THE PROCOAGULANT FROM PSEUDONAJA SPECIES.

ISOLATION AND BIOCHEMICAL CHARACTERISATION AND COMMENTS ON VENOM VARIABILITY

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TABLE OF CONTENTS

ABSTRACT 3

DECLARATION 4
ACKNOWLEDGMENTS 5
LIST OF ABBREVIATIONS: 6

CHAPTER 1 7

INTRODUCTION – SNAKEBITE IN AUSTRALIA 7
ABORIGINAL AUSTRALIA 8
EUROPEAN ARRIVAL 9
FIRST AID 9
SNAKEBITE TREATMENT 10
ENVENOMATION 13
REFERENCES 15

CHAPTER 2 19

VENOM COAGULANTS 19
CLINICAL OBSERVATIONS 19
EXPERIMENTAL OBSERVATIONS 29
OVERVIEW OF THESIS 45
REFERENCES 46

CHAPTER 3 52

OBSERVATIONS ON WHOLE VENOM COAGULATION ACTIVITY AND ISOLATION OF THE PROCOAGULANT FROM THE VENOM OF *PSEUDONAJA TEXTILIS* 52
INTRODUCTION 52
MATERIALS AND METHODS 57
Venoms: 57
Protein estimation: 57
Liquid chromatography of the venom: 57
Electrophoresis: 58
Blotting: 58
Fold purification analysis of procoagulant: 59
RESULTS 59
Protein estimation 59
Purification 60
Electrophoresis 63
DISCUSSION 65
REFERENCES 67

CHAPTER 4 70

CHARACTERISATION OF THE PROCOAGULANT PT 1 - EXAMINATION OF THE COMPLETE PROCOAGULANT .. 70
INTRODUCTION 70
MATERIALS AND METHODS 71
Procoagulant: 71
Electrophoresis: 71
Active-site labelling: 71
Carbohydrate detection: 72
Determination of the inhibitory effect of various reagents on procoagulant activity: 72
Chromogenic substrate conversion: 73
Immunoprecipitation: 73

RESULTS 73
β-mercaptoethanol reduction of procoagulant bands 73
Active-site labelling 74
Chromogenic substrate conversion 76
The effect of inhibitors on the procoagulant conversion of chromogenic substrate S2222 78
Glycan detection 78
Immunoprecipitation 79
DISCUSSION 80
REFERENCES 84

CHAPTER 5 .... **85**

CHARACTERISATION OF THE PROCOAGULANT PT 2 - EXAMINATION OF THE PROCOAGULANT SUBUNITS 85
INTRODUCTION 85
MATERIALS AND METHODS 88
Procoagulant: 88
Isolation of individual bands: 89
Isolation of Textarin from procoagulant: 89
Carbohydrate analysis of subunits: 90
End terminal analysis of carbohydrates: 90
Plasma substrate requirements for Textarin: 91
N-terminal amino acid sequencing of the 43, 48 and 52kDa subunits: 91
RESULTS 92
Isolation of individual subunits (bands) 92
Textarin isolation 94
Carbohydrate analysis 94
Textarin substrate requirements for coagulant activity 96
N-terminal amino acid analysis 100
DISCUSSION 104
REFERENCES 111

CHAPTER 6 .... **114**

VARIATION IN THE VENOM COMPOSITION OF THE BROWN SNAKES 114
INTRODUCTION 114
MATERIALS AND METHODS 117
Seasonal variability: 117
Geographic variability: 118
Comparison of the procoagulant from the venom of the brown snake species: 118
Comparison of the procoagulant from P. textilis and Oxyuranus microlepidotus: 119
RESULTS 120
Seasonal variability 120
Geographic variability 121
Procoagulant species comparison 124
Comparison of the procoagulant from P. textilis and Oxyuranus microlepidotus 127
DISCUSSION 128
REFERENCES 133

CHAPTER 7 .... **136**

CONCLUSIONS AND FUTURE DIRECTIONS 136
SUMMARY 143
FUTURE DIRECTIONS 144
REFERENCES 147

EPILOGUE **149**

PUBLICATIONS **150**

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ABSTRACT

The venom from the Australian brown snakes (*Pseudonaja* spp.) contains a strong procoagulant component that produces bleeding due to consumption of clotting factors in bite victims. Whole venom from *P textilis* clotted citrate plasma rapidly while EDTA, heparin and warfarin plasmas were also clotted by the venom. Investigation of the role of phospholipid and calcium found neither was essential for activity, but calcium could shorten the clotting time. Factor X and V deficient plasmas were clotted by the venom thus the procoagulant was a direct prothrombin converter. It was hypothesised that the procoagulant consisted of the equivalent of the prothrombinase complex, factor X, V, calcium and phospholipid. Investigation of venom variability due to season, geographic origin or species found no differences in venom composition. There were differences in the activity of these components although this appeared to be no greater than that expected due to individual variability. The procoagulant was found to be conserved across species and geographic range and therefore must provide the snakes with a selective advantage. The procoagulant was isolated from whole venom by gel filtration and anion exchange chromatography. The isolated procoagulant produced a strong slow-moving band and a faster faintly staining band on native PAGE, both of which were active-site labelled. SDS-PAGE of the procoagulant resulted in a number of bands (subunits), divided into a high and low molecular weight group. The active-site label was confined to two distinct bands with molecular weights of 48 and 52kDa (the molecular weight and activity of these subunits was consistent with that for *Textarin*). In isolation these subunits required factor V and calcium for activity while phospholipid in combination with calcium improved the activity dramatically. The activity was thus the equivalent of factor Xa although heparin plasma and descarboxyprothrombin were still activated by these subunits. The activity of these subunits alone however was insufficient to account for the rapid clotting noted with all subunits intact, therefore either disruption of these subunits or dissociation of an amplifying subunit reduced the activity. Sequencing of the 48 and 52kDa subunits revealed homology to factor Xa, while the sequence of a 43kDa subunit showed homology to factor V. This provided the evidence of a cofactor to support the hypothesis that the procoagulant resembles the prothrombinase complex.

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

9/2/2000

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LIST OF ABBREVIATIONS:

BAEE	N_{α}-benzoyl-L- arginine ethyl ester
BME	β-mercaptoethanol
CSL	Commonwealth Serum Laboratories
dansyl-GGACK	dansyl-Glu-Gly-Arg-CH_2Cl
DFP	Diisopropylfluorophosphate
DSA	*Datura stramonium* agglutinin
EDTA	Ethylenediaminetetra-acetic acid
GNA	*Galanthus nivalis* agglutinin
INR	International Normalised Ratio
MAA	*Maakia amurensis* agglutinin
PAGE	Polyacrylamide gel electrophoresis
PLDPA	Phospholipid dependent prothrombin activator
PLIPA	Phospholipid independent prothrombin activator
PMSF	Phenylmethylsulfonyl fluoride
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SNA	*Sambucus nigra* agglutinin
TAME	N_{α}-p-tosyl-L-arginine methyl ester

CHAPTER 1

INTRODUCTION – SNAKEBITE IN AUSTRALIA

Australia is reputed to have the most dangerous snakes in the world and yet despite this, the literature has had a heavy bias in venom research towards the Northern Hemisphere. Awareness of the potential therapeutic agents and research tools that can be discovered in venoms, has focused interest back onto Australian venoms as a source of such components.

Our current understanding of the action of various venom constituents has improved over the last twenty years, but of interest is the historical background that has allowed us to arrive at this point, particularly the early understanding of the process of envenomation, treatments based on this understanding and the early experimentation. The early work did not link particular activities with individual components present in venom, but as this was realised, the neurotoxins, myolysins and coagulants became distinct entities in research, no longer clumped under the general heading of venom activities. Tidswell (1906) reported that with identification of differing chemical composition and physiological action, "venoms are not the comparatively simple substances they were formerly supposed to be". The work of Flexner and Noguchi (1902, 1903), Lamb (1902) and others indicated the action of venoms on the body were due to independent toxic principles, including, 'fibrin ferments' and 'antifibrin ferments'.

This thesis is to concentrate on the coagulant activity predominantly and the venom variability to a lesser extent of the brown snake(s) (*Pseudonaja* spp.), however in the opening chapters, the background of theories of envenomation, treatment, clinical observation and laboratory investigation are reviewed.

ABORIGINAL AUSTRALIA

The Aboriginal inhabitants of Australia had great respect for snakes and they played a significant role in Dreamtime stories (Wilson, 1972; Hercus and Potezny, 1993).
It has been suggested that the occurrence of snakebite in the Aboriginal population was a rare event despite the life-style, with hunting and gathering placing them at risk of encountering snakes and thus also at risk of being bitten. Beddoe (1878) reported that snakes made up a good portion of their food, with all kinds being eaten, both venomous and harmless, but suggested that in hunting them they were careful to smash the head of the snake to a pulp with a stone. The Aborigines were obviously conversant with the possible effect of venomous snakebite and a funeral of an Aboriginal who died as a result of a snake bite has been described (Anon, 1973). Irrespective of the frequency of snakebite in the Aboriginal population, snakebite treatments were prominent in their medical protocols (Cleland, 1962; Cawte, 1975; Covacevich, 1990). One such treatment was to tie a ligature round the bitten limb and then to deeply scarify the limb in a circle above the wound. It is uncertain however if this was an original treatment of the Aboriginal population or performed as a result of the common practice of the European colonists in excising the bite site after envenomation. The Aborigines certainly warned the European settlers against contact with particular snakes because of the danger they posed (Sutherland, 1983).

The traditional owners of the land around Goyder's Lagoon, in South Australia, were certainly aware of the possibly fatal bite from a western taipan, (*Oxyuranus microlepidotus*) or "dandarabilla" as it was known (Covacevich, 1990). However the symptomatology does not appear to have been addressed, although reeds were sometimes used to blow wind into

the navel of the victim, if breathing became difficult. Thus despite fatal bites there are no reports that I am currently aware of recounting episodes of bleeding as a result of a bite.

EUROPEAN ARRIVAL

The incidence of snakebite among the settlers was small and most likely reflected the small population and therefore interaction with snakes. In the early days of the colonies, incidents of snakebite were reported in the local press and were well dramatised. With increasing population and dissemination of that population into the rural areas, contact with snakes increased.

FIRST AID

Early colonial methods

Any number of first aid methods were developed during the mid to late 19th century, including cautery of the bitten area using gun powder (Hall, 1859), amputation of the bitten limb, to the extreme of blasting out the bitten part with gunfire (Anon,1867). On many of these occasions treatment was probably unnecessary, as many of the bites were possibly dry strikes or glancing blows, although a glancing blow does not exclude the possibility of severe envenomation.

Current concepts

The application of arterial tourniquets as a means of stopping venom movement is painful and potentially dangerous, with the possibility of tissue and nerve damage. The efficacy of an 'arterial' tourniquet is also questionable, particularly in view of the difficulty of applying same to achieve stasis. Barnes and Trueta (1941) indicated the important role that the lymphatics play in the movement of venom and in 1979, Sutherland et al investigated pressure and immobilisation as a means of impeding venom movement. This method was further investigated by examining the movement of venom in the monkey *Macaca fascicularis*

(Sutherland et al, 1981) and the recommendation in the case of Australian snakebite was to apply firm pressure via a bandage over the bite site and up the bitten limb and then to immobilise the limb via a splint. Neither immobilisation nor pressure alone was sufficient to reduce venom movement.

Murrel (1981) reported a case of tiger snake bite where the use of the pressure/immobilisation technique appeared to delay onset of envenomation symptoms. The effectiveness of the technique was also confirmed by Pearn et al (1981), when removal of the constrictive bandage from a bitten limb resulted in symptoms of significant envenomation developing. The effects of a rush of venom on release of the constriction and the development of subsequent symptoms within a short period has since been noted on many occasions and is supportive of the effectiveness of the procedure.

The pressure/immobilisation technique is now well established as a means of reducing venom movement until the patient can be transported to a facility with an adequate intensive care unit capable of dealing with the consequences of envenomation.

SNAKEBITE TREATMENT

Theories on envenomation

Two interesting theories were held to be true during the last part of the 19th century, which hindered advancement of knowledge on envenomation, venom composition and the development of antivenoms. The first, was the 'germ theory' of envenomation. Halford (1867, 1873) had determined that envenomation was akin to an infection and reported on the presence of poison cells in venom demonstrable under a microscope. Creed (1884) referred to the work of Fayrer in India where the serum/blood from an animal killed by venom was introduced into another animal which subsequently died. He then took the serum from this animal and injected into a third, with the same fatal result. The estimate of a dilutional effect of some $9x10^6$ suggested to him that the only explanation for the continued strength of the

toxin was that it had multiplied, "...a substance with properties akin to a ferment, which under favourable conditions multiplies itself.."

Under the circumstances the conclusions were not unreasonable, but were responsible for the development of some bizarre snakebite treatments.

The second point of misunderstanding was the broadly held theory that all snake venoms are analogous. The differences noted in the symptoms displayed were due to the depth the fangs penetrated and the subsequent volume of venom injected (Berncastle, 1869). The belief that venoms are analogous was dispelled when Calmette's serum (Calmette, 1894 a, b, c; Tidswell, 1902) - developed against the cobra and French viper - was shown to be ineffective against tiger snake venom (Martin, 1897; Tidswell, 1906), although Bill (1902) described treatment of a snakebite case with Calmette's 'Antivenine' with a satisfactory outcome, although the snake responsible was not seen. However what part the treatment played in the recovery of the patient is uncertain.

Treatment - pre 1930

The lack of understanding regarding these concepts led people to develop treatments which were inappropriate, dangerous and distracted researchers attentions from developing more effective treatment and first aid.

The germ theory provided the basis for Professor Halford's ammonia treatment (Halford, 1869, 1873), whereby "saturation of the blood cells and its deoxygenation prevents the growth of new cells". The use of the ammonia treatment in Australia caused considerable political furore, but with the deaths of patients, despite treatment, and growing evidence of it being of no use from overseas, the technique lapsed. The germ theory did provide the basis for one other treatment of interest, that devised by Clutterbuck (1864), which involved the use of mercury. Under the belief current at the time that two specific diseases rarely coexist "...if an attempt is made to introduce another poison into the circulating system - the

primary morbid process might be so influenced or disturbed as to dispose it to decline...". Fortunately this form of treatment failed to gain favour and disappeared quickly.

The use of strychnine was also advocated in the colonies as a treatment for snakebite (Mueller, 1890; Creed, 1891). Mueller was so convinced that the treatment with strychnine was infallible "..I do not hesitate to say that, in my opinion, the life of any patient who dies from the effects of snake-poison, who has not been treated with adequate doses of strychnia, has been sacrificed to ignorance or prejudice". Fortunately again this method of treatment failed to be taken up in an enthusiastic manner and excessive use of the strychnine in the case of a snakebite resulted in the patient's death (Croll, 1912).

Excision of the bite site was a commonly employed method of treatment and this practice survived well into modern times even with the availability of antivenoms, despite its attendant dangers of infection, spread of the venom and the possibility of haemorrhage. Excision was pursued as it was believed that the short fangs of the Australian snakes would fail to produce deep injection of the venom and thus the venom should be removed with the excision of the bitten area.

Antivenom therapy

The development of an effective antivenom did not occur until 1930. Advancement in this area showed early progress in the wake of Calmette's pioneering work, with Tidswell (1902) producing immunization of an old ambulance horse against tiger snake venom in 1898. The final challenge of venom given to the animal was a massive 600 mg (multiple lethal doses) and despite the immunity of the animal these experiments were not followed up until Fairley (1929) encouraged Kellaway and Morgan to re-examine the possibility of producing antivenom. Tiger snake antivenom was first produced in 1930 and Tisdall and Sewell (1931) used the antivenom in a bite case while Kellaway and Morgan (1931) reported on the presentation and administration of the antivenom. Antivenom was then produced against the other major clinically significant venom types, (Table 1.1). Initially it was felt that it would

be unlikely that an antivenom to brown snake would be ever produced because of the small fangs of the snake and the fact that very small quantities of venom are produced, however persistence triumphed, with the release of the antivenom in 1956.

Table 1.1 Production of antivenom in Australia.

Antivenom	Year of Production
Tiger	1930
Taipan	1955
Brown	1956
Death Adder	1958
Polyvalent	1962

ENVENOMATION

Prey acquisition

Snake venoms are a complex mixture of reactive proteins, nucleotides(sides) and inorganic ions. This mixture is well developed to enable the snake to acquire prey, assist in digestion of that prey and as a means of defending itself against other predators.

In acquiring prey, the snake needs to protect itself against the prey as it may inflict life-threatening wounds, prior to its own demise or escape. The snake can overcome these attempts by the prey by firmly grasping the prey (in a manner similar to constrictors), ensuring the prey is unable to move while the venom takes effect. Alternatively, a fast strike on the prey and injection of copious amounts of venom with quick release, even to the point of only inflicting a glancing blow with the fangs, may be sufficient to introduce enough venom to immobilise or kill the prey.

The introduction of the venom needs to bring about either the death or immobilisation of the prey animal within a period that the prey cannot escape beyond an area that the snake will be able to find it. Thus the neurotoxins may help in immobilising the prey, however this may take some time to take effect, while the haemostatically active components may be able to

bring about rapid death of the prey (Tidswell, 1906; Fairley, 1929). There is also the possibility of direct organ toxins such as cardiotoxins and nephrotoxins that may aid in the demise of the prey.

Diagnosis

The occurrence of a snakebite is perhaps more difficult to diagnose than would be expected, and anecdotally this would appear to be the case. There are a number of cases where snakebite was least suspected (Hilton, 1989). Confusion may arise with a possible spiderbite, or 'bites' occurring in thickly vegetated areas may be nothing more than a wound inflicted by the undergrowth, and the influence alcohol can play in the perception of the victim must not be underestimated (Sutherland, 1992; White, 1992). When young children are the victims a history of snakebite may be unavailable; however a bite should be considered as a differential diagnosis with uncertain symptoms (White et al, 1983-4; Underhill, 1987). Details about suspected / possible snakebites should be gathered as these can assist when deciding on possible treatment strategies. One of the most important things to be performed is a venom detection on the bite site, to assist in identifying the offending species, such that, should antivenom be required, monospecific can be employed.

Symptomatology

Envenomation may result in a number of general symptoms which include headache, nausea, vomiting, abdominal or chest pain. On occasion dizziness may be encountered with possible collapse or convulsions or both.

Specific symptoms may be evident as a result of individual toxins acting on their targets (Table1.2).

Table1.2 Systemic effects of venom toxins.

TOXIN	TARGET	SPECIFIC SYMPTOMS	TIME TO APPEARANCE
Neurotoxin	Neuromuscular junction	Ptosis, ophthalmoplegia, weakness, ataxia, respiratory failure	Initial paralysis >1hour Respiratory failure usually > 12 hour
Myolysins	Skeletal muscle	Muscle weakness, pain on movement, myoglobinuria	Initial early attachment to muscle, symptoms delayed for several hours
Haemotoxins	Coagulation factors, platelets	Oozing from mucosal surfaces and drip sites, bruising, haematemesis, per rectal bleeding, haematuria	May appear as early as 15 min

There is the possibility that cardiotoxins and nephrotoxins may also be present in venoms, acting directly on these target organs.

REFERENCES

Anon (1867). Local topics. Aust. Med. J. **12**, 286.

Anon (1973). An Aboriginal funeral in the late 1860's. Richmond River Historical Soc. Bull. **67**, 13-14.

Barnes, J.M., Trueta, J. (1941). Absorption of bacteria, toxins and snake venoms from the tissues. Importance of the lymphatic circulation. Lancet1, 623-626.

Beddoe, J. (1878). On the Aborigines of central Queensland. J. Anthropol. Instit. **7**, 145-148.

Berncastle, J. (1869). The treatment of snake bites. Aust. Med. Gaz. **1** , 31-35.

Bill, G. (1902). Notes on a case of snake-bite, treated with antivenine. Intercol. Med. J.**7**, 346-348.

Calmette, A. (1894a). Propriétés du sérum des animaux immunisés contre le venin des serpents; thérapeutique de l'envenimation. C.R. des Séances de L'Academie des Sciences **118**, 720-722.

Calmette, A. (1894b). Propriétés du sérum des animaux immunisés contre les venins de diverses espéces de serpents. C. R. des Séances de L'Academie des Sciences **118**, 1004-1006.

Calmette, A. (1894c). Contribution a l'étude du venin des serpents. Immunisation des animaux et traitement de l'envenimation. Ann. de L'Instit. Pasteur **8**, 275-291.

Cawte, J.E. (1975). Australian Aboriginal medicine before European contact. Ann. Intern. Med. **82**, 422-423.

Cleland, J.B. (1962). Aborigines: Diseases and medicines. Aust. Encycl. 2nd ed. pp. 82-87. Angus and Robertson, Sydney.

Clutterbuck, J.B. (1864). On two cases of snake-bite successfully treated. Aust. Med. J. **9**, 195-196.

Covacevich, J. (1990). Phangs and physic. 40,000 years of risky business. Pp 60-72. In: Fevers and Frontiers. Eds. Pearn, J., Cobcroft, M. Amphion Press, Brisbane.

Creed, J.M. (1884). Australian snakebite - Fear as a factor in producing many of the alarming symptoms following. Australas. Med. Gaz. **3**, 224-229.

Creed, J.M. (1891). Strychnine in snake-bite. Australas. Med. Gaz. **11**, 84-89.

Croll, G. (1912). Death from strychnine poisoning after snake bite. Australas. Med. Gaz. **32**, 189.

Fairley, N.H. (1929). The present position of snake bite and the snake bitten in Australia. Med. J. Aust. **1**, 296-313.

Flexner, S., Noguchi, H. (1902). Snake venom in relation to haemolysis, bacteriolysis and toxicity. J. Exp. Med. **6**, 277-301.

Flexner, S., Noguchi, H. (1903). The constitution of snake venom and snake sera. J. Path. Bact. **8**, 379-410.

Halford, G.B. (1867). On the condition of the blood after death from snake-bite, as a probable clue to the further study of zymotic diseases, and of cholera especially. Trans. Proc. Roy. Soc. Vict. **8**, 73-94.

Halford, G.B. (1869). On the injection of ammonia into the circulation. Aust. Med. J. **14**, 100-112.

Halford, G.B. (1873). Snake-poisoning and its treatment. Med. Tms. Gaz. pp. 90-91, 170-171, 224-226, 323-324, 461-463, 575-577, 712-714.

Hall, E.S. (1859). On snake bites. Aust. Med. J. **4**, 81-96.

Hercus, L.A., Potezny, V. (1993). Arkaya. The Kestrel history. Report to the Heritage Commission, Australian National Library, Canberra.

Hilton, J.M.N. (1989). A tragic case of snakebite. Med. J. Aust. **151**, 600-601.

Kellaway, C.H., Morgan, F.G. (1931). The treatment of snake-bite in Australia. Med. J. Aust. **2**, 482-485.

Lamb, G. (1902). On the precipitin of cobra venom: a means of distinguishing between the proteins of different snake poisons. Lancet **2**, 431-435.

Martin, C.J. (1897). The curative value of Calmette's anti-venomous serum in the treatment of inoculations with the poisons of Australian snakes. Intercol. Med. J. Australas. **II**, 527-536.

Mueller, A. (1890). A contribution to the history of medicine in Australia. Australas. Med. Gaz. **9**, 153-156.

Murrell, G. (1981). The effectiveness of the pressure/immobilization first aid technique in the case of a tiger snake bite. Med. J. Aust. **2**, 295.

Pearn, J., Morrison, J., Charles, N., Muir, V. (1981). Efficacy of a constrictive bandage with limb immobilization in the management of human envenomation. Med. J. Aust. **2**, 293-295.

Sutherland, S.K., Coulter, A.R., Harris, R.D. (1979). Rationalisation of first-aid measures for elapid snakebite. Lancet **1**, 183-186.

Sutherland, S.K., Coulter, A.R., Harris, R.D., Lovering, K.E., Roberts, I.D. (1981). A study of the major Australian snake venoms in the monkey (*Macaca fascicularis*) 1. The movement of injected venom, methods which retard this movement and the response to antivenoms. Pathology **13**, 13-27.

Sutherland, S.K. (1983). Australian animal toxins, the creature, their toxins and care of envenomed patient. Oxford University Press, Melbourne.

Sutherland, S.K. (1992). Deaths from snakebite in Australia, 1981-1991. Med. J. Aust. **157**, 740-746.

Tidswell, F. (1902). A preliminary note on the serum-therapy of snake-bite. Australas. Med. Gaz. **21**, 177-181.

Tidswell, F. (1906). Researches on Australian venoms. Snake-bite, snake-venom and anti-venine. The poison of the platypus. The poison of the red-spotted spider. Dept. Public Health, N.S.W. Govt. Printer, Sydney.

Tisdall, H.T., Sewell, J.E. (1931). Treatment with antivenine twenty-four hours after a bite by a tiger snake. Med. J. Aust. **1**, 604-605.

Underhill, D. (1987). Australia's dangerous creatures. p.133. Reader's Digest, Surrey Hills, N.S.W.

White, J., Tomkins, D., Steven. I., Williams, V. (1983-4). Tiger snake bite. Rec. Adelaide Child. Hosp. **3**, 169-173.

White, J. (1992). A review of snakebites and suspected snakebites in South Australia with particular reference to reptile handlers. In: Recent advances in toxinology research, **1**, 367-377. (Eds. Gopalakrishnakone, P. Tan, C. K.), National University of Singapore, Singapore.

Wilson, B.K. (1972). Tales told to Kabbarli. Angus and Robertson, Publishers, Australia.

N.B. The volumes of the Australasian Medical Gazette referenced are in accordance with the Barr Smith Library, University of Adelaide, South Australia. If difficulty is encountered in locating the article, the volume or year either side of that referenced should be consulted.

CHAPTER 2

VENOM COAGULANTS

CLINICAL OBSERVATIONS

(The classification of the Australian elapid snakes can be found in Table 2.1for reference.)

In the Editorial comments of the Medical Journal of Australia (1973), the presence of a haemostatic defect after snakebite could be characterised by excessive bleeding from sites of trauma, such as venepuncture sites, or perhaps even through haematemesis. Sutherland (1975) extended the list of likely general effects seen as a result of coagulopathy to include

- haematemesis
- haemoptysis
- haematuria
- large bowel haemorrhage
- menorrhagia

Case reports pre 1930

The early reports of envenoming generally appeared to concentrate on the symptoms in the victims that were obviously of a neurotoxic origin. Clutterbuck (1864) was consulted on two cases of snakebite where his description of the envenomation was that “great disorder of the sensorium was manifest”. Creed (1884) reported ten cases of supposed snakebite and reported the condition of a 12 y.o. girl as being semi-comatose.

Table 2.1 Taxonomy of the Australian elapid snakes.

Scientific name	Abbreviation	Previous scientific names	Common names
Pseudonaja textilis	*P. textilis*	*Demansia textilis* *Diemenia*	Brown snake Common brown Eastern brown
Pseudonaja nuchalis	*P. nuchalis*	*Demansia nuchalis*	Western brown Brown Gwardar
Pseudonaja affinis	*P. affinis*	-	Dugite
Pseudonaja inframacula	*P. inframacula*	-	Peninsula brown
Oxyuranus scutellatus	*O. scutellatus*	-	Taipan Coastal taipan
Oyuranus microlepidotus	*O. microlepidotus*	*Parademansia microlepidotus*	Inland taipan Small -scaled snake Fierce snake
Notechis scutatus	*N. scutatus*	-	Tiger snake
Notechis ater niger	*N. niger*	-	Black tiger Peninsula tiger
Tropidechis carinatus	*T. carinatus*	-	Rough-scaled snake
Pseudechis porphyriacus	*P. porphyriacus*	-	Red bellied black Black snake
Acanthophis antarcticus	*A. antarcticus*	-	Death adder Common death adder Early usage: Deaf adder
Austrelaps superbus	*A. superbus*	*Hoplocephalus superbus*	Copperhead

She could only be roused with difficulty, with dilated pupils, cold clammy skin, feeble and irregular pulse, with convulsive twitchings of the muscles of the whole body. Similar descriptions are found in other reports such as that from a series of three patients seen by Dr. Weekes of Lithgow (see Creed, 1891), where all three patients were comatose and in one, the patient had also had a convulsion. Another read '...her countenance was swollen and dusky, cornea glassy and insensible and stupor was complete'. There was a fatal case reported by Hedley and Rees (1874) however, where a child presumably bitten by a tiger snake, had vomited some blood and at post mortem the lungs were congested with dark venous blood and the blood was fluid in the heart.

In this series of bite reports though, in most cases the site of the bite had been excised without any reports of excess bleeding, although it is debatable as to how many of the victims had in fact been envenomed. Scarification of bite sites was also often performed but no reports suggest that the bleeding as a result was untoward. Creed (1884), when extolling the virtues of excision suggested that padding should suffice in curbing the bleeding, but that a moderate amount of bleeding was to be encouraged.

Tidswell (1906) reviewed the symptoms reported from fifty cases of snakebite where some notes were made by the attending physician. Table 2.2 shows a summary of the symptoms most often encountered.

Mueller (1893), reported the finding of haematuria following snakebite and commented that "It appears strange that so important a symptom as haematuria has not been previously observed and recorded here, and only within the last few months attracted my attention". He further added that it was no surprise that it had been overlooked as there was no reason to suspect an abnormality, since the haemorrhagic process of the Indian vipers was absent in the Australian colubrines and only indicated *exceptionally* by blood vomiting. Kellaway (1938) suggested that many of the aberrant symptoms seen in man are unquestionably caused by

thrombosis in various sites, which may give rise to the haemorrhages from the mucous membranes.

Table2.2 Summary of symptoms from snakebite (Tidswell, 1906).

Mental condition	-restless, excited, somnolent, lethargic, or comatose -drowsiness
Sensory function	-general sensation blunted, needle pricks not felt
Reflexes	-lack of response to light
Motor functions	-weakness, collapse, tremors, convulsions, paralysis of speech, swallowing and eyelids
Circulation	-rapid, feeble or irregular pulse, cold clammy skin, pallor, faintness
Bleeding	-occasional record of profuse bleeding from amputated or scarified bitten parts -occasional report of blood in sputum, vomit or urine -2 of 50 reports indicated the blood did not clot in the usual manner

Perhaps the first report to indicate the potential significance for a bite victim of the coagulant in venoms of Australian snakes was that of Foxton (1914). The victim was a 29 y.o. male, bitten on the hand by a snake (not identified other than by colour, as brown or copper). Ligatures were applied within minutes of the bite and the bite site was scarified and potassium permanganate rubbed into the wound. Within an hour the patient complained of headache and three hours after the removal of the ligatures (4 hr post bite) the patient vomited, the vomit containing altered blood. The patient vomited on several more occasions and each contained blood and he also showed haemorrhage from the gums. The patient died 20 hr after the bite and a post mortem showed intracranial haemorrhage into the ventricles and pons, the ventral surface structure of the pons and adjacent part of the medulla almost obliterated by haemorrhages. Foxton concluded that the haemorrhages were of such extent to have caused rapid death.

Coagulopathy due to brown snake (Pseudonaja) envenomation

More recent snake bite cases where a brown snake (*Pseudonaja* spp.) has been responsible show definite bleeding tendencies as reported in the literature. Schapel et al (1971), described a bite in which there was generalised extensive submucosal and subcutaneous bruising. There was persistent bleeding from skin puncture wounds and approximately 500ml of blood was vomited by the patient. Herrmann et al (1972), related the effects of bites from the dugite, *Pseudonaja (nuchalis) affinis*. In all three cases some evidence of interference with normal coagulation was present. One victim showed bleeding from around venepuncture sites, the second, a female, was menstruating at the time of the bite and showed an increase in flow, while the third had a shower of petechiae appear after having a blood pressure cuff applied to the bitten limb.

Sutherland et al (1975) reported the case of a 42 y.o. woman who had trodden on 'a stick', which was later confirmed as in fact having been a brown snake, which had bitten her. Bleeding from the site of intravenous access, increased menstrual loss and numerous bruises at discharge from the hospital, were the obvious signs in this case of interference with coagulation. Envenomation by a juvenile gwardar (*Pseudonaja nuchalis*) produced no neurological effects in a 57 y.o. male, however there was haemorrhaging at the venepuncture sites (Chester and Crawford, 1980).

Fatalities

Despite the advances in first aid and medical facilities and the availability of mono or poly specific antivenoms, deaths from snakebite still do occur. The cases reported below are due to snakes of the *Pseudonaja* genus (brown snakes).

Hilton (1989), reported the case of a child who was diagnosed as a snakebite at post mortem when venom was detected. The child had shown drowsiness prior to being found comatose and apnoeic and unable to be revived. The influence the venom coagulant played in this death

is difficult to assess. At post mortem, there was modest congestion and oedema of each lung and numerous petechial haemorrhages were present on the visceral pleura. Two petechiae were observed on the epicardium. Thus there was obvious interference with coagulation, although it is impossible to determine whether this was responsible for, or had hastened the child's death.

Sutherland (1992) reviewed 18 cases of snakebite death in the period 1981-1991 and of these 11 were attributed to brown snake bites. Of the 11 brown snake cases, two had fluid blood at autopsy and one of these also showed a small retroperitoneal haemorrhage. Although not specifically stated in these cases, there was no implication that the coagulopathy had contributed to their deaths. One of the 11 cases however could certainly implicate the coagulant in being responsible for the patient's death. A 53 y.o. male was bitten multiple times by a brown snake and 2 hr after the bite he had a severe coagulopathy and received antivenom. He continued to bleed slowly from his tongue, where he had bitten it. At 7 hr post bite, his mental state deteriorated rapidly and a CT scan revealed an extensive cerebral haemorrhage, and eventually the patient was declared brain dead.

Henderson et al (1993) warned that snakebite particularly by brown snake should be treated seriously, with the death of two patients. Both patients showed clinical evidence of severe coagulopathy - with one passing large quantities of fresh blood per rectum and the second presenting with epistaxis, only controlled with difficulty, after an attempt had been made at nasotracheal intubation. While there was no evidence to directly implicate the coagulopathy in their deaths, both had myocardial depression, the mechanism for which was unclear, but microemboli within the myocardial capillary bed, may be a possibility (Tibballs et al 1989; Tibballs et al 1992).

