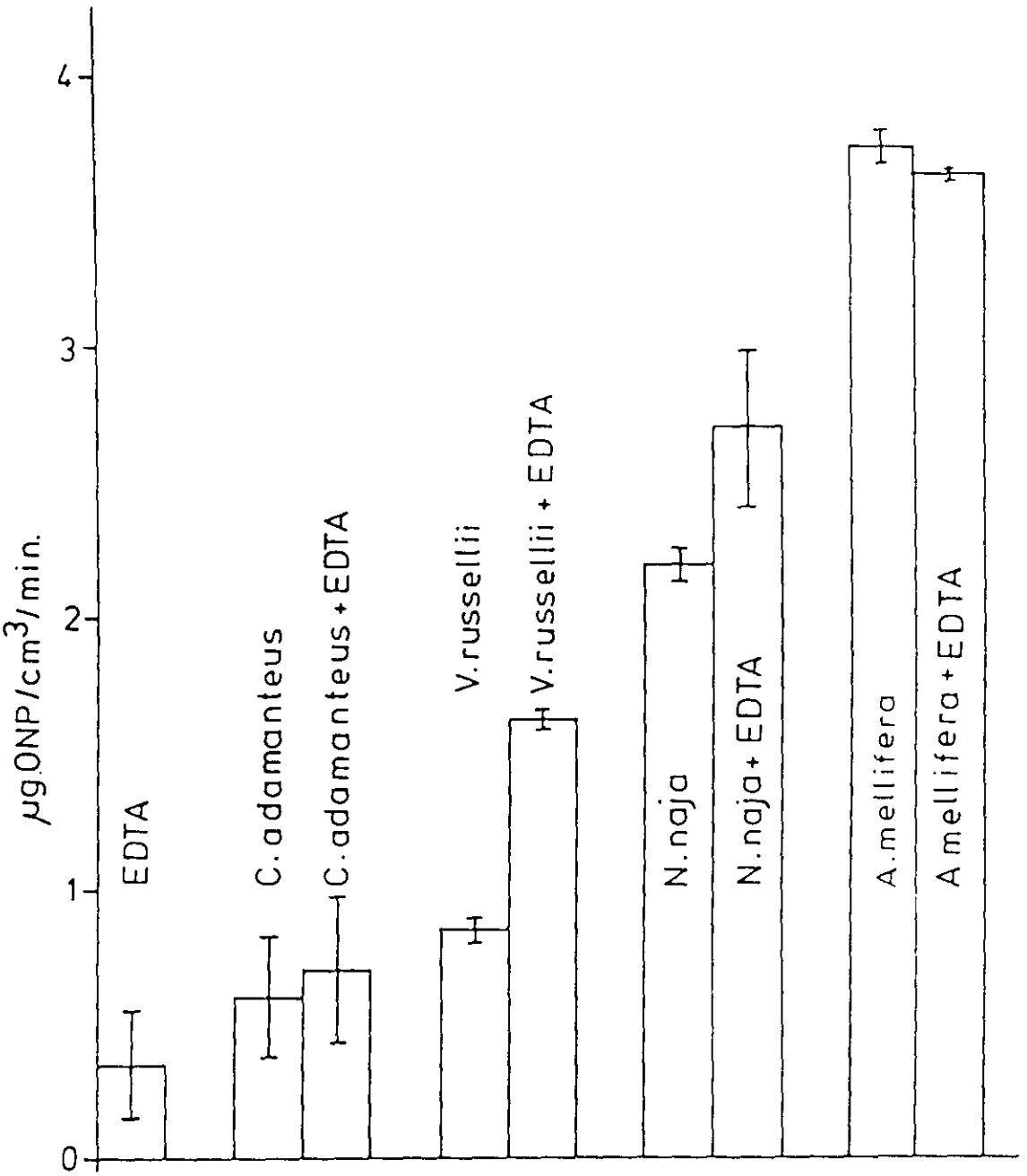


FIGURE 20

The effect of EDTA on ß-galactosidase released from *E. coli* by venom treatment at 37°C. Conditions were 625 μg venom/cm^3; 1 mM EDTA; and cell treatment as figure 19.

β-galactosidase release. This effect was also seen with incubation of the cells with EDTA, $MnCl_2$ and venom regardless of the concentration of venom.

(iii) $\underline{CaCl_2}$

Basal β-galactosidase release from *E. coli* was not affected by incubation with $CaCl_2$ (1-5 mM) whether or not the cells were pretreated with EDTA. When $CaCl_2$ was included during incubation with crude venoms, the β-galactosidase release was lowered, particularly with *N. naja sputatrix* and *A. mellifera* venoms (Figure 21). This inhibition was not altered in the presence of EDTA (1 mM). Increasing the concentration of $CaCl_2$ with 250 $\mu g/cm^3$ venom increasingly inhibited the β-galactosidase release both in the presence or absence of EDTA (Figure 22).

(iv) Fatty-acid-free Albumin.

Inclusion of fatty-acid-free albumin (125 $\mu g/cm^3$) with *E. coli* increased the basal β-galactosidase release by up to 0.25 μg ONP/cm^3/min (26%). The presence of albumin (up to 12.5 mg/cm^3) during treatment of *E. coli* by *A. mellifera* and *N. naja sputatrix* venoms (625 $\mu g/cm^3$) produced no alteration of β-galactosidase release.

(v) L - α - Phosphatidylcholine.

The presence of L - α - phosphatidylcholine (0-250 $\mu g/cm^3$) during cell treatment by either *V. russellii* or *C. adamanteus* venoms had no effect on the release of β-galactosidase.

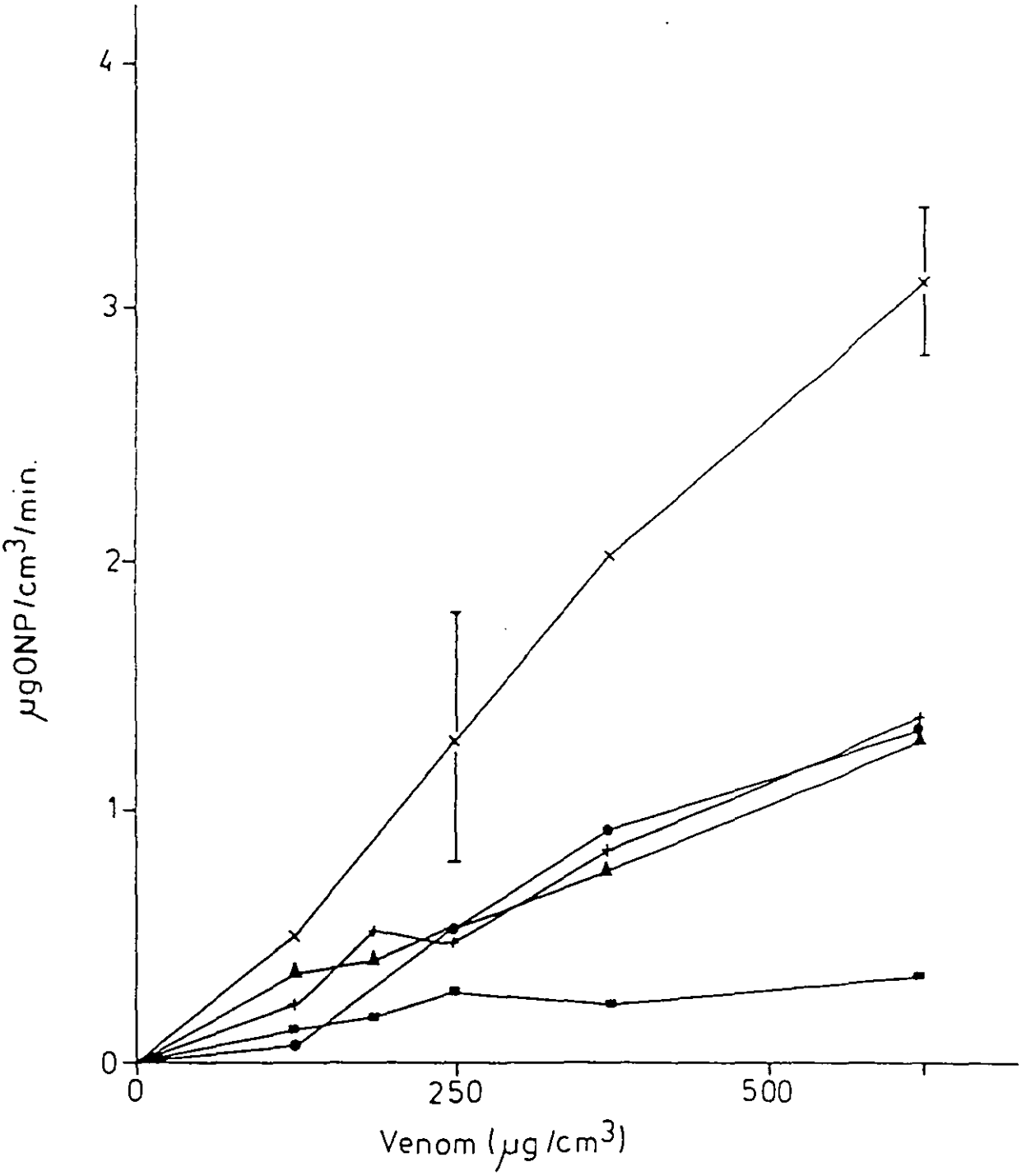


FIGURE 21

Inhibition by $CaCl_2$ of venom-induced ß-galactosidase release from *E. coli* and the effect of EDTA on the inhibition. Concentrations were 2.5 mM $CaCl_2$ and 1 mM EDTA. Addition of $CaCl_2$ to cells was followed after 1 minute by venom addition, incubation being continued at 37°C for 20 minutes. Pretreatment by EDTA was for 2 minutes where applicable.

- ● —— ● *N. naja sputatrix* venom
- ■ —— ■ *N. naja sputatrix* venom, $CaCl_2$
- × —— × *A. mellifera* venom
- ▲ —— ▲ *A. mellifera* venom $CaCl_2$
- \+ —— \+ *A. mellifera* venom $CaCl_2$, EDTA

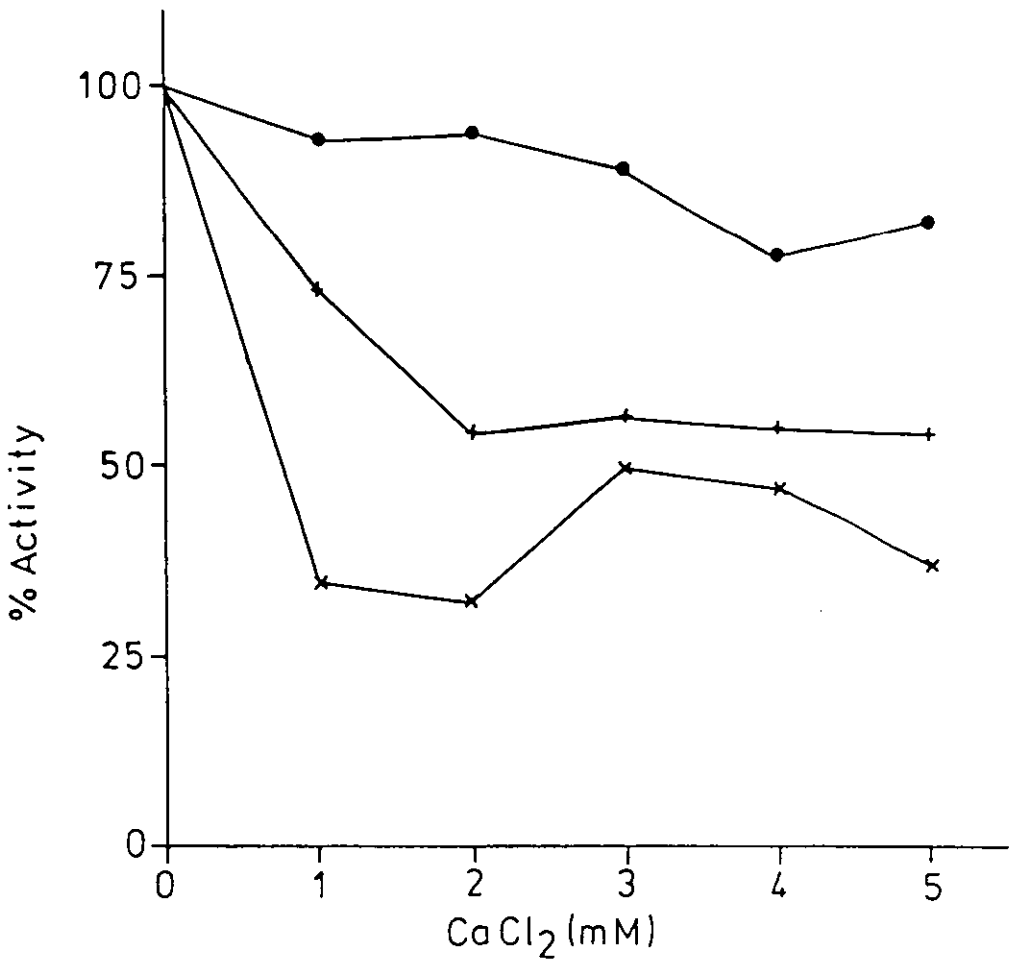


FIGURE 22

Inhibition by $CaCl_2$ of venom-induced β-galactosidase release from *E. coli*. Venom concentrations were 250 μg/cm^3, EDTA concentration was 1 mM. Conditions were as figure 21. Release by venoms alone was (μg ONP/cm^3/min)
1.10 by *N. naja sputatrix* venom; 2.90 by *A. mellifera* venom; 4.45 by *A. mellifera* venom with EDTA pretreatment.

●——● *N. naja sputatrix* venom, $CaCl_2$

+——+ *A. mellifera* venom, $CaCl_2$

×——× *A. mellifera* venom, $CaCl_2$, EDTA

3.3 RELEASE INDUCED BY PHOSPHOLIPASES A_2.

Dose-response curves for the phospholipases A_2 from *A. mellifera* and *N. naja sputatrix* venoms (0-100 $\mu g/cm^3$) against *E. coli* cell suspensions were obtained (Figure 23).

β-galactosidase release caused by PLA_2 *(A. mellifera)* appeared to be increased by pretreatment of cells with EDTA and decreased by $CaC\ell_2$ (2.5 mM) and $MnC\ell_2$ (2.5 mM) (Figure 24) but reproducibility was poor. Inclusion of $MnC\ell_2$ following EDTA treatment blocked the effect of the chelator on β-galactosidase release caused by *A. mellifera* PLA_2 (Figure 24).

Release of β-galactosidase caused by *N. naja sputatrix* PLA_2 (100 $\mu g/cm^3$) (0.35 ± 0.3 μg ONP/cm^3/min) was not significantly affected by pretreatment with EDTA (0.5 ± 0.4 μg ONP/cm^3/min).

Incubation of fatty-acid-free albumin (0-12.5 mg/cm^3) with either PLA_2 had no effect on the β-galactosidase released from *E. coli* cells.

3.4 RELEASE INDUCED BY MELITTIN.

Addition of melittin (0-1250 $\mu g/cm^3$) caused a dose-dependent increase in the release of β-galactosidase from *E. coli* (Figure 25).

Pretreatment by EDTA (1 mM) and/or $CaC\ell_2$ (2.5 mM) had no significant effect on β-galactosidase release induced by melittin (125 $\mu g/cm^3$) (Figure 26).

No effect other than additive of *A. mellifera* PLA_2 (100 $\mu g/cm^3$) activity by melittin (125 $\mu g/cm^3$) was observed (Figure 27a). Inclusion of EDTA increased the release cause by *A. mellifera* PLA_2 and melittin. Addition of $CaCl_2$ decreased this release (Figure 27a).