Brown snakes were responsible for envenomation resulting in fatal intracranial haematomas in two patients in Western Australia (Sprivulis and Jelinek, 1995). Both showed severe coagulopathy which no doubt was a contributing factor in their bleeding and subsequent

death, however one patient had undergone collapse and lacerated his occiput, which at autopsy showed as an area of brain trauma. This suggested that either the coagulopathy was present at the time of collapse and the trauma bled from that time on or that the developing coagulopathy resulted in bleeding from the area at a later date when the coagulopathy was well established. If the former is correct it would be further evidence for the rapidity with which coagulopathy can develop after bites by brown snakes (see Table 2.5).

Laboratory findings in coagulopathy

The likelihood of finding thrombocytopenia in an uncomplicated envenomation is debatable. In the majority of cases there is no evidence of thrombocytopenia although in cases where there is concomitant renal damage, thrombocytopenia would appear to be more likely. Lymphopenia has been reported in many cases although the cause of this remains uninvestigated at this point (White et al, 1989), although it may reflect the response at the draining lymph nodes which often become enlarged and tender.

The results of interest are those from the clotting studies. A number of tests are recommended that will aid in determining-:

- if envenomation has occurred and the severity of any resultant coagulopathy
- the effectiveness of antivenom therapy

The most basic test that can be effective in assessing coagulation status is the 20 minute Whole Blood Clotting Test (Warrell et al, 1977, 1986: Sano-Martins et al, 1994). This involves simply placing freshly drawn blood into a glass tube, leaving it upright and undisturbed and at 20 min observing if the blood has clotted. This can be very useful in giving a gross estimation of clotting integrity, when laboratory facilities are unavailable. If necessary it could also be used as a crude marker of the effectiveness of antivenom treatment if this is required to be provided in the field.

If laboratory facilities are available then a number of tests should ideally be performed to determine the patient's clotting status.

Table2.3 Laboratory tests in cases of envenomation.

Test	Description
20 min whole blood clotting test (WBCT 20) or (whole blood) clotting time	- crude but effective screen of the intrinsic pathway and an estimate of fibrinogen levels
Prothrombin time (INR)	- specific test of the extrinsic pathway (factors VII, X, V, prothrombin and fibrinogen)
APTT (activated partial thromboplastin time)	- specific test of the intrinsic pathway (factors XII, XI, IX, VIII, X, V, prothrombin and fibrinogen)
Thrombin time	- specific test of fibrinogen level and conversion by thrombin
Fibrinogen estimation	- quantitation of fibrinogen levels
XDP or D-dimer (cross-linked fibrin degradation products)	-quantitation of factor XIII stabilised fibrin breakdown products
FDP (Fibrin(ogen) degradation products)	- important for some non-Australian snakes - release of fibrinopeptides A or B

It is likely that each of these parameters will be markedly abnormal with an inability to obtain a time for either the prothrombin time or APTT, an undetectable level of fibrinogen and massively increased levels of cross-linked fibrin degradation products. Typical clotting profiles obtained from an envenomed patient are seen in Table 2.4. Table 2.5 shows the rapidity with which coagulopathy can develop.

Table 2.4 Coagulation profile in patient with marked coagulopathy after envenomation by brown snake.

Time after bite (h:min)	**2.25**	**2.55**	**4.25**	**6.55**	**7.55**	**10.55**	**43.55**
Antivenom (no. vials)	-	1	2	-	2	-	-
(Whole blood) CT (min) N < 10	-	-	-	13.5	-	5	4
PT (INR) N < 1.4	>10	-	-	>10	-	1.7	1.0
APTT (sec) N 28-39	>200	-	-	135	-	47	31
Fibrinogen (g/l) N 1.8-4.0	<1.8	-	-	0.02	-	0.45	2.31
XDP (mg/l) N <0.2	-	-	-	32	-	5	0.3
Platelet count (10^9/l) N 150-400		-	-	235	-	210	276

Abbrev. White and Williams, 1989

Treatment of coagulopathy

The accidental envenomation of a human victim by the brown snakes (also tigers and taipans) will often result in a massive disturbance of the coagulation pathways as has been shown in the clinical reports.

Although in most cases the coagulopathy is not life-threatening, deaths do occur (see Fatalities above). The likelihood of bleeding can be problematic as well when attempting to establish an access line through which to administer antivenom and to allow blood to be withdrawn for laboratory testing to determine the patient's status and to monitor treatment. If these lines are not strategically placed, there is the possibility of dangerous haematomas developing around the I.V. site.

Treatment of the coagulopathy that may develop is best achieved by neutralising the venom with the specific antivenom. The use of replacement factors is counterproductive if

neutralisation has not been achieved as these will also be consumed by the activating effect of the venom. Treatment with heparin in cases of Australian envenomation as a means to halt or reverse the coagulopathy is questionable. As some of the venom procoagulants are unaffected by heparin, its use would appear to be of little value (Williams and White, 1989; Tibballs and Sutherland, 1992; Tibballs et al, 1992). With neutralisation by the antivenom there is a reasonably rapid recovery of the coagulation factors, particularly fibrinogen.

Table 2.5 Defibrination syndrome showing rapidity of development in 7 year old male after envenomation by brown snake.

	Time after bite						
	25 min	1 hour	5 hours	7 hours	~ 18 hrs	~ 40 hrs	~ 64 hrs
Antivenom brown snake		1000 U					
(Whole blood) Clotting time N<10 min	unclotted		20 min	5.5 min	5 min	5.5 min	5.5 min
PT (Ratio) N< 1.3	-		>10	2.3	1.4	1.0	1.0
APTT N 28-39 (sec)	-		74	51	46	34	32
Fibrinogen (g/l)	-		0.14	0.19	-	0.99	1.56
FDP N <10 (mg/ml)	-		2000	1000	500	10	6
Platelet count $10^9/l$ N 150-400	-	-	265	-	-	187	253
Clinical state	irritable drowsy	irritable drowsy	irritable less drowsy	awake not irritable	normal	normal	normal

White, 1980-81

The clinical experience of unclottable blood from bite victims due to a venom induced coagulopathy is however at variance with laboratory investigations, where injection of venom into experimental animals or adding venom to plasma (or whole blood) promotes rapid clotting.

EXPERIMENTAL OBSERVATIONS

Historical origins

The first serious study of snake bite can perhaps be attributed to Francesco Redi (1626-1697), who showed that the injection of the contents of viper venom sacs into animals resulted in physical effects characteristic of envenomation. However beyond demonstrating the anatomy of the vipers, the toxic nature of the venom and the mechanism of its delivery, he admitted that he was unable to decide 'upon the manner in which the poison brought about the death of the victim after it had entered the body'.

Fontana (1730-1805) took up the problem left by Redi. He established that there was a dose dependency between venom concentration and the severity of the effects and there was a distinct difference between local and systemic effects, although he failed to come to a satisfactory conclusion on the cause of these differences. His important contributions in terms of this thesis relate to his investigations of the effect of the venom on blood and he recognised the possibility that the venom was responsible for coagulating the blood in the vessels of the bitten animal. Injection of venom directly into the jugular vein of rabbits resulted in convulsions and death in less than two minutes. At post mortem clotted blood was found in the heart and the larger vessels. Further experiments convinced him of the importance of circulation in the distribution of the venom and drew him back to the disparity between in vivo and in vitro effects on blood. In his experiments he mixed blood from fowls with venom and found it failed to clot compared with the control. He also found this to be the case with blood from frogs and guinea pigs. Thus he had expected to find clotting to be the result of the addition of the venom but had in fact found the opposite. He believed that the venom when distributed in the circulation, coagulated blood in the heart, lungs, liver and larger vessels, while dissolving the blood in other parts. This partial coagulation and dissolution of

the blood 'produced violent derangement of the vital organs and a serious impediment to circulation', with the result that the animal died.

Mitchell and Reichert (1886) when investigating the effect of Crotalus and Moccasin venoms on living animals made the observation that the animals suffered from haemorrhaging and the blood when exposed to the air, remained in a liquid state. However in cases where high concentrations of venom were used the animal succumbed rapidly and the blood was solidly clotted.

Lamb (1901) found a similar situation when working with the venom of Russell's viper. Rapidly fatal doses of venom when injected produced in each instance an extensive thromboses and in extreme cases the whole vascular system was essentially a solid mass. The situation with delayed death however was quite different, with careful dissection required to find any small clots and fluid blood collected from the heart remaining unclotted.

In vitro experiments where venom was added to citrated whole blood, consistently resulted in clotting of the blood and Lamb reported that he had tried to reproduce in vitro the lack of clotting found in slow-death experimental animals without success. He also recognised a significant difference in the effect the venoms from Russell's viper and cobra venom had on blood in vitro, and concluded the venoms had quite different physiological actions.

Many venoms have now been investigated for the presence of haemostatically active components and these can be grouped into a number of different categories (Markland, 1998): (1) enzymes that clot fibrinogen (2) enzymes that degrade fibrin(ogen) (3) plasminogen activators (4) prothrombin activators (5) factor V activators (6) factor X activators (7) anticoagulant activities (8) enzymes with haemorrhagic activity (9) enzymes that degrade plasma serine proteinase inhibitors (10) platelet aggregation inducers and inhibitors.

The known mechanisms by which the above mentioned components interfere with haemostasis have been well-reviewed recently (see: Hutton and Warrell, 1993: Marsh, 1994 and Markland, 1998). The actions of interest within the context of this thesis is the prothrombin activating action of the (Australian) venoms.

Early Australian experimentation

While controversy raged over treatment in Australia in the late 19th century, experimental work was pioneered by Martin.

C.J. Martin performed some of the ground breaking experiments on the clotting activity in Australian venoms. In 1894, Martin reported the results of some fascinating experiments. These involved the use of venom from the black snake (*Pseudechis porphyriacus*), but the observations made are extremely relevant to the effects noted with a large number of the Australian venoms. While injecting small quantities of venom (10-20 µg/kg body weight) into dogs, he discovered that on drawing blood, it either failed to clot or took considerable time for this to occur. On other occasions, when stronger doses were employed, intravascular coagulation resulted, and at post mortem, the blood in the whole vascular system, except for the pulmonary veins and the left auricle, was found to be solid. The clot in the portal venous system was particularly solid.

The coagulability of the blood was described in phases, with a negative phase referring to an inability of the blood to clot on withdrawal and a positive phase (activated state) which resulted in a clot forming faster than in a matched control. In the animal experiments, a low dose of venom resulted in a negative phase of coagulation, whereby the clotting ability of the blood disappeared, while high doses resulted in intravascular coagulation (positive phase). Although these observations were not new and the fluidity of the blood at post mortem or intravascular coagulation had been described, the novelty of Martin's experiments was the description of both the positive and negative phase of coagulability in an animal given a low

dose of venom. Injecting a small dose (< 0.1 mg/kg body) produced an initial increased coagulability, however he noted that this increase was transient, and it would be missed if blood samples were not collected from the animal in the first two minutes after injecting the venom. After about three minutes however, the negative phase of coagulation would dominate and the blood would fail to clot or would only do so after an extended period of time.

Another point of interest from this work was the comment by Martin, when he had performed numerous experiments involving observations on clotting activity in dogs, that "Dogs evince varying degrees of susceptibility in this respect, the same dose per kilo. producing in one animal intravascular clotting, in another inhibition of the coagulation of the shed blood". Thus the variability of response to venom was recognised by Martin and this variability has been and continues to be problematic for basic venom research and also in the presentation of symptoms in snakebite victims.

Martin also discovered that if venom is injected slowly into the circulation, the blood does not clot but becomes unclottable when withdrawn, while rapid injection (bolus dose) produced intravascular coagulation. Thus slow infusion of the venom reproduced the effects seen with subcutaneous injection of the venom or more truly represented the situation with snake bite.

He also investigated the effect of a second injection of venom after an initial low dose. At about twenty minutes after the initial low dose of venom, the blood from the dog was unclottable. At this point a second injection containing a much larger dose (10-20 times the first dose, in normal circumstances capable of producing intravascular coagulation) was introduced into the animal with no evidence of increased coagulability and in fact the blood does not clot any sooner than those drawn prior to the second injection of venom. Martin concluded that "the establishment of a negative variation confers an immunity, as far as the intravascular clotting is concerned, against further injections of the venom". However, as we are now aware, no such immunity had been conferred. The lack of clotting in blood drawn on

the second occasion was only a reflection of the lack of available fibrinogen, which had been consumed in the activation of the clotting pathway by the initial injection of the venom.

Martin (1894) also worked with the venoms of the tiger snake and black snake and in further experiments injected these directly into the blood stream of dogs, cats and rabbits with resultant intravascular coagulation. This phenomenon was also found in small animals such as frogs and mice.

Initially Martin interpreted the results as suggesting the release of a 'nucleo-proteid'-like substance by the venom was responsible for the clotting effects. The other interesting observation was that the venoms were able to clot oxalate, citrate, fluoride and magnesium sulphate plasmas. Although this only questioned the requirement for calcium presence, Martin concluded '..the venoms appear to contain veritable fibrin ferments and their clotting power is not due to their action upon prothrombins or thrombogens contained in the plasma as the presence of calcium is unnecessary'

Martin (1905), attempted to physically characterise the ferment solution. He found that

- ferment is not used up in causing clotting as the activity could be then transferred causing further clotting to occur
- the clotting activity is heat labile and its activity could be destroyed by heating at 75°C for 10-15 min
- despite destruction at high temperatures the clotting activity was at its greatest in a temperature range around 40°C
- there was a degree of specificity in the clotting activity, as the serum from a horse immunised with tiger snake venom also inhibited the effect of *Pseudechis porphyriacus* but failed to have an effect on *Echis carinatus* venom

Tidswell (1906) produced his classic paper on the researches on Australian venoms, in which he made similar observations to those of Fontana and Martin. Injecting snake venom in to the circulation produced results reliant on the circumstances. In some cases the animal showed violent convulsions, collapse and death within minutes. In these instances the blood was extensively clotted, including the portal vein and pulmonary artery, the animal's death being equated to the arrest of circulation.

In other instances however the animal again died within minutes but without convulsions or at most a feeble twitch, however in these cases examination of the body revealed fluid blood and when removed showed a decreased tendency to clot. If the animal took considerable time to die, the blood was definitely fluid and after removal in these cases slowly but imperfectly clotted. At post mortem there were signs of haemorrhage in the viscera, especially in the lungs and endocardium.

With the conclusion of the work of Martin and Tidswell, venom research came to standstill and in 1929, Fairley lamented that the excellent work of Martin and Tidswell may be forgotten and stimulated Charles Kellaway to re-examine Australian snake venoms with the intention of producing antivenenes. Fairley reviewed the current knowledge and reported the important Australian venomous snakes were the death adder (*Acanthophis antarcticus*), the tiger snake (*Notechis scutatus*), the copper-head (*Austrelaps superbus*), the brown snake (*Pseudonaja textilis*) and the black snake (*Pseudechis porphyriacus*). He repeated some of the lethality experiments of Tidswell and added to the known information on venom yields (Fairley and Splatt, 1929), but the brown snake was neglected in this study, the majority of the work focusing on the black snake (*P. porphyriacus*) as had the work of Martin and Tidswell, despite Ferguson (1926) having reported the occurrence of brown snake bite equalling that of the black snakes. The toxicity was disregarded to some extent, as it was known that the brown snakes had the least efficient biting mechanism, the smallest venom glands and the lowest venom yields and these factors combined to produce recovery in a large

number of bite victims, although Fairley did concede that when venom is efficiently inoculated, death may result (Fairley, 1929).

Fairley also investigated the influence of the venoms on the clotting activity by intravenous injection of tiger snake, copperhead and death adder venom into sheep, chosen primarily by Fairley as they had a body weight most closely aligned to man. Again the findings were of thrombotic episodes as a result of intravenous administration of tiger snake venom, while subcutaneous injection resulted in the negative phase of coagulation being manifest. Examination of sheep at post mortem after intravenous injection of venom showed the usual thrombi in the heart and major vessels, but due to the observations of liquid blood after subcutaneous injection of the venom, Fairley concluded that ''thrombases' play a role in killing small prey such as birds and mice rapidly, in large sized animals including man, its action in this direction is insignificant. Neurotoxin is the killing constituent in all Australian venoms'.

Brown snakes (**Pseudonaja** ***spp.) -whole venom studies***

Kellaway (1930), performed a series of experiments on the effect of whole venom on animals and determined the results of injecting sheep with various venoms, but of interest were his observations with *P. textilis*. In most of these experiments venom was injected subcutaneously at slightly greater than estimated certainly lethal dose. The animals at death (which occurred rapidly) showed thromboses in the heart, pulmonary veins, portal veins and inferior vena cava. Kellaway made an interesting observation in that the 'thrombase' of *Pseudonaja* was more potent in sheep than the other venoms and post mortem evidence of thrombosis, suggested the venom appeared more diffusible than the other venoms.

In his investigations on the certainly lethal dose of the venom from common brown snake in a variety of laboratory animals (horse, monkey, cat, rabbit, guinea pig, rats and mice) Kellaway (1931) found there was evidence of interference with the clotting mechanism of that animal at

post mortem. In the horse, the blood was found to be fluid and there were numerous petechial haemorrhages in the heart muscle plus haemorrhagic oedema in both lungs. The kidneys also showed areas of intense haemorrhage with haemorrhagic petechiae in the bladder mucosa.
In the monkey at post mortem the blood was again fluid although the areas of haemorrhage were not as pronounced as in the horse. In the cat, the blood was fluid, while in the rabbit a different picture was obtained. In the rabbit 'whipped fibrin' was found in the right side of the heart, however despite this, no thrombi were found in the pulmonary vessels, portal vein nor the inferior vena cava, and in fact these vessels contained liquid blood. Guinea pigs in his series showed some congestion and haemorrhages in the lungs and the rats showed marked thrombosis in the heart, lungs or portal vein when they succumbed quickly. In those animals that took longer to die, a direct relationship between clotting abnormalities and their death was more difficult to establish. The mice however showed rapid evidence of intravascular coagulation and rapid death.

This series of experiments showed the venom to be responsible for a dramatic effect on the coagulation system of a number of laboratory animals. Whether the venom interfering with the coagulation system was entirely responsible for the demise of the animal is debatable but in the rabbit, rat and mouse the evidence of thrombi in the major vessels is suggestive that the rapid onset of death associated with collapse and convulsion seen in these animals and on occasion in human victims may be due to a massive thrombotic episode capable of killing the victim.

Kellaway performed an in vitro appraisal of the 'thrombin' activity of the venom from *P. textilis* as well, using plasma from human, horse, sheep, rabbit and guinea pig as the substrate. The experiments only involved determination of the extent of dilution the venom could undergo and still bring about clotting against these plasmas but was probably the beginning of studies aimed at more closely understanding the effect of the venom on clotting per se.

Little work was done after Kellaway, in terms of investigating the clotting activity in Australian venoms, until in 1967, Macfarlane and Denson classified venoms into four categories, (Table 2.6) and equated the action of snake venoms on plasma with that of the action of the components of the latter part of the clotting cascade. Denson (1969), investigated the venoms of *Notechis scutatus, Acanthophis antarcticus, Oxyuranus scutellatus* and *Pseudonaja textilis* for their ability to produce clotting in normal (citrated) plasma and the requirement for cofactor presence by testing the clotting ability of the venoms on factor V deficient plasma. Although the experimental detail was lacking, two points of interest could be drawn from this paper. The first was that the venoms from the taipan and the brown snake appeared to have a more active venom than that from the tiger or death adder and secondly that the venom from the tiger and death adder were incomplete prothrombin activators, while that from the brown and taipan was a complete activator.

Herrmann et al (1972), performed two stage clotting tests to determine the effect of varying cofactors on the clotting activity of the venom from the Western Australian tiger snake (*Notechis occidentalis*), the dugite (*Pseudonaja affinis*), and the western brown or gwardar (*Pseudonaja nuchalis*). (Table 2.7). The results indicated that although both the venom from *P. nuchalis* and *P. affinis* were capable of clotting fibrinogen in the absence of factor V, there was a significant difference in these venoms as far as their ability to convert fibrinogen, with and without, calcium and phospholipid being present. This result suggested that the venom from *P. affinis* was dependent on both calcium and phospholipid to convert prothrombin to thrombin. This is an interesting result in view of the observation that the coagulant activity of the venom from this snake is more difficult to neutralise with antivenom than that from either *Pseudonaja nuchalis*, or *Pseudonaja textilis*.

Table 2.6 Classification of venoms according to clotting ability.

Classification	Characteristics
Type 1	Ability to convert factor X to activated factor X
Type 2	Ability to convert prothrombin to thrombin in the presence of factor V (incomplete activator)
Type 3	Ability to convert prothrombin to thrombin without the requirement for factor V (complete converter)
Type 4	Ability to convert fibrinogen directly to fibrin

Macfarlane and Denson, 1967

Table 2.7 Clotting times of substrate mixtures

Mixture	Clotting time (sec) after the addition of fibrinogen and calcium		
	Tiger (*N.occidentalis*)	**Dugite (*P. affinis*)**	**Gwardar (*P. nuchalis*)**
Prothrombin, factor V, phospholipid calcium, venom.	35	43	28
Prothrombin, phospholipid, calcium, venom. *(Factor V - absent)*	379	33	24
Prothrombin, factor V, calcium, venom. *(Phospholipid - absent)*	144	335	33
Prothrombin, factor V, phospholipid, venom. *(Calcium - absent)*	270	405	40
Factor V, phospholipid, calcium, venom. *(Prothrombin - absent)*	330	180	234
Prothrombin, factor V, phospholipid, calcium. *(Venom - absent)*	439	360	330

Herrmann, Davey, Skidmore (1972).

Chester and Crawford (1982), continued these experiments to determine the effect that the various cofactors may have on the venom activity and also established the ability of various venoms to activate descarboxyprothrombin. From these experiments they concluded that the venoms from the taipans (*Oxyuranus scutellatus, Oxyuranus microlepidotus*) and the common brown (*P. textilis*) were capable of converting descarboxyprothrombin. They also established that the addition of calcium, phospholipid or the use of factor V deficient plasma had little effect on the clotting time produced by the venom from *P. textilis*, implying that the venom was able to supply all of these requirements for clotting by itself and was therefore a complete prothrombin converter according to Macfarlane and Denson's classification.

Marshall and Herrmann (1983) examined the clotting requirements of a large number of Australian venoms with a number of species represented for each genus. They examined the venoms from the common brown, (*P. textilis*), western brown or gwardar (*P. nuchalis*) and the dugite (*P. affinis*). From these testings the venom from *P. textilis* was extremely strong in its clotting ability, with some influence resulting from the presence of calcium or phospholipid, the times for clot formation showing a small but definite change from 23 sec in the absence of calcium and phospholipid to 6 sec in the presence of both.

The effect on the venoms from *P. nuchalis* and *P. affinis* was more dramatic however, and suggested that there was a difference in the way these venoms responded to cofactors. *P. affinis* showed an improved clotting time in the presence of calcium and some further improvement in the presence of phospholipid, while *P. nuchalis* appeared to show little improvement in response in the presence of calcium but a considerable shortening of the clotting time when phospholipid was available. These results seemed to be at variance with those previously reported, where the venom of *P. affinis* needed calcium and phospholipid, while that from *P. nuchalis* was able to clot without these being present. The difficulty in comparing these results also comes from the lack of knowledge regarding the origin of the venoms and whether they were from individual snakes or from pools of venom. The

individual variation and the number of animals and geographic origin of these snakes may have had an influence on these results (see Chapter 6).

Classification of prothrombin activators

Prothrombin activators were classified into 5 groups by Rosing and Tans (1991). Group 5 originate from sources other than snake venoms and will not be considered. The classification of the remaining 4 groups was based on the structural and functional properties of the activator (Table 2.8, c.f. Macfarlane and Denson, 1967, Table 2.6).

Table 2.8 Classification of prothrombin activators

Group	Enzymatic action	Cofactors	Product
I	Metalloproteinase	Nil (Ca^{2+})	Meizothrombin
II	Serine proteinase	Phospholipid, Ca^{2+}, factor Va	Meizothrombin, thrombin
III	Serine proteinase	Phospholipid, Ca^{2+}	Meizothrombin, thrombin
IV	Specific or broad acting proteinase	(Ca^{2+})	Enzymatically inactive product

Rosing and Tans, 1991

Group I prothrombin activators are not influenced by the non-enzymatic cofactors of the prothrombinase complex (factor Va and phospholipid) and are metalloproteinases, inhibited by chelating agents. Prothrombin is converted to meizothrombin and thrombin is then produced through autocatalysis of the meizothrombin.

Group II activators resemble factor Xa both structurally and functionally and as such the presence of phospholipid, factor Va and Ca^{2+} enhances the activity. The products of prothrombin activation in this group are meizothrombin and thrombin.

Group III activity is stimulated by the presence of phospholipid and Ca^{2+}. The activators consist of subunits which have been hypothesised to equate to factor Xa and factor Va. Members of Groups II and III have only been identified in Australian venoms at this stage (see Table 2.9).

Group IV members cleave peptide bonds in prothrombin, however the resultant product is not itself enzymatically active, and is usually prethrombin I and prethrombin II.

Table 2.9 Classification of exogenous prothrombin activators.

Source	Molecular weight	Subunits	Cofactors	Reference
Group II				
Notechis scutatus	54,000	32,000 23,000	PL, Va, Ca^{2+}	Jobin & Esnouf (1966) Tans et al (1985) Herrmann et al (1972) Chester & Crawford (1982) Marshall & Herrmann (1983)
Notechis ater niger	58,000	37,000 23,000	PL, Va, Ca^{2+}	Marshall & Herrmann (1983) Speijer et al (1986) Williams & White (1989)
Notechis ater humphreysi			PL, Va, Ca^{2+}	Marshall & Herrmann (1983) Speijer et al (1986)
Notechis ater serventyi			PL, Va, Ca^{2+}	Marshall & Herrmann (1983)
Notechis flinders			PL, Va, Ca2+	Marshall & Herrmann (1983)
Notechis occidentalis			PL, Va, Ca^{2+}	Marshall & Herrmann (1983)
Tropidechis carinatus	54,000 64,000 46,515	41,500 17,000 30,000	PL, Va, Ca^{2+}	Chester & Crawford (1982) Marshall & Herrmann (1983) Morrison et al (1987) Marsh et al (1997) Joseph et al (1999)
Pseudechis porphyriacus			PL, Va, Ca^{2+}	Chester & Crawford (1982) Marshall & Herrmann (1983) Speijer et al (1986)

Source	Molecular weight	Subunits	Cofactors	Reference
Group III				
Oxyuranus scutellatus	300,000	multiple subunits	PL, Ca^{2+}	Marshall & Herrmann (1983) Speijer et al (1986)
	380,000	multiple subunits		Pirkle et al (1972) Owen & Jackson (1973) Walker et al (1980)
Oxyuranus microlepidotus			PL, Ca^{2+}	Marshall & Herrmann (1983) Speijer et al (1986)
Pseudonaja affinis (Dugite)			PL, Ca^{2+}	Herrmann et al (1972) Marshall & Herrmann (1983)
Pseudonaja nuchalis (Gwardar)			PL, Ca^{2+}	Herrmann et al (1972) Marshall & Herrmann (1983)
Pseudonaja textilis	$\geq$200,000	multiple subunits	PL, Ca^{2+}	Marshall & Herrmann (1983) Speijer et al (1986) Masci et al (1987, 1988)

Expansion of the knowledge regarding the prothrombin activators of Group III, specifically, *Pseudonaja textilis* is the object of this thesis.

The classification of the Australian snake venom prothrombin activators as Group II or III was dependent on the requirement for the cofactors calcium, phospholipid and factor Va. A comparison of the relative rates of activation of prothrombin by factor Xa, *Notechis scutatus* and *Oxyuranus scutellatus* crude venom (Table 2.10) shows that when both calcium and phospholipid are present the rate of activation of prothrombin is increased. However the presence of factor Va is only effective in increasing the rate of activation of prothrombin by factor Xa and *N. scutatus* venom. There is essentially no change in the relative rate of prothrombin activation by *O. scutellatus* venom when factor Va is present, suggesting that factor V is not required or that the venom contains a procoagulant which has not only a factor Xa - like action but also has a factor Va equivalent component. This also applies to the venom of *P. textilis* as a member of group III.

Table 2.10 Relative rates of prothrombin activation by coagulation factor Xa or the crude venom from *Notechis scutatus* and *Oxyuranus scutellatus*

Activator mixture	Xa *	*N. scutatus* *	*O. scutellatus* †
-	1	1	1
Ca^{2+}	1.7	1.5	7.8
Ca^{2+}, phospholipid	4.8×10^2	9.8×10^2	3.58×10^3
Ca^{2+}, phospholipid, factor Va	3.5×10^5	3×10^6	3.64×10^3

*** Tans and Rosing (1993), † Speijer et al (1986)**

***Isolation and characteristics of the prothrombin activator from* P.textilis**

Masci et al (1987,1988) reported the first isolation of the procoagulant from *P. textilis*. The procoagulant had a high molecular weight >200,000 and consisted of a number of subunits when examined by SDS-PAGE. The isolated procoagulant was able to coagulate citrate, warfarin and factor X and factor V deficient plasmas. Calcium and phospholipid had little effect on the clotting ability of the procoagulant.

Triplett et al (1993) and Stocker et al (1994) reported the isolation of two separate prothrombin activators from the venom of *P. textilis*. These activators could be identified by their requirement for phospholipid. A phospholipid independent prothrombin activator (PLIPA) and phospholipid dependent prothrombin activator (PLDPA) also named Textarin®, were isolated. It remained uncertain as to whether factor V was necessary for Textarin to be active. The work of Masci et al (1987, 1988), Triplett et al (1993) and Stocker et al (1994) are considered in more detail in the appropriate chapters.

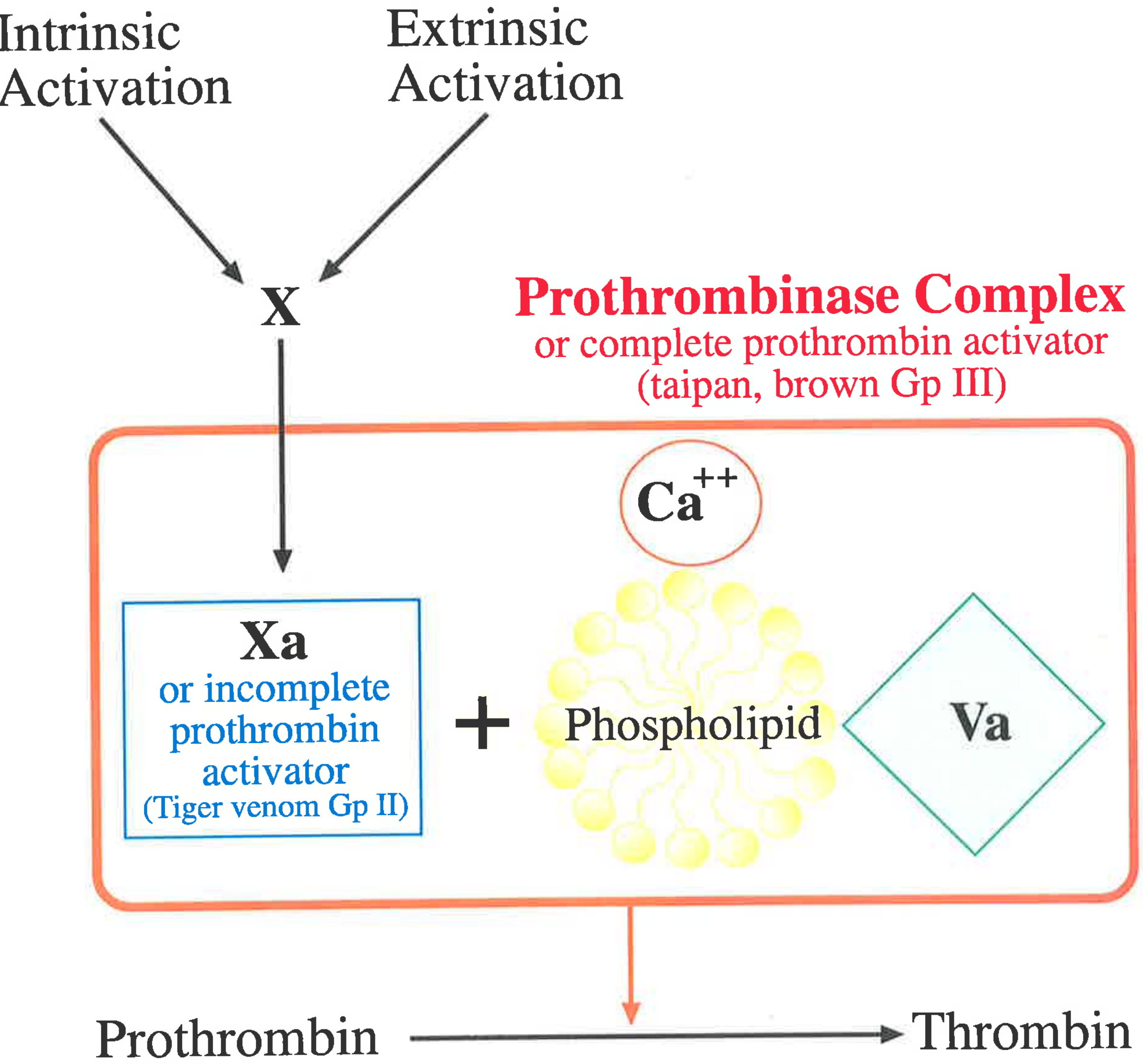

Fig 2.1 Point of action of the prothrombin activators in Australian snake venoms. Incomplete activators (Group II, see Table 2.9) mimic the action of Xa, while complete activators (Group III) hypothetically mimic the action of the prothrombinase complex. Group II activators are found in tiger snake venom (*Notechis* species) and the rough scaled snake (*Tropidechis carinatus*), group III are found in the venom from taipan and brown snakes (*Oxyuranus* and *Pseudonaja* species).

OVERVIEW OF THESIS

The procoagulant of the venom of *Pseudonaja textilis* is an extremely active component of the venom and is responsible for the clinical bleeding noted in bite victims. The action of the procoagulant has been reported to be consistent with a complete prothrombin activator, able to activate prothrombin to thrombin by mimicking the 'prothrombinase complex', of factor Xa - factor V - phospholipid and calcium.

At present an analogy between the venom procoagulant and the clotting factors of the prothrombinase complex is only speculative. The claim for such an analogy remains to be proven. Currently the requirement by the procoagulant for calcium and phospholipid still remains uncertain. The studies of Herrmann et al (1972) and Marshall and Herrmann (1983) suggested that calcium and phospholipid requirement may have been species dependent, while the studies of Chester and Crawford (1982) and Masci (1988) found that calcium and phospholipid had little effect. A factor V-like component in the venom would also be supportive of the procoagulant being analogous to the prothrombinase complex. However, while the majority of studies have found there is no requirement for factor V (suggesting the procoagulant does contain such a factor V-like component) the work of Triplett et al (1993) and Stocker et al (1994) has now suggested a differential requirement for phospholipid.

The procoagulant has been shown to be inhibited by benzamidine, indicating that it is a serine protease however the active site has not been identified. Masci et al (1988) reported the procoagulant consisted of a number of subunits, although this has been challenged (Stocker et al 1994). Thus the existence of any subunits or the presence of two distinct procoagulants remains unconfirmed. If the procoagulant is composed of subunits, the subunits may be factor X and V-like, supportive of the prothrombinase theory, although currently, no activities, physical characteristics or interactions of any subunits have been elucidated.

The aim of this thesis was twofold. The major part of the study was to investigate the hypothesis that the procoagulant from *P. textilis* is composed of a number of subunits and that these subunits physically resemble and have activities consistent with factor X and V and/or the prothrombinase complex. Chapter 3 reaffirms the findings on the requirements for the cofactors, factor V, calcium and phospholipid and the isolation of the procoagulant and initial characterisation are reported. Further physical characteristics are examined in chapter 4 and the subunit components of the procoagulant were isolated and homology between the subunits and factor Xa and factor V was examined and reported in chapter 5.

In chapter 6, the secondary related study of the effect that season, geographic origin and species may have on venom composition and hence the implications this has for researchers in terms of consistent experimental results and for antivenom therapy is reported. Chapter 7 summarises the experimental findings, discusses the difficulties encountered in performing the study and suggests possible directions for future investigations.

REFERENCES

Chester, A., Crawford, G.P.M. (1980). Envenomation by a juvenile gwardar. Med. J. Aust. **2**, 462.

Chester, A., Crawford, G.P.M. (1982). In vitro coagulant properties of venoms from Australian snakes. Toxicon **20**, 501-504.

Clutterbuck, J.B. (1864). On two cases of snake-bite successfully treated. Aust. Med. J. **9**,195-196.

Creed, J.M. (1884). Australian snakebite. - Fear as a factor in producing many of the alarming symptoms following. Australas. Med. Gaz. **3**, 224-229.

Creed, J.M. (1891). Strychnine in snake-bite. Australas. Med. Gaz. **10**, 84-89.

Denson, K.W.E. (1969). Coagulant and anticoagulant action of snake venoms. Toxicon **7**, 5-11.

Editorial. (1973). Bleeding after snakebite. Med. J. Aust. **1**, 322-323.

Fairley, N.H. (1929). The present position of snake bite and the snake bitten in Australia. Med. J. Aust. **1**, 296-313.

Fairley, N.H., Splatt, B. (1929). Venom yields in Australian poisonous snakes. Med. J. Aust. **1**, 336-348.

Ferguson, E.W. (1926). Cases of snake bite in New South Wales. Report of the Director-General of Public Health, New South Wales for the year 1924, Govt. Printer, Sect. IV, Number 5, 171-184.

Fontana, F. (1781). Traitæ sur le vænin de la vip¿re. Florence.

Foxton, H.V. (1914). A case of fatal snake-bite, (with autopsy.) Med. J. Aust. **1**, 108.

Hedley, G.D., Rees, W.C. (1874). Fatal case of snake-bite, presenting some curious points of interest. Aust. Med. J. **19**, 49-53.

Henderson, A., Baldwin, L.N., May, C. (1993). Fatal brown snake (*Pseudonaja textilis*) envenomation despite the use of antivenom. Med. J. Aust. **158**, 709-710.

Herrmann, R.P., Davey, M.G., Skidmore, P.H. (1972). The coagulation defect after envenomation by the bite of the dugite (*Demansia nuchalis affinis*), a Western Australian brown snake. Med. J. Aust. **2**, 183-186.

Hilton, J.M.N. (1989). A tragic case of snake bite. Med. J. Aust. **151**, 600-601.

Hutton, R.A., Warrell, D.A. (1993). Action of snake venom components on the haemostatic system. Blood Rev. **7**, 176-189.

Jobin, F., Esnouf, M.P. (1966). Coagulant activity of tiger snake (*Notechis scutatus scutatus*) venom. Nature **211**, 873-5.

Joseph, J.S., Chung, M.C.M., Jeyaseelan, K., Manjunatha Kini, R. (1999). Amino acid sequence of Trocarin, a prothrombin activator from *Tropidechis carinatus* venom: Its structural similarity to coagulation factor Xa. Blood **94**, 621-631.

Kellaway, C.H. (1930). Local venesection in the treatment of snake bite: an experimental study. Med. J. Aust. **2**, 351-360.

Kellaway, C.H. (1931). Observations on the certainly lethal dose of the venom of the common brown snake (*Demansia textilis*) for the common laboratory animals. Med. J. Aust. **2**, 747-751.

Kellaway, C.H. (1938). The symptomatology and treatment of the bite of Australian snakes. Med. J. Aust. **2**, 585-589.

Lamb, G. (1901). On the action of snake venom on the coagulability of the blood. Indian Med. Gaz. **36**, 443-447.

Macfarlane, R.G., Denson, K.W.E. (1967). General techniques used in the study of snake venoms. In: Proc. 10th Congr. Europ. Soc. Haemat. , Strassberg 1965, Part II, 1074-1081.

Markland, F.S. (1998). Snake venoms and the hemostatic system. Toxicon **36**, 1749-1800.

Marsh, N.A. (1994). Snake venoms affecting the haemostatic mechanism - a consideration of their mechanisms, practical applications and biological significance. Blood Coag. Fibrinol. **5**, 399-410.

Marsh, N.A., Fyffe, T.L., Bennett, E.A. (1997). Isolation and partial characterization of a prothrombin activating enzyme from the venom of the Australian rough-scaled snake (*Tropidechis carinatus*). Toxicon **35**, 563-571.

Marshall, L.R., Herrmann, R.P. (1983). Coagulant and anticoagulant actions of Australian snake venoms. Thromb. Haemost. **50**, 707-711.

Martin, C.J. (1894). On some effects upon the blood produced by the injection of the venom of the Australian black snake (*Pseudechis porphyriacus*). J.Physiol. **15**, 380-400.

Martin, C.J. (1905). Observations upon fibrin-ferments in the venoms of snakes and the time-relations of their action. J. Physiol. **32**, 207-215.

Masci, P.P., Whitaker, A.N., deJersey, J. (1987). Purification and characterization of the prothrombin activator of the venom of *Pseudonaja textilis*. In: Progress in venom and toxin research. pp. 209-219. Eds. Gopalakrishnakone, P. , Tan, C.K. Proceedings of the first Asia-Pacific Congress of Animal, Plant and Microbial Toxins.

Masci, P.P., Whitaker, A.N., deJersey, J. (1988). Purification and characterization of a prothrombin activator from the venom of *Pseudonaja textilis textilis*. Biochem. Int. **17**, 825-835.

Mitchell, S.W., Reichert, E.T. (1886). Researches upon the venoms of poisonous serpents. Smithson. Contrib. Knowl. **26**, 1-186.

Morrison, J.J., Masci, P.P., Bennett, E.A., Gauci, M., Pearn, J., Whitaker, A.N., Korschenko, L.P. (1987). Studies of the venom and clinical features of the Australian rough-scaled snake (*Tropidechis carinatus*). In: Progress in venom and toxin research. pp 220-233. Eds: Gopalakrishnakone, P., Tan, C.K. National University of Singapore, Singapore.

Mueller, M.D. (1893). On haematuria in snakebite poisoning. Australas. Med. Gaz. **12**, 247-249.

Owen, W.G., Jackson, C.M. (1973). Activation of prothrombin with *Oxyuranus scutellatus scutellatus* (taipan snake) venom. Thromb. Res. **3**, 705-714.

Pirkle, H., McIntosh, M., Theodor, J., Vernon, S. (1972). Activation of prothrombin with taipan snake venom. Thromb. Res. **1**, 559-568.

Redi, F. (1664). Osservazioni intorno alle vipere. Florence.

Rosing, J., Tans,G. (1991). Inventory of exogenous prothrombin activators. Thromb. Haemost. **65**, 627-630.

Sano-Martins, I.S., Fan, H.W., Castro, S.C.B., Tomy, S.C., Franca, F.O.S., Jarge, M.T., Kamaguti, A.S., Warrell, D.A., Theakston, R.D.G. and BIASG. (1994). Reliability of the simple 20 minute whole blood clotting test (WBCT 20) as an indicator of low plasma fibrinogen concentration in patients envenomed by *Bothrops* snakes. Toxicon **32**, 1045-1050.

Schapel, G.J., Utley, D., Wilson, G.C. (1971). Envenomation by the Australian common brown snake - *Pseudonaja* (*Demansia*) *textilis textilis*. Med. J. Aust. **1**, 142-144.

Speijer, H., Govers-Riemslag, J.W.P., Zwaal, R.F.A., Rosing, J. (1986). Prothrombin activation by an activator from the venom of *Oxyuranus scutellatus* (Taipan snake). J. Biol. Chem. **261**,13258-13267.

Sprivulis, P., Jelinek, G.A. (1995). Fatal intracranial haematomas in two patients with brown snake envenomation. Med. J. Aust. **162**, 215-216.

Stocker, K., Hauer, H., Muller, C., Triplett, D.A. (1994). Isolation and characterization of Textarin®, a prothrombin activator from eastern brown snake (*Pseudonaja textilis*) venom. Toxicon **32**, 1227-1236.

Sutherland, S.K. (1975). Treatment of snakebite in Australia. Some observations and recommendations. Med. J. Aust. **1**, 30-32.

Sutherland, S.K., Coulter, A.R., Broad, A.J., Hilton, J.M.N., Lane, L.H.D. (1975). Human snakebite victims. The successful detection of circulating snake venom by radioimmuno- assay. Med. J. Aust. **1**, 27-29.

Sutherland, S.K. (1992). Deaths from snakebite in Australia, 1981-1991. Med. J. Aust. **157**, 740-746.

Tans, G., Govers-Riemslag, J.W.P., van Rijn, J.L.M.L., Rosing, J. (1985). Purification and properties of a prothrombin activator from the venom of *Notechis scutatus scutatus*. J. Biol. Chem. **260**, 9366-9372.

Tans, G., Rosing, J. (1993). Prothrombin activation by snake venom proteases. J. Toxicol.-Toxin Rev. **12**, 155-173.

Tibballs, J., Sutherland, S.K., Kerr, S. (1989). Studies on Australian snake venoms, Part I: The haemodynamic effects of brown snake (*Pseudonaja*) species in the dog. Anaesth. Intens. Care **17**, 466-469.

Tibballs, J., Sutherland, S.K. (1992). The efficacy of heparin in the treatment of common brown snake (*Pseudonaja textilis*) envenomation. Anaesth. Intens. Care **20**, 33-37.

Tibballs, J., Sutherland, S.K., Rivera, R.A., Masci, P.P. (1992). The cardiovascular and haematological effects of purified prothrombin activator from the common brown snake (*Pseudonaja textilis*) and their antagonism with heparin. Anaesth. Intens. Care **20**, 28-32.

Tidswell, F. (1906). Researches on Australian venoms. Snake-bite, snake-venom and antivenine. The poison of the platypus. The poison of the red-spotted spider. Dept. Public Health, N.S.W. Govt. Printer, Sydney.

Triplett, D.A., Stocker, K.F., Unger, G.A., Barna, L.K. (1993). The Textarin/Ecarin ratio: a confirmatory test for lupus anticoagulants. Thromb. Haemost. **70**, 925-931.

Walker, F.J., Owen, W.G., Esmon, C.T. (1980). Characterization of the prothrombin activator from the venom of *Oxyuranus scutellatus scutellatus* (taipan venom). Biochem. **19**, 1020-1023.

Warrell, D.A., Davidson, N.M.C.D., Greenwood, B.M., Ormerod, L.D., Pope, H.M., Watkins, B.J., Prentice, C.R.M. (1977). Poisoning by bites of the saw-scaled or carpet viper (*Echis carinatus*) in Nigeria. Q. J. Med. **46**, 33-62.

Warrell, D.A., Looareesuwan, S., Theakston, R.D.G., Phillips, R.E., Chanthavanich, P., Virivan, C., Supanaranond, W., Karbwang, J., Ho, M., Hutton, R.A., Vejcho, S. (1986). Randomized comparative trial of three monospecific antivenoms for bites by the Malayan pit viper (*Calloselasma rhodostoma*) in southern Thailand: clinical and laboratory correlations. Am. J. trop. Med. Hyg. **35**, 1235-1247.

White, J. (1980-81). Ophidian envenomation. A South Australian perspective. Rec. Adel. Children's Hosp. **2**, 311-421.

White, J., Williams, V., Duncan, B. (1989). Lymphopenia after snakebite. *Lancet*, Dec. **16**, 1448 - 1449.

White, J., Williams, V. (1989). Severe envenomation with convulsion following multiple bites by a common brown snake, *Pseudonaja textilis*. Aust. Paediatr. J. **25**, 109-111.

Williams, V., White, J. (1989). Purification and properties of a procoagulant from Peninsula tiger snake (*Notechis ater niger*) venom. Toxicon **27**, 773-779.

CHAPTER 3

OBSERVATIONS ON WHOLE VENOM COAGULATION ACTIVITY AND ISOLATION OF THE PROCOAGULANT FROM THE VENOM OF PSEUDONAJA TEXTILIS

INTRODUCTION

Snake venoms consist of a mixture of components which are particularly adept at bringing about the death of a prey item and also in aiding in the digestion of the prey. In examining the activity of a particular toxin of the venom, the presence of synergistic or inhibitory components within the whole venom should be considered. In the case of the coagulant activity, an isolated system of plasmas allows the effect of the whole venom to be examined without the difficulties experienced in using animal experiments where the total effect of all components are expressed, be they neurotoxins, myolysins or coagulants. This system has lent itself to examination of coagulant cofactor requirements in research.

Denson (1969) stated that *O. scutellatus* and *P. textilis* venoms contained complete prothrombin activators, with no requirement for cofactors and capable of clotting factor V deficient plasma. Whole venom clotting activity has been shown to be independent of a requirement for factor V (and factor X) in all studies and this was confirmed in preliminary experiments for this thesis. The requirement for calcium and phospholipid however is less clear cut. Herrmann et al (1972) found a difference in the requirement of *P. affinis* and *P. nuchalis* for calcium and phospholipid (Table 2.7) but this was not confirmed in the later

study of Marshall Herrmann (1983), where the presence of calcium alone and then calcium with phospholipid shortened the clotting times of citrated plasma with venom from *P. textilis*, *P. nuchalis* and *P. affinis*. Chester and Crawford (1982) failed to show any effect of calcium, phospholipid or both on the citrate plasma clotting times of *P. textilis* venom. Although using tiger and taipan venoms, Tans and Rosing (1993) showed an increased relative rate of prothrombin activation in the presence of calcium and phospholipid, compared with calcium alone. Unfortunately phospholipid alone was not tested.

Phospholipid appears to have little influence on the clotting times obtained (Masci et al, 1988), although a marginal shortening may be found when both calcium and phospholipid are present, compared with calcium alone. Normal assembly of the prothrombinase complex is reliant on the presence of a phospholipid membrane to anchor factor Va and subsequently factor Xa (Rosing et al, 1980) and thereby localise the conversion of prothrombin to thrombin at the site of tissue damage. In the case of venom, there is no requirement for the localisation of the clotting and therefore the role of phospholipid may be diminished. Preliminary whole venom experiments have confirmed that phospholipid alone has no effect on clotting times (Table 3.1).

Table 3.1 Preliminary confirmatory experiments on calcium and phospholipid cofactor requirements of *P. textilis* whole venom

Venom concentration	15μg/ml		0.8μg/ml	
	Citrate	EDTA	Citrate	EDTA
No addition	21.6 ± 2	27.5 ± 0.4	36 ± 2	82 ± 5
Calcium	19.1 ± 1.1	23.2 ± 1.5	29 ± 4	43 ± 3
Phospholipid	22.1 ± 0.6	27 ± 0.4	38 ± 3	89 ± 10
Calcium + phospholipid	18.6 ± 0.6	22.8 ± 1.5	29 ± 3	41 ± 2

25μl whole venom (diluted to 15 or 0.8μg/ml in saline) was added to 200μl of citrate plasma (9 parts whole blood added to 1 part 3.8% w/v tri sodium citrate) or 200μl of EDTA plasma (EDTA final concentration ~ $4x10^{-3}$M) and the clotting time recorded. (N = 6)

The traditional determination of venom calcium dependence is to add calcium to a citrate plasma and note any shortening of the clotting time and if noted thereby infer calcium dependence (Marshall and Herrmann, 1983). Masci et al (1987) noted that the venom of *P. textilis* clotted EDTA plasma, although the significance of this in terms of calcium requirement by the venom was not pursued. The fact that whole venom can clot a calcium chelated EDTA plasma suggested the clotting mechanism is calcium independent, although the efficiency of prothrombin activation may be reduced in the absence of calcium. Reducing the venom concentration magnifies the decreased efficiency of prothrombin activation in the absence of calcium (Table 3.1). The lack of difference in clotting times in the presence or absence of calcium in the study of Chester and Crawford (1982) was most likely a reflection of the venom concentration employed. At a lower venom concentration possibly a difference may have been noted in the effect of calcium and phospholipid.

When present however calcium will stimulate the clotting activity and this is consistent with a factor X-like component being available to bind calcium via γ-carboxyglutamic acid residues which have been found in the procoagulant of both tiger (*Notechis* spp.) and taipan (*O. scutellatus*) venoms.

Masci et al (1987) reported that heparin had no effect on the clotting activity of the isolated procoagulant from brown venom and this was confirmed in the preliminary studies for the thesis. Again however, the activity was concentration dependent, with the clotting action almost eliminated at low venom concentrations, while citrate plasma was still readily clotted. Overcoming the effect of heparin is most likely due to the procoagulant converting prothrombin to meizothrombin which is insensitive to heparin (Schoen and Lindhout, 1987). At high concentrations of venom, the meizothrombin produced is in sufficient quantity to produce clotting rapidly, while the inefficiency of meizothrombin at converting fibrinogen to fibrin becomes more evident at lower concentrations of venom.

This ability to clot heparinised plasma calls to question the therapeutic use of heparin in combating coagulopathy due to snakebite (Warrell et al, 1976). Is the use of heparin in these situations counterproductive? Tibballs et al (1992) showed that pre-injection of heparin (100U/kg) into dogs was capable of eliminating the effect of a later injection of prothrombin activator (purified), however in cases of an established coagulopathy after introduction of the purified procoagulant, heparin had no effect (Tibballs and Sutherland, 1992). In bites by *Pseudonaja textilis* and *nuchalis*, treatment with heparin failed to show any improvement of the coagulopathy (Schapel et al, 1971; White and Fassett, 1983). Thus the in vitro finding of insensitivity to heparin by the whole venom, appears to mirror clinical experience.

In further confirmatory studies of the work of Chester and Crawford (1982) and Masci et al (1988), warfarin and aluminium hydroxide adsorbed plasma were clotted with almost the same ease as citrate plasma. The fact that the venom was not reliant on calcium for activity and was able to convert prothrombin to meizothrombin were findings consistent with the likelihood of venom activating descarboxyprothrombin. Thus the use of whole venom provides some insight into the action of the proocoagulant, however to fully understand the mechanism of action and physical characterisation of the procoagulants in venoms, they must be isolated and purified.

The procoagulant from the tiger snake (*N. scutatus*) (Tans et al, 1985) and the black tiger snake (*N. ater niger*) (Williams and White, 1989) were isolated and their properties examined. The procoagulant from both species were shown to be almost identical and to consist of a single chain protein, made up of two subunits held together by a disulphide bridge. The clotting profile of this component equated its action to that of factor Xa, requiring factor V to be present and the activity being stimulated by calcium and phospholipid. The procoagulant from *T. carinatus* has been isolated and the sequence of the procoagulant shown to be homologous to factor Xa (Joseph et al, 1999).

The taipan (*O. scutellatus*) venom procoagulant was initially isolated by Walker et al (1980) and then re-isolated by Speijer et al (1986). Walker at al suggested the prothrombin activator may have had two activities either as separate parts of the venom or as physically associated units, showing amidolytic and clotting action. They suggested the possibility of the procoagulant consisting of a two chain structure with molecular weights of 220kDa and 160kDa. Speijer et al showed the procoagulant to consist of subunits and as defined by Macfarlane and Denson (1967) was a complete prothrombin activator, not requiring the presence of cofactors for its activity. The authors speculated that the subunits consisted of a factor Xa - like part and a factor V - like part. They also speculated that the procoagulant in brown snake may be similar, based on the characteristics for the brown snake venom reported by Marshall and Herrmann (1983).

Masci et al (1987, 1988) isolated the procoagulant from the common brown snake (*P. textilis*). The method employed was a two stage technique, involving initial separation from the whole venom on a Con-A sepharose column, identification of the active fraction and application of that fraction to a Sephacryl S-300 column, resulting in a purified procoagulant fraction. The purified prothrombin activator on native PAGE, pH 8.6, gave a heavy-staining slow-moving band, and two minor bands, when the gel was heavily loaded. Activity against the chromogenic substrate S2222, was found in the main heavy-staining slow moving band, while activity in the close minor band was uncertain, the fast moving minor band appeared to be inactive. The prothrombin activator comprised approximately one third of the crude venom and when applied to SDS-PAGE produced multiple banding, with approximate molecular weights of 200 kDa, 170 kDa, 65 kDa and 60 kDa. The appearance of a number of bands is presumably due to the detergent releasing closely associated proteins. β-mercaptoethanol reduction reduced the band numbers to two, although a considerable amount of protein aggregated and therefore was unable to enter the gel.

In this chapter an alternative method of isolation and a preliminary characterisation of the procoagulant thus isolated are presented.

MATERIALS AND METHODS

Venoms:

Pseudonaja textilis venom was a pool of venom from specimens collected around Adelaide and near environs and was purchased from Venom Supplies, Tanunda, South Australia.

Protein estimation:

Three methods for protein estimation were employed, Bradford (Bradford, 1976) (Bio-Rad, Ca), Bicinchoninic Acid (Smith et al, 1985) (Pierce, Rockford, Il) and Lowry (Lowry et al, 1951) (Sigma, 690-A). As each of these methods has a particular bias in estimating protein concentration due to the presence of particular amino acids, this allowed an evaluation of the methods to provide the most accurate estimate of protein concentration and therefore the method most appropriate for future protein estimates on the procoagulant.

Liquid chromatography of the venom:

The procoagulant was isolated from whole venom according to the method reported in Williams et al (1994), with slight modifications.

Whole venom was dissolved in 0.05M NaOAc/).15M NaCl, pH 8.35, at varying concentrations dependent on availability, (typical concentration 100mg/ml), and 200µl loads applied to a TSK G3000SW, 8x300 mm column. The peaks were eluted with the same buffer at a flow of 0.5 ml/min and 0.5 ml fractions were collected and screened for coagulant activity. Twenty five microlitres of each fraction was added to 200µl of pooled normal citrate plasma and the time for appearance of a clot recorded. Observation was abandoned at 60 sec if no clot had developed. A gel filtration standard (Bio-Rad, Ca) was also run through the column under identical conditions. The fractions containing procoagulant activity were pooled, concentrated (Amicon 10, Microconcentrators) and applied to an anion exchange column,

TSK DEAE 5PW, 8x75 mm, and eluted with a linear gradient developed between 0.05M NaOAc/ 0.15M NaCl, pH 8.35 and 0.05M NaOAc/ 0.6M NaCl, pH 8.35. The fractions were again screened for coagulant activity and those most active pooled. The method of Masci et al (1988) was also performed with an initial gel filtration as described above followed by Con-A Sepaharose chromatography with elution from the column by 45mM α methylmannopyranoside in 0.05M NaOAc/ 0.15M NaCl, pH 8.35.

Electrophoresis:

Native-PAGE or SDS-PAGE were performed on a Hoeffer Mighty Small II, (Hoeffer,Ca) using 1.0 mm thick gels. Procoagulant loads were between 3-10 µg.

Native PAGE: A 7% polyacrylamide gel with a 4% stacking gel, was run in the absence of SDS essentially according to the method of Laemmli (1970), using a Tris Glycine buffer.

SDS-PAGE: In the presence of SDS, a 10% separating gel was used with a 4% stacking gel. 20 mAMP was applied per gel until the bromophenol marker had reached the bottom of the gel. At the completion of the run the bands were visualised with 0.125% Coomassie blue (R250, Sigma, B-7920).

Blotting:

Western blotting was performed using a Milliblot semi-dry apparatus (MilliBlot - SDE). Anode 1 buffer (3M Tris, 20% methanol, pH 10.4) , Anode 2 buffer (0.025M Tris, 20% methanol, pH 10.4) and Cathode buffer (0.025M Tris, 0.04M aminohexanoic acid, 20% methanol, pH 9.4). Proteins were transferred to nitrocellulose membrane (Hybond-C, Amersham) using 8 $mAmp/cm^2$, for 1 hour. The nitrocellulose was then blocked with 5% skim milk for 1hr. The membrane was then washed 3x with PBS and overlaid overnight with 1/1000 monoclonal antibody (PT-1D3) raised against prothrombin activator from *P. textilis* (Gift from P. Masci, Qld). The monoclonal is directed against the active site of the procoagulant (Monoclonal Pt-1D3 inhibits S2222 chromogenic substrate conversion and

citrate plasma clotting time - personal communication). After 3 washes with PBS the bands were overlaid with peroxidase-conjugated anti mouse immunoglobulin, and visualised after the conversion of the peroxidase substrate (3,3'-diaminobenzidine tetrahydrochloride/hydrogen peroxide).

Fold purification analysis of procoagulant:

The ability to convert the chromogenic substrate S2444 was used as a measure of activity of the procoagulant. Twenty five microlitres of whole venom, gel filtration or anion exchange fraction were added to 950µl of 0.05M Tris buffer, pH8.3 and with the addition of 25µl of chromogenic substrate, a timer was started and at exactly 60 sec the reaction was stopped by the addition of 100µl of 50% glacial acetic acid. The change in OD 405nm was recorded and the number of moles of paranitroaniline released / min calculated using Beer's Law, with ε S2444 = 1.3×10^4 and the molecular weight of S2444 = 498.9.

RESULTS

Protein estimation

The comparison of the methods of protein estimation produced considerable difference between the result from the Bradford method compared to that for the Lowry and Bicinchoninic Acid techniques, but this effect was only noted in the case of the whole venom (Table 3.2). Little difference was noted in the protein concentrations obtained after the purification step of gel filtration and no difference was seen after anion exchange between the results obtained from the three methods.

Table 3.2 Protein estimation results (mg/ml) using the Bradford, Bicinchoninic acid and Lowry methods, on whole venom, gel filtration fraction and anion exchange fraction.

	Bradford	**Bicinchoninic Acid**	**Lowry**
Whole venom (53 mg/ml)	23.6 (44%)*	52 (98%)*	56 (>100%)*
Gel filtration fraction	1.16	1.23	1.32
Anion exchange fraction	1.84	1.84	1.84

*** % of whole venom contributed by protein as determined by the three protein estimation techniques.**

The Bradford method is highly dependent on the particular protein under investigation and therefore it was not surprising to find the disparity in the protein estimation. The Lowry method also falsely raised the protein concentration and gave a result greater than the starting material. The important point to note however was that there was no discrepancy between the three methods when used to estimate the protein concentration of the purified procoagulant (Anion exchange fraction), and therefore all three methods could be used with equal validity.

Purification

Fig 3.1 shows the elution profile of the gel filtration and the shaded area indicates the procoagulant activity. The profile is similar to that obtained by Masci et al (1988) and they estimated the procoagulant to be some 30% of the crude venom. The recovery data from the purification of the procoagulant over a series of separate runs indicated the procoagulant contributed 23 - 35% of the whole venom. This range in percent contribution may be a reflection of differences between purification runs or occur as a result of variability between

batches of crude venom. An estimate of the molecular weight of the purified procoagulant from the gel filtration standard elution profile was 169 kDa.

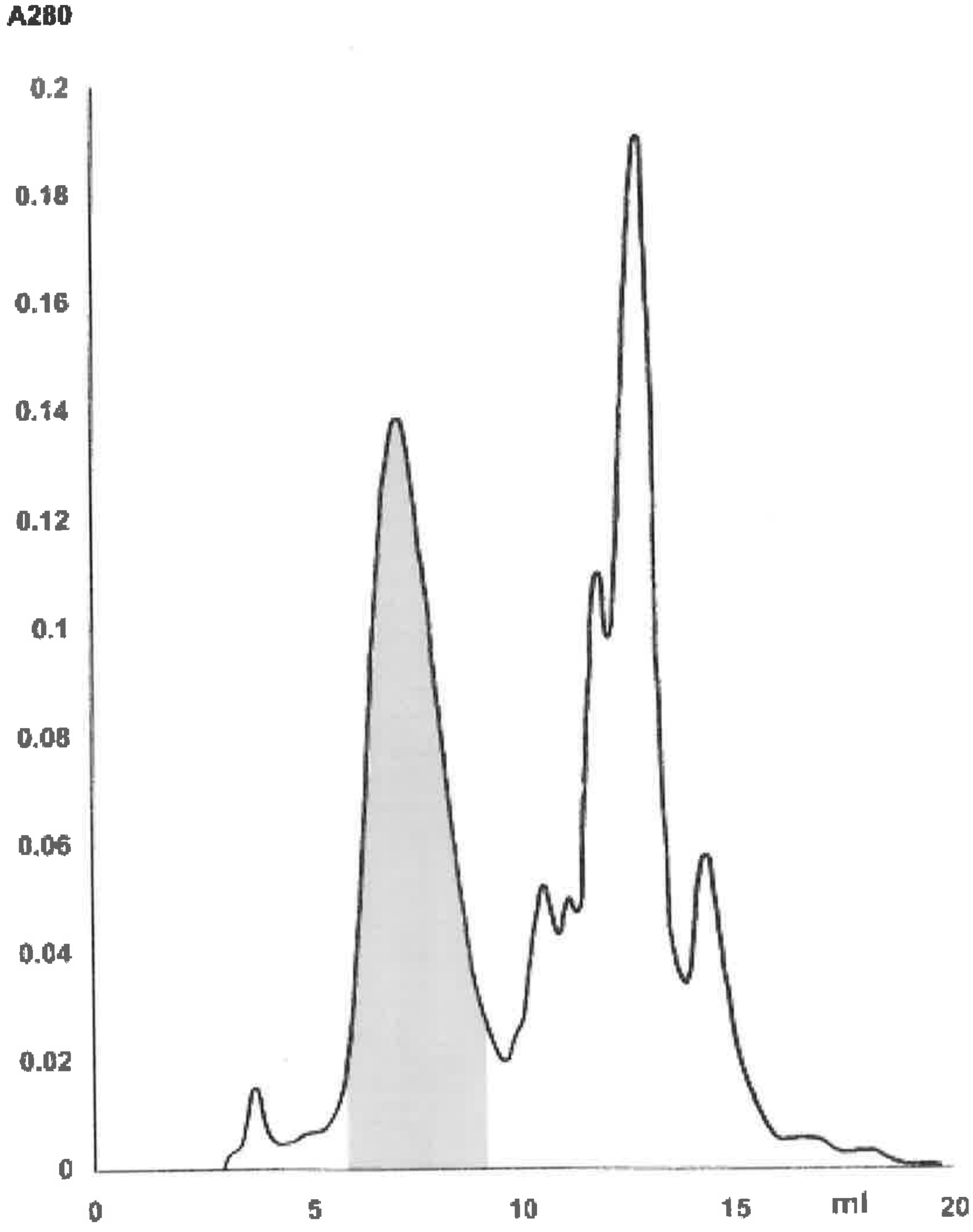

Fig 3.1 Gel filtration elution profile. *Pseudonaja textilis* whole venom was dissolved in 0.05M NaOAc/0.15M NaCl, pH 8.35 and applied to a TSKG3000SW (8x300mm) column. Elution was performed at 0.5ml/min with the same buffer and fractions collected at 1 tube/min. All fractions were screened for clotting activity and those found to contain the clotting activity (shaded) were combined, concentrated and applied to an anion exchange column.

Fig 3.2 shows the elution profile obtained from the anion exchange. There was a contaminant present after the gel filtration but most of this was eliminated essentially at the void volume, the procoagulant attaching to the column and being eluted at ~ 0.35M salt. The peak was symmetrical in the majority of the runs although occasionally a slight shoulder was noticeable, however the origin of this shoulder was not evident on SDS-PAGE with no specific change in the banding patterns being found.