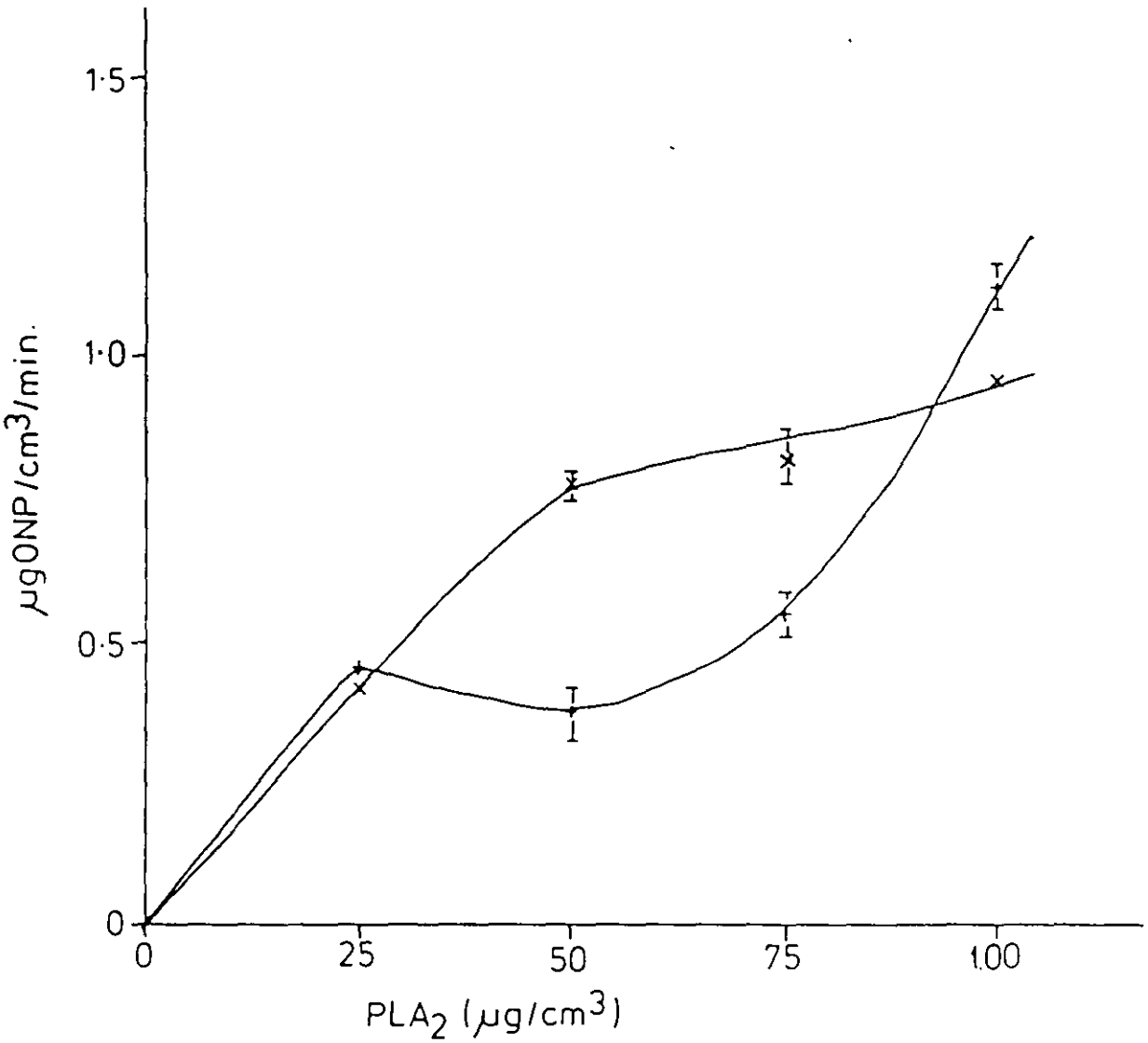


FIGURE 23

Dose-response curves for phospholipase A_2 (PLA_2) - induced β-galactosidase release from *E. coli*. Treatment was for 20 minutes at 37°C.

×—— × *A. mellifera* PLA_2

+ —— + *N. nàja sputatrix* PLA_2

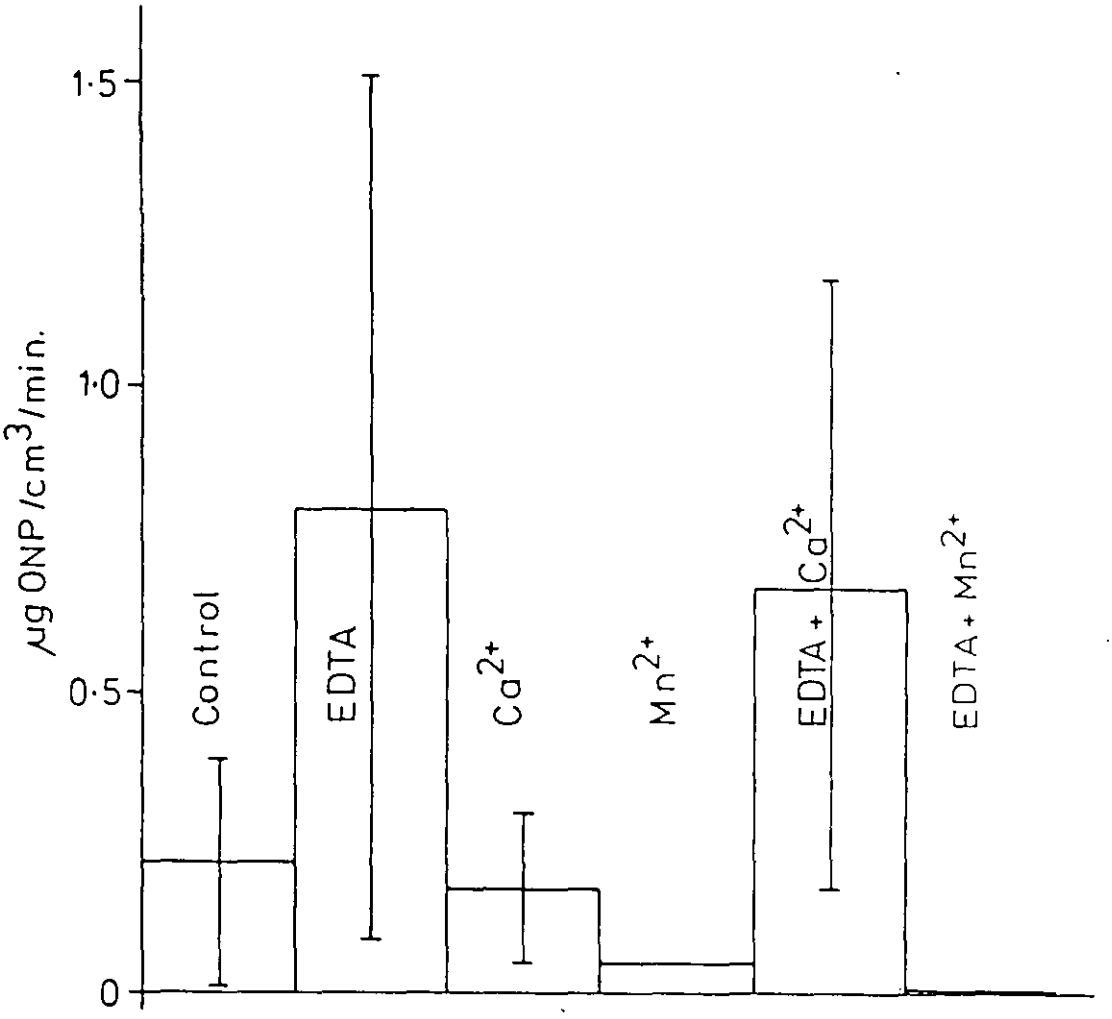


FIGURE 24

The effect of ions on β-galactosidase release from *E. coli* induced by PLA_2 from *A. mellifera* venom (100 μg/cm^3). EDTA pretreatment for 2 minutes was followed by 1 minute treatment with ions before addition of PLA_2, incubation being continued at 37°C for 20 minutes. Concentrations used were 1 mM EDTA, 2.5 mM $CaCl_2$, 2.5 mM $MnCl_2$.

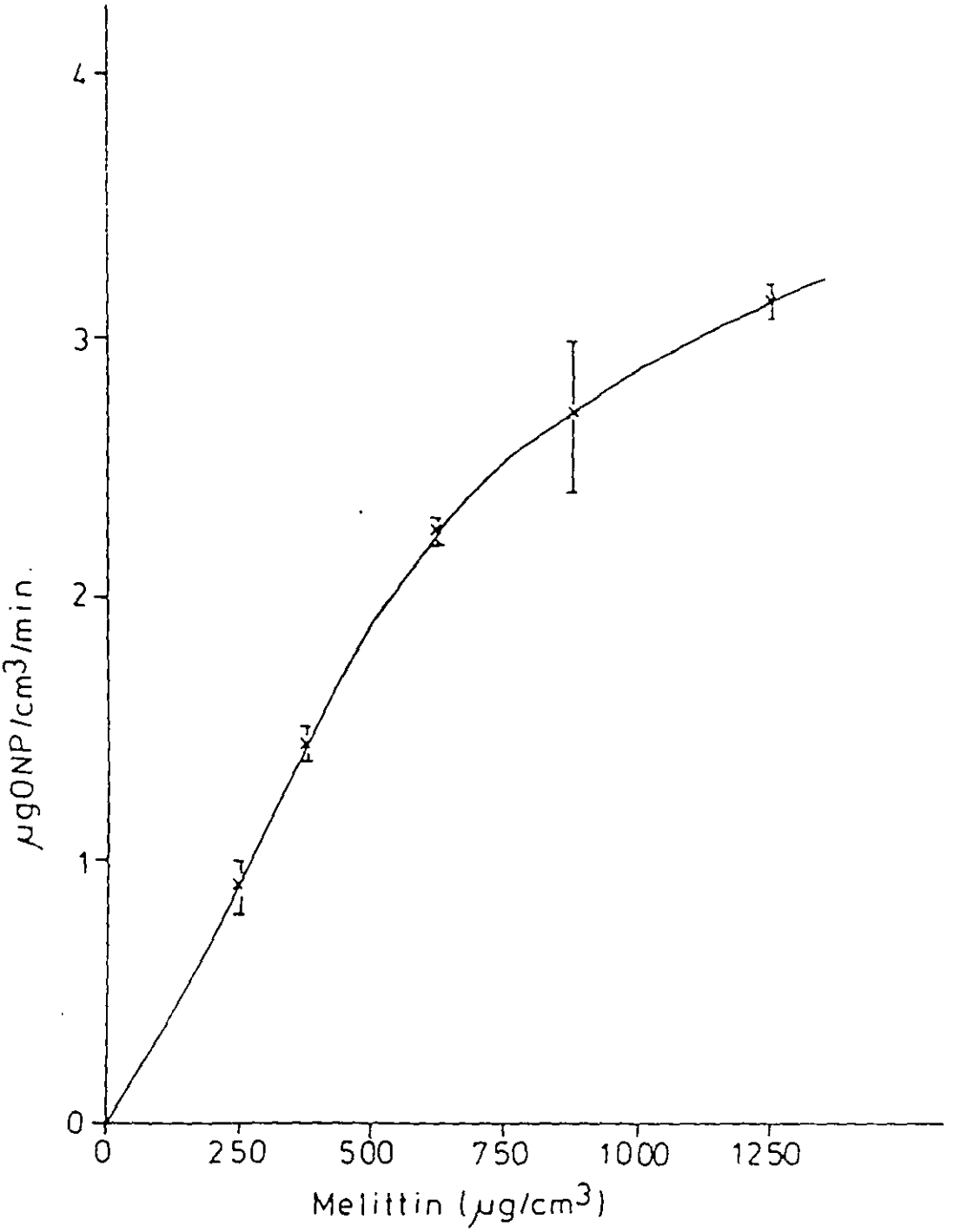


FIGURE 25

Dose-response curve for melittin-induced ß-galactosidase release from *E. coli* incubated for 20 minutes at 37°C.

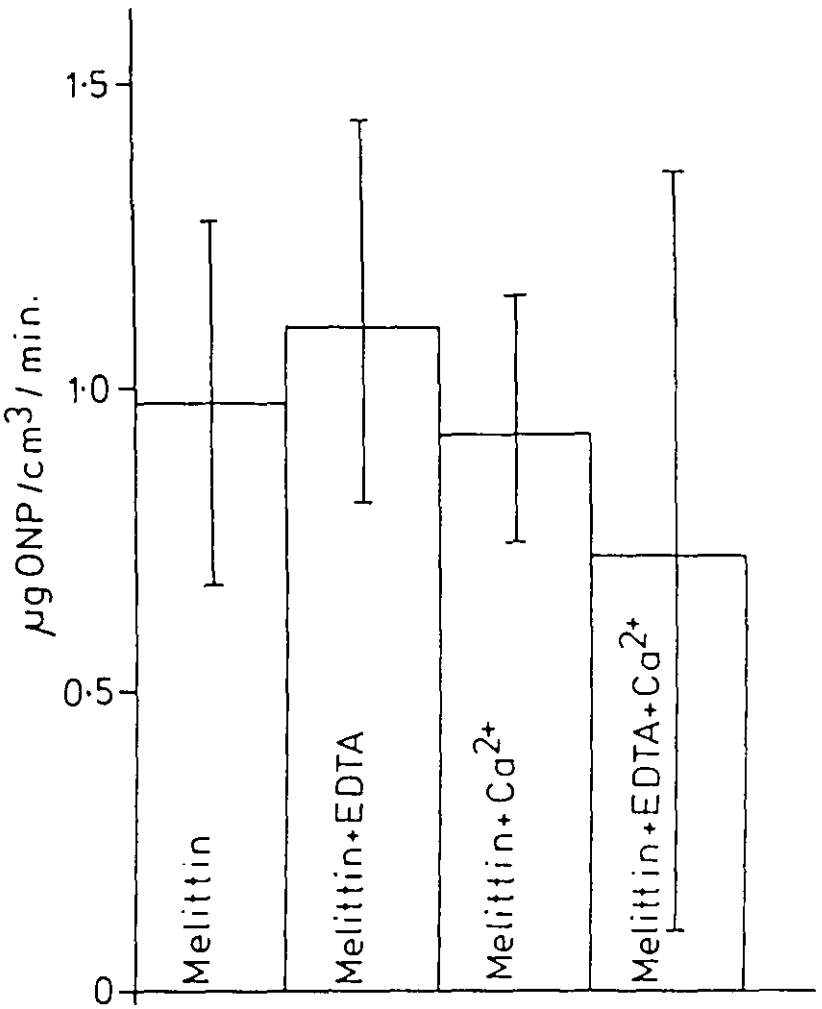


FIGURE 26

The effect of ions on β-galactosidase release from *E. coli* by melittin (125 μg/cm^3). Conditions and concentrations used were as figure 24.

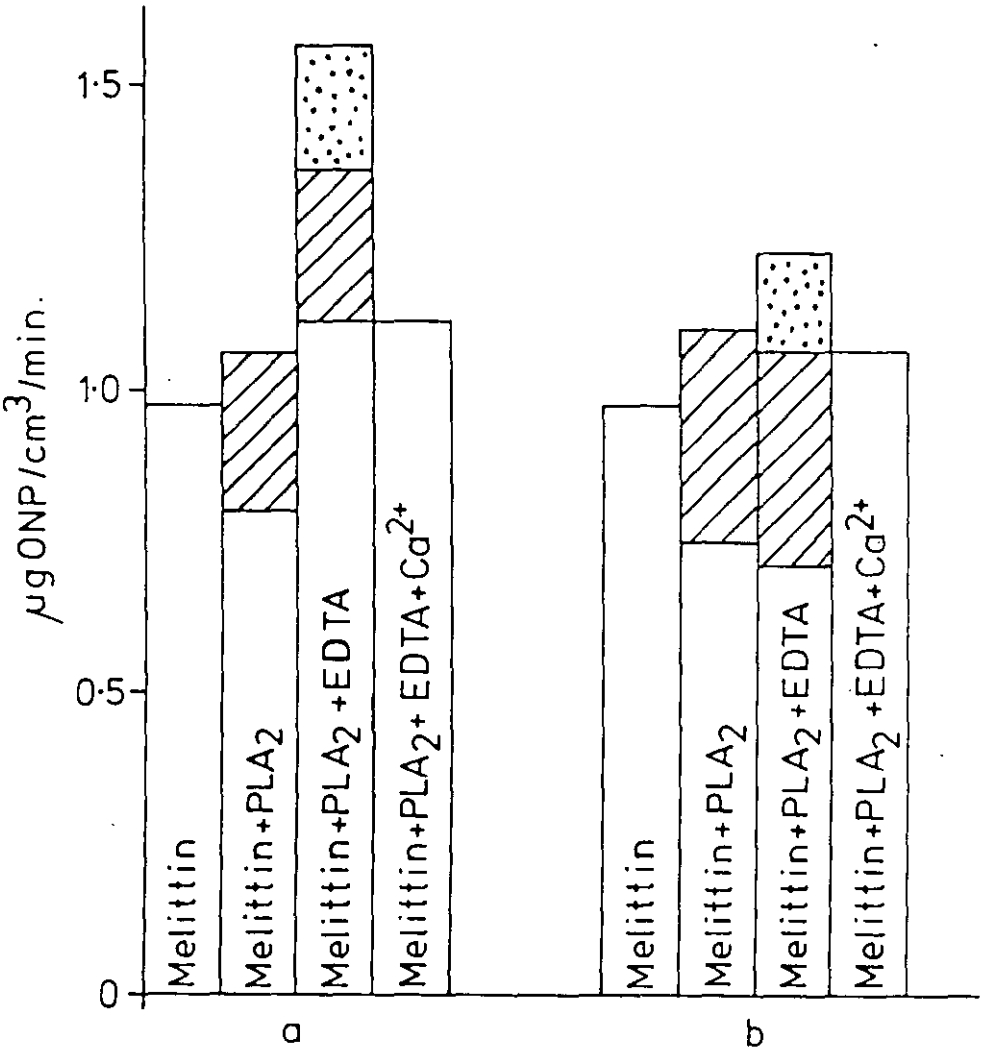


FIGURE 27

The effect of melittin (125 μg/cm^3) and ions on β-galactosidase release from *E. coli* induced by PLA_2 (100 μg/cm^3). Conditions and concentrations used were as figure 24.

a *A. mellifera* PLA_2

b *N. naja sputatrix* PLA_2

Contribution from PLA_2 alone

Contribution from EDTA alone

Similar but smaller effects on release were seen with *N. naja sputatrix* PLA_2 (Figure 27b).

Release of β-galactosidase by crude venoms (0-625 $\mu g/cm^3$) was not affected by simultaneous incubation with melittin (0-125 $\mu g/cm^3$). The presence of EDTA, $CaCl_2$ or $MnCl_2$ did not alter β-galactosidase release by melittin and crude venoms.

3.5 RELEASE INDUCED BY POLYMIXIN B.

Release of β-galactosidase caused by incubation with varying doses of polymixin B is shown in Figure 28.

In the presence of a submaximal level of *A. mellifera* venom polymixin B added either before, together with or after the venom showed an additive effect (Figure 29).

With *N.naja sputatrix* venom no obvious additive effect was observed (Figure 29).

In the case of the purified PLA_2 from these venoms, a similar though less easy to discern pattern occurred (Figure 30).

4. OTHER EFFECTS ON NON-PROLIFERATING CELLS.

4.1 Cell Viability.

A. mellifera and *N. naja sputatrix* venoms lowered cell viability of *E. coli* suspensions. No such effect was caused by *V. russellii* or *C. adamanteus* venoms (see Table 4).

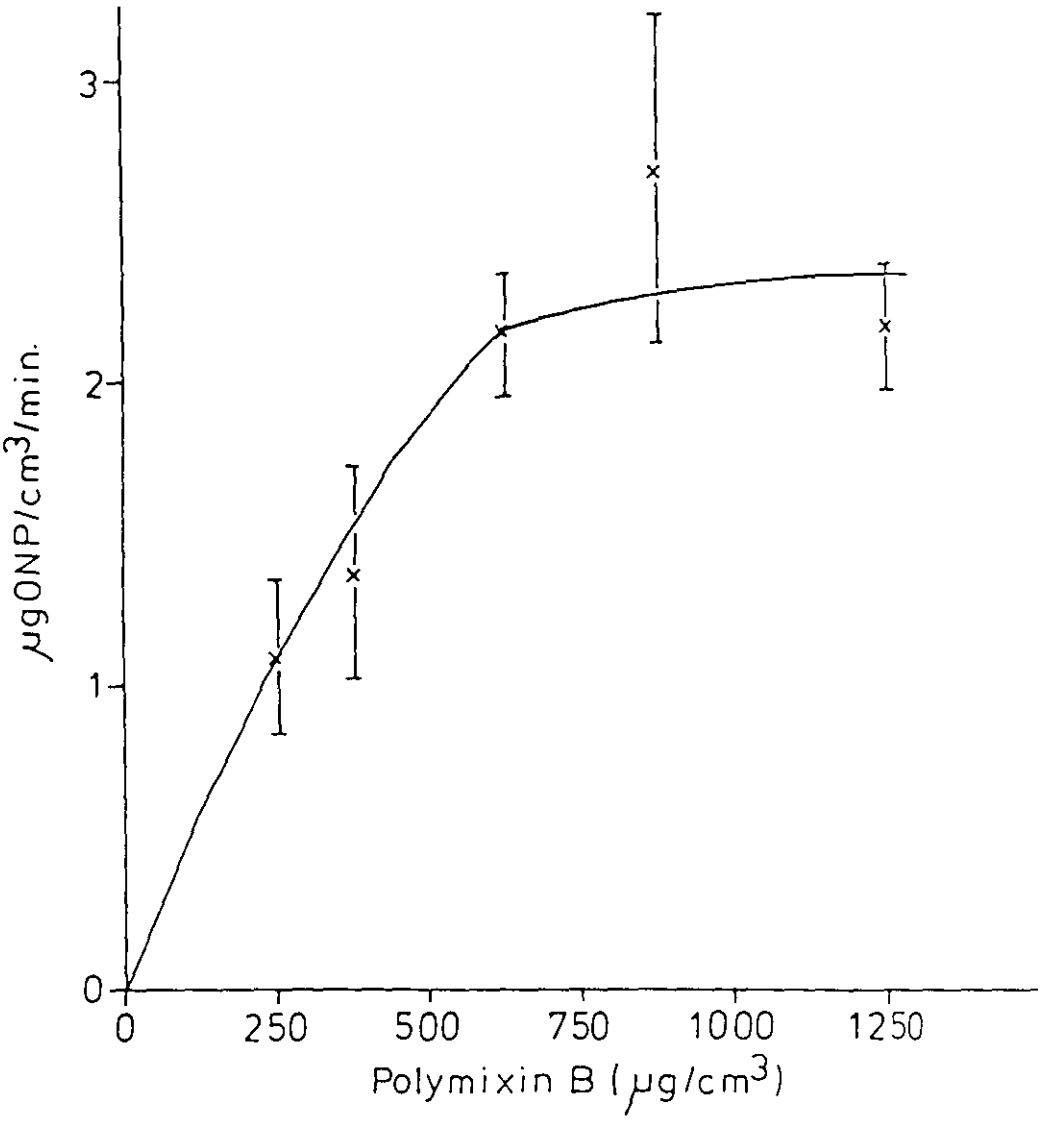


FIGURE 28

Dose-response curve for polymixin B - induced ß-galactosidase release from *E. coli* incubated for 20 minutes at 37°C.

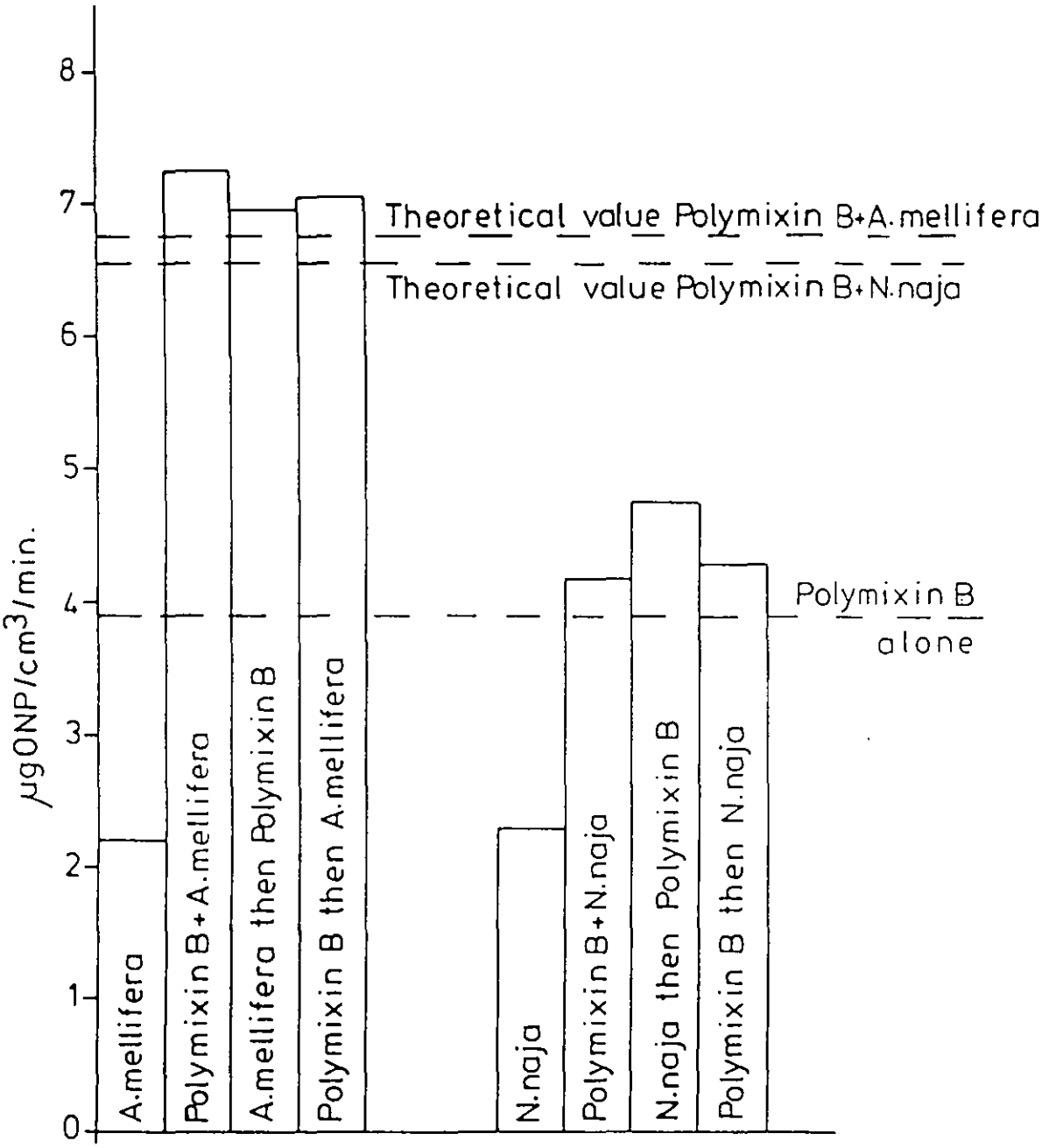


FIGURE 29

The effect of polymixin B on venom-induced β-galactosidase release from *E. coli* at 37°C. Concentrations used were 250 µg venom/cm^3 and 250 µg polymixin B/cm^3. Treatment was for 20 minutes except when venom or polymixin B was added after 20 minutes treatment with the initial substance, when incubation was continued for a further 20 minutes.

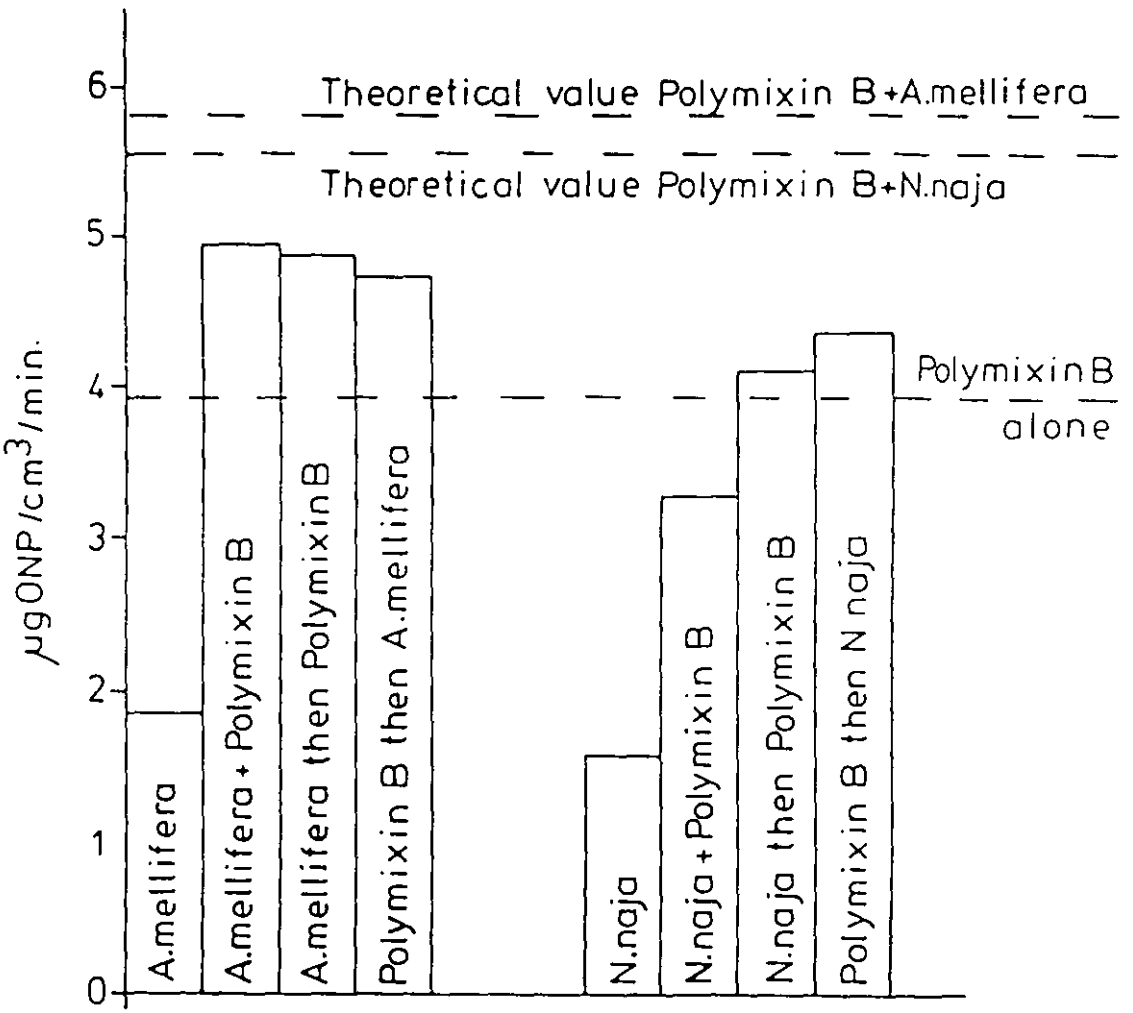


FIGURE 30

The effect of polymixin B on PLA_2 - induced β-galactosidase release from *E. coli*. Concentrations used were 50 µg PLA_2/cm^3 and 250 µg polymixin B/cm^3. Conditions were as figure 29.

TABLE 4

Effect of Venom Treatment (1250 $\mu g/cm^3$) on Cell Viability of *E. coli* suspensions.

Venom	Viable Count × 10^7
Control	75 ± 6
C. adamanteus	99 ± 2
V. russellii	96 ± 4
N. naja sputatrix	39 ± 3
A. mellifera	15 ± 1

4.2 Cell Lysis.

Cell lysis was seen to occur in cell suspensions treated with *A. mellifera* and *N. naja sputatrix* but not *V. russellii* or *C. adamanteus* venoms (each at 625 $\mu g/cm^3$) (Figure 31).

Treatment of cells with purified PLA_2 of either *A. mellifera* or *N. naja sputatrix* did not cause any decrease in cell absorbancy.

Melittin caused a decrease in cell absorbancy, reversible at higher doses, whilst cells treated with polymixin B showed a small increase in absorbancy compared with control cells (Figure 32).

4.3 Loss of 260 nm - absorbing Cell Constituents.

Leakage of 260 nm - absorbing cell constituents due to treatment by *A. mellifera* and *N. naja sputatrix* venoms was observed (Figure 33). Neither PLA_2 caused loss of these constituents (Figure 34).