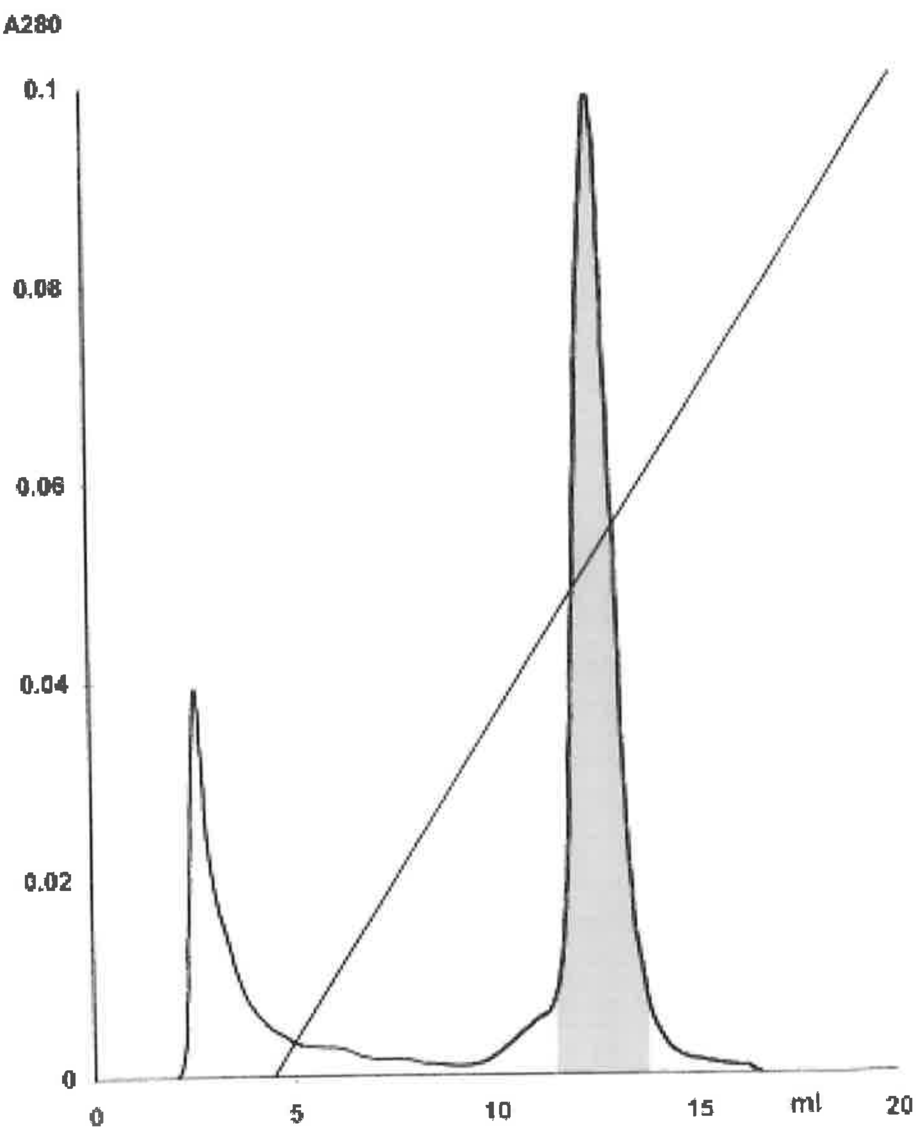

Fig 3.2 Anion exchange of the concentrated coagulant peak from gel filtration. A gradient was developed from 0.05M NaOAc/0.15M NaCl, pH 8.35 to 0.05M NaOAc/0.6M NaCl, pH 8.35, flow rate 0.5ml/min and fractions collected at 1 tube/min. Coagulant activity is shaded.

The chromatography steps resulted in just over a twofold purification of the procoagulant (Table 3.3) and this was a typical result for all purifications performed. The small increase in

fold purification indicated the gel filtration alone produced an almost pure activator. The increase in purity may be marginal with anion exchange, however the material removed in the void volume had no activity. It was anticipated that the increase in purification should have been slightly higher and was expected to be between 2.5 to 3.5. This may have been due to the difficulty in accurately assaying the activity through particularly a clotting based assay or through the chromogenic assay employed. The fold purification was comparable however to that found by Masci et al (1988) at 3.2.

Table 3.3 Purification of the procoagulant from *P. textilis* crude venom

Fraction	Total protein (mg)	Activity Total nmoles pNa released/min	Specific activity nmoles pNa released/min/mg	Purification
Crude venom	40.8	46.1	0.9	-
Gel filtration	9.53	18.58	1.95	2.16
Anion exchange	5.49	11.36	2.07	2.3

Use of gel filtration and subsequent Con A Sepharose chromatography (Masci et al, 1988) produced a result no different to that found with gel filtration followed by anion exchange.

Electrophoresis

The banding pattern of the procoagulant on native PAGE was dependent on the amount of procoagulant loaded. At low concentrations a single slow moving band was found while if the concentration was increased then two bands were noted. The second, a fast moving but less obvious band, showed considerable distance separation from the main band (Fig 3.3). Both bands were not discrete sharp bands but tended to appear more as smudges. Monoclonal antibody detection of the blotted native PAGE produced banding at the regions of both the fast and slow moving bands. The fast moving band was observed by Masci et al (1988) but was not considered to be significant.

Fig 3.3 Native PAGE (7%) of the isolated procoagulant (3.0µg) from *P.textilis* venom.
Left lane: Coomassie Blue staining.
Right lane: Detection of monoclonal antibody (Masci) directed against procoagulant..

SDS-PAGE (Fig 3.4) produced a large number of bands, however eight principle bands were discernible. The high molecular weight region appeared to be quite variable between batches of venom and bands were also found at 208, 190 and 125kDa. At the low molecular weight end an extra band was sometimes present in the isolate at 39kDa. The bands at 52,48 and 43kDa were constantly present. (For comparison with SDS-PAGE banding of Masci et al, (1988): Triplett et al, (1993): Stocker et al, (1994): see table 7.1).

The pattern obtained after blotting the SDS-PAGE and developing against the monoclonal antibody showed only the low molecular weight species were recognised (Fig 3.4).

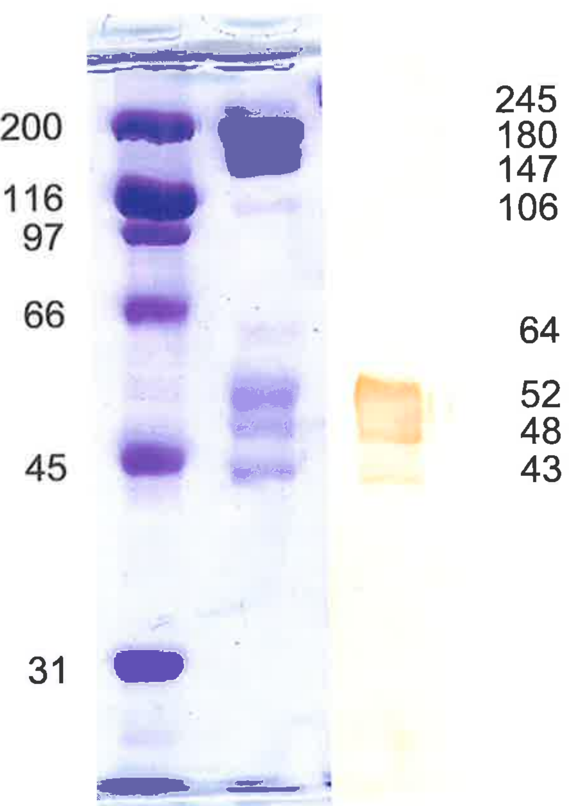

Fig 3.4 SDS-PAGE (10%) of the isolated procoagulant (3.0µg) from *P.textilis* venom.
Left lane: Molecular weight markers.
Centre lane: Isolated procoagulant stained with Coomassie Blue.
Right lane: Detection of monoclonal antibody (Masci) directed against procoagulant.

DISCUSSION

The procoagulant was isolated using a combination of gel filtration and anion exchange chromatography. The method was simple, quick and resulted in a distinct peak cut off. The active fraction was obtained in a small volume with a minimum number of tubes. The Con A Sepharose elution was slow and occurred over a wide volume due to the relative inefficiency of binding to Con A of the eluant α-mannopyranoside compared with the more efficient binding of the complex mannose-containing sugars on the protein, with difficulty encountered due to the activity being extended over a large number of tubes. The two methods however isolated the procoagulant and gave a fold purification of similar proportion. When run onto native PAGE they gave banding with the same characteristics, a slow moving large band and a fast moving less distinct band.

The significance of these bands is discussed in much greater detail in later chapters, but Masci et al (1988) indicated that the fast moving band appeared to be inactive against the chromogenic substrate S2222 and hence disregarded it. However the fast moving band was detected with Masci's monoclonal antibody and stained quite strongly. Therefore the fast and slow moving bands had epitopes in common (Fig 3.3). This finding indicated that the fast moving band was of considerable significance.

SDS-PAGE of the procoagulant gave a surprising result in that a number of bands became visible, and these could be conveniently divided into a group referred to as high molecular weight (HMWG > 100 kDa) and a group referred to as low molecular weight (LMWG <100 kDa). This result was unlike that obtained from tiger and black tiger snake procoagulant where there was a single band found on SDS-PAGE corresponding to the procoagulant and has a 'low' molecular weight of 54 kDa (Tans et al, 1985; Williams and White, 1989). The banding does show a similarity to that obtained from taipan venom (Speijer et al, 1986) with a characteristic high molecular weight group ~100 kDa and a low molecular weight group at ~ 60 kDa. The similarity of the brown and the taipan is probably not unexpected as Masci et al found cross reactivity between the taipan and brown proteins and compared the molecular weights of the bands obtained for the brown and taipan.

The presence of the high and low molecular weight components also raised the question about their interaction in producing a powerful clotting entity. Speijer et al (1986) suggested that in the taipan the high molecular weight material most likely acted as a cofactor as the active site could be located in the low molecular weight bands. In the case of the brown procoagulant, the antibody supplied by Masci was considered to be directed against the active site and only the low molecular weight bands were able to be recognised.

The separation of the bands from the purified procoagulant into a high and low molecular weight group and the identification of the possible active site in the low molecular weight

group was supportive of the hypothesis that the procoagulant consists of subunits with protease activity and cofactors for this activity. On the native PAGE immunoblot, the antibody stained the 'heavier' slow moving band as well as the fast moving band suggesting that in normal circumstances the high and low molecular weight species are possibly closely associated. This close association would allow the cofactor and active parts to be in close physical proximity to bring about rapid conversion of prothrombin under various conditions which may be adverse to normal clotting factors found in the circulation. The characteristics of these subunit bands were investigated and are reported in the following chapters.

REFERENCES

Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**, 248-254.

Chester, A., Crawford, G.P.M. (1982). In vitro coagulant properties of venoms from Australian snakes. Toxicon **20**, 501-504.

Denson, K.W.E. (1969). Coagulant and anticoagulant action of snake venoms. Toxicon **7**, 5-11.

Herrmann, R.P., Davey, M.G., Skidmore, P.H. (1972). The coagulation defect after envenomation by the bite of the dugite (*Demansia nuchalis affinis*), a Western Australian brown snake. Med. J. Aust. **2**, 183-186.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. (1951). Protein measurement with the folin phenol reagent. J. Biol. Chem. **193**, 265-275.

Macfarlane, R.G., Denson, K.W.E. (1967). General techniques used in the study of snake venoms. In: Proc. 10th Congr. Europ. Soc. Haemat. , Strassberg 1965, Part II, 1074-1081.

Marshall, L.R., Herrmann, R.P. (1983). Coagulant and anticoagulant actions of Australian snake venoms. Thromb. Haemost. **50**, 707-711.

Masci, P.P., Whitaker, A.N., deJersey, J. (1987). Purification and characterization of the prothrombin activator of the venom of *Pseudonaja textilis*. In: Progress in venom and toxin research. pp. 209-219. Eds. Gopalakrishnakone, P. , Tan, C.K. Proceedings of the first Asia-Pacific Congress of Animal, Plant and Microbial Toxins.

Masci, P.P., Whitaker, A.N., deJersey, J. (1988). Purification and characterization of a prothrombin activator from the venom of *Pseudonaja textilis textilis*. Biochem. Int. **17**, 825-835.

Rosing, J., Tans, G., Govers-Riemslag, J.W.P., Zwaal, R.F.A., Hemker, H.C. (1980). The role of phospholipids and factor Va in the prothrombinase complex. J. Biol. Chem. **255**, 274-283.

Schapel, G.J., Utley, D., Wilson, G.C. (1971). Envenomation by the Australian common brown snake - *Pseudonaja (Demansia) textilis textilis*. Med. J. Aust. **1**, 142-144.

Schoen, P., Lindhout, T. (1987). The in situ inhibition of prothrombinase-formed alpha thrombin and meizothrombin (des F1) by antithrombin III and heparin. J. Biol. Chem. **262**, 11268-11274.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., Klenk, D.C. (1985). Measurement of protein using bicinchoninic acid. Anal. Biochem. **150**, 76-85.

Speijer, H., Govers-Riemslag, J.W.P., Zwaal, R.F.A., Rosing, J. (1986). Prothrombin activation by an activator from the venom of *Oxyuranus scutellatus* (Taipan snake). J. Biol. Chem. **261**,13258-13267.

Tans, G., Govers-Riemslag, J.W.P., van Rijn, J.L.M.L., Rosing, J. (1985). Purification and properties of a prothrombin activator from the venom of *Notechis scutatus scutatus*. J. Biol. Chem. **260**, 9366-9372.

Tans, G., Rosing, J. (1993). Prothrombin activation by snake venom proteases. J. Toxicol.-Toxin Rev. **12**, 155-173.

Tibballs, J., Sutherland, S.K. (1992). The efficacy of heparin in the treatment of common brown snake (*Pseudonaja textilis*) envenomation. Anaesth. Intens. Care **20**, 33-37.

Tibballs, J., Sutherland, S.K., Rivera, R.A., Masci, P.P. (1992). The cardiovascular and haematological effects of purified prothrombin activator from the common brown snake (*Pseudonaja textilis*) and their antagonism with heparin. Anaesth. Intens. Care **20**, 28-32.

Walker, F.J., Owen, W.G., Esmon, C.T. (1980). Characterization of the prothrombin activator from the venom of *Oxyuranus scutellatus scutellatus* (Taipan venom). Biochem. **19**, 1020-1023.

Warrell, D.A., Pope, H.M., Prentice, C.R.M. (1976). Disseminated intravascular coagulation caused by the carpet viper (*Echis carinatus*): Trila of heparin. Br. J. Haemat. **33**, 335-342.

White, J., Fassett, R. (1983). Acute renal failure and coagulopathy after snakebite. Med. J. Aust. **2**, 142-143.

Williams,V., White, J. (1989). Purification and properties of a procoagulant from peninsula tiger snake (*Notechis ater niger*) venom. Toxicon **27**, 773-779.

Williams, V., White, J., Mirtschin, P. (1994). Comparative study on the procoagulant from the venom of the Australian brown snakes (Elapidae: *Pseudonaja* spp.). Toxicon **32**, 453-459.

CHAPTER 4

CHARACTERISATION OF THE PROCOAGULANT PT 1 - EXAMINATION OF THE COMPLETE PROCOAGULANT

INTRODUCTION

The procoagulant was simply and rapidly isolated from the whole venom of the brown snake (*P. textilis*) by gel filtration followed by anion exchange chromatography. The procoagulant thus isolated was extremely active and rapidly clotted plasmas. Masci et al (1987) showed that a bolus injection of the procoagulant was capable of bringing about the death of injected rats within minutes, while with injections of increasing quantities of the procoagulant, starting with low concentration, the animals survived, mirroring the experiments of Martin (1894). Martin had found that an animal that had survived an initial challenge with venom, when rechallenged, showed no further effect. The results again suggested that the low doses of procoagulant produced a defibrination without any evidence of thrombus formation, and subsequent challenges failed to have any effect as the fibrinogen had been consumed. An initial massive bolus resulted in a widespread thrombosis and the subsequent rapid death of the subject.

The procoagulant was capable of producing clotting in a test system, irrespective of the presence of calcium and phospholipid, had the ability to clot plasma containing descarboxyprothrombin and from the evidence of its insensitivity to heparin, was able to convert prothrombin to thrombin via the intermediary of meizothrombin. Isolation of the

procoagulant showed it consisted of distinctive high and low molecular weight subunits and it is hypothesised that these will possess physical characteristics and activity consistent with a factor X - factor V complex. In this chapter the physical characterisation of the procoagulant was continued and the activity investigated.

MATERIALS AND METHODS

Procoagulant:

Isolated as previously described (Chapter 3).

Electrophoresis:

Native PAGE and SDS-PAGE were performed according to the methods described in chapter 3.

β-mercaptoethanol reduction: Procoagulant (~370μg/ml) was diluted 1:2 in reducing buffer consisting of 475μl of sample buffer (4.8ml distilled water: 1.2ml 0.5M Tris-HCl, pH 6.8: 1.0ml Glycerol: 2.0ml 10% (W/V) SDS: 0.5ml 0.1% bromophenol blue) and 25μl of β-mercaptoethanol. The sample was boiled for at least 2 min prior to loading onto the gel. 20μl loads were used (~3.2 μg of protein).

Active-site labelling:

Twenty five microlitres of the active site directed chloromethyl ketone (Calbiochem), dansyl-Glu-Gly-Arg-CH_2Cl (dansyl-GGACK) (80μM) was incubated with 25μl of the procoagulant (9.5μg) at room temperature for at least 20 min. Excess label was removed by washing the procoagulant in saline and concentrating in an Amicon 10 Microconcentrator (Amicon Ca), to a volume of 40μl. Twenty five microlitres of this was then applied to a native gel or SDS

containing electrophoretic gel. The gels after running were observed under a UV lamp and photographed.

Carbohydrate detection:

The procoagulant was electrophoresed on a 10% SDS gel as before and transferred to nitrocellulose as previously described (Chapter 3). The bands were then developed on the nitrocellulose according to the Glycan detection method of Boehringer Mannheim, using digoxigenin labelling of the glycoconjugates. An alkaline phosphatase conjugated anti-digoxigenin specific antibody was overlaid and the bands visualised by the development of the converted substrate.

Determination of the inhibitory effect of various reagents on procoagulant activity:

A battery of reagents were tested for their ability to inhibit the chromogenic substrate conversion activity of the procoagulant. The clotting activity was not used as the inhibitors would interfere with the conversion of fibrinogen to fibrin. Twenty five microlitres of the inhibitor was incubated with 25µl of the procoagulant (9.5µg) for 3 min at room temperature. Endoglycosidase D, which removes complex type oligosaccharides, was incubated with the procoagulant overnight at 37°C. Then 925µl of 0.05M Tris, pH 8.3 was added followed by 25µl of the chromogenic substrate S2222, 3mM and the reaction allowed to proceed for 3 min. Saline was substituted for inhibitor in the control. The reaction was stopped by the addition of 100µl of 50% glacial acetic acid and the OD 405nm recorded. The % inhibition was calculated from the reduction in OD from the saline control. The following inhibitors were used:

Aprotinin (1000U/ml), benzamidine (10mM), hydroxylamine (10mM), di-isopropyl-fluorophosphate (DFP, 25mM), leupeptin (20µg/ml), phenylmethylsulphonylfluoride (PMSF, 10mM), N-tosyl-arginine methyl ester (TAME, 10mM): Sigma, St. Louis, Mo.

N-benzoyl-L-arginine ethyl ester (BAEE, 10mM), dansyl-GGACK (40µM): Calbiochem, Germany.

Endoglycosidase D (100mU/ml): Boehringer Mannheim, Germany.

β-mercaptoethanol (electrophoretic grade). Bio Rad, Hurcules, Ca.

Chromogenic substrate conversion:

The ability to convert the chromogenic substrates S2444, S2238, S2222, S2366, S2302 and S2251 was determined. Twenty five microlitres (9.5ug) of procoagulant was added to 950μl of 0.05M Tris buffer, pH8.3 and with the addition of 25μl of chromogenic substrate, a timer was started and at exactly 60 sec the reaction was stopped by the addition of 100μl of 50% glacial acetic acid. The change in OD 405nm was recorded.

Immunoprecipitation:

A simple Ouchterlony immunodiffusion plate was prepared with a 1% agarose gel poured in a petri dish and wells punched. 100μl of brown, tiger and taipan antivenom (CSL, Ltd Melbourne) was added to the peripheral wells and 50μl of procoagulant (19 μg) to the central well. The precipitin lines were allowed to develop for two days prior to being photographed.

RESULTS

β-mercaptoethanol reduction of procoagulant bands

The procoagulant was reduced with β-mercaptoethanol to determine the presence of any disulphide linkages and the bands that resulted from the reduction can be seen in Fig 4.1. The wide range in molecular weights obtained after β-mercaptoethanol reduction has reduced the accuracy of the molecular weight determinations, therefore the weights quoted below are approximations only. However the lower molecular weight bands were pale and not easily defined.

Major bands were found in the range 16-100kDa. It appeared that all bands had undergone a reduction in molecular weight from those obtained with untreated procoagulant, with both the HMWG and LMWG containing proteins with disulphide linkages present. This was confirmed for all bands except the 43kDa native species by the reduction of the individually isolated bands shown in Fig 5.1 (Results not shown).

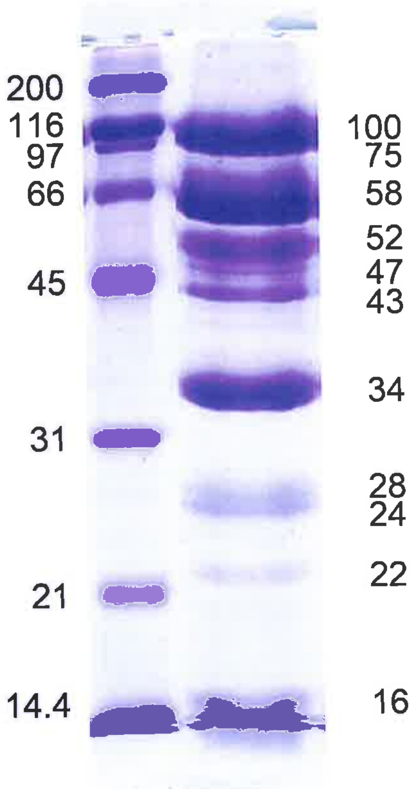

Fig 4.1 SDS-PAGE (10%) of β-mercaptoethanol reduced isolated procoagulant (3.2μg) from *P. textilis* venom .
Left lane: Molecular weight markers.
Right lane: β-mercaptoethanol reduced isolated procoagulant.

Active-site labelling

To determine which subunits contained the catalytic site, the procoagulant was stained with dansyl-GGACK. This active-site labelling produced two fluorescent bands on the native gel which corresponded to the fast moving, indistinct band and the heavy staining slow moving band found with Coomassie Blue staining. Despite the difference in the concentrations of protein in these bands, this was not reflected in the fluorescence, the fast moving band showed a strong disproportionate degree of fluorescence (Fig 4.2).

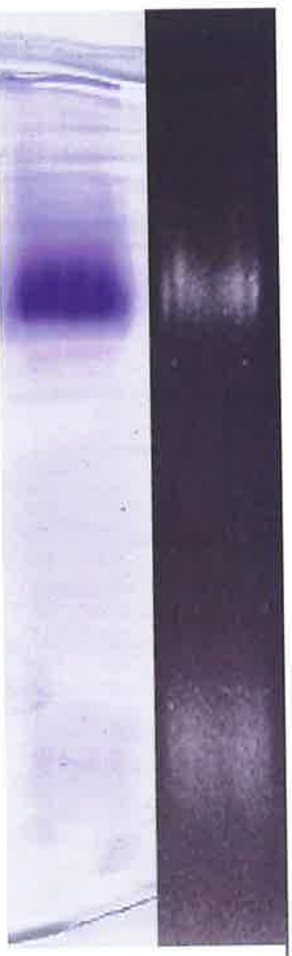

Fig 4.2 Native (7%) PAGE of active-site labelled isolated procoagulant (3.5μg).
Left lane: Coomassie Blue staining of procoagulant.
Right lane: dansyl-GGACK active-site fluorescent label of procoagulant.
The pale, diffuse, fast-moving band stained with Coomassie Blue is easily visualised with the fluorescent active-site label, dansyl-GGACK.

The fluorescent banding found when the procoagulant was run onto an SDS gel was only associated with the low molecular weight group. Two distinct bands were found (Fig 4.3a), although when the gel was over-loaded, up to four bands were visualised (Fig 4.3b). It is debatable if the fluorescence found when the gel was overloaded was legitimate or carry-over, the latter appearing more likely. When the procoagulant was treated with β-mercaptoethanol a single labelled band was found. The molecular weight of the bands found were 52kDa and 48kDa in the native procoagulant and when reduced the fluorescent band was at ~ 34kDa (Fig 4.4).

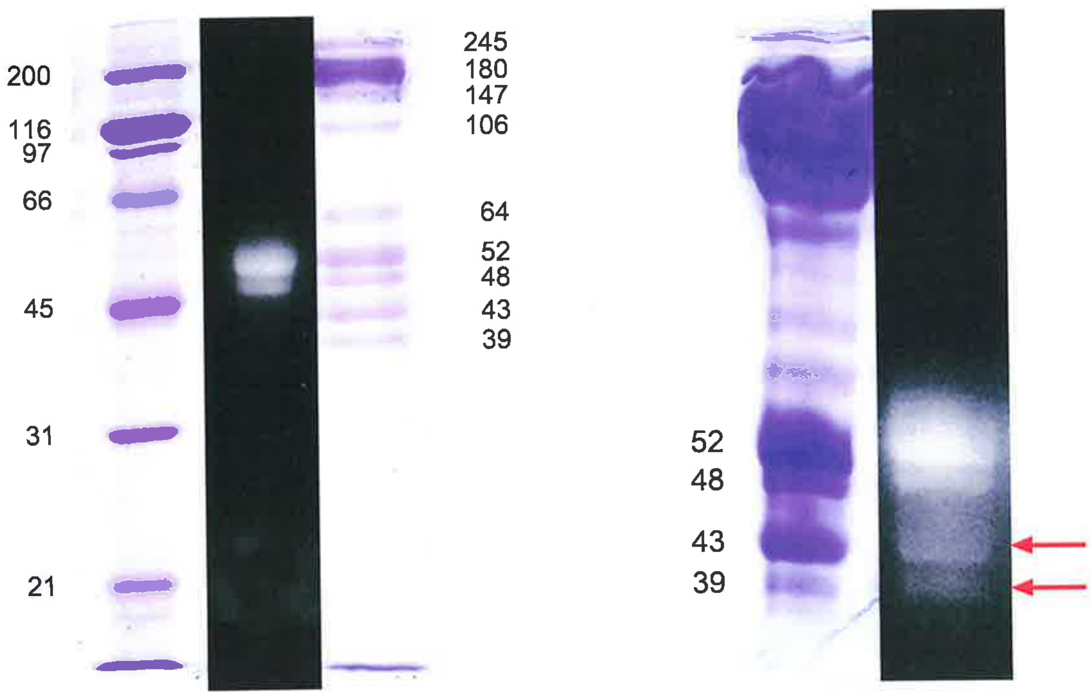

Fig 4.3a SDS-PAGE (10%) of active-site labelled isolated procoagulant (3.5μg).
Left lane: Molecular weight markers.
Centre lane: dansyl-GGACK active-site fluorescent label of procoagulant.
Right lane: Coomassie Blue staining of isolated procoagulant.
The active-site label aligns with the bands at 52 and 48kDa.

Fig 4.3b SDS-PAGE (10%) of heavily overloaded isolated procoagulant (>20μg). Dansyl-GGACK fluorescence is noted in bands corresponding to a molecular weight of 43 and 39 kDa.

The band at 39kDa equated with a band found when the 43kDa band was deglycosylated. The presence of this band was intermittent and no specific treatment differences predicted the likelihood of the band being present.

Chromogenic substrate conversion

Masci et al (1988) reported that the procoagulant was able to convert the following chromogenic substrates and rated their comparative activities in the order shown S2222 > S2444 > S2288 > S2238. It was also reported that there was negligible activity to S2251. The conversion of the chromogenic substrates is reliant on the ability of the venom to break the critical bond between the terminal amino acids incorporated into the substrate. In this study the substrates S2366 and S2302 not tested by Masci et al were also included. The target bond

for each of these substrates is Arg-PNA except for the substrate S2251 where Lys is the terminal amino acid.

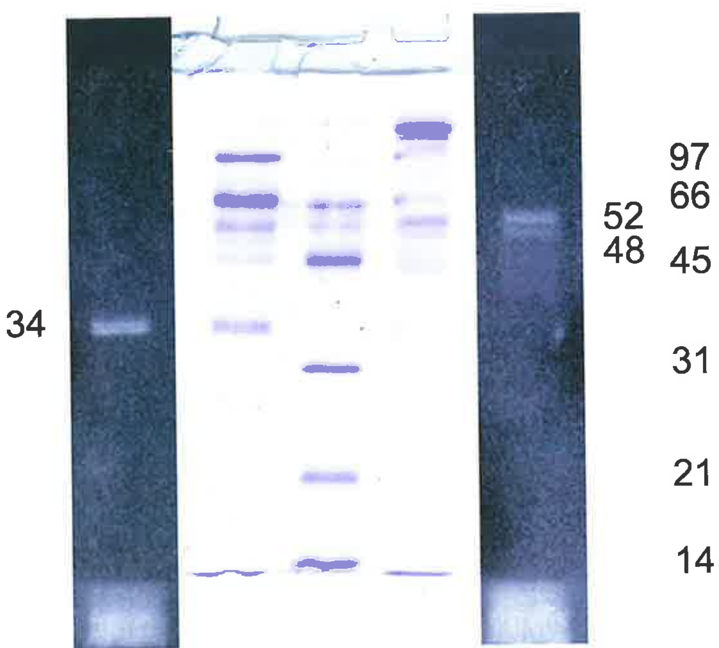

Fig 4.4 SDS-PAGE (15%) of native and reduced isolated procoagulant (3.0μg and 3.5μg respectively).
Lane 1&2: β-mercaptoethanol reduced isolated procoagulant, active-site labelled (left lane) and Coomassie Blue stained (right lane).
Centre lane: Molecular weight markers.
Lane 3&4: Native procoagulant, Coomassie Blue stained (left lane) and active site labelled (right lane).
The active site associated with bands at 52 and 48kDa is found in a single band at 34kDa after β-mercaptoethanol reduction.

In this series, the procoagulant was able to release p-nitroaniline from all the chromogenic substrates tested, except, as above, S2251at either 3 or 30mM was not converted. The chromogenic substrates S2366 and S2302 not tested by Masci et al were also able to be converted. Although an end-point method was used in this testing, the difference in conversion rates previously reported was not as obvious, with S2222, S2238 and S2302 being of equivalent activity, while S2444 was less easily converted and S2366, the least active.

The effect of inhibitors on the procoagulant conversion of chromogenic substrate S2222

The procoagulant activity has been shown to be inhibited by benzamidine previously, but in this study the serine protease inhibitors DFP and PMSF were also included and the inhibition produced by β-mercaptoethanol, and the removal of complex oligosaccharides was also investigated. The results of the inhibition studies are shown in table 4.1. Strong inhibition was found with dansyl-GGACK, benzamidine and β-mercaptoethanol. Aprotinin and DFP produced partial inhibition as did leupeptin. However, the venom showed considerable variability in sensitivity to leupeptin with each testing. The remaining agents had essentially no effect.

Table 4.1 Percentage inhibition by various reagents of the procoagulant-induced conversion of chromogenic substrate S2222.

Inhibitor	**% Inhibition**
Aprotinin (1000U/ml)	35 ± 5
Benzamidine (10mM)	76 ± 7
BAEE (10mM)	8 ± 13
dansyl-GGACK (40µM)	100
Endoglycosidase D (100mU/ml)	0
Hydroxylamine (10mM)	10
DFP (25mM)	63 ± 14
Leupeptin (20µg/ml)	40 ± 15
β-mercaptoethanol	93 ± 9
PMSF (10mM)	0
TAME (10mM)	0

The % inhibition was calculated from the reduction in OD from the saline control. (N = 4)

Glycan detection

Carbohydrate was detected in the majority of the bands from the procoagulant when run onto SDS-PAGE. The band normally noted at 64kDa with Commassie staining is extremely pale

but readily visualised by the carbohydrate stain. The low molecular weight group are heavily stained, while the very high moleculer weight entities have not stained (Fig 4.5).

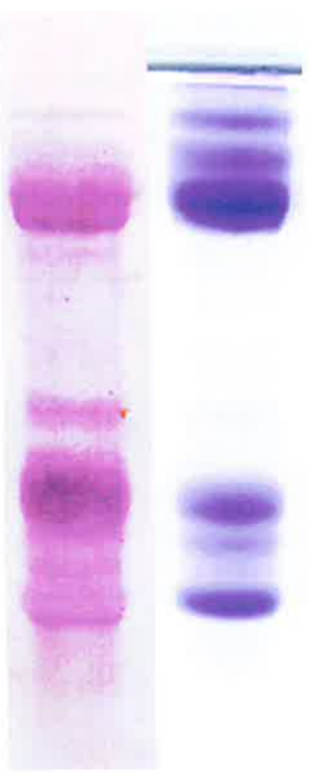

Fig 4.5 10% SDS-PAGE. Presence of carbohydrate in isolated procoagulant (6.0μg load).
Left lane: Glycan detection of isolated procoagulant.
Right lane: Coomassie Blue staining of isolated procoagulant.
The glycan detection is extremely sensitive and the pale bands found with Coomassie staining are heavily stained. Carbohydrate is present in the majority of the bands.

Immunoprecipitation

The simple immunodiffusion produced a precipitin line between the procoagulant and brown snake antivenom. The taipan antivenom produced a precipitin line against the procoagulant, which showed identity to that produced with brown snake antivenom but also produced a less distinct second precipitin line. No line was evident with tiger snake antivenom (Fig 4.6).