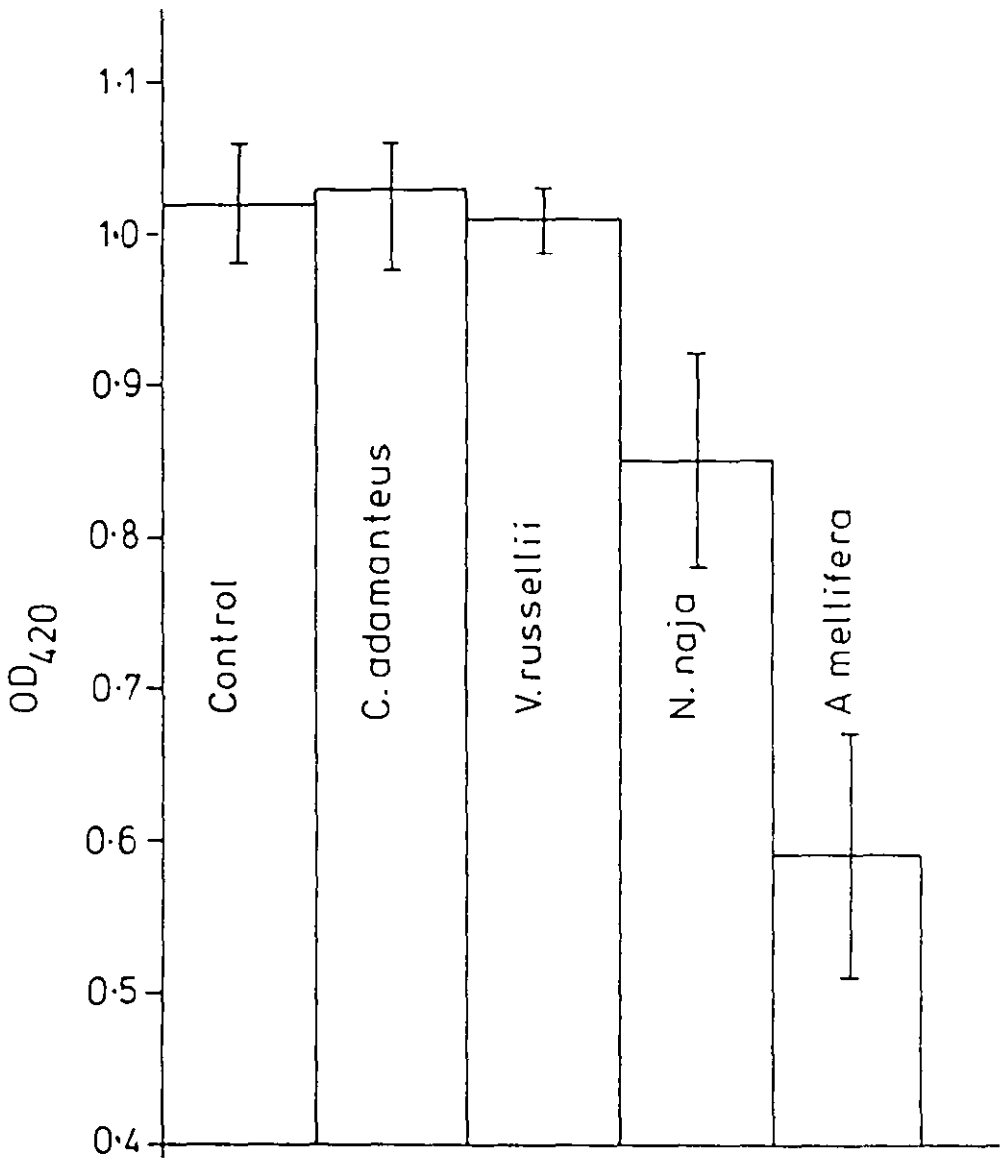


FIGURE 31

Absorbancy of *E. coli* cell suspensions after treatment with venom (625 μg/cm^3) for 20 minutes at 37°C.

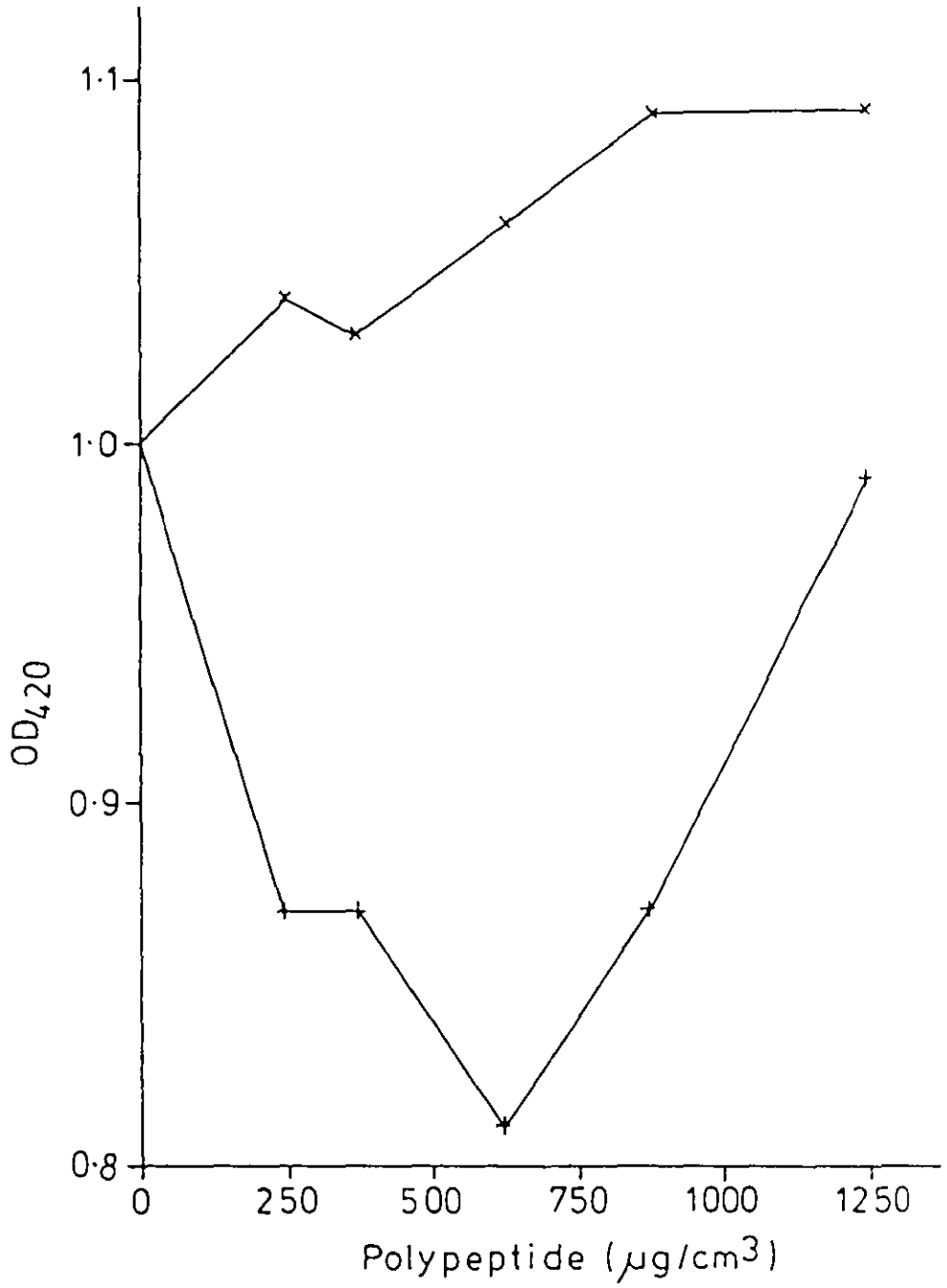


FIGURE 32

Absorbancy of *E. coli* cell suspensions after polypeptide treatment for 20 minutes at 37°C.

+——+ melittin

×—— × polymixin B

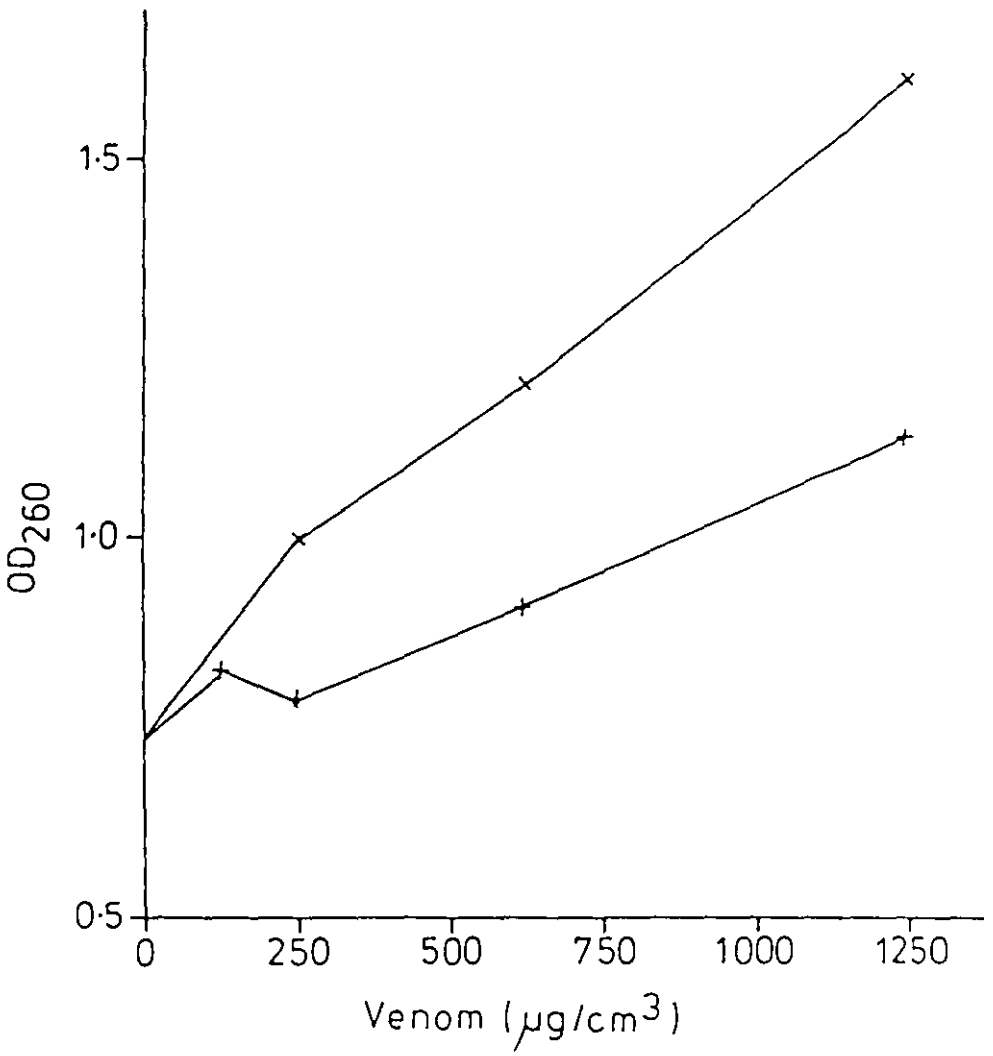


FIGURE 33

Loss of 260 nm - absorbing cell constituents from *E. coli* after venom treatment for 20 minutes at 37°C.

×—— × *A. mellifera* venom

+—— + *N. naja sputatrix* venom

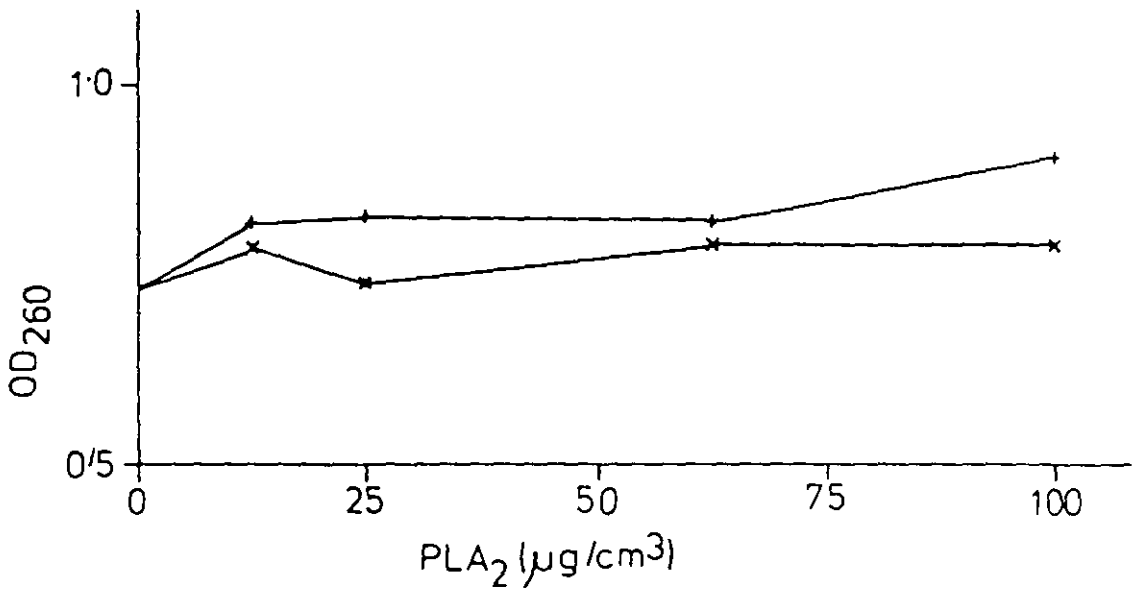


FIGURE 34

The effect of PLA_2 on the loss of 260 nm - absorbing cell constituents from *E. coli* after 20 minutes incubation at 37°C.

×—× *A. mellifera* PLA_2

+—+ *N. naja sputatrix* PLA_2

5. SEPARATION AND ACTION OF VENOM COMPONENTS.

5.1 Gel Filtration.

(a) *N. naja sputatrix* venom.

Separation of *N. naja sputatrix* venom on Sephadex G75 fine gave two peaks (Figure 35). Fractions 2-5 (A2) and 14-15 (A3) respectively were pooled, the large first peak containing most of the solid material on freeze drying. Both fractions caused release of β-galactosidase from *E. coli* cell suspensions (Figure 36). A second separation gave one peak and fractions 1-6 (B2) also caused β-galactosidase release (Figure 36).

N. naja sputatrix venom separated on Sephadex G75 superfine gave three peaks, fractions 1-3 (C1), 7-12 (C2) and 21 (C3) being freeze dried. Samples C1 and C2 caused release of β-galactosidase (Figure 36).

(b) *A. mellifera* venom.

Similar separation of *A. mellifera* venom on Sephadex G75 fine apparently gave one major peak, fractions 4-7 (Figure 37). In addition fraction 14 was collected for further examination. The fractions (4 cm^3 aliquots) were freeze dried individually as the venom components would be eluted close together (Shipman and Cole 1969).

All the fractions were shown to cause β-galactosidase release although the activity of fractions 7 and 14 was very low. (Figure 38).

5.2 Electrophoresis.

The components of all four venoms, melittin and cardiotoxin *(N. naja siamensis)* moved towards the cathode with the exception

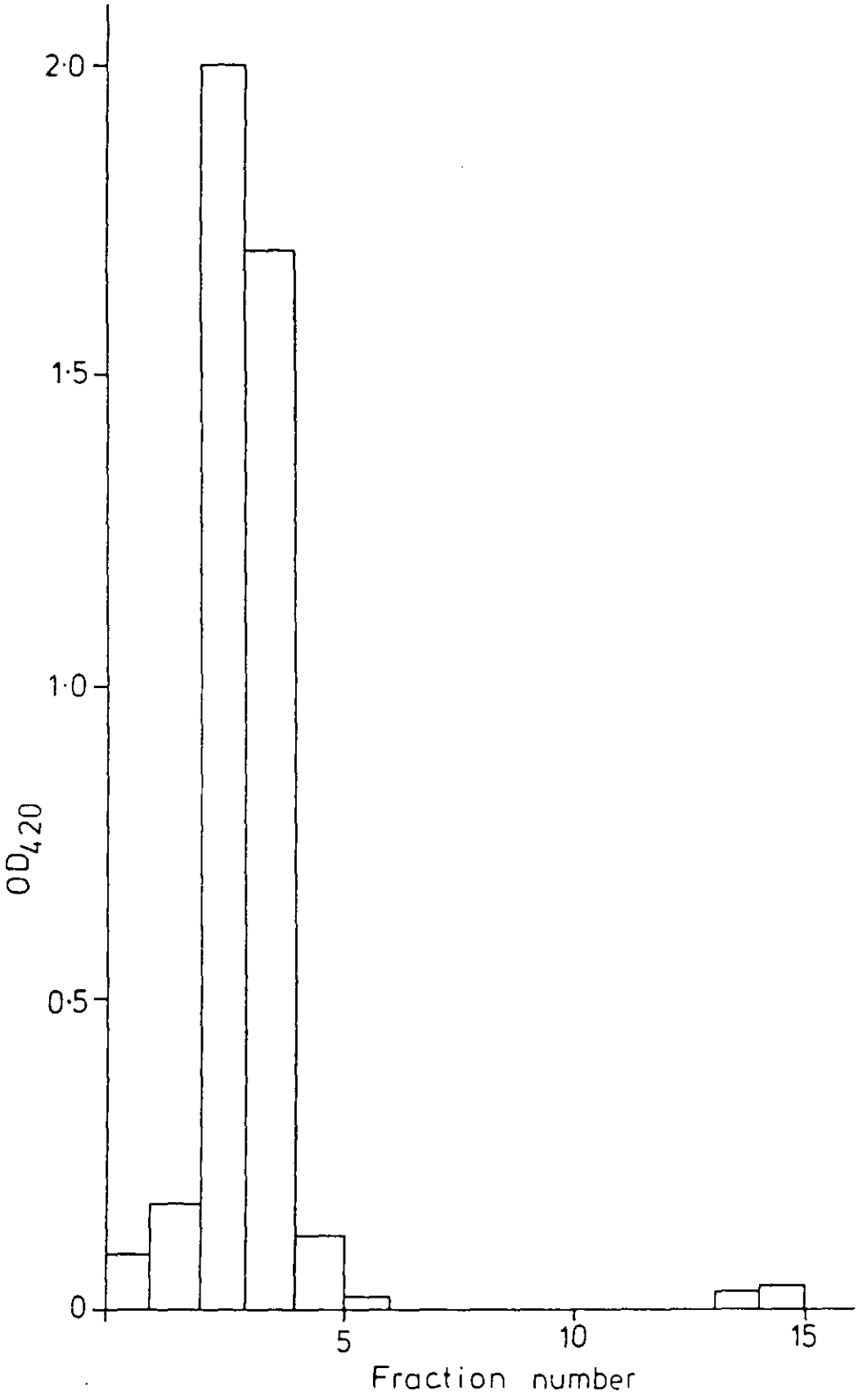


FIGURE 35

Separation of *N. naja sputatrix* venom by column chromatography using Sephadex G75. The venom sample (2 mg/cm^3) was eluted with distilled water, and collected as 10 cm^3 aliquots.

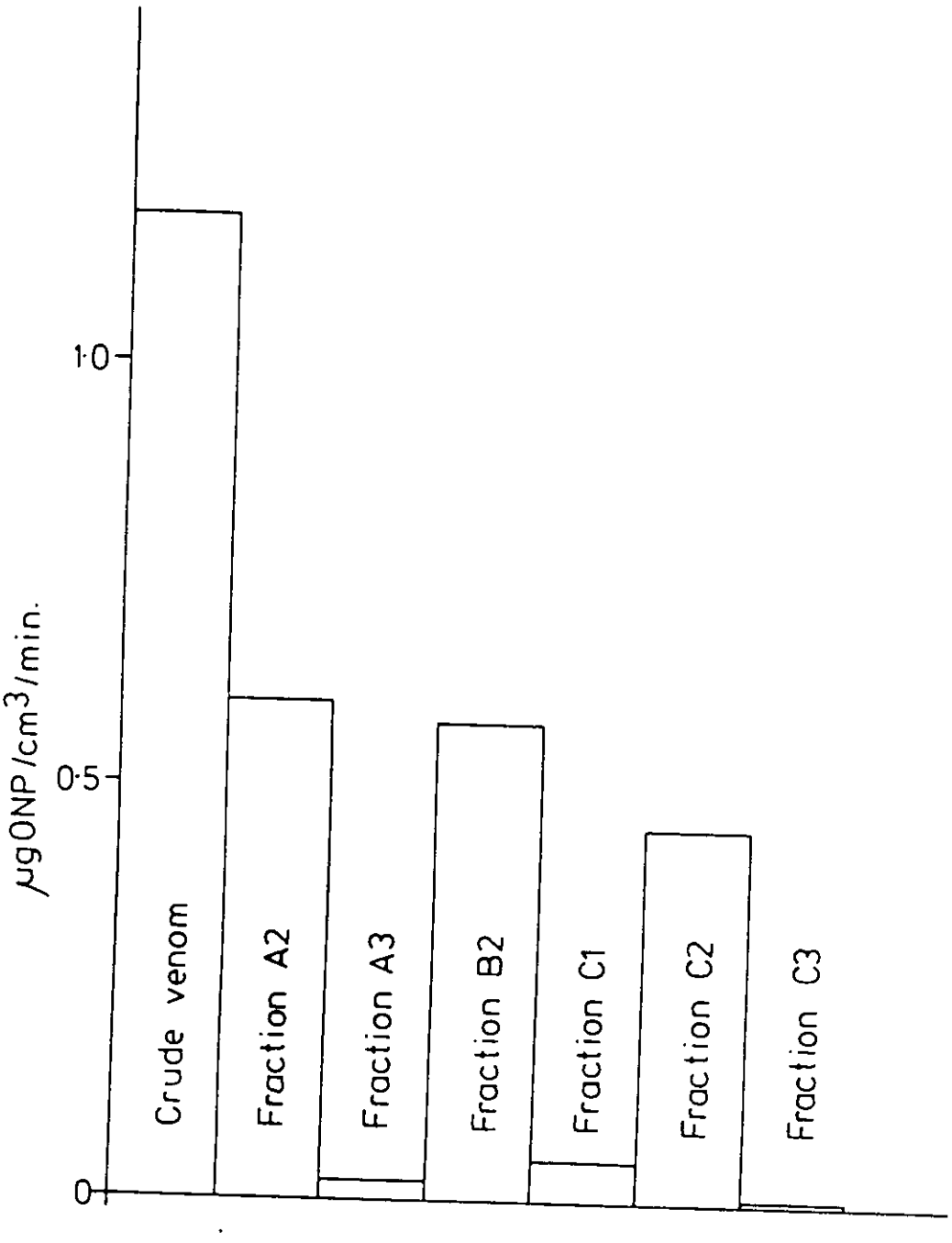


FIGURE 36

Release of β-galactosidase from *E. coli* induced by *N. naja sputatrix* venom fractions after column chromatography. Fractions A2 and A3 were the pooled volumes 2-5 and 14-15 respectively from the separation shown in figure 35. Fraction B2 and fractions C1, C2 and C3 were equivalent aliquots from the separation of other *N. naja sputatrix* venom samples (2 mg/cm^3). *E. coli* cell suspensions were treated with venom fractions for 20 minutes at 37°C.

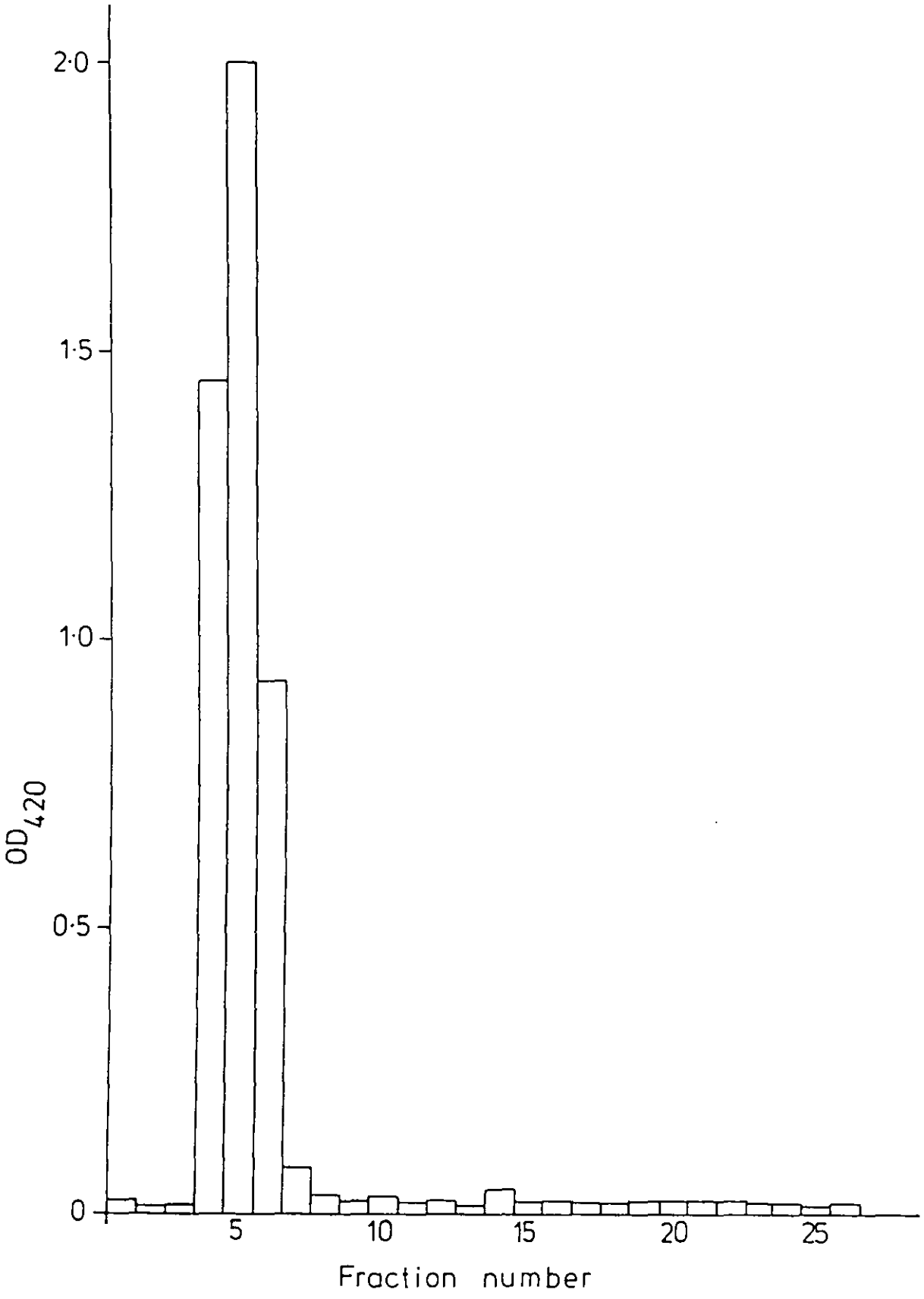


FIGURE 37

Separation of *A. mellifera* venom by column chromatography using Sephadex G75. The venom sample (2 mg/cm^3) was eluted with distilled water and collected as 4 cm^3 aliquots.

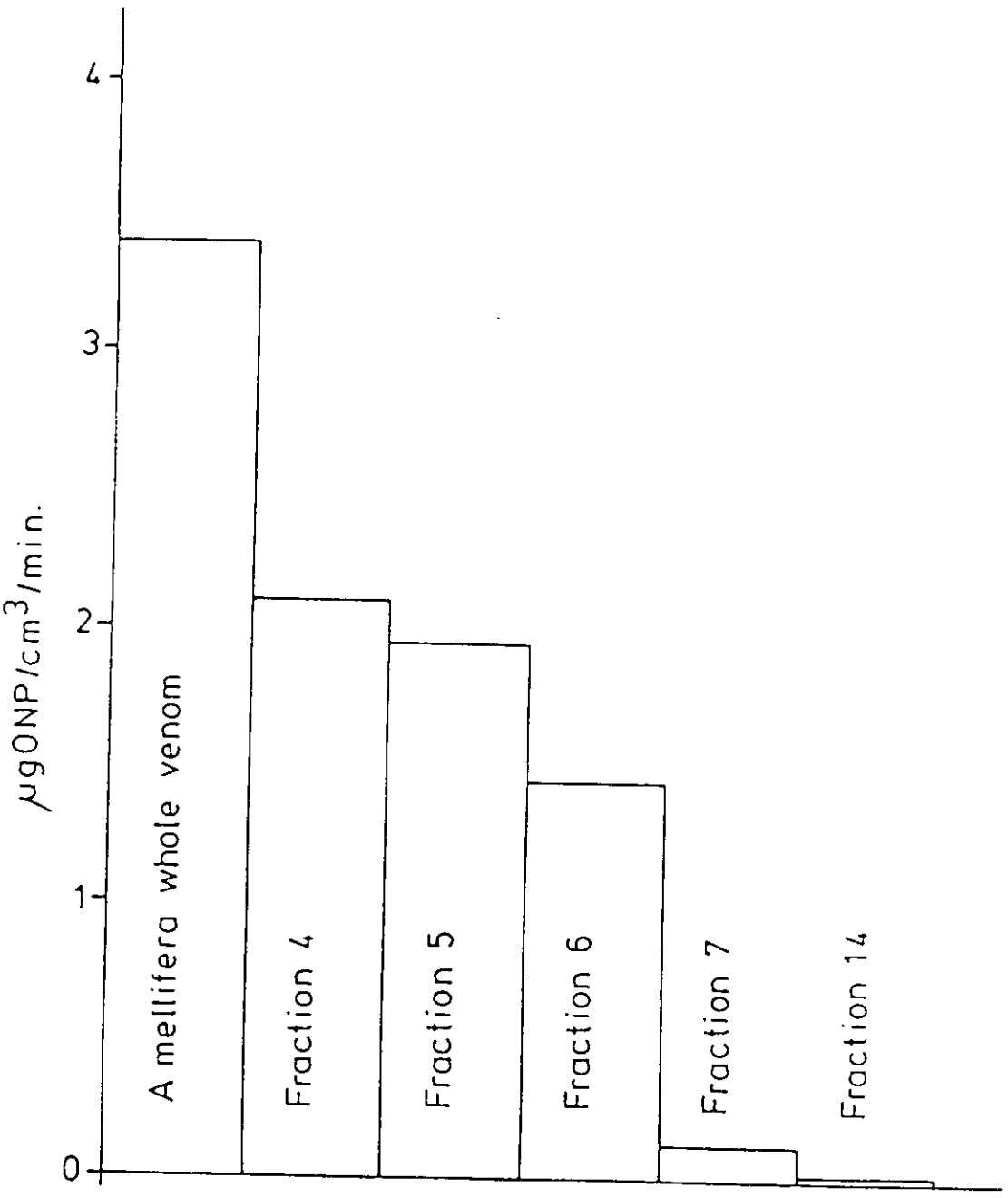


FIGURE 38

Release of β-galactosidase from *E. coli* induced by *A. mellifera* venom fractions after column chromatography. Individual aliquots from the separation shown in figure 37 were tested by incubation with *E. coli* for 20 minutes at 37°C.

of two bands in *V. russellii* and *C. adamanteus* venoms (Figure 39).

Inhibition of *E. coli* growth was caused by melittin, by various components of *A. mellifera* venom and to a lesser extent by components of *N. naja sputatrix* venom as shown on Figure 39. No evidence of growth inhibition by *V. russellii* or *C. adamanteus* venoms was observed.

Melittin and some of the venom components caused a white precipitate to form in the agar similar to that observed in the agar diffusion assay. Inhibition of *E. coli* growth occurred in these areas.

A better separation of the venoms was obtained using a KOH/acetic acid buffer pH 4.3. However this altered the pH of the agar during incubation at $4^{o}C$ with the cellulose acetate strips such that *E. coli* growth over the entire area covered by the strips was inhibited.

6. ELECTRON MICROSCOPY OF CELLS TREATED WITH VENOMS AND CONSTITUENT MATERIALS.

6.1 Scanning Electron Microscopy.

Differences in cell morphology of log-phase cultures of *E. coli* after treatment by *N. naja sputatrix* and *A. mellifera* venoms and melittin were observed by scanning electron microscopy. The concentrations used were those which had been found to be active against growing cultures (section 2.3 above).