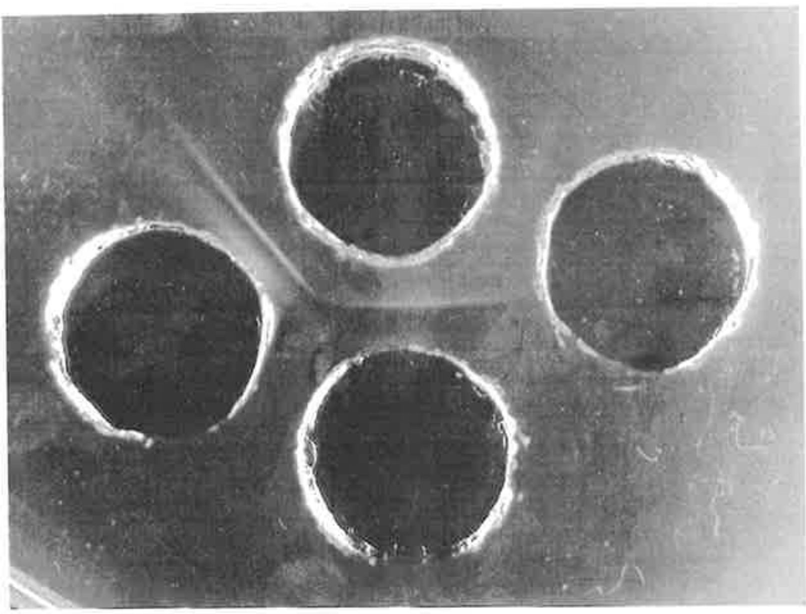

Fig 4.6 Immunoprecipitin lines developed between isolated procoagulant (*P. textilis*): Central upper well and left to right: taipan, brown snake and tiger snake antivenom (CSL).

DISCUSSION

The procoagulant contained a number of disulphide linkages, both in the high molecular weight group and in the low molecular weight group. There was no evidence of the aggregated material which failed to enter the gel in the Masci et al (1988) study and nor were there less bands after reduction, in fact in this study the number of bands had increased. The molecular weight of the high group dropped from ~245-180kDa down to ~100kDa, while the low molecular weight group dropped from 52 kDa down to a maximum of 43 kDa, with a number of bands much lower than this. In the reduced state the lowest molecular weight obtained in this study was 16kDa, while a band at 65 kDa was the lowest recognised by Masci et al (1988). The loss of chromogenic activity after reduction with β-mercaptoethanol indicated the disruption of the active site. It is uncertain if this disrupted a necessary sequence for activity or whether the protein conformation was disrupted, thereby removing a receptor site. However if the active site in the procoagulant was equated with factor X, the inhibition of the activity was expected. Factor X is comprised of two chains held together by disulphide bonds and for activity the bonds must be intact (Di Scipio, 1977).

Active-site labelling of the bands on native PAGE showed the fast-moving band contained the active site, as this was stained by dansyl-GGACK, which attaches to the active histidine residue in the catalytic triad of a serine protease (Kettner and Shaw, 1981a,b). Thus the active site(s) of the procoagulant were distributed between both the slow and fast moving bands. The other observation regarding this staining of the native gel was the disproportionate stain taken up by the fast moving band, which had considerably less protein present as determined by the intensity of Coomassie staining. This suggested that perhaps two or more proteins present had serine protease activity. Alternatively this may have reflected the fact that the active site containing protein was found in the fast moving band but due to an unknown force holding the subunits together not all of the active site was able to be separated from the slow moving band.

In the presence of SDS the situation became somewhat clearer. The SDS was obviously able to overcome whatever force held the procoagulant subunits together and separated the individual constituent subunit bands. The active site was now confined to two distinct bands corresponding to the Coomassie-stained bands with molecular weights of 52 kDA and 48 kDa. There was no active-site labelling of the high molecular weight group and this was consistent with the monoclonal antibody (see Fig 3.4) only being associated with the low molecular weight group. As the high molecular weight subunits had no activity associated with them they may perform one of two tasks. Firstly, it is possible that they provide a cofactor portion for the procoagulant, consistent with a prothrombinase complex. The other possibility is that they provide stability for the procoagulant acting either as a carrier molecule or as a protective device in the venom gland.

The reduction of the procoagulant by β-mercaptoethanol resulted in the active-site label being now found in a single band, at a molecular weight of 34 kDa, approximately 14 - 18 kDa lighter than either band before reduction. Thus the molecules containing the active site must contain at least two (or more) chains connected by a disulphide linkage, with the chain

containing the active site being common to both of these bands. This is unlike the situation with the taipan (Speijer et al, 1986) where a single band only is stained with the active-site label prior to reduction, although the molecular weight of the chain containing the active-site after reduction at ~30 kDa is close to that of the common brown at ~34 kDa. (The single active site in the taipan was confirmed, see Fig 6.9).

In converting prothrombin to thrombin or meizothrombin the cleavage site of the venom is Arg_{322}-Ile_{323}. Therefore any chromogenic substrates that present arginine as a cleavage site should release para-nitroaniline in the presence of the procoagulant. This was found to be the case with the substrates S2222, S2238, S2302 and S2444 releasing p-nitroaniline in the presence of the procoagulant, while the substrate S2251, with a terminal lysine, failed to be converted at either 3 or 30mM. (Chromogenic substrate sequences Fig 4.7). The second amino acid in the sequence from arginine appeared to have little influence on the rate that the chromogenic substrate was converted.

Table 4.7 Chromogenic substrate sequences

Substrate	Sequence
S-2302	H-D-Pro-Phe-Arg-NH-⬡-NO_2 .2HCl
S-2238	H-D-Phe-Pip-Arg-NH-⬡-NO_2 .2HCl
S-2251	H-D-Val-Leu-Lys-NH-⬡-NO_2 .2HCl
S-2222	⬡-CO-Ile-Glu-(γ-OR)-Gly-Arg-NH-⬡-NO_2.HCl
S-2444	<Glu-Gly-Arg-NH-⬡-NO_2.HCl

The activity of the procoagulant was consistent with a serine protease. Dansyl-GGACK was able to both label the active site and also completely abolish the activity of the procoagulant. However a number of serine protease inhibitors failed to have an influence on the activity, but there was no evidence to suggest the venom activity was due to anything other than a serine

protease, a metalloprotease eliminated by the lack of inhibition by EDTA. DFP produced 58% inhibition while PMSF was unable to inhibit the activity. This most likely represents an effect of the polarity of these inhibitors on the ease with which they can react with the active site. Benzamidine was most effective in inhibiting the activity.

The glycan detection showed that all the proteins that constitute the whole procoagulant contain carbohydrate. Removal of the carbohydrate failed to inhibit the procoagulant activity, and therefore instead of contributing to the activity it may reflect a protective mechanism for the venom proteins, in view of the highly proteolytic environment of the venom gland.

The results of the immunoprecipitation were in keeping with the notion that the procoagulant from the brown snakes and the taipan are in fact similar. The significance of a second precipitin line found with the taipan venom was uncertain but there was no evidence of any action of the tiger antivenom against the brown procoagulant. The active-site labelling indicated that after reduction with β-mercaptoethanol a single band was obtained from the brown snake at a molecular weight close to that of the band found in the taipan. The similarity was also noted in the fact that there was a high and low molecular weight group found in both venom 'whole' procoagulants.

The hypothesis that the procoagulant is made up of factor Xa and factor V-like subunits was reinforced in this chapter. Two active-site containing subunits were found after treatment with SDS, in keeping with the speculation from the immunoblot results in the previous chapter, and these were serine proteases, as predicted for a factor Xa-like subunit. The assumption from this was that the high molecular weight subunits and those not associated with the active-site in the low molecular weight range were involved in the clotting process as cofactors. The physical characterisation and determination of the activities of the isolated subunits should help clarify their contribution to the procoagulant complex.

REFERENCES

Di Scipio, R.G., Hermodson, M.A., Yates, S.C., Davie, E.W. (1977). A comparison of human prothrombin, factor IX (Christmas factor), Factor X (Stuart factor) and protein S. Biochem. **16**, 698-706.

Kettner, C., Shaw, E. (1981a). Inactivation of trypsin-like enzymes with peptides of arginine chloromethyl ketone. Meth. Enzym. **80**, 826-842.

Kettner, C., Shaw, E. (1981b). The selective affinity labeling of factor Xa by peptides of arginine chloromethyl ketone. Thromb. Res. **22**, 645-652.

Martin, C.J. (1894). On some effects upon the blood produced by the injection of the venom of the Australian black snake (*Pseudechis porphyriacus*). J.Physiol. **15**, 380-400.

Masci, P.P., Whitaker, A.N., deJersey, J. (1987). Purification and characterization of the prothrombin activator of the venom of *Pseudonaja textilis*. In: Progress in venom and toxin research. pp. 209-219. Eds. Gopalakrishnakone, P. , Tan, C.K. Proceedings of the first Asia-Pacific Congress of Animal, Plant and Microbial Toxins.

Masci, P.P., Whitaker, A.N., deJersey, J. (1988). Purification and characterization of a prothrombin activator from the venom of *Pseudonaja textilis textilis*. Biochem. Int. **17**, 825-835.

Speijer, H., Govers-Riemslag, J.W.P., Zwaal, R.F.A., Rosing, J. (1986). Prothrombin activation by an activator from the venom of *Oxyuranus scutellatus* (Taipan snake). J. Biol. Chem. **261**,13258-13267.

CHAPTER 5

CHARACTERISATION OF THE PROCOAGULANT PT 2 - EXAMINATION OF THE PROCOAGULANT SUBUNITS

INTRODUCTION

In the previous chapters the whole venom as a procoagulant and its characteristics were explored and those of the isolated procoagulant were examined. It was noted that the isolated procoagulant consisted of a number of bands in the high molecular weight region (>100kDa) and a number of low molecular weight bands (<50kDa and below). This was in keeping with the findings of Masci et al (1988) with brown snakes and is similar to that reported for the taipan (Speijer et al, 1986). Active-site labelling confined the proteolytic activity to the low molecular weight proteins of the procoagulant, and the procoagulant was capable of converting both heparinsed plasma and descarboxy prothrombin .

Triplett et al (1993), reported the isolation of *Textarin* from the venom of brown snakes, which was capable of activating prothrombin and required phospholipid to optimise its activity. *Textarin* could be separated from whole venom by barium citrate absorption, leaving a phospholipid independent activator in the supernatant. The *Textarin* isolated in this manner produced a major band at 52kDa, with two minor bands at high molecular weight (> 100kDa). After reduction a major band at 32kDa was isolated with a number of minor bands, ranging from 19 - 99 kDa, also present.

Masci et al (1988) and this present study contend that the procoagulant action of the venom from *P. textilis* is capable of activating both normal and descarboxyprothrombin, can clot heparinised plasmas and is independent of phospholipid, calcium and factor V. *Textarin* on the other hand is capable of converting descarboxyprothrombin as evidenced by the use of warfarinised plasmas, however *Textarin* appears severely limited in the presence of heparin, is dependent on the presence of phospholipid, calcium and factor V. In the study, inhibitors to factor V present in plasma blocked the clotting activity of the *Textarin* while factor V deficient plasma was also not easily clotted by *Textarin,* indicative of a requirement for factor V to be present for *Textarin* to bring about clotting.

Stocker et al (1994) extended and refined the work on the venom. Hydrophobic interaction chromatography of the crude venom produced a semi-purified activator, and examination of phospholipid and calcium requirements suggested the presence of two different prothrombin activators, namely a Ca^{2+}- phospholipid - dependent prothrombin activator (PLDPA - *Textarin*) and a Ca^{2+}- phospholipid - independent activator (PLIPA). The PLIPA was capable of converting EDTA specimens, indicating the activity was not that of a metallo-protease. *Textarin* (PLDPA) was shown to have the characteristics of a serine protease from inhibitor studies. The action of serine protease inhibitors on the procoagulant in suppressing all coagulation activity, suggests that both the phospholipid dependent and independent activators (PLDPA/PLIPA) are serine proteases.

Textarin was isolated from the whole venom by using a hydroxyl apatite column SDS gel electrophoresis of the *Textarin* produced two bands at 53kDa and 50kDa, in the native state and a single band at 33kDa in the reduced state.

The ability of *Textarin* to be absorbed by barium citrate and to attach to a hydroxyl apatite column is suggestive of a calcium binding molecule, most likely due to the presence of γ - carboxyglutamic acid residues which would be consistent with the findings of Tans et al (1985) for tiger and taipan prothrombin activators. The co-factor requirements of *Textarin*

indicated that the longest clotting time obtained for the barium-absorption prepared product was in the absence of calcium, (essentially producing no clotting) reinforcing the likelihood of a calcium binding site being present on the molecule and the calcium dependence of *Textarin*. This result appeared to contradict the fact that *Textarin* was capable of converting EDTA plasmas, where little or no calcium is available (PLIPA is also capable of converting EDTA plasma).

The requirement for factor V is also debatable. Where Triplett et al (1993) reported that factor V was necessary for *Textarin* to have full activity, Stocker et al (1994) suggested that factor V had little stimulatory effect. It was concluded that the high molecular weight components noted in the procoagulant (see Fig 3.4, 4.3a) and removed through barium citrate absorption of whole venom (Triplett et al, 1993) showed no factor V-like activity and Stocker et al (1994) commented on the fact that *Textarin* neither contains nor depends on a factor V-like venom component.

The postulated presence of two separate activators in the venom was suggested by Stocker et al (1994) to be the explanation for contradictory statements on properties and actions of the prothrombin activator. They also stated that SDS-PAGE of *Textarin* did not show any evidence of the sub-unit structure considered characteristic of the *Oxyuranus* and *Pseudonaja* species (Masci et al 1988; Speijer et al, 1986). The possibility of a sub-unit structure is dismissed as most likely reflecting a high affinity of *Textarin* for other venom components, resulting in a stable complex formation.

The isolation of the procoagulant by liquid chromatography, produced a single peak of coagulant activity across the fractions (Fig 3.2) and native electrophoresis of the procoagulant produced a single, heavy diffuse band and on occasion a shadowy second band. SDS-PAGE of the procoagulant however produced a number of bands (Fig 3.4) consistent with a sub-unit - like composition. This however does not eliminate the suggestion of Stocker at al (1994) that an affinity between the venom components and *Textarin* is responsible for the number of

bands seen or the possibility that PLIPA and PLDPA (as defined by Triplett et al, 1993) closely associate and therefore co-elute, giving the impression of a sub-unit composition.

Initial characterisation of the procoagulant through active site labelling of the procoagulant showed two bands only on SDS-PAGE, at 52kDa and 48 kDa and in the reduced state a single band was noted at 34kDa (Figs 4.3a, 4.4). These figures were consistent with the fraction of the venom referred to as *Textarin*. Thus the possibility of two distinct serine protease prothrombin activators being present in the venom would appear unlikely, but the situation was still supportive of the concept of a factor Xa - like active fraction, acting in concert with the remainder of the procoagulant. This is in agreement with the Speijer et al (1986) original model of prothrombin activation by the taipan, with a factor X-like and factor V-like fraction acting in synergy to produce rapid clotting. The discrepancy between the existence of a single active group and two activators as postulated by Triplett et al may be due to contamination of PLIPA by the factor X-like component, resulting in what may appear to be a second activator with serine protease characteristics.

In this chapter the physical characterisations and activity studies were performed on the isolated subunits. The contribution that these subunits could make to a 'prothrombinase complex' was considered and reconciled with current knowledge.

MATERIALS AND METHODS

Procoagulant:

Isolated as previously described (Chap 3).

Isolation of individual bands:

Procoagulant was loaded onto a slab gel (120 x 80 x 2 mm). Approximately 1-1.5 ml (360-500μg of protein) of procoagulant was loaded per run. The bands were identified by Coomassie blue R250 staining and each was excised and electroeluted using a modification of the small-volume concentrator of Rhodes and Yphantis (1981). The eluted bands were maintained stained in electrophoresis buffer, (0.25M Tris, 0.192M glycine, 0.1% SDS) and in this form the proteins were stable without further addition for up to three months. In most cases re-electrophoresis of the bands produced a single distinct band. In this form the bands were amenable to further analysis.

The electroeluted bands were treated with β mercaptoethanol and then re-electrophoresed. The bands were diluted 1:2 in reducing buffer (475μl sample buffer + 25μl β-mercaptoethanol) and boiled for 2 min prior to application to the gel.

Isolation of Textarin from procoagulant:

The molecular weights reported by Triplett et al (1993) and Stocker et al (1994) for *Textarin* were consistent with those obtained in this study for the active site containing subunits. Thus it was possible to isolate *Textarin* from the procoagulant (when all subunits are present) by harvesting the early elution from a native PAGE tube gel. This protocol was developed from the observation that on native gels the fast moving band contained the active site from dansyl-GGACK labelling and showed considerable separation from the slow moving band allowing for easy harvesting and when re-electrophoresed this produced the two bands of interest free of contaminants. The proteins eluted were collected into dialysis tubing, containing electrophoresis buffer. The eluted *Textarin* was maintained in the buffer for a maximum of two days after which the proteins showed the 'stickiness' reported by Triplett et al (1993). The latter eluted bands still had *Textarin* associated with them and showed the high and low molecular weight split (HMWG, LMWG) previously noted with SDS-PAGE of the

procoagulant. Thus although electrophoresis was able to separate the *Textarin* bands from the other subunits this was incomplete. The forces holding the subunits together made isolation of individual subunits in a manner which retained activity extremely difficult. The *Textarin*-depleted fraction was re-electrophoresed to reduce further the *Textarin* content although the complete removal of *Textarin* was not possible at this stage.

Carbohydrate analysis of subunits:

The high and low molecular weight bands were treated with endoglycosidase F (Boehringer Mannheim, N-Glycosidase F, Deglycosylation Kit) to determine O or N linkage of the carbohydrate.

End terminal analysis of carbohydrates:

The subunits identified at 43, 48 and 52kDa on SDS-PAGE were used in most further analysis only and the high molecular weight bands were not further characterised. The subunits 43, 48 and 52kDa were re-electrophoresed and then transferred to nitrocellulose using a semi dry process (Milliblot system). Three separate runs were performed and the DIG Glycan Differentiation Kit (Boehringer Mannheim) employed to identify the terminal carbohydrates of each of these species. The lectins SNA, DSA, GNA and MAA were employed , which recognise the following terminal carbohydrates-:

- SNA (*Sambucus nigra* agglutinin)
    - sialic acid linked α(2-6) to galactose
- DSA (*Datura stramonium* agglutinin)
    - Galβ-(1-4)GlcNAc in complex and hybrid N-glycans
- GNA (*Galanthus nivalis* agglutinin)
    - mannose α(1-3), α(1-6) or α(1-2) linked to mannose ('high mannose' structures)
- MAA (*Maakia amurensis* agglutinin)
    - sialic acid linked α(2-3) to galactose

Plasma substrate requirements for Textarin:

The clot promoting activity of this fraction (20µg/ml) of the procoagulant was tested against a variety of plasma substrates. Plasma where prepared, was a pool of at least 20 healthy adult donors. Citrate plasma: 9 parts blood to 1 part 3.8% w/v tri-sodium citrate. Heparin plasma: To the citrate plasma, heparin (David Bull Laboratories, Vic. Australia) was added to a concentration of 1 U/ml. EDTA plasma was prepared from blood collected into commercially prepared EDTA containers (Disposable Products, Sth. Australia). Warfarin plasma: Plasmas were prepared as pools of plasma from patients attending the anticoagulant clinic at the Institute of Medical and Veterinary Science, Adelaide. The pooled plasma was required to produce an INR of at least 2.8 - 3.5. Factor II, V and X deficient plasmas were commercial preparations from Dade Ltd. Calcium chloride when added was 0.025M and phospholipid was provided by the Bell and Alton platelet substitute, Diagen, Thames Pty Ltd.

Clotting tests: The clotting tests were performed in the following manner - 50µl of the venom component was added to 200µl of the appropriate plasma, in a 10 x 75mm glass tube at 37^0C and using a tilt-tube technique, the time for the appearance of a clot was recorded. Where the addition of calcium or phospholipid was also being investigated 50µl of the venom component was added to a tube containing 200µl of the appropriate plasma, plus 50µl of each additive or saline in the control tubes.

N-terminal amino acid sequencing of the 43, 48 and 52kDa subunits:

Amino acid sequencing was performed by the Australian Proteome Analysis Facility, Macquarie University, NSW, Australia. Twelve residues were identified for each of the three bands (43,48 and 52kDa) initially, to provide a basis for a non-specific homology search using the FASTA program search engine. When homology was found with human clotting factors, the 43kDa subunit was sequenced to 22 residues and the 52kDa subunit was extended to 32, to determine the extent of the homology with these extra residues.

RESULTS

Isolation of individual subunits (bands)

The further characterisation of the subunits required a means of separating these from one another. Electroelution was used, however the resultant fractions were not active due the presence of SDS in the elution buffer. SDS-PAGE of the electroeluted bands showed the resultant single bands (Fig 5.1). These showed the range of molecular weights 43kDa in the low molecular weight range through to 158kDa in the high molecular weight range. *Textarin* was isolated as bands at 48 and 52kDa..

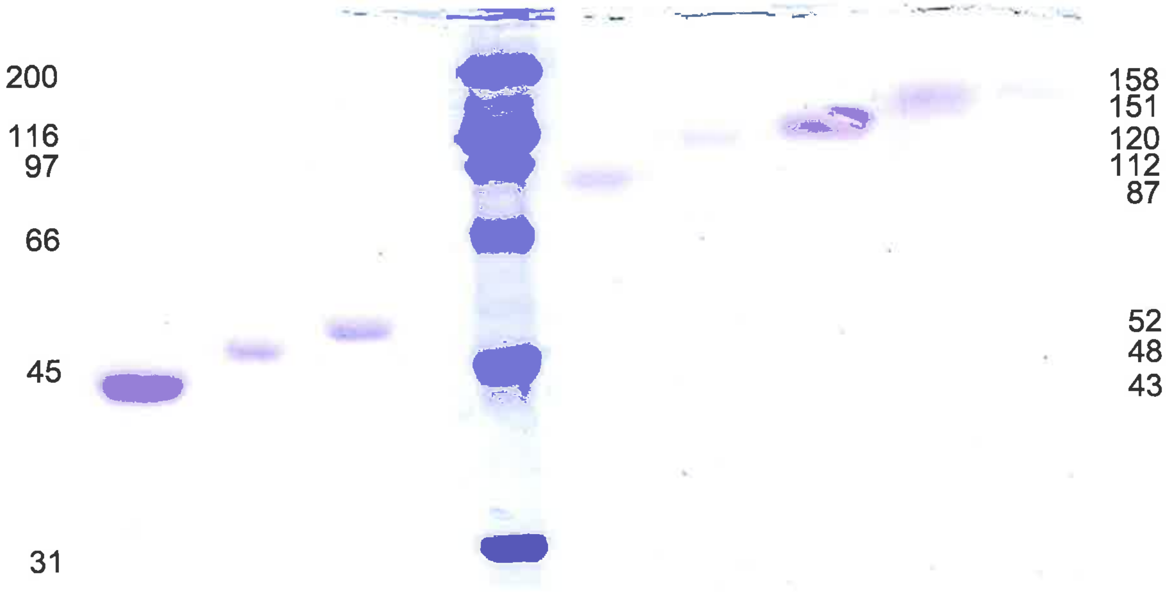

Fig 5.1 SDS-PAGE (10%) showing banding obtained from individually isolated 'subunits' of the procoagulant. The maximum molecular weight obtained through electro-elution of the individual 'subunits' is 158kDa, suggesting higher molecular weight bands obtained (Fig 3.4) may represent multimers of the 'subunits'.

The β-mercaptoethanol reduction of the bands appeared to have no effect on the 43kDa band. The 52 and 48kDa species produced a band at 34kDa in common and a band at 28 and 24kDa respectively (Fig 5.2). The 34kDa band was close to that reported by Stocker et al (1994) for reduced *Textarin*. The high molecular weight bands also showed an interesting

result in that the bands present in the native state, now produced only four major bands when reduced, suggesting that perhaps a number of multimers are present in the high molecular weight group.

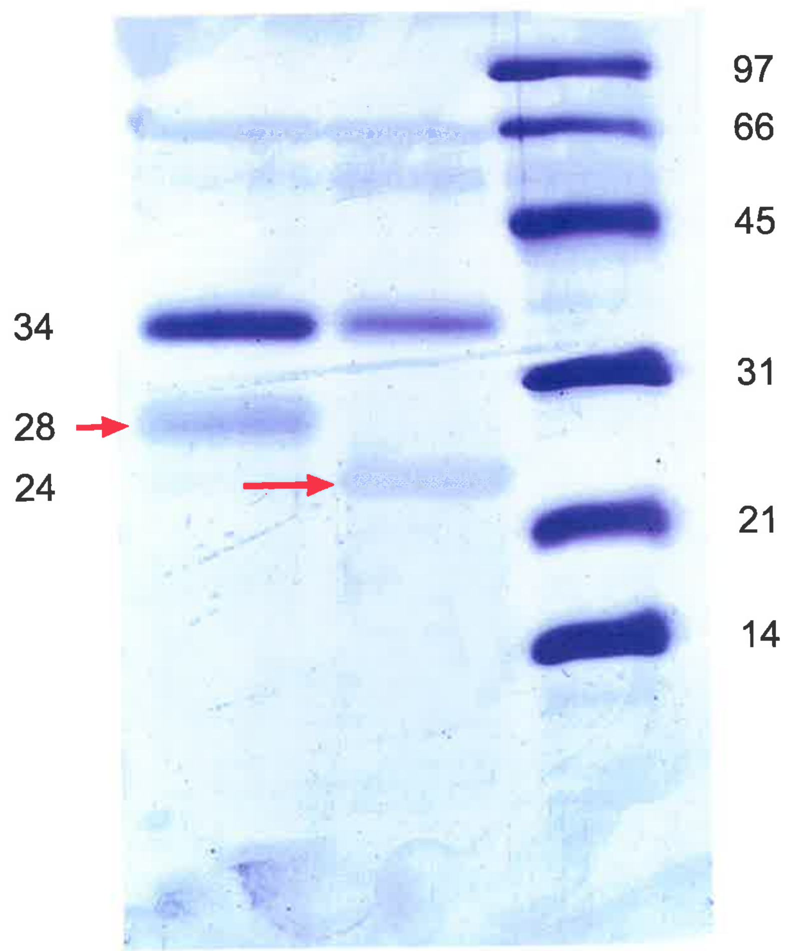

Fig 5.2 SDS-PAGE (15%) of β-mercaptoethanol reduced 52 and 48kDa bands (Textarin®).
Lane 1: 52kDa band and Lane2: 48kDa band reduced with β-mercaptoethanol.
Lane 3: Molecular weight markers.
(52 and 48kDa bands are the bands defined as Textarin®, Triplett et al, 1993).

Due to the uncertainty surrounding the basic unit of the HMWG (and the possibility of multimers confusing the situation) the majority of the further characterisation concentrated on the 43kDa species and *Textarin* because these were a size that was manageable and were consistently isolated on SDS-PAGE.

Textarin isolation

Isolation of *Textarin* without contamination from other subunits and in a solution without SDS allowed for the activity against a number of substrates and the requirement for cofactors to be determined.

Textarin was separated from the 'whole' procoagulant by running the bands off the bottom of a native gel, as described (Methods). When this technique was employed, the majority of the Textarin was separated from the whole procoagulant, however some of the *Textarin* still associated with the high molecular weight bands and this was seen when the elution was continued for a longer period (Fig 5.3). All bands associated with the 'whole' procoagulant were then visualised on SDS-PAGE in this prolonged elution. This meant that although a solution containing *Textarin* alone could be obtained, a solution not contaminated with *Textarin* was not possible at this stage. Thus because of this contamination the activity of the subunits devoid of *Textarin* could not be determined and only an approximation of the action was possible.

Carbohydrate analysis

The carbohydrate associated with the procoagulant was not involved in the activity, however carbohydrate analysis provided further physical information about the subunits and allowed a comparison between *Textarin* and the other components.

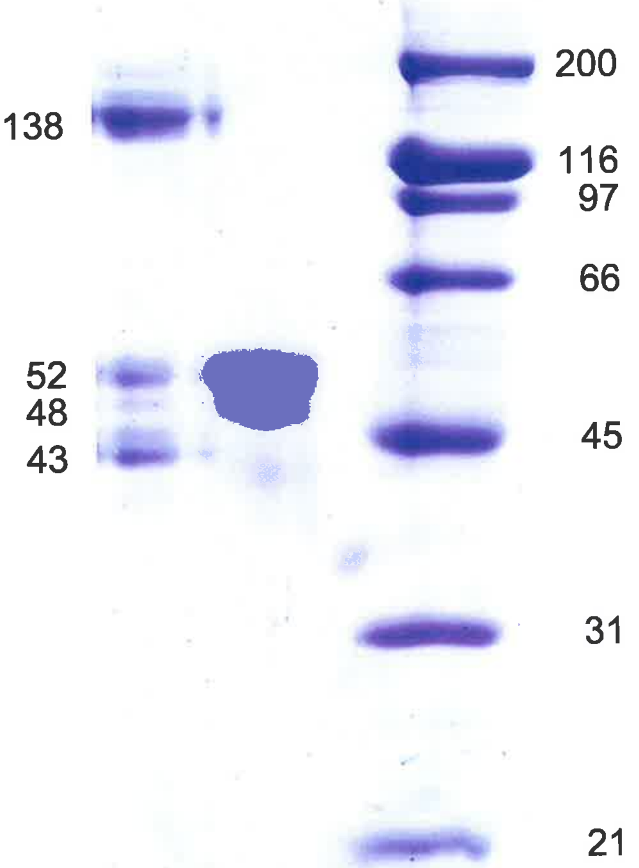

Fig 5.3 Isolation of the 52 and 48kDa bands (Textarin®) by electroelution of the isolated procoagulant from a native tube gel (as per methods).
Lane 1: Bands obtained from prolonged electroelution of the isolated procoagulant.
Lane 2: Harvested 52 and 48kDa bands (Textarin®) by electoelution.
Lane 3: Molecular weight markers.

The SDS-PAGE banding showed a molecular weight shift after treatment with endoglycosidase F, both for the high and low molecular entities. The *Textarin* bands when treated by endoglycosidase dropped back to a single band at 46kDa (Fig 5.4). This indicated that the difference between the *Textarin* bands at 52 and 48kDa was a post translational change related to carbohydrate content.

Terminal carbohydrate analysis showed that none of the three bands (43, 48 or 52kDa) reacted with SNA indicating that sialic acid linked α(2-6) to galactose was not present. *Textarin* (52 and 48kDa subunits) and the 43kDa subunit showed a combination of Galβ-(1-

4)GlcNAc and sialic acids linked α(2-3) to galactose, while the 43kDa subunit showed a reaction with GNA and thus the presence of 'high mannose' glycan chains.

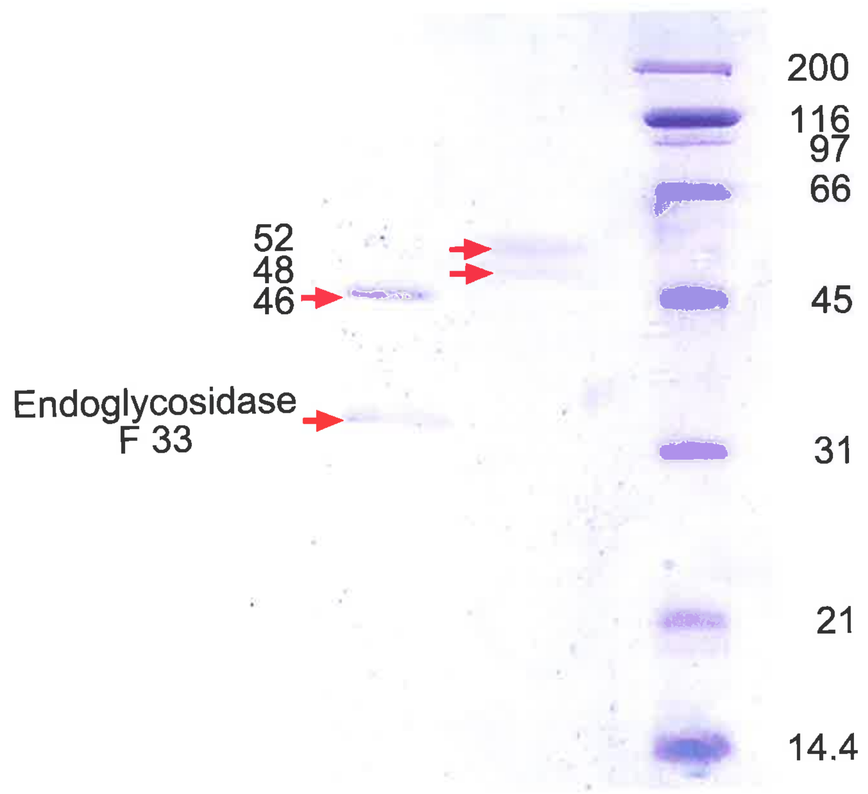

Fig 5.4 Endoglycosidase-F treatment of the 52 and 48kDa bands (Textarin®).
Lane 1: Deglycosylated Textarin®. (Endoglycosidase-F band at ~33kDa).
Lane 2: 52 and 48kDa bands in native state.
Lane 3: Molecular weight markers.

Textarin substrate requirements for coagulant activity

Due to the fact that the 52 and 48kDa (*Textarin*) bands contained the active site they were equated with factor Xa. To determine if the activity of these bands was consistent with factor Xa various plasma substrates and factor X and V deficient plasmas were employed in clotting tests. *Textarin* was able to clot citrated plasma (Table 5.1) and heparinised plasma (Table 5.2), but failed to clot EDTA (Table 5.3) or warfarin plasma within the 300 sec exclusion

limit placed on the test system (Table 5.4). The addition of phospholipid failed to change the clotting times in citrate, EDTA or warfarin but markedly reduced the clotting time of the heparin plasma. The addition of calcium shortened the clotting times of all substrates but was less effective in the case of heparin. The presence of both phospholipid and calcium produced a dramatic reduction in the clotting time of the citrate , EDTA, and warfarin plasmas, while there was less of an effect of the calcium in the case of the heparin plasma.

Factor X deficient (Table 5.5) and factor V deficient (Table 5.6) plasmas were both clotted by *Textarin* and the addition of phospholipid had essentially no effect on the clotting times. The difference noted between the deficient plasmas was in the clotting times obtained in the presence of calcium alone or in combination with phospholipid. Factor X deficient plasma showed a reduction in the clotting time with calcium and the combination of calcium and phospholipid, while the factor V deficient plasma showed essentially no improvement in the clotting times in the presence of calcium and/or phospholipid.

Table 5.1. The effect of phospholipid and calcium on the ability of the 52 and 48kDa bands to clot citrate plasma.

Plasma addition	Clotting time (sec)
Saline	137 ± 4.5
Calcium	68 ± 2.7
Phospholipid	119 ± 2.0
Calcium and phospholipid	20 ± 0.9

Controls: Plasma plus saline or phospholipid - no clot detected.
Plasma plus calcium or calcium and phospholipid - 269 and 137 sec respectively.
(N = 6)

Table 5.2. The effect of phospholipid and calcium on the ability of the 52 and 48kDa bands to clot heparin plasma.

Plasma addition	Clotting time (sec)
Saline	120 ± 1.6
Calcium	94 ± 1.5
Phospholipid	18 ± 0.58
Calcium and phospholipid	12 ± 0.37

Controls: Plasma plus saline, phospholipid, calcium or phospholipid and calcium - no clot detected.
(N = 6)

Table 5.3. The effect of phospholipid and calcium on the ability of the 52 and 48kDa bands to clot EDTA plasma.

Plasma addition	Clotting time (sec)
Saline	>300
Calcium	76 ± 2.0
Phospholipid	>300
Calcium and phospholipid	54 ± 1.0

Controls: Plasma plus saline or phospholipid - no clot detected.
Plasma plus calcium or calcium and phospholipid - 268 and 131 sec respectively.
(N = 6)

Table 5.4. The effect of phospholipid and calcium on the ability of the 52 and 48kDa bands to clot warfarin plasma.

Plasma addition	Clotting time (sec)
Saline	>300
Calcium	158 ± 1.3
Phospholipid	>300
Calcium and phospholipid	80 ± 2.4

Controls: Plasma plus saline, phospholipid, calcium or phospholipid and calcium - no clot detected.
(N = 6)

Table 5.5. The effect of phospholipid and calcium on the ability of the 52 and 48kDa bands to clot factor X deficient plasma.

Plasma addition	Clotting time (sec)
Saline	127 ± 1.4
Calcium	54 ± 1.2
Phospholipid	128 ± 1.4
Calcium and phospholipid	14 ± 0.4

Controls: Plasma plus saline or phospholipid - no clot detected.
Plasma plus calcium or calcium and phospholipid - >300 sec
(N = 6)

Table 5.6 The effect of phospholipid and calcium on the ability of the 52 and 48kDa bands to clot factor V deficient plasma.

Plasma addition	Clotting time (sec)
Saline	140 ± 2.5
Calcium	105 ± 1.2
Phospholipid	128 ± 0.9
Calcium and phospholipid	90 ± 2.4

Controls: Plasma plus saline or phospholipid - no clot detected.
Plasma plus calcium or calcium and phospholipid - >300sec.
(N = 6)

Factor II deficient plasma failed to clot in under 300 sec even in the presence of both calcium and phospholipid.

N-terminal amino acid analysis

The amino acid sequence obtained for the *Textarin* bands (52 and 48kDa) showed identity, in keeping with what had been expected from the carbohydrate analysis, where removal of the carbohydrate aligned these bands on SDS-PAGE (Fig 5.4). Sequence searches revealed a 50% or better homology with at least 10 proteins, 8 of which are represented in Table 5.7. Five of these showed a 54% homologywhile 3 produced 63% homology. The most important sequence homology was with coagulation factor X precursor and factor X.

Amino acid sequencing of the 43kDa band produced homology with at leaast 12 proteins represented in Table 5.8. Homology of 54% was found against 7 of these proteins, 63% with 4 and 1 showed 72% homology. The important homology, and of interest to this study, was with coagulation factor V.

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Table 5.7 Amino acid sequencing of the 52 and 48kDa bands. 12 residue - N-terminal analysis - most likely sequence represented, non-specific homology search.

Matching proteins	Sequence											
52 & 48kDa protein	_	**V**	**N**	**G**	**M**	**D**	**C**	**K**	**K**	**G**	**E**	**N**
Class I acidic chitinase	I	I	**N**	**G**	I	E	**C**	G	**K**	**G**	Y	**N**
Tolkin	H	E	**N**	**G**	H	**D**	**C**	**K**	E	**G**	**E**	C
Tolloid related	H	E	**N**	**G**	H	**D**	**C**	**K**	E	**G**	**E**	C
3'5'-cyclic phosphodiesterase	D	L	**N**	**G**	L	**D**	**C**	**K**	**K**	N	A	Y
DNA Topoisomerase II	I	I	**N**	**G**	V	N	**C**	V	**K**	**G**	**E**	H
EGF-like repeats	L	**V**	**N**	**G**	S	**D**	**C**	**K**	N	**G**	G	K
Factor X precursor	I	**V**	G	**G**	Q	**D**	**C**	R	D	**G**	**E**	C
Factor X	I	**V**	G	**G**	Q	E	**C**	**K**	R	**G**	**E**	C

Having established homology with factor X and factor V, the sequencing was extended to 32 residues for the 52kDa subunit and to 22 residues for the 43kDa subunit. A comparison of the residue sequence from the 52kDa subunit and human, bovine, chick and rabbit factor X showed at least a 56% homology with human factor X and 50% with bovine, chick and rabbit respectively (Table 5.9). If the possible conservative amino acid substitutions were taken into account the homology was extended to 78% for the human, 73% for rabbit and to 69% for the bovine and chick.

Table 5.8 Amino acid sequencing of the 43kDa band. 12 residue - N-terminal analysis - most likely sequence represented, non-specific homology search.

Matching proteins	Sequence											
43kDa band	_	**E**	**K**	**D**	**L**	**T**	**F**	**K**	**K**	**I**	**V**	**Y**
Hemolysin (Cytolysin II)	N	L	**K**	**D**	**L**	**T**	**F**	**K**	**K**	V	D	S
RTX II Toxin Determinant	N	L	**K**	**D**	**L**	**T**	**F**	**K**	**K**	V	D	S
YQZC Protein	V	K	S	**D**	M	S	**F**	**K**	**K**	**I**	**V**	K
Propolyphenol oxidase prec	Q	**E**	E	I	**L**	**T**	**F**	**K**	**K**	**I**	A	**Y**
Beta-Adaptin	A	T	R	**D**	I	**T**	L	**K**	**K**	L	**V**	**Y**
Leukotoxin A	N	L	**K**	**D**	**L**	**T**	**F**	E	**K**	V	N	H
Cosmid	V	**E**	T	**D**	**L**	**T**	**F**	S	**K**	I	T	D
Transcription elongation factor	I	**E**	**K**	**D**	**L**	G	**F**	**K**	**K**	A	I	V
MGPA Protein	S	L	A	**D**	**L**	K	**F**	**K**	**K**	Y	**V**	**Y**
Factor V Bovine	K	P	F	E	T	S	**F**	**K**	**K**	**I**	**V**	**Y**
Factor V Human	N	L	S	V	T	S	**F**	**K**	**K**	**I**	**V**	**Y**
Chick Lamin 1	L	I	E	D	L	E	**F**	**R**	**K**	**N**	**V**	**Y**

The sequence homology with factor V was also further investigated for the 43kDa subunit with extra residues being identified. Initially twelve residues gave an approximate homology of 50%. A further 10 residues were identified to determine the extent of the homology. The results of the sequencing are shown in table 5.10.The first six residues were not distinct and suggested that there were a number of proteins present with differing N-terminal amino acids. The signal after residue six, however was much larger and therefore supported the likelihood of a number of similar proteins that had the same residues at position 7 onwards. Allowing

for a match with one of the amino acids identified at position 3 and conservative substitutions, the resultant homology across the 22 residues identified was 72% against the human sequence and 68% against the bovine. If the first 6 residues were ignored then the homology of the sequence between residue 7 and 22 was as high as 87%.

Table 5.9 52kDa species sequence comparison with human, bovine, chick and rabbit factor X.

Residue			Human FX	Bovine FX	Chick FX	Rabbit FX
1	GAF	**I**	I	I	I	I
2	N	**V**	V	V	V	V
3	DN		G	G	G	G
4	L	**G**	G	G	G	G
5	MV		Q	R	D	Q
6		**D**	E	D	E	D
7	ED	**C?**	C	C	C	C
8		**K**	K	A	R	R
9	KL		D	E	P	D
10		**G**	G	G	G	G
11		**E**	E	E	E	E
12	N		C	C	C	C
13		**P**	P	P	P	P
14		**W**	W	W	W	W
15		**Q**	Q	Q	Q	Q
16		**A**	A	A	A	A
17	G		L	L	V	L
18	POGT	**L**	L	L	L	L
19		**V**	I	V	I	V
20	D		N	N	N	N
21	D		E	E	E	E
22		**K**	E	E	K	E
23	K		N	N	G	N
24	G		E	E	E	E
25	V		G	G	E	G
26	D	**F**	F	F	F	F
27	C?		C	C	C	C
28		**G**	G	G	G	G
29		**G**	G	G	G	G
30		**T**	T	T	T	T
31		**I**	I	I	I	I
32		**L**	L	L	L	L

Matching sequences are heavily shaded while those where conservative substitution is possible are lightly shaded.

Table 5.10 Sequence homology between factor V and the 43kDa subunit.

Residue	43kDa	Factor V Human	Factor V Bovine
1	-	N	K
2	E	L	P
3	**KNS**	S	F
4	**DGRL**	V	E
5	LMV	T	T
6	TDY	S	S
7	**F**	F	F
8	**K**	K	K
9	**K**	K	K
10	**I**	I	I
11	**V**	V	V
12	**Y**	Y	Y
13	**R**	R	R
14	**E**	E	E
15	**Y**	Y	Y
16	**E**	E	E
17	L	P	A
18	D	Y	Y
19	**F**	F	F
20	**K**	K	Q
21	**Q**	K	K
22	**E**	E	E

Matching sequences are heavily shaded while those where conservative substitution is possible are lightly shaded.

DISCUSSION

The individual bands from the procoagulant were separated by electroelution and this allowed further investigation of the biochemical characteristics to be performed. Thus the change in molecular weight of bands noted after reduction with β-mercaptoethanol when observing the whole procoagulant complex could now be equated to individual bands. Likewise the association of carbohydrate with particular bands can be determined and the linkage of the carbohydrate (O or N - linked) assessed. The isolation of the bands also allowed the terminal carbohydrates to be analysed and the likely structure of the moiety to be postulated. The

isolation of these bands however was problematic as the resultant isolates were invariably dramatically reduced in their activity or absent of activity. This inhibited the chance of relating each individual band with the activity of the entire complex.

The isolation of the individual bands by electroelution showed an interesting phenomenon in the high molecular weight region when the bands were re-electrophoresed. The extremely high molecular weight fractions (>180kDa-250kDa) disappeared and were replaced by a band with the highest molecular weight of ~160kDa This supported the theory that the high molecular weight bands seen at greater than 120kDa may in fact be multimers of the bands electroeluted with molecular weights of 60kDa and greater. This phenomenon was also encountered when the procoagulant was electroeluted from the native PAGE tube gel. On this occasion the extremely high molecular weight species were found to be missing with a similar maximum molecular weight of around 160kDa. This value was closer to that reported by Triplett et al (1993) for the high molecular weight contaminants initially found in their isolates of *Textarin*.

The β-mercaptoethanol reduction of the individual bands showed that the bands at 52 and 48kDa produced a common single band at 34kDa, as was predicted from the movement of the active-site labelled band (Fig 4.4), and the difference in molecular weight was due to a disparity in the light chains. The molecular weights of the light chains however when combined with the heavy chain gave a result that was greater than the unreduced glycoprotein. The reason for this was uncertain but most likely was the result of the associated carbohydrate influencing the electrophoretic properties of the isolated subunits. The band at 43kDa appeared to be unaffected by reduction with β-mercaptoethanol, indicating it was a single chain molecule.

The molecular weight shifts of the high molecular weight bands were also of considerable interest. Masci et al (1988) found that the reduction of the procoagulant complex resulted in a number of apparent aggregates that failed to enter a SDS-PAGE gel. This was not the case

when the complex was reduced, but the results obtained suggested that as opposed to aggregates formed due to the presence of the reduced fractions, the high molecular weight entities found on a native SDS-PAGE may be the result of combinations of the reduced peptides

The carbohydrate associated with all bands was N-linked (ie attached at asparagine residues). Removal of the carbohydrate from the 52 and 48kDa bands now aligned these bands at 46kDa, and thus indicated that the difference was not due to the amino acid sequence but was post translational in origin. Although this is not an uncommon finding in biologically active glycoproteins, the purpose in this situation is uncertain. The presence of the two active proteins may provide extra stimulus to the prothrombin conversion or may have resulted as a protective mechanism against other proteolytic enzymes in the venom gland.

The use of citrate, heparin, EDTA and warfarin plasmas showed the 52 and 48kDa bands (*Textarin*) were capable of converting prothrombin in citrate and heparin but were not able to clot EDTA or warfarin plasma. In citrate, the addition of calcium shortened the clotting time but phospholipid alone had no effect, while together the clotting time was substantially shortened. This suggested the activity was calcium dependent, and that when calcium was present phospholipid also further shortened the clotting time. The clotting times obtained with EDTA bore this out. In the absence of calcium no clotting occurred within the observation period, however when calcium was available in the system clotting did occur, confirming the dependence on the presence of calcium for activity. The phospholipid again had no effect, but when both calcium and phospholipid were added, the shortening found with citrate plasma was observed.

The result with heparin was also of interest with a clotting time obtained for the saline control and in the presence of calcium, although these times were reasonably long. The addition of phospholipid dramatically reduced the time and implied a dependence on phospholipid, however when both calcium and phospholipid were available the time was

only marginally better. The results reflected the fact that calcium was available in each of the tests as heparin will not bind calcium in the plasma. The more important point from the heparin plasma clotting was the fact that the activity was not inhibited by the substantial quantity of heparin present (1U/ml). The use of warfarin plasma as a substrate again illustrated the reliance on calcium for activity and the secondary nature of the phospholipid. It also illustrated the ability of *Textarin* to clot descarboxyprothrombin, although this was not as rapid as when normal prothrombin containing γ-carboxyglutamic acid residues was the substrate.

Thus the activity was dependent on calcium but not reliant on phospholipid, but when present, the clotting action was augmented. The clotting ability therefore mimicked to some extent the prothrombinase complex of factor Xa and factor Va, requiring the presence of calcium and using phospholipid to direct the action of the clotting activity to a surface. The clotting results from the deficient plasmas however indicated that the action was more akin to factor Xa alone, with the clotting times of factor X deficient plasma being essentially the same as normal citrate. This concept was reinforced when factor V deficient plasma was used as the substrate and there was no great improvement in the clotting time either in the presence of calcium or both calcium and phospholipid, as was seen with all other substrates. This implied the clotting times were rate limited by the absence of factor V rather than the availability of calcium or phospholipid. *Textarin* therefore had actions explained by the fact that it was a mimic of factor Xa, requiring calcium, stimulated by the presence of phospholipid and more active when factor V was also present. The action against heparin and warfarin however are not those expected of factor Xa, but these actions have been noted before in the prothrombin activator from black tiger snake, which has also been equated with factor Xa as a result of its clotting characteristics. This concept necessarily implied also that the venom may contain a factor V-like action as well or that *Textarin* relied on endogenous factor V in a bite victim's plasma to significantly amplify the clotting ability. The amplification ability of the remaining components of the procoagulant was seen when the plasma substrates were clotted by the procoagulant which had had most of the *Textarin,* (but

not all) removed. The clotting times obtained were not significantly longer than the procoagulant with all subunits intact and therefore appeared calcium and phospholipid independent. Thus the influence of the non-*Textarin* subunits was such that the procoagulant was no longer as dependent on calcium and was also capable of clotting descarboxyprothrombin in a more efficient manner. Speijer et al (1986) have previously speculated on the presence of a factor V-like protein in taipan venom. The N-terminal analysis of the 52 and 48kDa and the 43kDa entity, showed this speculation to have a sound basis in the composition of these three proteins.

The amino acid sequences of the *Textarin* bands (52 and 48kDa) were expected to be the same in view of the carbohydrate analysis which suggested the difference in molecular weight was due to carbohydrate, and this was found to be the case. The fascinating result was the homology found between these bands and factor X. This homology was the evidence sought to in part confirm the hypothesis that the procoagulant mimicked the prothrombinase complex, with the discovery of a factor X-like entity among the subunits of the procoagulant. Joseph et al (1999) have recently reported on the highly homologous structure of the procoagulant from the venom of *T. carinatus* (Trocarin) and factor Xa. This was the first description of homology between a Group II prothrombin activator and factor Xa. However this thesis provides the first report of homology between a subunit of a Group III prothrombin activator and factor Xa. The hypothesis of mimicry of the prothrombinase complex by the venom procoagulant thus also required evidence for a factor V-like entity and this was found in the sequence homology of the 43kDa band with factor V.

Irrespective of the sequence, a comparison of the 52 and 48kDa bands from the brown snake with human and bovine factor X showed similar physical characteristics. Human factor X has a molecular weight of 59,000 and when isolated from plasma is composed of a heavy chain (43kDa) and a light chain (16kDa) held together by a disulphide bond (DiScipio et al 1977a), while bovine factor X has a molecular weight of 58kDa and consists of a heavy chain (39.3kDa) and light chain (16.5kDa) linked by a disulphide bond (Jackson 1972; Fujikawa et

al 1972a). The brown snake factor X analogue had a molecular weight of 52kDa (or depending on the carbohydrate attached, 48kDa) and this consisted of a heavy chain of 30kDa and light chain(s) of 24 and 28kDa . Interestingly, both human and bovine factor X also exist in two forms as well, with similar biochemical and biological properties.

The sequence homology with factor X was exceptional as the reported homology between bovine and human factor X is 65% (Fung et al, 1985). The complete amino acid sequence of bovine factor X was reported by Titani et al (1975) and Enfield et al (1980) and the disulphide arrangement was described by Hojrup and Magnusson (1987). In the case of human factor X a number of direct sequences have been reported (Di Scipio et al 1977a) and deduced sequences from cDNA cloning studies (Leytus et al, 1986; Reddy et al, 1989). The sequence for factor X is shown in Fig 5.5.

Factor X is activated to Xa by the release of an activation peptide from the N-terminal end of the heavy chain at residue 52 by extrinsic or intrinsic activation complexes, or by the activator from Russell's viper venom, (Fujikawa et al 1972b, Kisiel et al 1976, Di Scipio et al 1977b). The point of activation of factor X to Xa also corresponded to the beginning of the homologous sequence between factor X and the 52kDa species from the procoagulant of the brown snake. It would thus appear that the venom contained an active form of factor X capable of prothrombin conversion to thrombin without prior processing. The cysteine at position 64 in the heavy chain which is involved in a disulphide linkage does not appear to have an equivalent in the sequence of the 52kDa subunit, although this may not have a great influence on the overall activity of the factor Xa. The second disulphide linkage would appear to be of greater importance for the action of the factor Xa, since it is involved in delineating the catalytic triad. The dansyl-GGACK staining of the procoagulant has indicated that the active catalytic triad is associated with the heavy chain as with factor X.

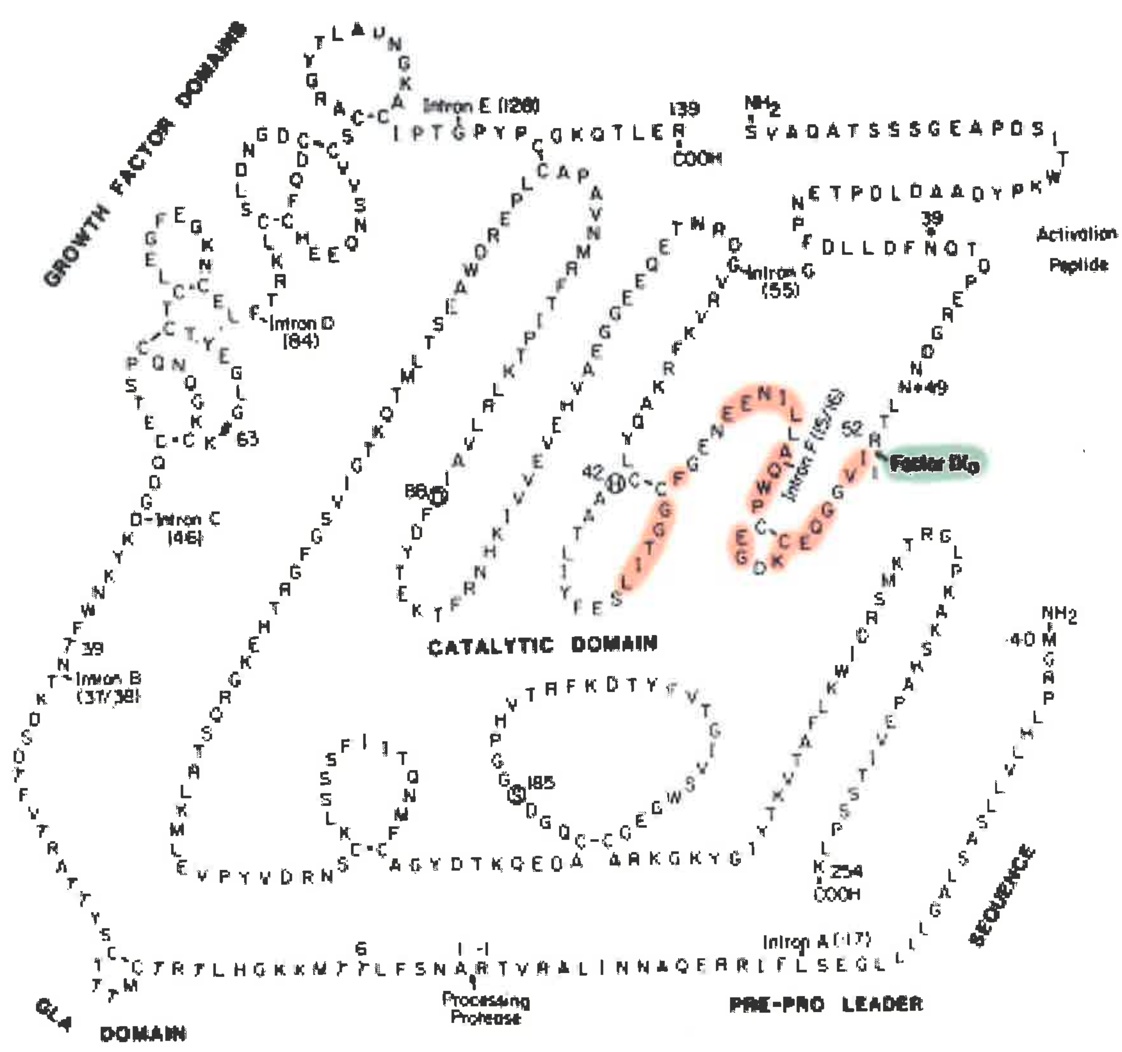

Fig 5.5 Amino acid sequence for human factor X (after Leytus et al, 1986). The 52 and 48kDa band sequence homology with factor X is highlighted.

The sequence homology with the 43kDa subunit occurred in the region of residues 55 - 76 of the factor V molecule. This forms part of the 105kDa heavy chain of factor Va released from the factor V molecule by thrombin or factor Xa. When non-covalently linked to the 74kDa light chain derived from the C-terminal, it produces in the presence of calcium ions, the active cofactor, factor Va. The action of the venom derived protein can only be speculated upon, however in view of the sequence homology to factor V and particularly the fact that the homology occured in the heavy chain region of factor Va, it is not unreasonable to suggest it is capable of acting as a cofactor to the factor X subunit.

The use of factor V deficient plasma as a substrate for the 52 and 48kDa subunits showed a reduced clotting response even in the presence of both calcium and phospholipid (Table 5.6). Although not conclusive it did imply that for maximum activity, factor V was required and that this was quite likely supplied by the factor Va-like subunit present in the venom. Attempts to isolate the factor Va containing fraction without the factor Xa subunits failed. However with a series of electrophoretic runs to reduce the factor X-like subunit content of the 'whole procoagulant', the clotting times obtained still remained far quicker than when the factor X-like subunit alone was used to clot plasma. This supported the theory that the factor Va-like subunit was responsible for magnifying the clotting effect of the procoagulant.

Thus the hypothesis was confirmed, with the factor components of the prothrombinase complex being isolated from the procoagulant. The factor Xa -like activity was associated with the *Textarin* subunit and this activity was amplified and modified, when combined with all subunits, consistent with the cofactor effect of factor V. There was no evidence to suggest the venom contained two distinct prothrombin activators.

REFERENCES

Di Scipio, R.G., Hermodson, M.A., Yates, S.C., Davie, E.W. (1977a). A comparison of human prothrombin, factor IX (Christmas factor), factor X (Stuart factor) and protein S. Biochem. **16**, 698-706.

Di Scipio, R.G., Hermodson, M.A., Davie, E.W. (1977b). Activation of human factor X (Stuart factor) by a protease from Russell's viper venom. Biochem. **16**, 5253-5260.

Enfield, D., Ericsson, L.H., Fujikawa, D., Walsh, K.A., Neurath, H., Titani, K. (1980). Amino acid sequence of the light chain of bovine factor X (Stuart factor). Biochem. **19**, 659-667.

Fujikawa, K., Legaz, M.E., Davie, E.W. (1972a). Bovine factors X_1 and X_2 (Stuart factor), isolation and characterization. Biochem. **11**, 4882-4891.

Fujikawa, K., Legaz, M.E., Davie, E.W. (1972b). Bovine factor X_1 (Stuart factor): mechanism of activation by a protein from Russell's viper venom. Biochem. **11**, 4892-4899.

Fung, M.R., Hay, C.W., MacGillivray, R.T.A. (1985). Characterization of an almost full-length cDNA for human blood coagulation factor X. Proc. Nat. Acad. Sci. USA, **82**, 3591-3595.

Hojrup, P., Magnusson, S. (1987). Disulphide bridges of bovine factor X. Biochem. J. **245**, 887-892.

Jackson, C.M. (1972). Characterization of two glycoprotein variants of bovine factor X and demonstration that the factor X zymogen contains two polypeptide chains. Biochem. **11**, 4873-4882.

Joseph, J.S., Chung, M.C.M., Jeyaseelan, K., Manjunatha Kini, R. (1999). Amino acid sequence of Trocarin, a protrhombin activator from *Tropidechis carinatus* venom: Its structural similarity to coagulation factor Xa. Blood **94**, 621-631.

Kisiel, W., Hermodson, M.A., Davie, E.S. (1976). Factor X activating enzyme from Russell's viper venom: isolation and characterization. Biochem. **15**, 4901-4906.

Leytus S.P., Foster, D.C., Kurachi, K., Davie, E.W. (1986). Gene for human factor X: A blood coagulation factor whose gene organization is essentially identical with that of factor IX and protein C. Biochem. **25**, 5098-5102.

Masci, P.P., Whitaker, A.N., deJersey, J. (1988). Purification and characterization of a prothrombin activator from the venom of *Pseudonaja textilis textilis*. Biochem. Int. **17**, 825-835.

Reddy, S.V., Zhou, Z.Q., Rao, K.J., Scott, J.P., Watzke, H., High, K.A., Jagadeeswaran, P. (1989). Molecular characterization of human factor $X_{San\ Antonio}$. Blood **74**, 1486-1490.

Rhodes, D.G., Yphantis, D.A. (1981). A small-volume electrophoresis concentrator. Anal. Biochem. **116**, 379-382.

Speijer, H., Govers-Riemslag, J.W.P., Zwaal, R.F.A. Rosing, J. (1986). Prothrombin activation by an activator from the venom of *Oxyuranus scutellatus* (Taipan snake). J. Biol. Chem. **261**,13258-13267.

Stocker, K. , Hauer, H., Müller, C., Triplett, D.A. (1994). Isolation and characterization of Textarin®, a prothrombin activator from eastern brown snake (*Pseudonaja textilis*) venom. Toxicon **32**, 1227-1236.

Tans, G., Govers-Riemslag, J.W.P., van Rijn, J.L.M.L., Rosing, J. (1985). Purification and properties of a prothrombin activator from the venom of *Notechis scutatus scutatus*. J. Biol. Chem. **260**, 9366-9372.

Titani, K., Fujikawa, K., Enfield, D.L., Ericsson, L.H., Walsh, K.A., Neurath, H. (1975). Bovine factor X_1 (Stuart factor): amino acid sequence of heavy chain. Proc. Nat. Acad. Sci. USA **72**, 3082-3086.

Triplett, D.A., Stocker, K.F., Unger, G.A., Barna, L.K. (1993). The Textarin/Ecarin ratio: a confirmatory test for lupus anticoagulants. Thromb. Haemost. **70**, 925-931.

CHAPTER 6

VARIATION IN THE VENOM COMPOSITION OF THE BROWN SNAKES

INTRODUCTION

The variability in venom composition is perhaps an under-estimated influence in both venom research and clinical case presentation. Glenn and Straight (1978) reported the geographic variation found in the venom of *Crotalus scutulatus scutulatus*, with the presence or absence of Mojave toxin being the predominant finding in these venoms. Clinically, Hardy (1983) noted this variability in the presentation of the bite victims with either neurotoxic or tissue damage symptoms being displayed.

Variability in venom composition is also of great importance in developing effective antivenoms and this was best illustrated with the antivenom against Russell's viper developed at the Indian Haffkine Institute being of no value in cases of envenomation by Sri Lankan Russell's vipers (Theakston et al, 1989).

In Australia the brown snakes have shown similar clinical variability across their geographic range. It is most common that the clinically predominant symptom will be coagulopathy, although rarely without obvious cause neurotoxic symptoms are present without coagulopathy. Clinical experience with brown snake bites also suggests that possibly there may be inter-species variability in the extent of the coagulopathy and the response to

antivenom therapy (Jelinek and Breheny, 1990). This poses the question of whether the coagulant component from these venoms exhibit characteristics sufficiently different to explain the clinical manifestations and the greatly increased quantities of antivenom required for neutralisation of the coagulopathy.

The large distribution of the two major brown species of *P. nuchalis* and *P. textilis* suggests the possibility that they may form a large interbreeding population with few natural barriers to interrupt this. Thus a large number of venom enzymes could be investigated to determine any influence geographic origin may have on activity.

Seasonal variability in venom composition may also be a contributory factor in clinical manifestation of symptoms. Gubensek et al (1974) reported the disappearance of two basic low molecular weight proteins from the venoms of *Vipera ammodytes* specimens during the winter months and that these proteins were toxic. Generally however little evidence has been found to support seasonal variability (Gregory-Dwyer et al 1986: Latifi, 1984: Williams and Mirtschin , unpublished observations on *Notechis scutatus*).

The ability to take native venom for research purposes and to isolate a component of interest, confident that the component will (a) be present and (b) will constitute a certain percentage of the venom at each testing would simplify research programmes. Unfortunately this is not always the case and indeed Stocker et al (1994) reported that commercially prepared brown snake prothrombin activator showed considerable variability in its clotting activity under their test system.

In an attempt to reduce the possible sources of variation in the isolated procoagulant, in this study the venom was obtained from snakes from a constrained geographic area although no attempt was made to ensure the venom was collected at a particular time of year. The protocols for the isolation of the procoagulant were rigidly maintained. In the previous chapters some of the inconsistency associated with venom research was encountered, with

some bands being present or absent on SDS-PAGE of the procoagulant without any obvious variation in the preparation procedure. However it must be remembered that the venoms contain extremely active proteolytic enzymes and these may be responsible for considerable autocatalysis. To reduce this, inhibitors can be used, although the difficulty with this is the loss of the activity of interest through these inhibitors.

Thus the investigation of venom variability is important from a number of aspects. Clinically, knowledge of the genus, species and possibly the geographic origin of a snake involved in an envenomation may aid in the appropriate choice of treatment or development of new treatments. Knowledge of the variability possible in the venom may also be advantageous in research to ensure consistency of results and with the taxonomy of the Australian snakes constantly under review any differences in venom composition may be of assistance in separating species or sub-species.

In this chapter the sources of variability in venom are explored. Seasonal variability is examined in the enzyme activity changes noted over a year in the venom of a single specimen of *P. textilis*. The geographic influence on venom enzymes is investigated for both *P. nuchalis* and *P. textilis* through venoms collected across Australia, with a number of clustered specimens as a basis for comparison. The procoagulant from *P. nuchalis*, *P. textilis*, *P.affinis* and *P. inframacula* venoms is compared to determine differences that may explain the response to antivenom in neutralising the coagulant activity found between the species. Finally the procoagulant action of the *Pseudonaja* and *Oxyuranus* species have been equated (Rosing and Tans 1994) and the isolation and physical characteristics of the procoagulants are compared.

(The work in this chapter formed the basis of the papers: Williams and White, 1992; Williams et al, 1994; Williams and White, 1997)

MATERIALS AND METHODS

Seasonal variability:

Venom was collected from a single adult male (1.53 m) *P. textilis* specimen monthly over a twelve month period. The snake had been kept in captivity for a period of 6 years and maintained within a reasonably temperature-controlled environment (mean 23°C) with a seasonally adjusted photoperiod. The animal was offered 1-2 mice on a weekly basis and the period between feeding and milking was kept constant over the 12 months. The collected venom was dried and refrigerated at 4°C and prior to testing was adjusted to 1mg/ml of protein in normal saline and used at this concentration throughout the study.

Gel filtration elution profiles were obtained for the whole venom, as were SDS-PAGE banding patterns. Immunoprecipitation lines were developed in 1% agarose gels with 100µl of venom loaded into the peripheral wells for each month and 100µl of brown snake antivenom (20U, CSL, Melbourne, Australia) loaded into the central well. Precipitin lines were developed over 2 days prior to being photographed.