Cells treated with melittin (6-18 $\mu g/cm^3$) appeared shorter than control cells (Figure 40) and seemed to have a less smooth surface.

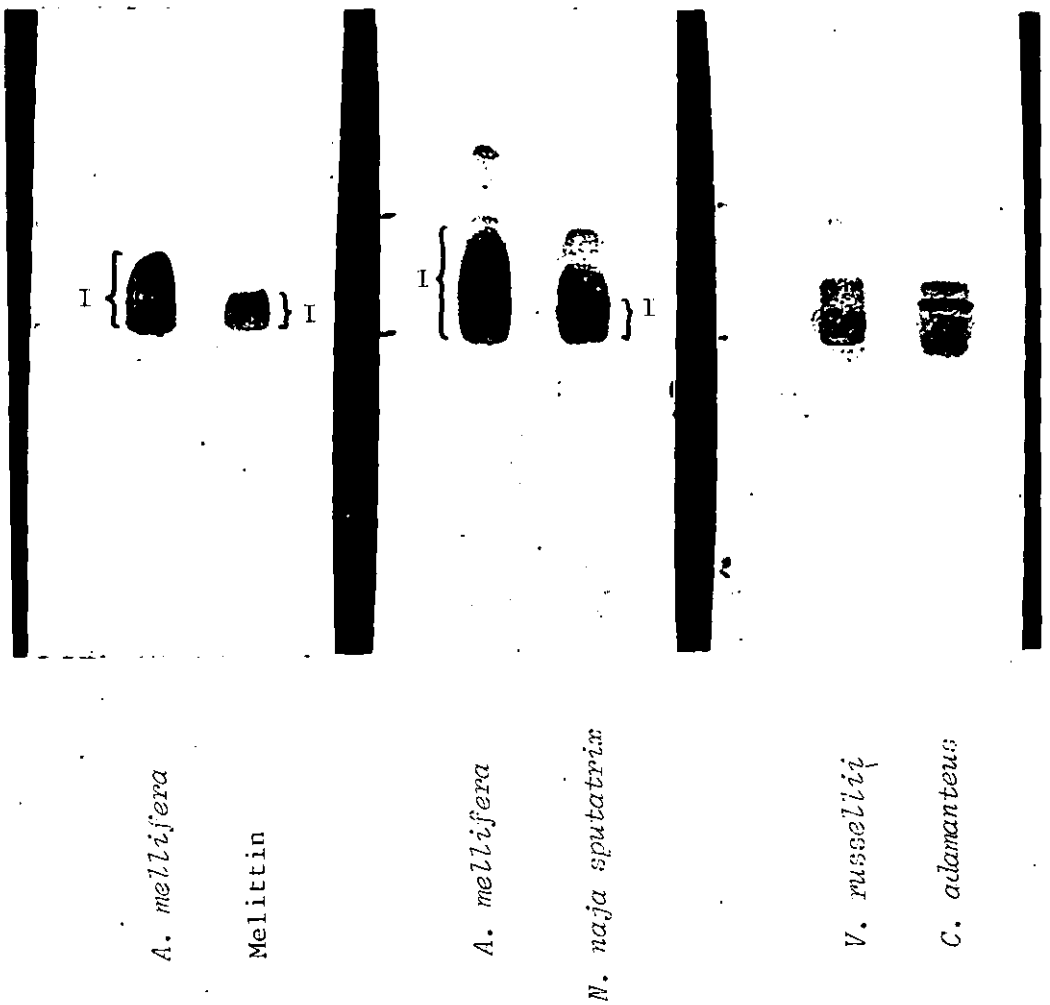
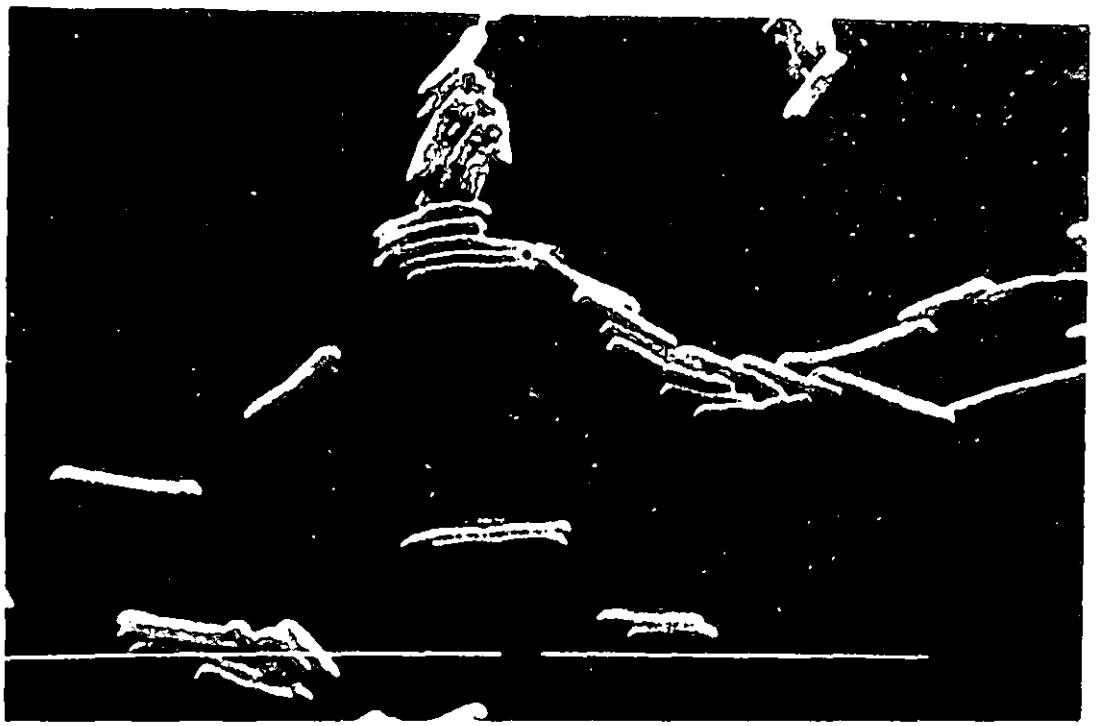


FIGURE 39

Electrophoretic separation of venoms and melittin. Electrophoresis was carried out using Cellogel II cellulose acetate strips, barbitone buffer pH 8.6 and a constant voltage of 200 V for 30 minutes with methyl green as a marker. Proteins were stained with Ponceau S.

I { Components causing inhibition of growth of *E. coli*.

a)



b)

FIGURE 40

Scanning electron micrographs of *E. coli* cells in log phase culture. Cell cultures were incubated at 37°C for 2½ hours following addition of test substance before being fixed for 16 hours at 4°C in 5% w/v glutaraldehyde. They were then washed with distilled water three times, air dried, dehydrated over calcium chloride under partial pressure and gold coated (10 nm layer). The cells were examined under a 15 kv beam. The gap in the marker bar represents 1μ.

a) Control cells × 5,000

b) Cells treated with melittin (12 $\mu g/cm^3$) × 5,000.

The general appearance of individual *E. coli* cells treated with *N. naja sputatrix* venom (200–600 $\mu g/cm^3$) was similar to that of control cells, however the formation of filaments was observed (Figure 41).

The greatest effect was seen with *A. mellifera* venom (4–12 $\mu g/cm^3$). This produced filaments and appeared to damage cells as adjudged by comparison with control cells. Such damaged cells were surrounded by debris which could have been due to leakage of cellular material (Figure 42).

6.2 Transmission Electron Microscopy.

Log-phase cultures of *E. coli* were treated with *N. naja sputatrix* and *A. mellifera* venoms and melittin as for scanning electron microscopy.

Control cells (Figure 43) compared with melittin-treated cells (Figure 44) showed little difference.

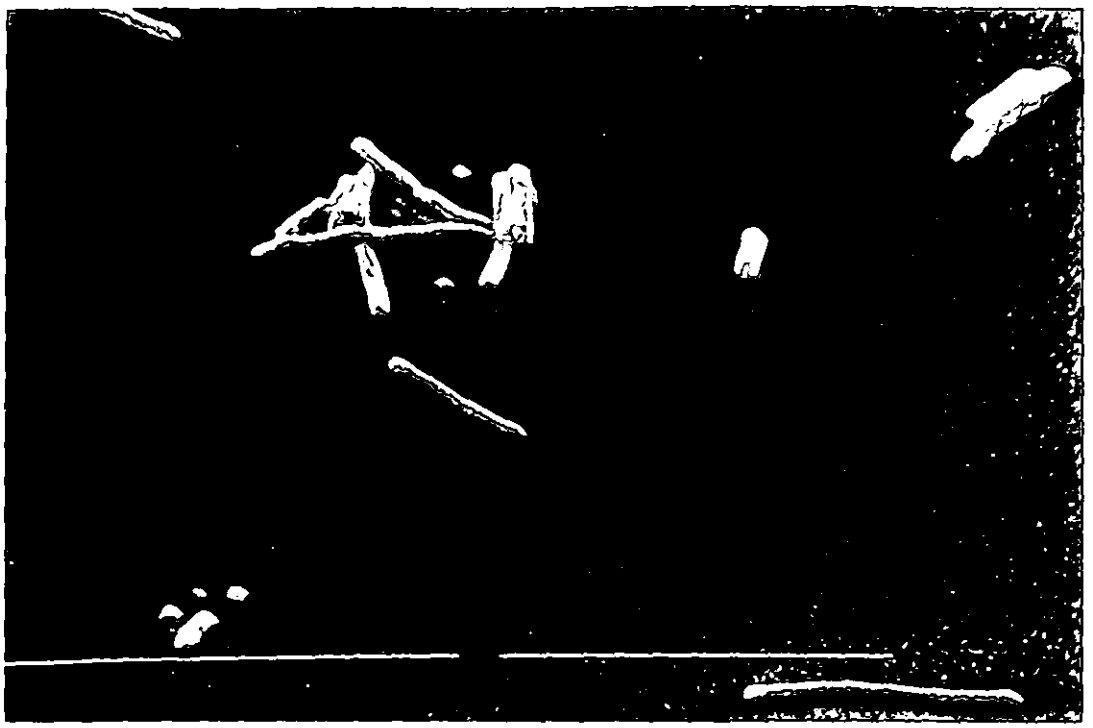
Cell integrity after treatment by *N. naja sputatrix* venom appears to be reduced (Figure 45) when the cells are compared with the control cells.

The greatest difference could be seen in cells subjected to the action of *A. mellifera* venom. Filamentous cells are produced (Figure 46) and cell ghosts are present (Figure 47).

7. JOB PLOTS.

The interaction of all four venoms, melittin and cardiotoxin with Ca^{2+} was evaluated by means of Job plots (Figure 48). No evidence of a stable complex of the venoms or venom components with

a)



b)

FIGURE 41

Scanning electron micrographs of *E. coli* treated with *N. naja sputatrix* venom (400 μg/cm^3). Conditions as figure 40.

a) × 5,000

b) × 2,000

a)

b)

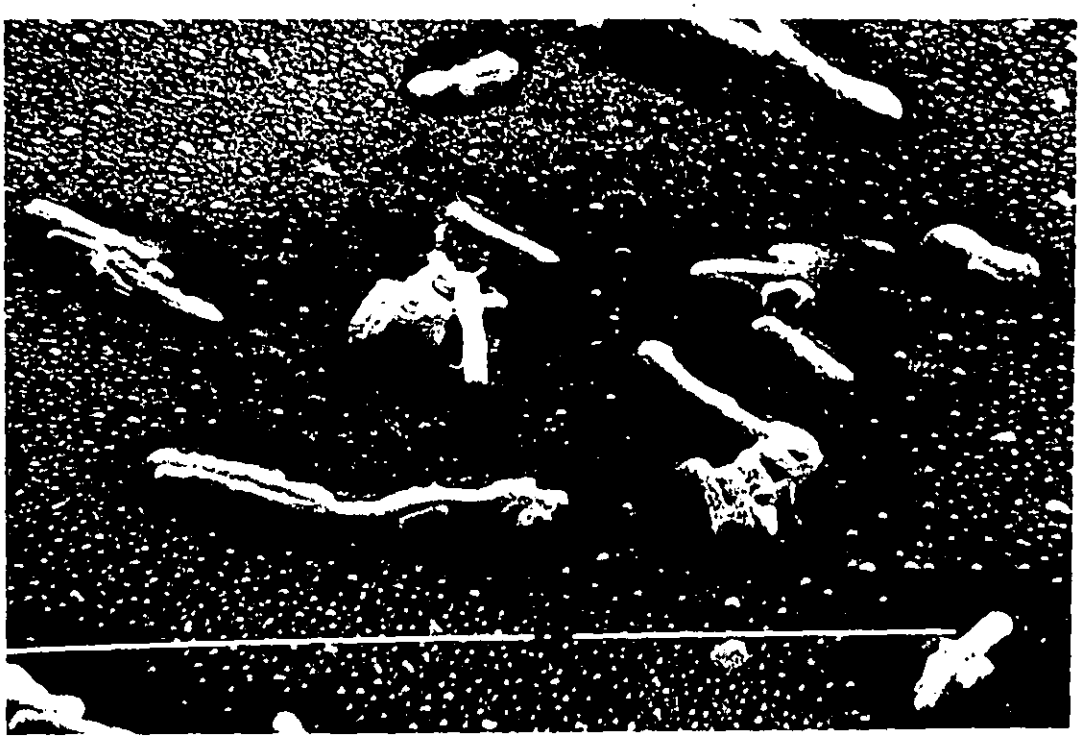


FIGURE 42

Scanning electron micrographs of *E. coli* treated with *A. mellifera* venom (8 μg/cm^3). Conditions as figure 40.

a) × 5,000

b) × 5,000

a)

b)

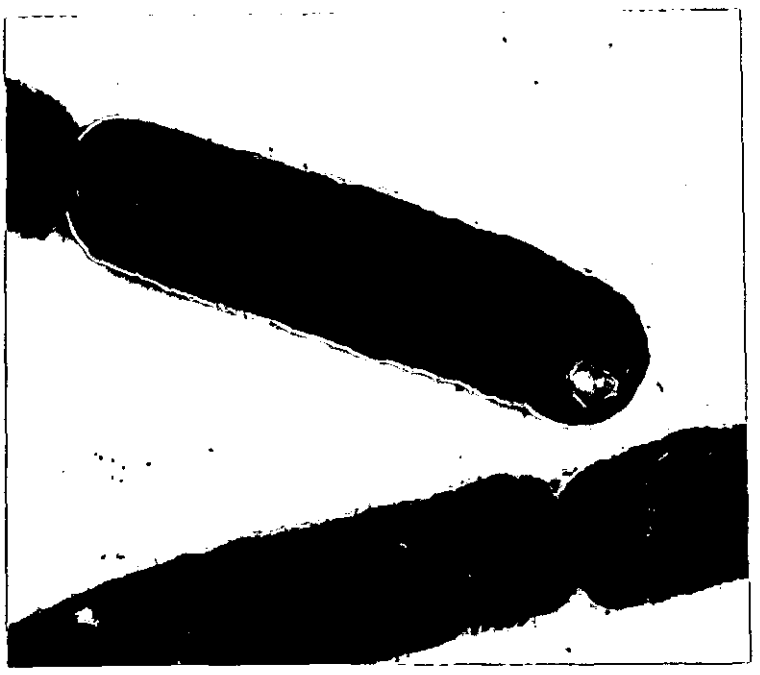


FIGURE 43

Transmission electron micrographs of *E. coli* cells (log phase) fixed in 5% w/v glutaraldehyde for 16 hours at 4°C after 2½ hours contact with the test substance. Cells were prestained with uranylacetate (2% w/v) and osmium tetroxide (1% w/v), embedded in araldite resin, poststained with Reynolds lead citrate and examined under a 60 kv beam.

a) Control cells × 40,000

b) Control cells × 40,000

a)

b)

FIGURE 44

Transmission electron micrographs of *E. coli* cells prepared as figure 43.

a) Cells treated with melittin (12 $\mu g/cm^3$) × 80,000

b) Cells treated with melittin (12 $\mu g/cm^3$ × 30,000

a)

b)