Amidolytic activity was determined against the chromogenic substrates S2222, S2238 (Kabi Vitrum, Stockholm, Sweden), Chromozym TRY and Chromozym TH (Boehringer, Mannheim, Germany). To 950µl of Tris 0.05M (pH 8.4), 25µl of venom was added and then 25µl of 3mM chromogenic substrate and the time noted. At exactly 5 min the reaction was stopped by the addition of 100µl of 50% acetic acid. The OD 450nm was recorded. 5' nucleotidase and non-specific phosphatase activity were determined by the method of Dixon and Purdom (1954), following the procedure described in the Sigma, 5'nucleotidase test kit, (Sigma procedure 675), 100µl venom per test. Phosphodiesterase activity was measured by the method of Sinsheimer and Koerner (1952), 40µl of whole venom preparation was used in each test. The method of Jayanthi and Veerabasappa Gowda (1988), with 100µl per test procedure was used to determine esterolytic activity against the substrates TAME and

BAEE. Coagulant activity was determined in arbitrary units (Williams and White, 1989), where 1 unit/ml is defined as being able to bring about the clotting of a pooled normal citrated plasma in 30 sec. Fifty microlitres of venom was added to 200µl of plasma and the clotting time recorded.

To facilitate the ease of representation of the large amount of enzymic data, the highest mean response obtained over the 12 months for each activity tested was considered 100% and all other months expressed as a percentage of this highest value. For each month for each activity measured, a minimum of 6 determinations were made and the result averaged.

Geographic variability:

Venoms were collected from 5 specimens of *P. textilis* and 9 specimens of *P. nuchalis*. The snakes originated from areas that gave a North-South coverage and also a cluster of close geographic origin was included as a comparative base (Fig 7.3 c,d). Each of the venoms was adjusted to 1mg/ml of protein in normal saline as per the seasonal study.

The coagulant activity, phosphodiesterase and 5'nucleotidase activity and amidolytic activity against the chromogenic substrate S2222 were determined as above (see seasonal variability).

As with the seasonal study the results were expressed as a percentage. The activity for each locale was expressed as a percentage against the highest responding locale (100%).

Comparison of the procoagulant from the venom of the brown snake species:

Crude pooled venom from specimens of *P. affinis* (23mg), *P. inframacula* (26mg), *P. nuchalis* (21mg) and *P. textilis* (28mg) was obtained from Venom Supplies, Tanunda, South Australia. The venoms were dissolved in 250µl of 0.05M NaOAc/ 0.15M Na Cl, pH 8.35 and the procoagulant isolated by gel filtration and anion exchange chromatography as described ion

Chapter 4. The procoagulant isolated from each species was adjusted to 384µg/ml for further testing.

The procoagulants (3.8µg) were loaded onto a 7% polyacrylamide gel in the absence of SDS according to the method of Laemmli (1970). The gel was 80x140mm and 140V was applied until the dye marker (bromophenol blue) was within 2 cm of the bottom of the gel. The bands were visualised with 0.125% Coomassie Blue. The procoagulant from *P. textilis*, *P. inframacula* and *P. affinis* was also applied to a 10% polyacrylamide gel in the presence of SDS and run on a Hoeffer Mighty Small, 20mA until the dye marker reached the bottom of the gel. Visualisation was again with 0.125% Coomassie Blue.

Arbitrary units of clotting were determined for each of the four venoms and converted to specific activities ie units of clotting activity/ mg procoagulant for comparison.

Immunoprecipitation was performed as described above in a 1% agarose gel, the central well containing 100µl (20U) of brown snake antivenom and the peripheral wells 100µl (38µg) of the procoagulant from each of the species. The gel was photographed after two days to allow for development of the precipitin lines.

Comparison of the procoagulant from P. textilis and Oxyuranus microlepidotus:

The procoagulant from both *P. textilis* and *O. microlepidotus* (inland taipan) were isolated according to the protocol developed in Chapter 4, using gel filtration and anion exchange chromatography. 50 mg of whole venom was used for each isolation and the isolated procoagulant was then run a 10% SDS-PAGE essentially according to the method of Laemmli (1970) with a 4% stacking gel. The gel was either stained with Coomassie Blue or blotted to nitrocellulose and stained using the Glycan detection kit Boehringer, Mannheim. The procoagulant from each was also labelled with dansyl-GGACK and photographed.

RESULTS

Seasonal variability

Sequential milking of the snake did not influence the dry weight of the venom collected, nor the protein content of the samples. The weight of venom milked ranged from 2.7 mg to 22.2 mg (mean weight 10.2 mg), while the protein content appeared to be regulated within a 15% range.

The gel filtration profiles and SDS-PAGE banding patterns (results not shown) produced considerable homogeneity, although as would be expected there were some quantitative differences noted, but qualitative differences were restricted to the minor peaks and bands.

The esterolytic activity assayed against BAEE and TAME showed drops in activity during both the winter and summer (Fig 6.1 A) while activity against the four chromogenic substrates (Fig 6.1 B) showed depression during the summer months, with the maximum activity for each of the above found during the spring. Non-specific phosphatase activity did not show any seasonal changes, with wide variability noted from month to month (Fig 6.1 C). The coagulant activity produced the weakest response during the summer months (Fig 6.1 C). The 5'-nucleotidase produced the maximum response in summer, and showed a single high response in spring. The phosphodiesterase response showed two high response months, one in summer and the other in spring (Fig 6.1 D).

The precipitin lines developed against the venoms by brown snake antivenom produced one line which was common to all twelve samples (Fig 6.2). However, a number of secondary precipitin lines were also present; some showed identity between samples, while others showed partial identity and some appeared to either lack antigens or to contain a unique but recognised antigen (eg Well 3,A Fig 6.2).

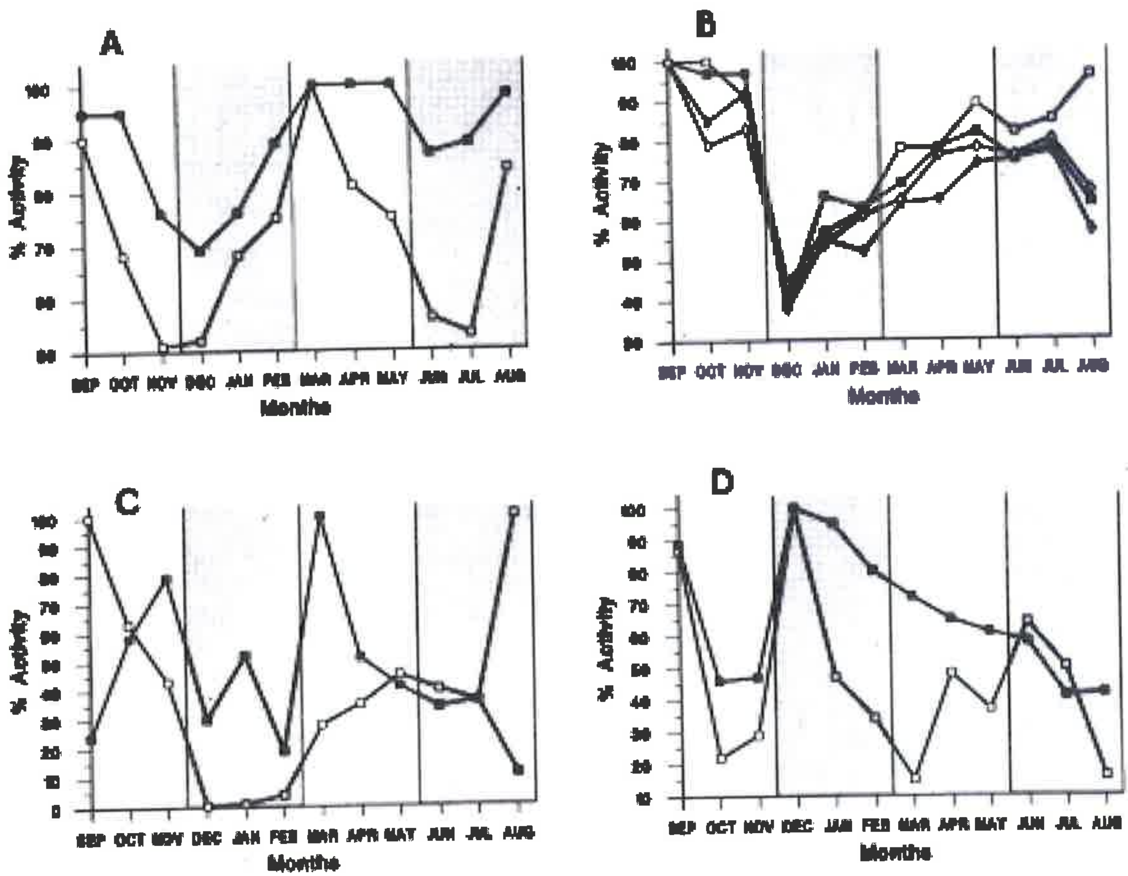

Fig 6.1 **The mean esterolytic, amidolytic, coagulant, non-specific phosphatase, 5'nucleotidase and phosphodiesterase activities determined for each month over the twelve month period. The activity was expressed as percentage of the highest responding month for each assay.**
A. BAEE ■, TAME □ B. Chromogenic substrates S2238 □ S2222 ■ Chromozym TRY ◆ Chromozym TH ✧ C. Coagulant activity □ non-specific phosphatase ■ D. 5'nucleotidase □ phosphodiesterase ■.

Geographic variability

The results of the activities per locality are shown in Fig 6.3 a,b. There was no greater variability in activity between snakes across the full geographic span of the study than there was between the clustered specimens.

The only activity that did not follow this trend was that obtained from the conversion of the chromogenic substrate S2222 and this was only in the venoms from specimens of *P nuchalis*. In this case, the cluster from the southern group of venoms all produced a reduced response (Fig 6.3 b).

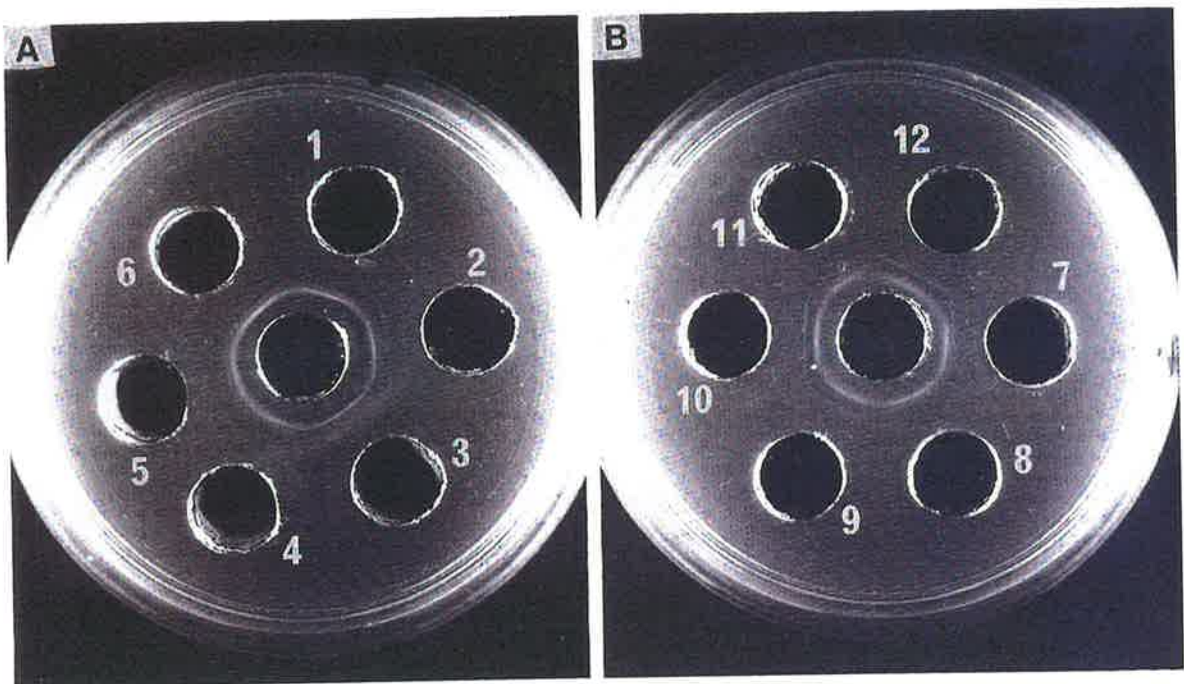

Fig 6.2 Immunopreciptin lines developed between brown snake antivenom (CSL Melbourne, central wells) and whole venom from each month over the 12-month period (peripheral wells).
A 1-6, October -March
B 7-12, April-September

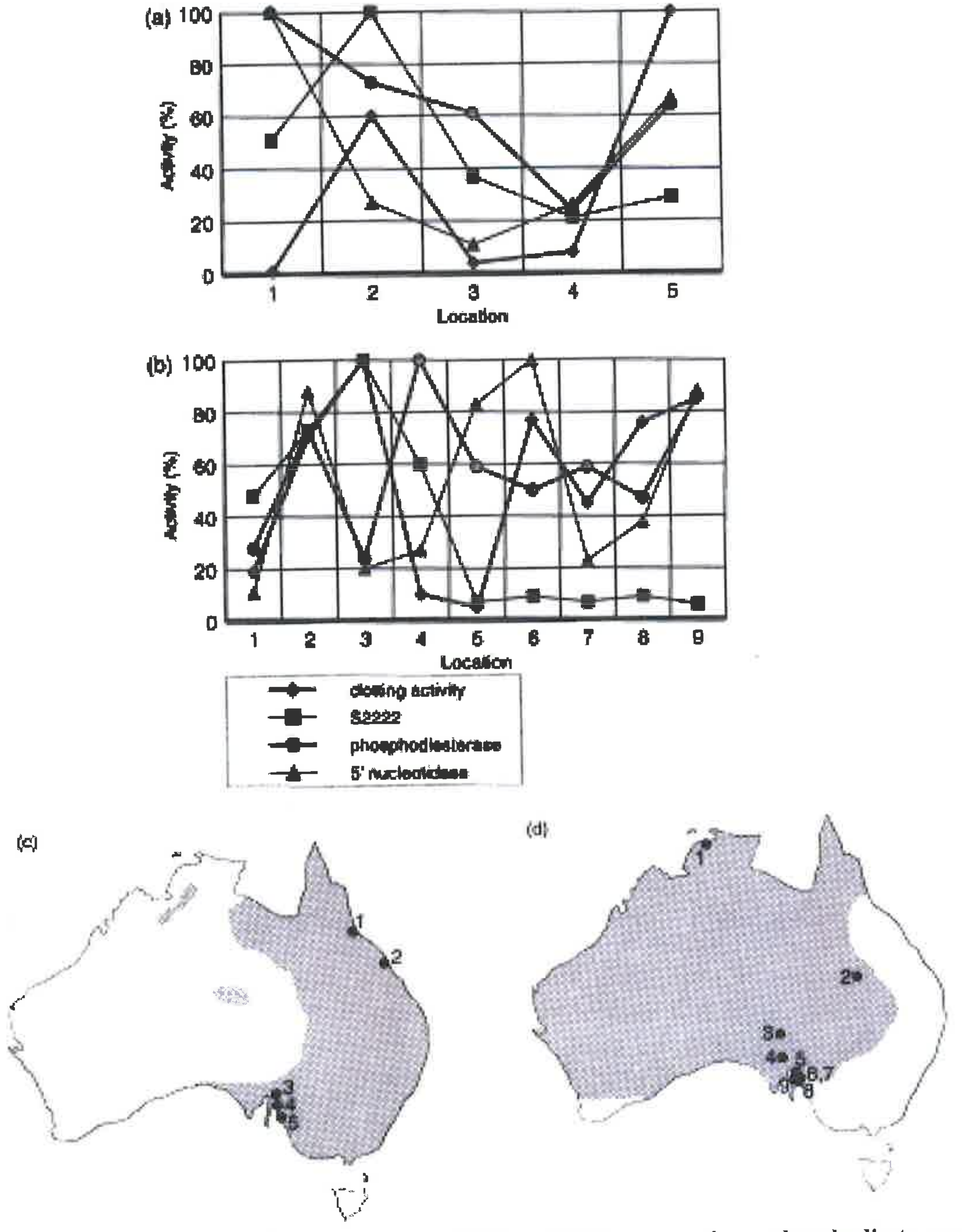

Fig 6.3 Geographical variability in clotting activity, S2222 conversion, phosphodiesterase and 5'nucleotidase activity of -

Upper graph (a) *P. textilis* venom: 1-: Townsville, 2-: Bundaberg, 3-: Crystal Brook, 4-: Wanderah, 5-: Adelaide.

Lower graph (b) *P. nuchalis* venom: 1-: Darwin, 2-: Charleville, 3-: Roxby, 4-: Depot Creek, 5-: Napperby, 6,7-: Port Pirie, 8-: Wanderah, 9-: Weeroona.

Activity of each population expressed as a percentage of the highest responder. Maps illustrate positions of each locality and species distribution (shaded region). (c) *P.textilis*. (d) *P. nuchalis*.

Procoagulant species comparison

All four species showed remarkably similar gel elution profiles (Fig 6.4) and clearly showed the elution of the coagulant activity at the same position. Elution from the anion exchange also produced equivalent profiles for all four species.

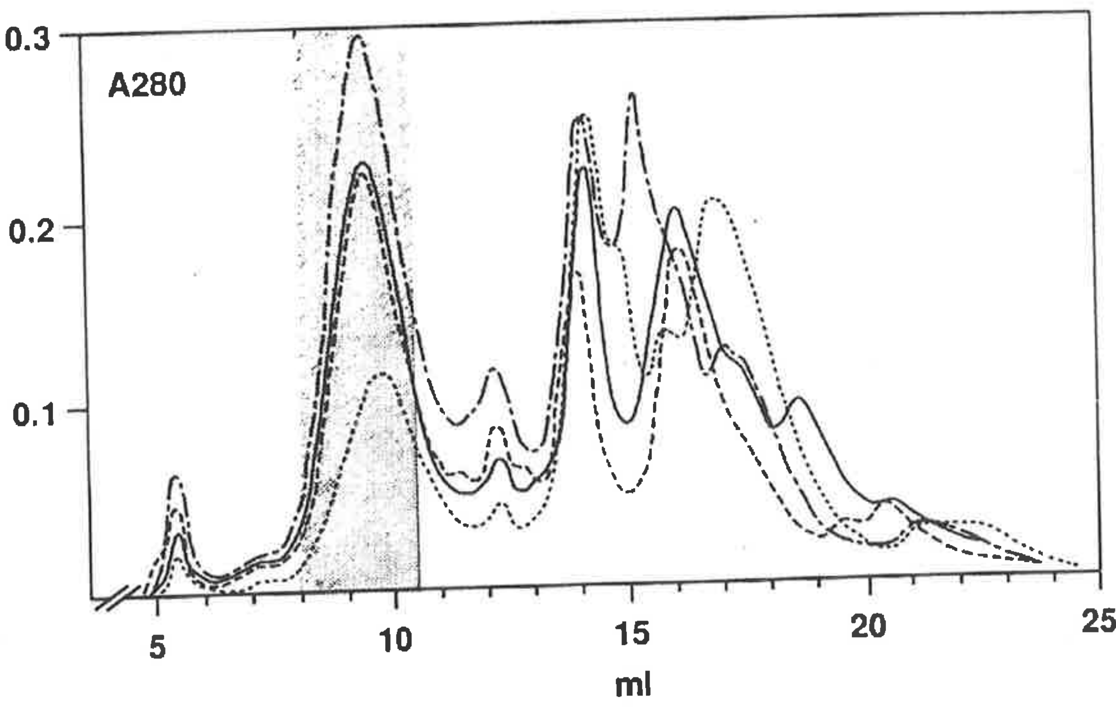

Fig 6.4 Superimposed gel filtration profiles of the whole venom from *P. affinis* (- - -), *P. inframacula* (—), *P. nuchalis* (...), *P.textilis* (▪•▪•). The region containing procoagulant activity is shaded.

Electrophoresis of the procoagulants in the absence of SDS produced a single but diffuse band for each of the species (Fig 6.5). *P. affinis* and *P. inframacula* showed a similar band, while there was a slight difference in the migration of these when compared to *P. nuchalis* and *P. textilis*. In the presence of SDS however (Fig 6.6) the similarity between each of the procoagulants is obvious with little to distinguish them.

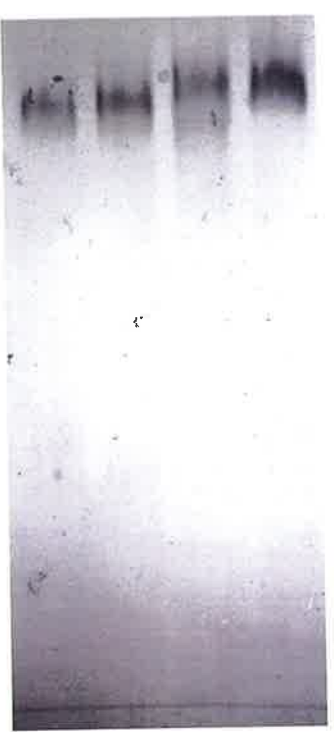

Fig 6.5 Native PAGE (7%, 3.5µg load) of the isolated procoagulant from the four *Pseudonaja* species. Left to right: *P. affinis*, *P. inframacula*, *P. nuchalis* and *P. textilis*.

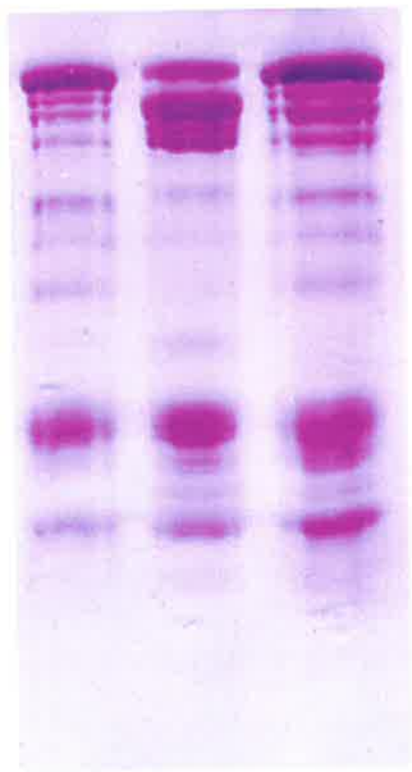

Fig 6.6 SDS-PAGE of the isolated procoagulants (3.0µg, load) from (left to right): *P. inframacula*, *P. textilis and P. affinis.*

Despite the co-elution and the similarity of the electrophoretic banding patterns there was a two-fold difference in the specific activity of *P. textilis* and *P. inframacula* when compared to *P. nuchalis* and *P.affinis* (Table 6.1). despite the difference each of the procoagulants was extremely powerful. The immunoprecipitin lines developed between the four procoagulants and brown snake antivenom showed identity (Fig 6.7).

Table 6.1 Specific activity of the procoagulants from the four species. (Activity expressed as arbitrary units of clotting activity/mg protein)

Species	Activity (arbitrary units/mg protein)
P affinis	6158
P inframacula	12000
P nuchalis	7700
P textilis	11000

Fig 6.7 Precipitin lines developed between CSL brown snake antivenom (Central well) and the isolated procoagulant from each of the four *Pseudonaja* species.
Left to right: *P. affinis, P. inframacula, P. nuchalis* and *P.textilis.*

Comparison of the procoagulant from P. textilis and Oxyuranus microlepidotus

The gel filtration and anion exchange elution profiles are shown in figure 6.8. The shaded areas indicate the peak associated with the procoagulant and the similarity of the gel filtration profiles between *P. textilis* (Fig 3.1, 3.2) and *O. microlepidotus* indicated the molecular weight of the procoagulants were extremely close. The contribution made to the total venom by the procoagulant of the taipan was less than in the brown snake.

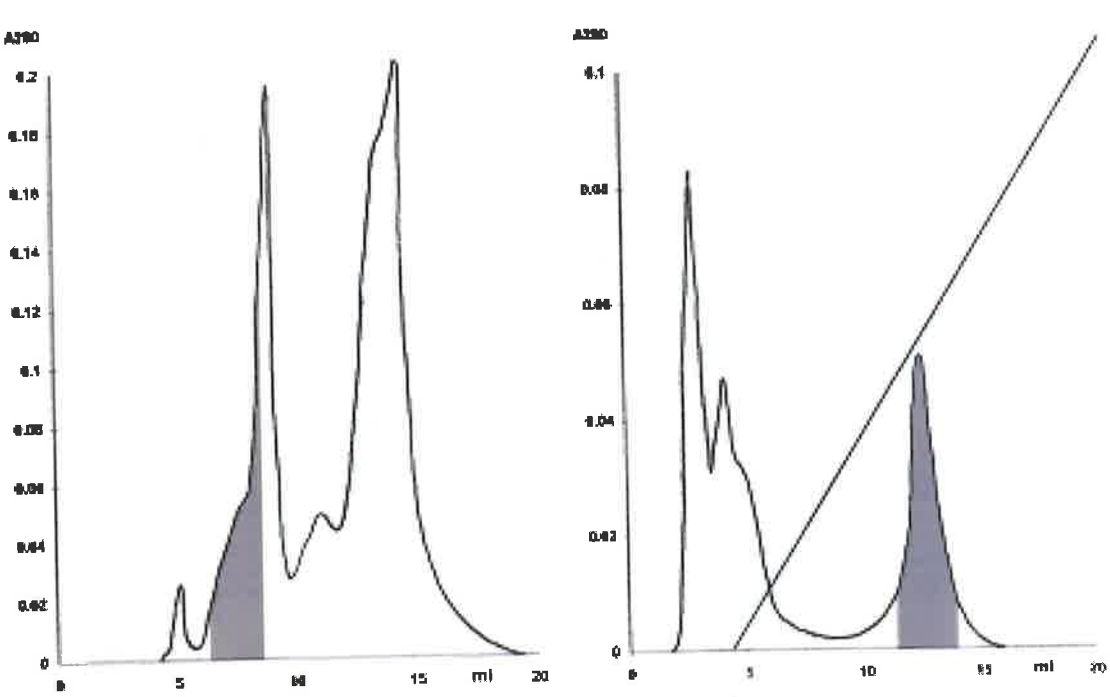

Fig 6.8 Gel filtration (left profile) of *Oxyuranus microlepidotus* whole venom. Coagulant activity shaded. Fractions containing activity were pooled, concentrated and applied to anion exchange column (right profile). Coagulant activity shaded. (Compare elution of procoagulant with that from *P. textilis*, Figs 3.1 & 3.2).

SDS-PAGE of the procoagulants (Fig 6.9) produced almost identical patterns in the high molecular weight range, while in the low molecular weight range there were some bands which appeared to be equivalent. Carbohydrate detection of these bands also showed the similarity of the banding. The major difference noted was the single band apparent after active-site labelling of the taipan compared with the double band present in the procoagulant from the brown snake.

The other important point to note is the presence of a band at 43kDa in the taipan procoagulant subunits, and thus by comparison with the brown snake it is likely that this band will show homology with factor V as well.

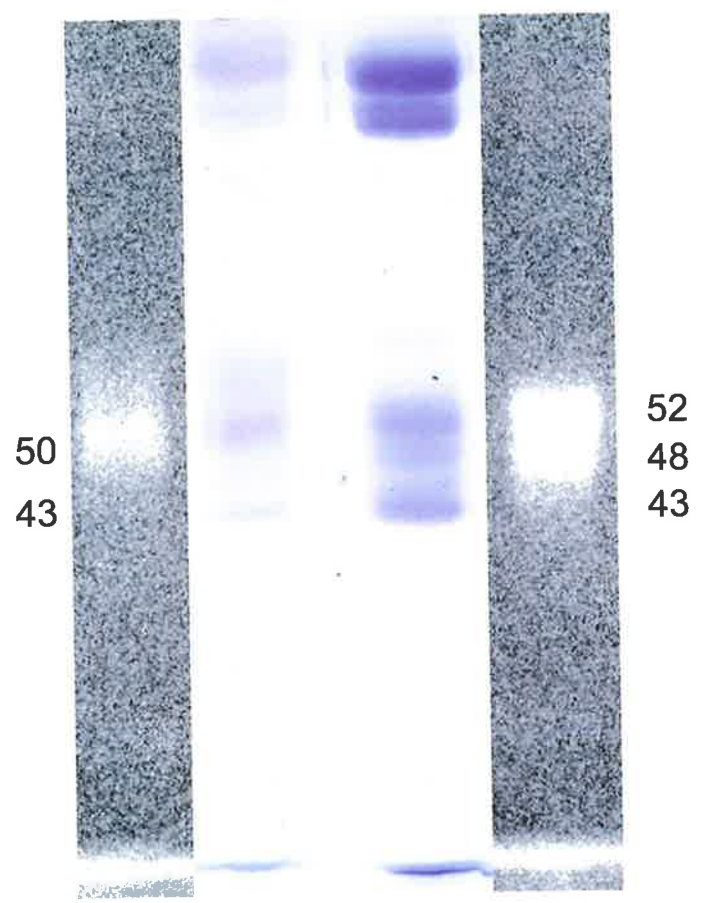

Fig 6.9 SDS-PAGE (10%) comparison of the isolated procoagulants from the inland taipan, *Oxyuranus microlepidotus* (2.0µg load) and *P. textilis* (3.5µg load).
Lane 1: dansyl-GGACK active-site labelled procoagulant from *O. microlepidotus*.
Lane 2: Coomassie Blue staining of isolated procoagulant from inland taipan.
Lane 3: Coomassie Blue staining of isolated procoagulant from *P. textilis*.
Lane 4: dansyl-GGACK active-site labelled procoagulant from *P. textilis*.

DISCUSSION

Although little work has been performed on the influence of season on venom composition, the results obtained from this single specimen of *P. textilis* were surprising, with some of the activities examined in the venom showing seasonal variation. Previously, apart from Gubensek (1974), studies had failed to find evidence of seasonal variation (Latifi 1984; Gregory-Dwyer et al , 1986). Milking the snake monthly appeared to have no influence on

the quantity of venom that might be expected nor did it appear to influence the protein content and in fact the protein content of the venom samples over the twelve months varied by as little as 15%.

While non-specific phosphatase showed no particular pattern of response a number of the remaining venom activities investigated produced close to the highest response during the spring months or at least at the beginning of spring, and showed some depression during the winter months. This was not an unexpected result as the winter is a period of minimal activity and feeding, while during spring the animal would begin to hunt prey and the ability to both capture the prey and aid in its digestion would be advantageous. However despite the expected rise and depression during spring and winter respectively, a number of results were surprising, particularly during summer. The coagulant activity was at its lowest during summer and this seasonal variability may be responsible for differences noted in plasma clotting times induced by the same venom concentration. The esterolytic activity was also at a minimum during summer, while 5'nucleotidase and phosphodiesterase showed peak activity at this time. This may reflect the activity of the snakes during the summer when they are more capable of catching prey and may show a predominance of digestive enzymes to assist the animals at a time of maximum feeding.

The precipitin lines also produced interesting results with considerable variability from month to month. In view of the homogeneity in the SDS-PAGE bandings and the chromatographic elution profiles it would suggest that the appearance of some of these precipitin lines was due to antibodies raised in response to minor components in the venom. The strength of some of these reactions suggested that these minor components were quite immunogenic.

The variation noted in the venom over the twelve months supported the case for consideration to be given to the time of the year that venom is collected for antivenom

production. It also suggested that venom collected year round and pooled was an appropriate mixture for immunising horses for antivenom production.

Geographic separation has been reported to produce differences in the venom composition or the concentration of particular components within the venom. This was noted in the venom composition of *Bothrops asper* specimens from the Atlantic and Pacific regions of Costa Rica where the populations are divided by a mountain range. Intermediate venom composition was found in the animals that were located in the passes through the mountains (Aragon-Ortiz and Gubensek, 1981). Variation was also noted in the isolated populations of the black tiger snakes from the islands around the coast of South Australia. In this case the length of isolation from what was presumably originally an interbreeding population, was reflected in the divergence in the venom composition (Williams et al 1988). In the case of the brown snakes however there would appear to be few natural barriers to the animals interbreeding and this is probably best seen in the distributions of *P. textilis* and *P. nuchalis* which are wide ranging. The activity differences noted between the widely separated populations was of no greater variation than that seen within the clustered populations. This suggested that individual variability was as great an influence on the venom composition as was any other factor such as geographic origin.

The fact that the animals have the same activities and the response of those activities was not influenced by geographical origin also suggested that envenomation by a member of these two species should respond to antivenom therapy equally well across the distribution of the animals. Also allowing for any influence from seasonal variation, each of these venoms should be of equal worth in contributing to antivenom production to be used anywhere across Australia against bites from these species.

While the clotting activity across the geographical range of the two species tested showed no particular pattern, it had been suggested that there may indeed be a difference in the coagulant ability of the venom from *P. affinis* compared with the other species as it appears more

antivenom is often required to bring about neutralisation of the coagulopathy. The comparison of the isolated procoagulants however did not support this theory. In fact the specific activity of the coagulant in the venom of *P. affinis* was half that of either *P. textilis* or *P. inframacula*. All other features of the procoagulants appeared equivalent, therefore some other explanation needed to be found for the requirement of extra antivenom in some cases of envenomation.

Possibly the simplest explanation for the disparity lay in the amount of venom that may be injected at a bite. Fairley and Splatt (1929), Freeman and Kellaway (1934) and Morrison et al (1983) have examined various Australian genera and species for the average yields that can be expected. These studies suggested that generally the quantity of venom injected by *Pseudonaja* species was quite small and that from this data 2-4 mg could be expected to be injected with a typical envenomation. The preliminary results of the study on the quantity of venom obtained at any milking from the various *Pseudonaja* species (Williams et al 1994, Table 6.2) suggested that a huge variation may be found in the amount of venom injected at a bite.

Table 6.2 Data on the quantity of venom milked from established captive specimens of four species of brown snake

Species	Origin of snakes	Ave wt per milking (mg)	Range (mg)	Number of snakes milked by number of milkings
P affinis	Perth, WA	21.2	8.5-34.2	3 x 2
P inframacula	Eyre Peninsula, SA	41.8	14.3-71.1	4 X 1
P nuchalis	Eyre Pen. SA	19.4	9.4-32.3	4 x 2
P textilis	Adelaide, SA	2.9	0.2-7.2	25 x 1
	Goyders Lagoon SA	18	-	1 x 1
	Gold Coast Qld	10.8	3.3-20	16 x 1

Weights are shown in mg dry weight.
All milkings performed by the same personnel, over a brief period in May 1993.
(Williams et al, 1994).

The quantity of antivenom per ampoule (CSL, Australia) is based on the amount needed to neutralise an average milking, which from above is 2-4 mg. Working on the average amounts milked for *P. textilis* and *P. nuchalis* from Table 6.2 anything up to 10 ampoules may be required to produce neutralisation, while in the case of *P. affinis,* at least 10 ampoules would not appear to be an uncommon requirement and bites from *P. inframacula* may require an even greater number. Thus the differences found clinically with coagulopathy are more likely due to quantity of venom injected than due to a qualitative difference in the procoagulants.

The similarity in the procoagulants from the taipan and the browns has been postulated from the characteristics of the coagulant activity, however this is the first physical comparison based on the same isolation protocol and under these conditions the similarity was quite obvious. The elution profiles (Fig 6.8) were almost interchangeable and the SDS-PAGE banding (Fig 6.9) showed considerable homology in the high molecular weight region and some similarity in the low molecular weight region. The major difference noted was the single band obtained with an active site stain for the taipan as opposed to the duel staining bands obtained with the brown snake. In view of the fact that the active-site containing fraction from brown snake procoagulant and that from *T. carinatus* show homology with factor Xa, it is not unreasonable to speculate that the active site fraction from the taipan will show similar homology to factor Xa. The homology of the 43kDa band in the brown snake procoagulant with factor V suggests the 43kDa band in the taipan procoagulant may also show such homology and this is consistent with a ‘complete prothrombin activator’.

The other area where there was a difference between the two procoagulants was the per cent contributed to the total venom composition. The procoagulant from the brown snake contributed around 30% of the total venom while in the case of the taipan this percentage was consistently smaller and at maximum was only 10% but generally slightly less than this. Despite this the strength of these procoagulants was such that envenomation will undoubtedly result in a coagulopathy in the victim. The implications of the similarity are also

obvious for taxonomists with the browns and taipans presumably originating from a common ancestor.

The venoms of the brown snake species showed the same enzymic activities and the geographic origin of the snakes had no influence on this activity. The procoagulants present in the venoms from each of the species were homologous and conserved and therefore represent a selective advantage to the snakes. The venom from *P. modesta* however has no coagulant activity (Williams and White, 1987) and this suggested that it should be reclassified, as the presence of the procoagulant is indicative of the genus. The seasonal variability found did not show loss of any activity over the twelve month period only a change in the strength of response. The procoagulant even at its lowest activity was still extremely active and therefore advantageous to the snake. Venom collected at any time of the year is no doubt capable of dispatching prey. Venom variability between individuals within a species was still a source of considerable difference (Chippaux et al 1991).

REFERENCES

Aragon-Ortiz, F., Gubensek, F. (1981). *Bothrops asper* venom from the Atlantic and Pacific zones of Costa Rica. Toxicon **19**, 797-805.

Chippaux, J-P., Williams, V., White, J. (1991). Snake venom variability: methods of study, results and interpretation. Toxicon, **29**, 1279-1303.

Dixon, T.F., Purdom, M. (1954). Serum 5'nucleotidase. J. Clin. Path. **7**, 341-343.

Fairley, N.H., Splatt, B. (1929). Venom yields in Australian poisonous snakes. Med. J. Aust. **1**, 336-348.

Freeman, M., Kellaway C.H. (1934). The venom yields of common Australian poisonous snakes in captivity. Med. J. Aust. **2**. 373-377.

Glenn, J.L., Straight, R.C. (1978). Mojave rattlesnake *Crotalus scutulatus scutulatus* venom: variation in toxicity with geographical origin. Toxicon **16**, 81-84.

Gregory-Dwyer, V.M., Egen, N., Bianchi Bosisio, A., Richetti, P.G., Russell, F.E. (1986). An isoelectric focusing study of seasonal variation in rattlesnake venom proteins. Toxicon **24**, 995-1000.

Gubensek, F., Sket, D., Turk, V., Lebez, D. (1974). Fractionation of *Vipera ammodytes* venom and seasonal variation of its composition. Toxicon **12**, 167-171.

Hardy, D.L. (1983). Envenomation by the Mojave rattlesnake (*Crotalus scutulatus scutulatus*) in southern Arizona, U.S.A. Toxicon **21**, 111-118.

Jayanthi, G.P., Veerabasappa Gowda, T. (1988). Geographical variation in India in the composition and the lethal potency of Russell's viper (*Vipera russelli*) venom. Toxicon **26**, 257-264.

Jelinek, G.A., Breheny, F.X. (1990). Ten years of snakebites at Fremantle Hospital. Med. J. Aust. **153**, 658-661.

Joseph, J.S., Chung, M.C.M., Jeyaseelan, K., Manjunatha Kini, R. (1999). Amino acid sequence of Trocarin, a protrhombin activator from *Tropidechis carinatus* venom: Its structural similarity to coagulation factor Xa. Blood **94**, 621-631.

Latifi, M. (1984).Variation in yield and lethality of venom from Iranian snakes. Toxicon, **22**, 373-380.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**, 680-685.

Morrison, J.J., Pearn, J., Charles, N.T., Coulter, A.R. (1983). Further studies on the mass of venom injected by elapid snakes. Toxicon **21**, 279-284.

Rosing, J., Tans, G. (1994). Structural and functional properties of snake venom prothrombin activators. Toxicon **30**, 1515-1527.

Sinsheimer, R.L., Koerner, J.F. (1952). A purification of venom phosphodiesterase. J. Biol. Chem. **198**, 293-295.

Stocker, K. , Hauer, H., Müller, C., Triplett, D.A. (1994). Isolation and characterization of Textarin, a prothrombin activator from eastern brown snake (*Pseudonaja textilis*) venom. Toxicon **32**, 1227-1236.

Theakston, R.D.G., Phillips, R.E., Warrell, D.A., Galigedera, Y., Abeysekera,. D.T., Dissanayake, P., Hutton, R.A., Aloysius, D.J. (1989). Failure of Indian (Haffkine) antivenom in treatment of *Vipera russelli pulchella* (Russell's viper) envenoming in Sri Lanka. Toxicon **27**, 82.

Williams, V., White, J. (1987). The five-ringed brown snake, *Pseudonaja modesta* Gunther): report of a bite and comments on the venom. Med. J. Aust. **147**, 603-605.

Williams, V., White, J., Schwaner, T.D., Sparrow, A. (1988). Variation in venom proteins from isolated populations of tiger snakes (*Notechis ater niger, N. scutatus*) in South Australia. *Toxicon* **26**, 1067 - 1075.

Williams, V., White, J. (1989). Purification and properties of a procoagulant from peninsula tiger snake (*Notechis ater niger*). Toxicon **27**, 773-779.

Williams, V., White, J. (1992). Variation in the composition of the venom from a single specimen of *Pseudonaja textilis* (common brown snake) over one year. Toxicon **30**, 202 - 206.

Williams, V., White, J., Mirtschin, P. (1994). Comparative study on the procoagulant from the venom of Australian brown snakes (Elapidae: *Pseudonaja* spp.). Toxicon **32**, 453-459.

Williams, V., White, J. (1997). Snake venom and snakebite in Australia. In: *Ecology, evolution and snakebite. Venomous snakes.* pp. 205-217. Eds. Thorpe, R. Wüster, W. and Malhotra, A. Oxford Science Publications and The Zoological Society of London.

CHAPTER 7

CONCLUSIONS AND FUTURE DIRECTIONS

The venoms of numerous snakes contain components that are able to interfere with the haemostatic pathway of man. This includes both activators and inhibitors of the coagulation cascade and also both activators and inhibitors of platelet function. These have been listed and classified by the International Society on Thrombosis and Haemostasis, subcommittees (Rosing and Tans, 1991: Markland, 1998: Smith and Brinkhous, 1991: Teng and Huang, 1991: Pirkle, 1998).

The venoms from the Australian elapid snakes have both coagulants and true anticoagulants such as that from *Pseudechis australis*. The coagulant fractions in the tiger snake and the black tiger and the taipan have been isolated and their clotting characteristics examined (Tans et al, 1985; Williams and White, 1989; Speijer et al, 1986). The coagulant activity of the brown snake has also been investigated (Masci et al, 1988: Stocker et al 1994). Many of the features of the coagulant activities described by these authors for the brown snakes showed conflicting results and thus the explanation of the characteristics noted were at variance. In this chapter an attempt will be made to reconcile the results they obtained with those found in this study and to review the implications of venom variability.

Although (a) a strict protocol was followed in isolating the procoagulant and (b) the elution profiles and SDS-PAGE banding patterns were essentially the same from each isolation and between those obtained from each of the species, SDS-PAGE banding variations were noted.

These involved the occasional appearance of certain bands, particularly a number of bands that were found between the high and low molecular weight delineation, although the core bands (the high and low molecular weight separation) were always present and the proportions of these remained quite constant. The appearance of these bands did not interfere with activity of the coagulant and the origin of these bands remains uncertain. The symmetry of the elution profile from the anion exchange suggested that their appearance was still in some manner associated with the procoagulant, possibly as a result of autocatalytic activity. Similarly this banding variation was not apparent when native PAGE was employed, with the two bands present, and this also supported the idea that the transient bands were related to the procoagulant in some manner. None of these bands were stained with active-site stain and were therefore not associated with the factor X like action of the whole procoagulant.

Despite the variable banding, if present at isolation, this remained quite stable in the isolation buffer of approximately 0.35 - 0.4 M NaCl / 0.05 m NaOAc, pH 8.35, for at least four weeks. Thus the variability in the banding obtained more likely related to multimers arising from the lower molecular weight entities as was suggested earlier.

Isolation of the procoagulant by the method of Masci et al (1988) produced a number of bands that equated to those found in this study. The use of con-A sepharose resulted in substantial tailing when the procoagulant was eluted which was not found when anion exchange was used as the second purification method. This second step in the purification protocol for both methods resulted only in a marginal increase in the purity of the procoagulant. Attempts to isolate the active fraction of the venom, employing either the barium citrate absorption or hydroxyl-apatite chromatography methods of Triplett et al (1993), Stocker et al (1994), failed to produce purified bands at 52 and 48kDa. On each occasion the resultant products of these isolations showed contamination with at least one other band, generally from the high molecular weight group. Thus activity associated with this isolation could not be ascribed directly to the 52 and 48kDa species as the contamination may have had a major impact in the resultant action on the various clotting substrates.

A number of approaches were used to improve the separation of the various bands and therefore give an opportunity to examine the action of each of the subunits or to determine any synergistic relationship between these components. Reverse phase and hydrophobic interaction chromatography were employed, but the separation was not an improvement on the gel filtration followed by anion exchange chromatography protocol. The high and low molecular weight fractions were still unable to be pulled apart, and the contamination noted with the barium citrate and hydroxyl-apatite separations was still apparent. Running the whole procoagulant isolated according to the gel filtration and anion exchange protocol on a hydroxyl-apatite column also proved to be disappointing. The intention had been to improve the possible separation of the subunits by already having them partially isolated from whole venom, however this prepared fraction appeared to be less effective in producing 'clean' subunits than had been the case using whole venom.

The most effective method of separating the subunits was to electroelute the bands from a SDS-PAGE slab gel. This allowed the physical characteristics of the subunits to be examined, although because of the Coomassie Blue staining associated with the bands the action of the fractions was severely inhibited and therefore specific activities could not be assigned to individual bands. Attempts to passively elute the proteins from the gels were unsuccessful and electroelution of the bands when they were not stained by Coomassie Blue produced extremely small yields suggesting that the Coomassie was perhaps acting as a carrier for the proteins thus aiding in their elution.

The similarity of the procoagulants from the brown snake and the taipan was also of interest both from the point of view of the origin of these animals and the likely composition of the subunits of the taipan procoagulant. The high molecular weight fractions of the taipan procoagulant were very close to those found in the brown snake, and the low molecular weight entities are also close. The major difference was the single band observed to contain the active site in the taipan as opposed to the brown snake with two. Speijer et al (1986),

reported that the prothrombin activator from *Oxyuranus scutellatus* both functionally and structurally resembled the factor Xa-Va complex. The enzymatic part of the prothrombin activator had a molecular weight of 60kDa and was composed of two 30kDa subunits. This was equated to the factor Xa heavy chain containing the active site with a molecular weight of 30kDa. The high molecular weight fractions isolated in the study (80kDa and 110kDa) were suggested to represent a possible cofactor portion similar to factor Va, and this analogy was extended by the comparison of the molecular weights of the polypeptides from Va at 115 and 73kDa.

This led to a hypothetical model of the prothrombin activator being constructed to show the likely combination of a factor Xa and factor Va -like subunit to form the total unit (Fig 7.1). In view of the sequencing of the subunits of the brown snake showing homology to factor X and factor V it would not be unreasonable to suggest that sequencing of the subunits from the taipan would reveal that the active site containing subunit has homology to factor X and that indeed the presumed factor V like subunits show homology to factor V.

The physical similarity of the procoagulant from *P. textilis* with the prothrombinase complex was taken a step further with the homology shown by the 52 and 48kDa bands with factor X. Although the activity of these fractions was in keeping with that expected from factor X and the procoagulant consisted of a heavy and light chain, the sequence homology was an exiting discovery. In conjunction with the homology to factor Xa shown by the procoagulant from *T. carinatus* (Joseph et al, 1999), it is likely that the 'active' component of all Group Ii and Group III prothrombin activators will prove to be factor Xa homologues.

A number of interesting points are raised by this finding, not the least of which is the origin of a clotting factor present in venom. The sequence homology would suggest that it is unlikely to have arisen independently, an alternative hypothesis for clotting activity being present in the venom. In this alternative hypothesis, the active site was a variation of the trypsin model, with any number of similar active components present in whole venom, which

would attack Lys, and or Arg bonds. However the specificity of the sequence would suggest a more likely origin was replication of the snakes own clotting factors. This finding, as mentioned above, has implications for the likely sequence of the active site containing fraction of taipan procoagulant and possibly for tiger snake procoagulant as well, since the clotting activity of the tiger snake procoagulant is also consistent with the action of factor Xa. The active site containing fraction for tiger and taipan has a molecular weight of around 30kDa. The fact that no cross-reactivity was found between the tiger antivenom and the brown snake procoagulant however would suggest that the procoagulant from tiger venom, although displaying factor Xa activity and an isolated active fraction with a similar molecular weight, has perhaps arisen from a different source.

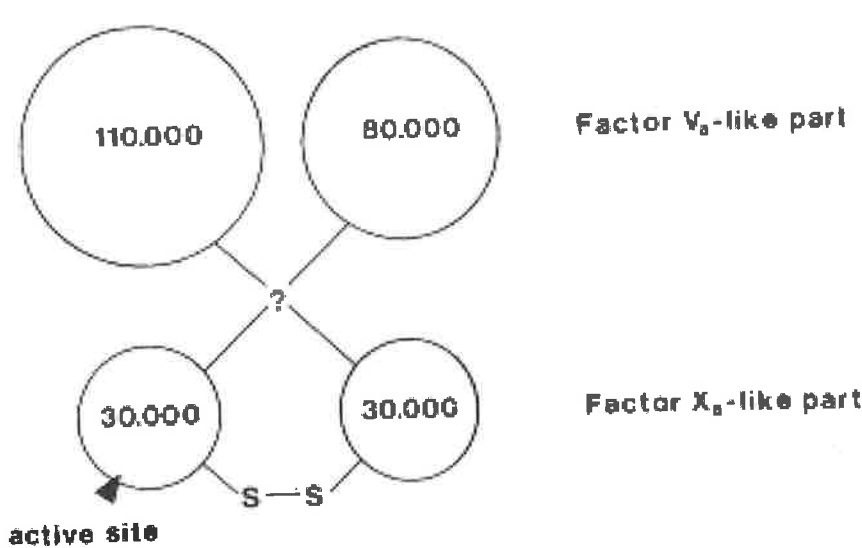

Fig 7.1 Hypothetical model of the procoagulant from the venom of *Oxyuranus scutellatus*. (Speijer et al, 1986). The active site has been identified in the low molecular weight subunits and these were equated with factor Xa. The high molecular weight subunits have been equated with the cofactor, factor V.

The contribution made by the high molecular weight fractions to the procoagulant remains unknown at this time. As previously mentioned there were a number of possible explanations for their presence. They may be a stabilising factor which assists in maintaining the integrity of the whole procoagulant, allowing the activator and cofactor portions to be kept in close contact for maximum clotting effect. Alternatively they may function in a protective role. The

environment of the venom gland is highly proteolytic and they may aid in protecting the procoagulant against premature destruction while still within the venom gland. The apparent multimeric nature of the high molecular weight fractions would perhaps assist in this protective role.

The final possibility is that they are in fact an integral part of the clotting mechanism and may act as part of a cofactor complex in concert with the 43kDa subunit. The model proposed by Speijer et al (1986) and Govers-Reimslag et al (1988) for the taipan (Fig 7.1) involved both high molecular weight species (110kDa and 80kDa) in the factor V cofactor-like function and this is an attractive proposal to explain the presence of these high molecular weight subunits. However at this stage there was no evidence to support any of these theories and it will require at least some sequencing to determine if there is any homology to factor V among these fractions. Currently an effective method has not been devised to allow a clean harvest of these fractions in a manner that will also preserve any activity.

Various aspects of venom variability were also examined, and in the seasonal study, although there was some variation in the strength of the reactions of the enzymic activities, no deletion of any of the components was noted over the twelve month period. It would be advantageous to run this study over a longer period to determine if this was a cyclic effect or a single observation. It would also be advantageous to determine if this was substantiated in other snakes of the same species. However it would appear that caution should be exercised when considering experimental procedures that may rely on a consistent quantity of a component in the venom, under experimental conditions. The variability over the year was of little concern when the activity to be investigated was the procoagulant though, as even when in this study it was at its lowest it was still extremely strong and the procoagulant contributed between 20-30% of the whole venom.

A number of points are worthy of further comment from the variability study that have significance for clinicians, taxonomists and researchers. The first is that the procoagulant

activity was found in the brown snakes from all areas of the geographical range of both *P. textilis* and *P. nuchalis*. The finding of the procoagulant across the range of the animals inferred the importance of this component in venom, conferring a selective advantage on the snakes, either due to its killing potential or its ability in prey to maintain liquid blood. The former is obvious and in smaller prey the strength of the coagulant activity is such that it could bring about the rapid demise of the prey through a massive thrombotic episode. Masci et al (1988) reported the sudden death of mice injected with the 'whole procoagulant' that he had isolated. Tibballs et al (1992) and Tibballs and Sutherland, (1992) also had implied the coagulant activity may be responsible for the sudden death and or collapse of bite victims. In this context however it must also be remembered that although this is particularly effective in the laboratory situation, the prey types being targeted by the snakes are of importance when considering the coagulation pathways that are to provide the substrates for this coagulant action. The brown snakes often target mice as prey and as a small mammal, one can assume the coagulation pathway is close to that of humans and therefore susceptible to the venom coagulant.

The second possibility for the venom containing the procoagulant is to maintain the blood in a fluid form post mortem. This would facilitate the movement of the venom enzymes around the circulatory system of the victim and effectively distribute digestive enzymes throughout the body of the prey. In this manner the prey item is then digested from the inside out, and in the case of large prey ensuring digestion will occur before putrefaction.

The preservation of the various activities in the venom of the brown snakes, independent of the geographic origin and the species involved, raises confidence in the ability of the current antivenoms to produce neutralisation of these components. There was no evidence to suggest that there is a difference in the procoagulants between the species sufficient to explain the difficulties encountered in the amount of antivenom to neutralise the coagulopathy, depending on the species involved. The possible maximum injection of venom at a bite is more important and the potential of the species to produce greater quantities of venom and to

inject those larger quantities is most likely the decisive factor. The ability to release large quantities of venom has also made even glancing blows potentially lethal when large quantities of venom are able to wash across the wound.

The consistency of the procoagulant across the geographic range of the *Pseudonaja* genus should allay fears that the present antivenom available is incapable of neutralising the coagulopathy found in most of the bite victims with substantial envenomation. A difference in the specific activities of the procoagulants from the four clinically important species of *Pseudonaja* was detected, although this was only a single fold dilution. Immunoprecipitation showed a line of identity between the procoagulants from the four species further strengthening the claim that neutralisation should be possible. The variability in the quantity of venom introduced into a victim must be taken into consideration, the larger quantities calling for considerably greater volumes of antivenom than currently recommended.

SUMMARY

The venom of the brown snakes contain a powerful procoagulant capable of rapid activation of prothrombin to thrombin. The activity was unaffected by the presence of heparin and was capable of clotting descarboxyprothrombin. EDTA had no effect on the whole venom nor on the isolated procoagulant, indicating the activity was not that of a metalloprotease. Inhibition studies showed that the activity was that of a serine protease and the active sites were confined to two proteins with molecular weights of 52 and 48kDa and known as *Textarin* (Triplett et al, 1993, Stocker et al, 1994). Sequence analysis of these fractions revealed homology to factor Xa and the action of these fractions was consistent with that associated with factor Xa. A fraction has also been identified that has a homologous sequence to factor V that suggests this fraction may act as a cofactor to magnify the clotting action of the factor Xa-like fraction. Venom variability in snakes due to geographical origin, species or season appeared to have no greater influence on venom composition than differences due to individual variation. In this study although variability was noted in the banding patterns

obtained between preparations of the isolated procoagulant, this did not affect the clotting activity nor the consistent appearance of the major active subunits.

A comparison of the findings of the studies of Masci et al (1988), Triplett et al (1993), Stocker et al (1994) and this study can be found in Table 7.1

FUTURE DIRECTIONS

The discovery of the two fractions with homology to factor Xa and V is an exciting basis for future investigations. Two important directions present themselves for such investigations.

The first is the complete sequencing of the factor Xa and factor V-like proteins. Only with the complete sequence can the modelling of the activity of the procoagulant be undertaken. The knowledge of the sequence and a better understanding of the means of activating prothrombin to thrombin, could provide options for therapeutics or further reagents for examining the clotting pathways.

In view of the homology to human, chick, rabbit and bovine clotting proteins, the proteins present in the venom gland are most likely themselves to be brown snake clotting factors X and V. Isolation and sequencing of these factors from the plasma of the snakes would allow this to be confirmed, however the question still remains as to how these plasma proteins are then incorporated into the venom gland. Does the venom gland itself have the ability to synthesise these proteins or are they sequestered from the circulation and subsequently incorporated in to the contents of the gland?

Table 7.1 Summary of findings of current studies on the procoagulant from *P.textilis* venom

	Masci et al (1988)	**Triplett et al (1993)**	**Stocker et al (1994)**	**This study**
Isolation	Gel filtration Con A sepharose	Barium citrate precipitation	Hydroxyl apatite	Gel filtration Anion exchange
Molecular weights (kDa)				
Low range	65, 60	52	53, 50	43, **48**, **52**, **64** (39)
High range	200, 170	150, 116	-	245,**180**,**147**,106 (**208**, 190, **125**)
Reduced species MW kDa				
Low range	65	32, (19-27)	33	43, **34**, **(16-28)**
High range	100	56, 67, 99.5	-	**100**, 75, **58**, 52, 47
Subunits	YES	-	NO	YES
Action	Serine protease	-	Serine protease	Serine protease
Active site	Unknown	Unknown	Unknown	52, 48 kDa native 34kDa reduced
Carbohydrates	-	-	-	Glycoproteins Sugars N-linked Terminal CHO subunit dependent
Sequencing	-	-	-	52, 48kDa homology Xa 43kDa homology V

Molecular weights in bold are those with an approximate equivalent from previous studies.

The second is to assign activities to each of the bands isolated on SDS-PAGE, both those in the low molecular weight range and those in the high molecular weight range. This necessarily involves developing a means of isolating the subunits or fractions in a manner that preserves their activity. The bands at 52 and 48kDa have an activity already assigned and although the band at 43kDa has shown homology with factor V, the activity has only been implied for this fraction by indirect means. The reason for the presence of the high molecular weight bands remains unknown at this stage and this hopefully can also be discovered.

Further investigation of the procoagulant may explain the ability of the procoagulant to convert prothrombin to thrombin in the presence of heparin and also to bring about clotting in an EDTA plasma. If the factor Xa homologue closely follows the sequence of the natural protein it would be anticipated that the protein should be inhibited by the presence of heparin, therefore some differences, at present unrecognised, must be responsible for this not being the case. The ability to clot heparinised plasma has also been found with the venom and isolated procoagulant from the black tiger snakes (Willams and White, 1989) and therefore suggests that the factor X-like activity found in both venoms is closely related.

The ability to convert EDTA plasma also remains an area for further investigation. The factor X-like fraction is calcium dependent, yet the whole procoagulant is capable of clotting EDTA plasma. If the 43kDa fraction is supplying the equivalent of factor V to the clotting complex, in normal circumstances the prothrombinase complex still requires calcium to be supplied for clotting to occur. However in the case of the venom, the factor V-like fraction, perhaps in concert with the high molecular weight entities, appears to be able to overcome the lack of calcium, to produce prothrombin activation. The role of phospholipid also remains somewhat uncertain. Phospholipid does not appear to be an essential ingredient for the procoagulant to be active, yet when present it may stimulate the activity. A screen of the procoagulant failed to reveal any phospholipid associated with any of the clotting fractions that may explain why it is not required, and therefore it suggests that it is unlikely to be of value to the venom in vivo. In the clotting cascade, phospholipid provides a focus for the conversion of

prothrombin to thrombin at the point of injury, through the presence of platelets, while in the case of the venom, dissemination of the procoagulant is advantageous to the snake, to either maintain the blood in a fluid condition or to allow the procoagulant to reach the important organs such as the heart where clotting will help dispatch the victim. Thus a number of avenues still exist for future investigation, with the intent of fully understanding the interactions of the procoagulant subunits and the actions of each, to explain the clotting actions reported.

This work also provides the basis for the further investigation of the (Group III) prothrombin activator in the venom of the taipan. The determination of the sequence of the 50kDa and 43kDa bands may reveal homology to factor Xa and factor V respectively, mirroring that found for the brown snake in this study.

REFERENCES

Govers-Riemslag, J.W.P., Speijer, H., Zwaal, R.F.A., Rosing, J. (1988). Purification and characterization of the prothrombin activator from *Oxyuranus scutellatus* (Taipan snake). In: Hemostasis and animal venoms. Eds: Pirkle, H., Markland, F.S. Marcel Dekker, Inc.

Joseph, J.S., Chung, M.C.M., Jeyaseelan, K., Manjunatha Kini, R. (1999). Amino acid sequence of Trocarin, a protrhombin activator from *Tropidechis carinatus* venom: Its structural similarity to coagulation factor Xa. Blood **94**, 621-631.

Markland, F.S. (1998). Snake venom fibrinogenolytic and fibrinolytic enzymes: An updated inventory. Thromb. Haemost. **79**, 668-674.

Masci, P.P., Whitaker, A.N., deJersey, J. (1988). Purification and characterization of a prothrombin activator from the venom of *Pseudonaja textilis textilis*. Biochem. Int. **17**, 825-835.

Pirkle, H. (1998). Thrombin-like enzymes from snake venoms: An updated inventory. Thromb. Haemost. **79**, 675-683.

Rosing, J., Tans, G. (1991). Inventory of exogenous prothrombin activators. Thromb. Haemost. **65**, 627-630.

Smith, S., Brinkhous, M. (1991). Inventory of exogenous platelet-aggregating agents derived from venoms. Thromb. Haemost. **66**, 259-263.

Speijer, H., Govers-Riemslag, J.W.P., Zwaal, R.F.A., Rosing, J. (1986). Prothrombin activation by an activator from the venom of *Oxyuranus scutellatus* (Taipan snake). J. Biol. Chem. **261**,13258-13267.

Stocker, K., Hauer, H., Müller, C., Triplett, D.A. (1994). Isolation and characterization of Textarin®, a prothrombin activator from eastern brown snake (*Pseudonaja textilis*) venom. Toxicon **32**, 1227-1236.

Tans, G., Govers-Riemslag, J.W.P., van Rijn, J.L.M.L., Rosing, J. (1985). Purification and properties of a prothrombin activator from the venom of *Notechis scutatus scutatus*. J. Biol. Chem. **260**, 9366-9372.

Teng, C-M., Huang, T-F. (1991). Inventory of exogenous inhibitors of platelet aggregation. Thromb. Haemost. **65**, 624-626.

Tibballs, J., Sutherland, S.K., Rivera, R.A., Masci, P.P. (1992). The cardiovascular and haematological effects of purified prothrombin activator from the common brown snake (*Pseudonaja textilis*) and their antagonism with heparin. Anaesth. Intens. Care **20**, 28-32.

Tibballs, J., Sutherland, S.K. (1992). The efficacy of heparin in the treatment of common brown snake (*Pseudonaja textilis*) envenomation. Anaesth. Intens. Care **20**, 33-37.

Triplett, D.A., Stocker, K.F., Unger, G.A., Barna, L.K. (1993). The Textarin/Ecarin ratio: a confirmatory test for lupus anticoagulants. Thromb. Haemost. **70**, 925-931.

Williams, V., White, J. (1989). Purification and properties of a procoagulant from Peninsula tiger snake (*Notechis ater niger*) venom. Toxicon **27**, 773-779.

EPILOGUE

'If by any accident, a man is bitten, it is his own fault if any bad effects follow (or the fault of those about him), as a penknife and a tumbler or two of pure brandy, with a few hours rest on the ground will suffice for treatment. As I can produce between three and four hundred cases of recovery from snake-poisoning in these colonies during the last fifteen years, I think this justifies me in arriving at this conclusion.'

Berncastle, J. (1870).

In discussing strychnine as a snakebite remedy.....
'...that its unfailing efficacy has been proven by other medical men during the last two years in numerous telling cases, and that in consequence deaths from snake-bite are almost events of the past in Australia....'

Mueller, A. (1890).

'The physician regards as a remedy for the disorder that medicament which has been followed by recovery, when in sound logic, no other deduction can be drawn than the vaunted remedy has not killed the patient: and we see that the physician quietly reasons and believes that the sick person would have certainly died had he not been treated by him, and with this supposes that which he does not know, and which is most likely altogether untrue.'

Fontana, F. (1782).

PUBLICATIONS

White, J. Tompkins, D. Steven, I. and **Williams, V**. (1983-4). Tiger snake bite. *Records of the Adelaide Children's Hospital* **3**, 169 - 173.

Sweeney, D. and **Williams, V**. (1987). The effect of halothane general anaesthesia on platelet function. *Anaesth. Intens. Care* **15**, 278 - 281.

Williams, V. and White, J. (1987). Variation in venom constituents within a single isolated population of Peninsula tiger snake (*Notechis ater niger*). *Toxicon* **25**, 1240 - 1243.

White, J. **Williams, V**. and Passehl, J. (1987). The five-ringed brown snake, *Pseudonaja modesta* (Gunther) : report of a bite and comments on the venom. *Med. J. Aust.***147**, 603 - 605.

Williams, V. White, J. Schwaner, T.D. and Sparrow, A. (1988). Variation in venom proteins from isolated populations of tiger snakes (*Notechis ater niger, N. scutatus*) in South Australia. *Toxicon* **26**, 1067 - 1075.

White, J. and **Williams, V**. (1989). Severe envenomation with convulsion following multiple bites by a common brown snake, *Pseudonaja textilis. Aust. Paediatr. J.* **25**, 109 -111.

Williams, V. and White, J. (1989). Purification and properties of a procoagulant from Peninsula tiger snake (*Notechis ater niger*). *Toxicon* **27**,773 - 779.

White, J. **Williams, V**. and Duncan, B. (1989). Lymphopenia after snakebite. *Lancet*, Dec. **16**, 1448 - 1449.

Williams, V. and White, J. (1990). Variation in venom composition and reactivity in two specimens of yellow-faced whip snake (*Demansia psammophis*) from the same geographical area. *Toxicon* **28**, 1351 - 1354.

Goldwater, P. **Williams, V**. Bourne, A. and Byard, R. (1990). Sudden infant death syndrome: a possible clue to causation. *Med. J. Aust.* **153**, 59.

Chippaux, J-P. **Williams, V**. and White, J. (1991). Snake venom variability: methods of study, results and interpretation. *Toxicon* **29**, 1279 - 1303.

Williams, V. and White, J. (1992). Variation in the composition of the venom from a single specimen of *Pseudonaja textilis* (common brown snake) over one year. *Toxicon* **30**, 202 - 206.

Williams, V. Berridge, D. Adams, M. and Griffiths, A. (1992). The appearance of a circulating anticoagulant in a patient on short term chlorpromazine. *Aust. J. Med. Sci.* **13**, 92 - 93.

White, J. Duncan, B. Wilson, C. **Williams, V**. and Lloyd, J. (1992). Coagulopathy following Australian elapid snakebite: A review of 20 cases. In: *Recent advances in toxinology research.* **Vol 1**. Eds. Gopalakrishnakone, P. and Tan, C. K. Venom and Toxin Group, National University of Singapore.

Williams, V. White, J. and Mirtschin, P. (1993). Comparative study on the procoagulant from the venom of Australian brown snakes (Elapidae: *Pseudonaja* spp.) In: *Advances in venom and toxin research.* Eds. Tan, N.H. Oo, S.L. Thambyrajah, V. and Azila, N. Malaysian Society on Toxinology, Kuala Lumpur, Malaysia.

Williams, V. White, J. and Mirtschin, P. (1994). Comparative study on the procoagulant from the venom of Australian brown snakes (Elapidae: *Pseudonaja* spp.). *Toxicon* **32**, 453 - 459.

Williams, V. and White, J. (1997). Snake venom and snakebite in Australia. In: *Ecology, evolution and snakebite. Venomous snakes.* pp. 205-217. Eds. Thorpe, R. Wüster, W. and Malhotra, A. Oxford Science Publications and The Zoological Society of London.

Williams, V. and Vining, R. (1998). Bioequivalence of Coumadin and Marevan. *Aust. Prescrib.* **21**, 61.

Williams, V. Currie, B. and Selvarajah, V. (1999). Snakebite? *Thromb. Haemost.* **81**, 478.