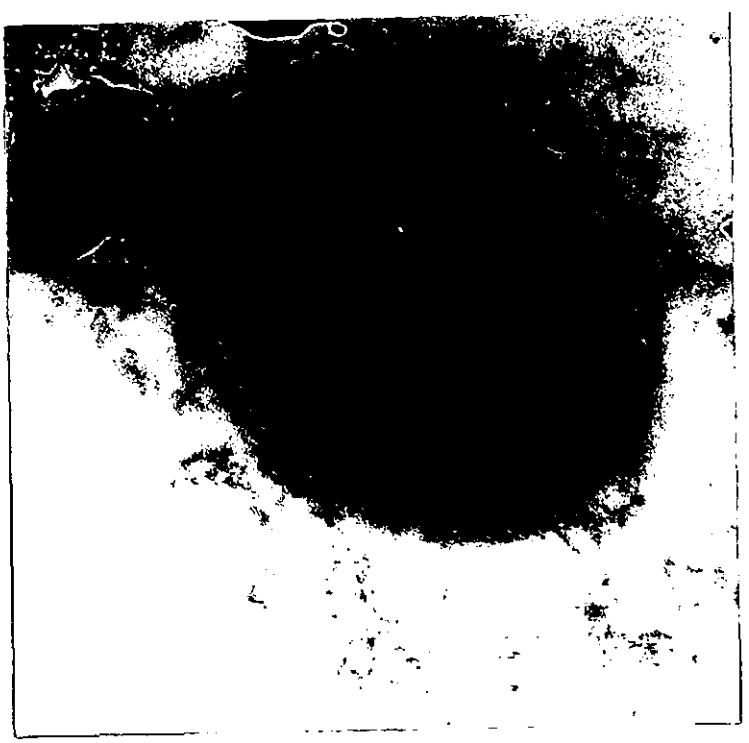


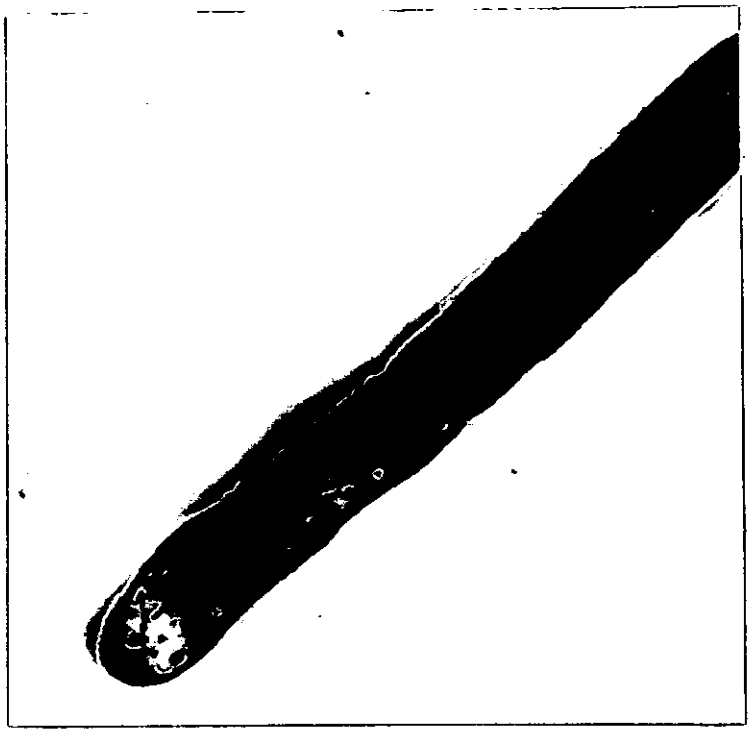
FIGURE 45

Transmission electron micrographs of *E. coli* cells prepared as figure 43.

a) Cells treated with *N. naja sputatrix* venom (400 $\mu g/cm^3$) × 24,000

b) Cells treated with *N. naja sputatrix* venom (400 $\mu g/cm^3$) × 80,000

a)



b)

FIGURE 46

Transmission electron micrographs of *E. coli* cells prepared as figure 43.

a) Cells treated with *A. mellifera* venom (8 $\mu g/cm^3$) × 30,000

b) Cells treated with *A. mellifera* venom (8 $\mu g/cm^3$) × 30,000

a)

b)

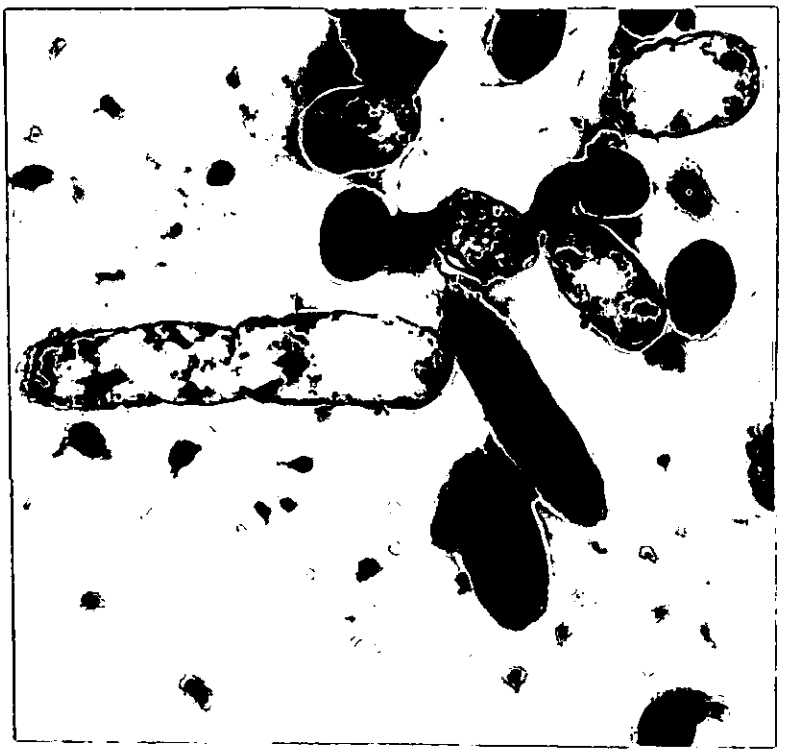


FIGURE 47

Transmission electron micrographs of *E. coli* cells prepared as figure 43.

a) Cells treated with *A. mellifera* venom (8 $\mu g/cm^3$) × 80,000

b) Cells treated with *A. mellifera* venom (8 $\mu g/cm^3$) × 20,000

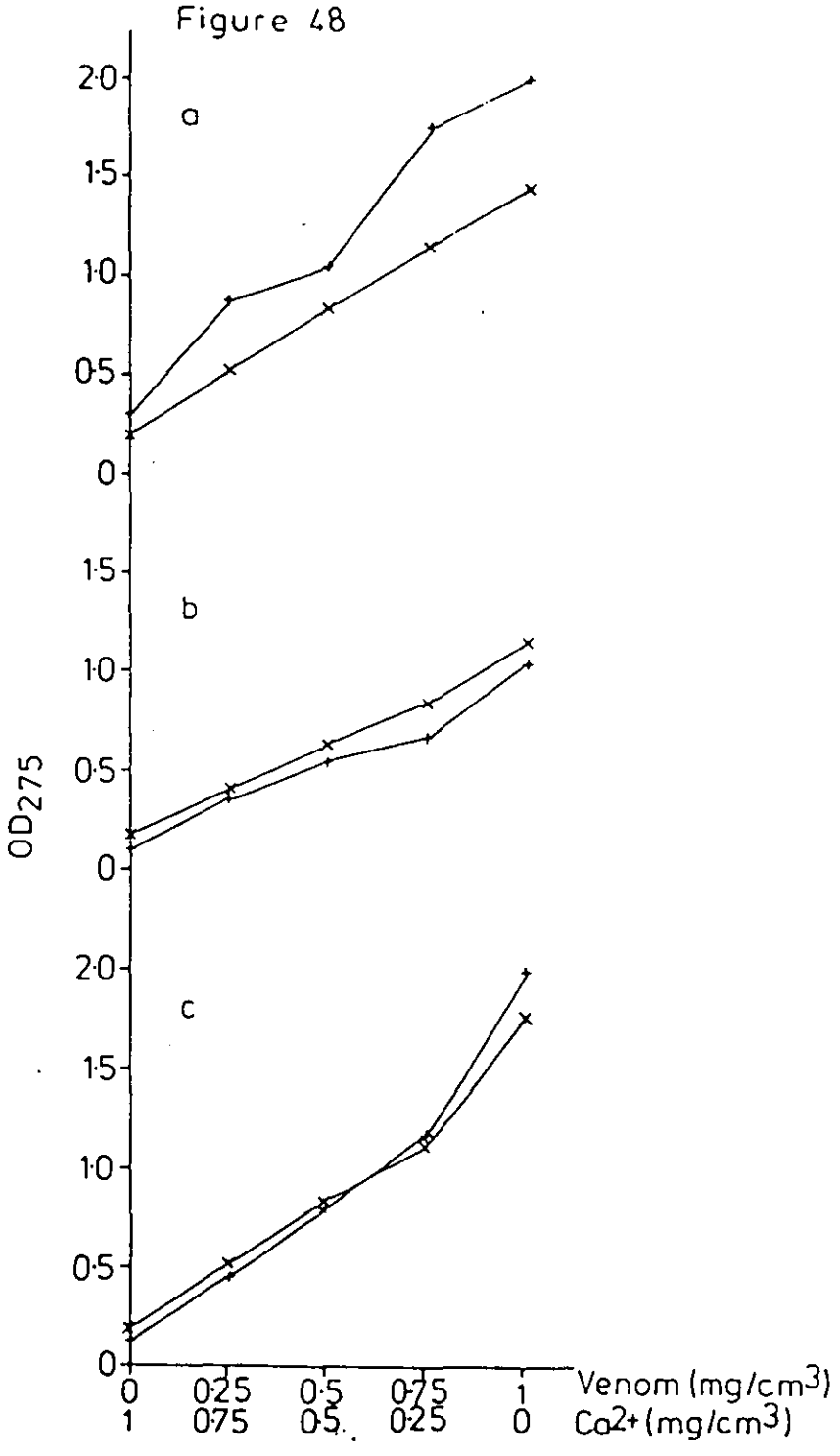


FIGURE 48

Job plots for venom with $CaCl_2$.

×——× Initial absorbancy of mixture.

+——+ Final absorbancy of mixture.

a) *A. mellifera* venom.

b) *N. naja sputatrix* venom.

c) *C. adamanteus* venom.

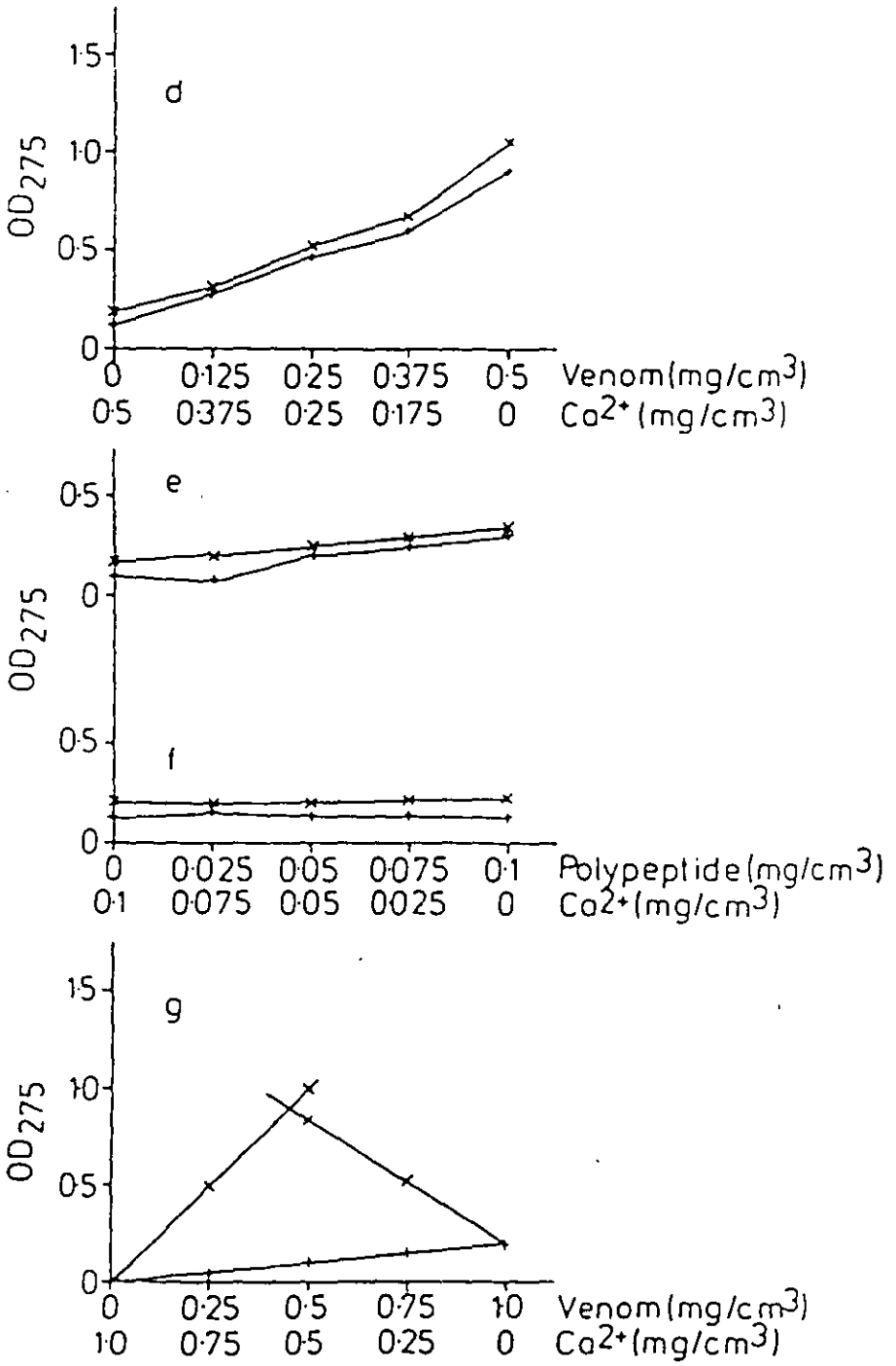


FIGURE 48

Job plots for venom and polypeptides with $CaCl_2$.

×—— × Initial absorbancy of mixture

+—— + Final absorbancy of mixture

d) *V. russellii* venom

e) Melittin

f) Cardiotoxin

g) Theoretical Job plot showing complex formation

Ca^{2+} was found by this means. However with *C. adamanteus* venom (1 mg/cm^3) a cloudy suspension is obtained which clarifies immediately on addition of $CaCl_2$ (Figure 49).

FIGURE 49

Clarification of *C. adamanteus* venom (1 mg/cm^3) upon addition of $CaCl_2$ (≡ 1 mg Ca^{2+}/cm^3)

a) venom

b) venom + $CaCl_2$

c) $CaCl_2$

Section 4

Discussion

Snake and bee venoms were shown to be bacteriostatic towards proliferating *E. coli* cells. The most active venoms are those of *A. mellifera* (honey bee) and *N. naja sputatrix* (cobra). *Vipera russellii* venom was much less active, whilst *C. adamanteus* venom was not observed to inhibit the growth of *E. coli* at concentrations up to 24 mg/cm^3. At higher levels, approximately twice the MIC values, the active venoms were bactericidal. Bee venom was as active against the gram positive organism *S. aureus* as against *E. coli*.

Inhibition of the growth of *E. coli* at effective concentrations of honey bee and cobra venoms occurred within an hour of addition to log phase cultures. The MIC values against aerobically proliferating cells were found to be 2-4 times lower than those obtained by the tube dilution method. It is possible that this could be due to oxygen limitation in the tube method. Alternatively the venoms may be more active against actively growing cells.

Damage to the cells caused by venom treatment was confirmed by the concurrent lowering of cell viability. The decrease in viability caused by *N. naja sputatrix* and *A. mellifera* venoms contrasted with *C. adamanteus* and *V. russellii* venoms which gave viable counts consistently equal to, or greater than, those of control cells. The larger numbers obtained were possibly due to the presence of surface - active components in the venoms, preventing clumping during diluting and plating out, leading to apparently greater numbers of colonies (Shipman and Cole, 1969).

Little work has previously been carried out with respect to the antibacterial effects of whole snake venoms towards *E. coli*, though Aloof-Hirsch (1968) reported inhibition of *E. coli* growth by the polypeptide DLF isolated from cobra venom. On the other hand *A. mellifera* venom has been the subject of some study. In the present work honey bee venom is 20 times more active than reported in earlier studies (Ortel and Markwardt, 1955; Dorman and Markley, 1971). In contrast Fennell et al. (1968) showed honey bee venom to be inactive towards *E. coli* grown on brain heart infusion plates. This group, however, did obtain antibacterial effects towards other gram negative and gram positive organisms. Although the present findings do differ from earlier work, the earlier results between laboratories are not consistent. This could be due to strain differences (no information), the growth medium or the source of venom.

All of the venoms tested were found to cause leakage of β-galactosidase from *E. coli* harvested in the log-phase of growth in the same order of potency as their antibacterial activity, that is *A. mellifera* the most potent and *C. adamanteus* the weakest. The venoms themselves did not exhibit β-galactosidase activity.

In order to measure β-galactosidase release accurately, certain conditions had to be fulfilled. Optimally the bacteria were grown on lactose as the sole carbon source and harvested in the log-phase of growth. Use of isopropyl - β - D - thiogalacto pyranoside as an additional inducer of β-galactosidase led to release which was not easily controllable. The optimal conditions were in agreement with the findings of former workers (Slein and Logan, 1967).

Release of β-galactosidase has been used as a measure of the antibacterial activity of phospholipase C (Slein and Logan, 1967) and polymixin B (Cerny and Teuber, 1971). These workers found cells to be susceptible only if harvested during the log-phase of growth.

The venoms also caused leakage of 260 nm absorbing material from *E. coli*. This leakage correlated with β-galactosidase release (Figures 13 and 33) and with a decrease in the optical density of the suspensions, a measure of cell lysis (Figure 31). This correlation was consistent over the range of activity which was accurately measurable and suggests β-galactosidase release to be related to the integrity of the cell envelope. Where no lysis was measured, for example with treatment by *V. russellii* and *C. adamanteus* venoms, there was virtually no measured leakage of 260 nm absorbing materials, indicating that the cell barrier function remained intact. However, these weaker venoms did cause β-galactosidase release, suggesting that the assay of this enzyme allows a sensitive method for determining effects on *E. coli* cells.

Examination of *E. coli* by both scanning and transmission electron microscopy (SEM and TEM respectively) confirmed that treatment by *N. naja sputatrix* and *A. mellifera* venoms damaged the cells. *Naja naja sputatrix* venom caused some filament formation and there was some evidence for membrane damage which could explain the increase in membrane permeability caused. Cellular damage by *A. mellifera* venom, the most active of the substances studied, was greater than that caused by *N. naja sputatrix* venom at equivalent concentration. In addition to the presence of filaments, honey bee venom treated

cells were seen to be rougher than control cells and many were surrounded by debris, probably leaked cell contents. This was supported by the observation of a high proportion of cell ghosts, containing breaks in both the outer and cytoplasmic membranes, in TEM photomicrographs. The findings suggest a direct lytic action of the more potent venoms, particularly from *A. mellifera* venom on *E. coli*. However, the formation of filaments suggests that these venoms also disrupt normal growth mechanisms, perhaps by acting on cell division or on septum-forming enzymes.

In all studies *A. mellifera* and *N. naja sputatrix* venoms were considerably more active than *V. russellii* or *C. adamanteus* venoms. Though very different in source and composition, both of the former venoms contain membrane-active polypeptides such as melittin, cardiotoxin and direct lytic factor. Indeed honey bee venom contains 50% melittin w/w dried venom (O'Connor and Peck, 1978) and Fennell et al. (1968) have suggested that this is the sole component responsible for the antibacterial action of these venoms on *E. coli*.

Melittin caused β-galactosidase release from *E. coli* and was antibacterial to a similar degree as whole honey bee venom. On SEM examination melittin treated cells appeared less smooth, less robust and shorter than control cells. By TEM, little difference between treated and control cells, with no obvious membrane damage to account for the antibacterial action, was discernable. Neither filaments nor cell ghosts were seen. The EM data would suggest that melittin is not solely responsible for the antibacterial action of bee venom. However biochemical and bacterial assay

suggests melittin to be of a similar order of potency as whole venom. It may be, therefore, that the ultrastructural changes observed with the whole venom are subsequent to melittin action and not primarily responsible for the antibacterial activity.

The action of melittin on *E. coli* was compared with that of another membrane-active polypeptide, polymixin B. Both are basic polypeptides with distinct hydrophobic and hydrophilic regions. Melittin consists of 26 amino acids in a straight chain and polymixin B of 10 residues in a heptapeptide ring with a hydrophobic side-chain (Yunes et al., 1977; Warner., 1961). Both peptides have strong membrane disrupting properties (Hsu Chen and Feingold, 1973) and are active against *E. coli* (Mollay et al., 1976; Storm et al., 1977). Polymixin B is believed to interact with lipid components of the membrane such as lipopolysaccharides and phosphatidyleth-anolamine (Feingold et al., 1974; Lopes and Inniss, 1969).

Polymixin B caused release of β-galactosidase from *E. coli* cells in a similar dose-range to melittin, although less maximal release was obtained. Previously Newton (1953) has reported that loss of cytoplasmic contents correlates with kill for polymixin B and Cerny and Teuber (1971) have measured the polymixin B-induced release of cytoplasmic enzymes from *E. coli* B cells. These workers obtained higher release (45%) than found in the present study (11%). Different strains of *E. coli* are known to have different susceptibilities to polymixin B (Storm et al. 1977).

Differences in cell absorbancy changes may indicate that the mode of action or potencies of melittin and polymixin B differ in the extent of physical damage caused. Though both polypeptides do

cause β-galactosidase release, a decrease in optical density of the treated suspension was obtained only with melittin.

Another important set of venom components which may contribute to action on *E. coli* cells and which have been the subject of much study are the phospholipases A_2. These enzymes have been shown to exhibit synergistic action with the polypeptide components of venoms.

Phospholipases A_2 from *A. mellifera* and *N. naja sputatrix* venoms were seen to cause some β-galactosidase release from *E. coli* cells. However no decrease in cell absorbancy and no loss of 260 nm absorbing material was observed. This suggests some membrane disruption occurs, but not enough to cause lysis. These findings contrast with those of Duckworth et al. (1974) who found highly purified porcine phospholipase A_2 caused no β-galactosidase release, despite almost complete degradation of membrane phospholipids. Cell disintegration did not occur, large molecular weight material being retained within the cytoplasmic membrane. The different findings may be due to the source and purity of the enzymes used. The work of Duckworth et al. (1974) used a highly purified mammalian enzyme whilst the present studies used commercially available partially purified venom enzyme. These enzymes do differ markedly in their actions on membrane targets (Brockerhoff and Jensen, 1974).

No potentiation of either bee or snake venom phospholipase A_2 by melittin or polymixin B was observed with the system used in this study. This is at variance with previous findings that melittin and DLF/cardiotoxins act synergistically with phospholipases A_2 (Condrea and De Vries, 1965; Klibansky et al., 1968; Slotta and Vick, 1969; Vogt et al., 1970). Much of this earlier research was carried out

using red blood cells or synthetic membrane systems and may not be comparable with actions on *E. coli* cells. However, Mollay et al. (1976) using cytoplasmic membranes prepared from *E. coli* K1060 measured a stimulation of both endogenous and added *A. mellifera* phospholipases A_2 by melittin. Also polymixin B stimulates phospholipases A_2 in *E. coli* W2252 cells (Kusano, 1976) and leucocytes (Weiss et al., 1979). The discrepancies may be explained in terms of molar ratios of melittin to membrane phospholipids (Mollay et al., 1976) or the need for a latent period before synergism is seen (Vogt et al., 1970). It may be that the peptides cause β-galactosidase release etc. by activation of endogenous phospholipases A_2 which exist in the outer membrane of *E. coli* (Raetz, 1978) rather than exogenous enzymes and the system is unable to show synergism. Polymixin B has been reported to inhibit the action of a phospholipase C (Saito et al., 1972).

In order to further characterize the effects of venoms, phospholipases and peptides on *E. coli*, in particular concerning β-galactosidase release, further biochemical experiments were performed.

It has been reported that some snake venoms require the addition of an exogenous source of phospholipid before causing lysis of red blood cells (Condrea, 1979). These venoms act by formation of membrane-damaging lysophospholipids. The effectiveness of the weaker venoms from *C. adamanteus* and *V. russellii* was not improved by including phospholipid in the reaction mixture, an effect which is probably explained by differences in the composition of the red blood cell and *E. coli* membranes. Membranes of both cells conform to the fluid mosaic model (Singer and Nicholson, 1972), though red cells have only one lipid bilayer, the cytoplasmic membrane (Weinstein, 1974) while

E. coli cell envelopes consist of two such bilayers, the outer and cytoplasmic membranes, separated by the peptidoglycan layer. Red blood cells contain approximately equal proportions of phosphatidylcholine, phosphatidylethanolamine and sphingomyelin with 30% cholesterol randomly distributed (Van Deenen and De Gier, 1974; Weinstein, 1974). *Escherichia coli* cell envelopes contain no cholesterol and the major phospholipid is phosphatidylethanolamine (69%) (Ames, 1968) with smaller amounts of phosphatidylglycerol (19%) and cardiolipin (6.5%). Importantly the outer half of the outer membrane contains lipopolysaccharide with localization of most phospholipid on the inner half (Nikaido and Nakae, 1979). These differences may render *E. coli* less susceptible to the action of venoms and while phospholipid hydrolysis may occur during *E. coli* treatment, lysis may be prevented by the rigidity of the lipopolysaccharide and peptidoglycan.

Long chain fatty acids, the product of phospholipase A_2 action on phospholipids, are often inhibitory to the enzyme thereby limiting its action. Albumin by binding such fatty acids prevents this feedback inhibition (Smith et al, 1972). Addition of albumin had no effect on the β-galactosidase release caused by *A. mellifera* or *N. naja sputatrix* venoms, or their purified phospholipases A_2, suggesting inhibitory fatty acids are not a contributing factor.

Ions and chelating agents are known to affect the action of polypeptides, phospholipases and many other antibacterial compounds. Introduction of the chelating agent EDTA into the *E. coli* suspension caused some leakage of intracellular material due to its effect on cellular permeability (Russell, 1971) but had little effect on

venom-induced permeability though it did improve slightly the action of *A. mellifera* phospholipase A_2. This is in contrast to the known ability of EDTA to increase the effectiveness of many antibacterial agents e.g. actinomycin (Leive, 1965), pencillin and cephaloridine (Fountain and Russell, 1970), polymixin and cationic agents (Muschel and Gustafson, 1968) and the phospholipid hydrolysing enzyme phospholipase C (Slein and Logan, 1967). There are, however, many antibiotics which are equipotent against EDTA-treated and untreated cells, including mitomycin C and chloramphenicol (Muschel and Gustafsen, 1968).

Addition of Ca^{2+} decreased markedly the ability of both *A. mellifera* and *N. naja sputatrix* venoms and the isolated phospholipases A_2 to cause release of intracellular β-galactosidase. This could be due to Ca^{2+} interacting with the venom components or with the *E. coli*. Study of the data by means of Job plots (Job, 1928) suggested no stable complex formation between Ca^{2+} and venom components. However, the immediate clearing of *C. adamanteus* venom solution in the presence of Ca^{2+} suggests some interaction is taking place. It is possible that Ca^{2+} has an effect on dynamic equilibria within the venom solution, rather than the formation of venom - component - Ca^{2+} complexes. Ca^{2+} shifts the equilibrium between monomer and dimer forms of crotalid phospholipase A_2 towards the dimer (Iwanaga and Suzuki, 1979) and this may apply to *C. adamanteus* phospholipase A_2 (Wells and Hanahan, 1969). Since Ca^{2+} also affects the phospholipase A_2-, in addition to whole venom-, induced release of β-galactosidase it would suggest perhaps that an important component of venom action is the phospholipase A_2, and that the monomer is the toxin form.

Ca^{2+} has a stabilising influence on the outer membrane of *E. coli* (Leive, 1974) and this could lead to blockage of venom antibacterial action if the outer membrane has first to be disrupted. However, the lack of effect of EDTA would argue against this proposal, though the possible increase in *A. mellifera* phospholipase A_2 activity caused by EDTA suggests the situation is not this simple.

Ca^{2+} may prevent the binding of polypeptide components to *E. coli* cells. The actions of cardiotoxin and DLF are prevented by Ca^{2+} (Chang, 1979) and Ca^{2+} prevents the binding of cardiotoxin to Ehrlich Ascites tumour cells (Leung, 1976). The results of the present study using melittin would be consistent with these findings.

The available evidence suggests interference with venom components is responsible for the antagonistic actions of Ca^{2+}. In the case of the phospholipases A_2 a major anomaly arises since such phospholipases have long been considered to have an absolute requirement for Ca^{2+}. However, Salach et al. (1971) have questioned the assumption that added Ca^{2+} is required for or potentiates phospholipase A_2 action on membrane-bound phospholipids, and Weiss et al. (1979) have shown that added Ca^{2+} is not required for the action of various phospholipases A_2 (*N. naja*, *A. mellifera*, porcine pancreas) on *E. coli* K12 phospholipids. It is possible that sufficient Ca^{2+} is available within the cell envelope and that additional Ca^{2+} becomes inhibitory.

Gel chromatographic separation of *N. naja sputatrix* venom yielded three fractions. The first fraction is likely to be a mixture of phospholipase A_2 and cardiotoxin since separation of

these two substances requires a pH gradient (Slotta et al., 1967; Iwanaga and Suzuki, 1979). This was confirmed by the potency of this fraction in causing β-galactosidase release from *E. coli*. Most of the protein in honey bee venom was eluted close together. The fractions eluted early were tentatively identified as phospholipase A_2, melittin and a mixture of the two substances (Shipman and Cole, 1969). All of these fractions released cytoplasmic contents, assayed as β-galactosidase, from *E. coli*. The non-membrane-active polypeptide apamin was also isolated and did not cause β-galactosidase release.

On electrophoretic separation at pH 8.6 all venom components exhibited cathodic mobility, with the exception of two bands in *V. russellii* and *C. adamanteus* venoms. This indicates isoelectric points greater than pH 8.6 as also found by Tu and Ganthavorn (1968) and Mebs (1969). The three slowest bands in *A. mellifera* and *N. naja sputatrix* venoms caused inhibition of *E. coli* growth. Melittin and cardiotoxin run separately were also antibacterial but did not account for the entire antibacterial action of the venoms.

No antibacterial activity was exhibited by any of the components of *V. russellii* or *C. adamanteus* venoms. We can conclude from this that the lack of antibacterial activity is due to an absence of active components, rather than the presence of an inhibitory factor.

This study has shown that venoms, particularly honey bee venom, exhibit antibacterial activity against *E. coli*, although that of *C. adamanteus* is very weak. The work was carried out using both proliferating and non-proliferating cells, rather than cytoplasmic

membrane preparations, liposomes or red blood cells used in earlier studies. The venoms and some of their constituents have a primary effect on the cell envelope causing permeability changes which is apparent by leakage of cytoplasmic constituents, particularly β-galactosidase. The changes may be of an order resulting in cell lysis. Scanning and transmission electron microscopy studies confirmed that membrane disruption occurred on venom treatment and also showed the presence of *E. coli* filaments, indicating action on cell growth mechanisms.

Phospholipases A_2 and membrane-active polypeptides have a direct effect on *E. coli* but it is possible that several other factors are also involved in the venom action against the bacterium. It is important to realise though that the two weak venoms are the ones which lack membrane-active polypeptides. It is possible, therefore, that the main effects of venom action could be due to disorganisation of lipopolysaccharide by the polypeptides similar to that caused by polymixin B and phospholipid hydrolysis by phospholipase A_2. Endogenous phospholipases in the outer membrane of *E. coli* may also be involved.

The order of increasing effectiveness against *E. coli* by the venoms is the opposite of increasing danger to mammals. It may be that the venom enzymes are more important in action against mammalian cells, while the toxins are instrumental in the antibacterial action of the venoms. It is intriguing to speculate that agents selectively toxic to bacterial cells might be isolated from venoms. However, the venoms are complex mixtures and the overall antibacterial action is likely to be due to many different components.

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