Pharmacological and Immunological Studies Aimed at Prevention of *Pimelea* Poisoning of Cattle

by

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Submitted for the Research Degree of Master of Applied Science

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ABSTRACT

Poisoning by *Pimelea* plants causes annual production losses exceeding $10 million to the Queensland cattle industry. Poisoning is caused either by ingestion of plants or by inhalation of dried plant debris. Daphnane and tigliane diterpenoid toxins found in the various *Pimelea* species are absorbed into the blood and cause pathological responses in target tissues through prolonged activation of protein kinase C isoenzymes. Simplexin, huratoxin and derivatives are the most toxic compounds prevalent in *Pimelea* species causing poisoning of cattle. Cattle are more vulnerable than sheep and horses to the toxins because the bovine pulmonary venous system has distinct sphincter -like arrangements in the smooth muscle coating of the venules. Prolonged contraction of the pulmonary venous system leads to oedema, jugular distension and right heart failure.

At the present time there is no effective means of controlling *Pimelea* poisoning in cattle, apart from removing animals from pastures infected with the plant. The main objectives of the research program described in this thesis was to determine whether the toxins could be made immunogenic and if so, investigate whether antibodies raised to the toxins offered any protection against *Pimelea* toxicity. The study also provided the opportunity to systematically examine the *in vitro* contractile effects of the toxins on bovine pulmonary venule preparations. These organ bath experiments also allowed investigation of the efficacy of purified anti-toxin antibodies and potential antagonists of the toxins.

The family of *Pimelea* diterpene toxins were isolated from dried Pimelea *trichostachya* plant material through a combination of solvent extraction and partition, column chromatography and preparative reverse phase HPLC. The

toxins were conjugated to carrier proteins (HSA or ovalbumin) through a succinylate linker. Two structurally similar and commercially available daphnane orthoesters, mezerein and resiniferinol were also conjugated to carrier protein.

Two groups of rabbits were vaccinated with the *Pimelea*- derived protein conjugates. ELISA methodology was developed to detect the presence of specific antibodies against *Pimelea* toxins from the serum of the vaccinated animals. After confirming the presence of specific IgG against *Pimelea* toxins in vaccinated rabbits, three groups of cattle were vaccinated with vaccines prepared from *Pimelea,* mezerein and resiniferinol protein conjugates. Cattle were vaccinated with the mezerein and resiniferinol conjugates to investigate the possibility of using commercially available compounds for vaccine preparation. The ELISA results for the vaccinated cattle showed the presence of specific antibodies which recognised toxin-protein conjugates coated to the plates. The ELISA cross reactivity data also showed that antibodies raised in animals vaccinated with mezerein and resiniferinol conjugates also recognised the *Pimelea* toxins.

Vaccinated and control cattle were exposed to *Pimelea trichostachya* plant material to establish whether the antibodies had any protective effects. Both groups of cattle developed symptoms of *Pimelea* poisoning over two weeks, although these animals were dosed with much higher *Pimelea* concentrations than would be expected under normal grazing conditions. Various blood parameters were monitored throughout this experiment and there were some trends evident under the lower dose regimes that vaccinated animals were less affected than controls.

The organ bath studies of isolated bovine pulmonary venule preparations showed that the EC_{50} toxin concentration required to elicit half maximal contraction (relative to the 1.0 μM 5-HT response) was in the sub-micromolar range. Purified rabbit anti-toxin IgG was shown to attenuate the contractibility of bovine pulmonary venules in response to *Pimelea* toxins (EC_{50}) in a dose dependent manner, whereas the purified bovine anti-toxin IgG lacked any efficacy. *Nux vomica* CM tincture (at doses above 200 μL in the 25 mL baths) completely inhibited the contraction in response to *Pimelea* toxins (EC_{50}).

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Author's Declaration

I declare that this thesis is my own work and has not been submitted in any form for another award at any other university of institution of tertiary education. Information derived from the published or unpublished work of others has been acknowledged in the text and a complete list of references is provided.

Signature Redacted

Geeta

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Publications

Book Chapter

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Oberoi, G., D'Occhio, M.J., Hoey, A. and Pegg, G.G. (1993) " *Nux Vomica* Attenuates Constriction of Bovine Pulmonary Venules induced by *Pimelea* Toxins." *Clinical and Experimental Pharmacology and Physiology*, Suppl. 1, 53.

(Note : The candidate enrolled originally as Geeta Oberoi, but during the course of her studies was divorced. Hence her name appears as G. Oberoi on these early publications, but the thesis is submitted under her maiden name, Geeta Nayyar.)

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CHAPTER ONE

PIMELEA POISONING OF CATTLE

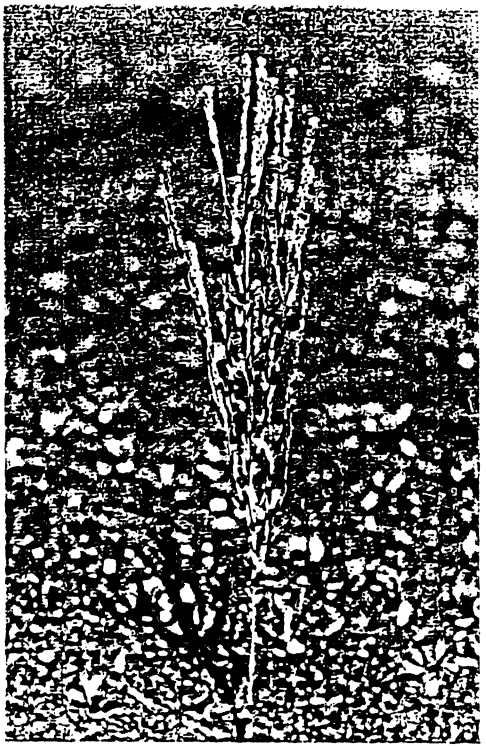
1.1 ECOLOGICAL, EPIDEMIOLOGICAL AND MANAGERIAL ASPECTS OF *Pimelea* POISONING IN QUEENSLAND

Pimelea poisoning is an often fatal sickness in cattle caused either by the ingestion of fresh or dried plant material and/or inhalation of the plant dust of poisonous *Pimelea* species (Cantello 1969; McClure and Farrow 1971, Roberts and Healy 1971, Clark 1971). It is a seasonal problem, usually occurring in the spring-early summer but the lasting toxic nature of the weed and plant debris presents a risk throughout the year. *Pimelea* poisoning is estimated to cost the Queensland beef industry between $7.25 million and $10 million (1990 values) per year in lost production (McKenzie,1985; Pressland and Dadswell, 1992). *Pimelea* poisoning is also known as St. George disease, big head, Marree disease, *Pimelea* or flax weed poisoning. It was first recorded in Australia in 1938 at St. George, Queensland, (Maunder, 1947) and the first outbreak occurred in South Australia in 1951. It was common then for cattle to be brought from the Northern Territory, delivered to Marree by rail and then walked to their destinations. The disease tended to affect cattle shortly after their departure from Marree, with drovers reporting up to half of the mob dying (Trengover, 1982; Dodson, 1965).

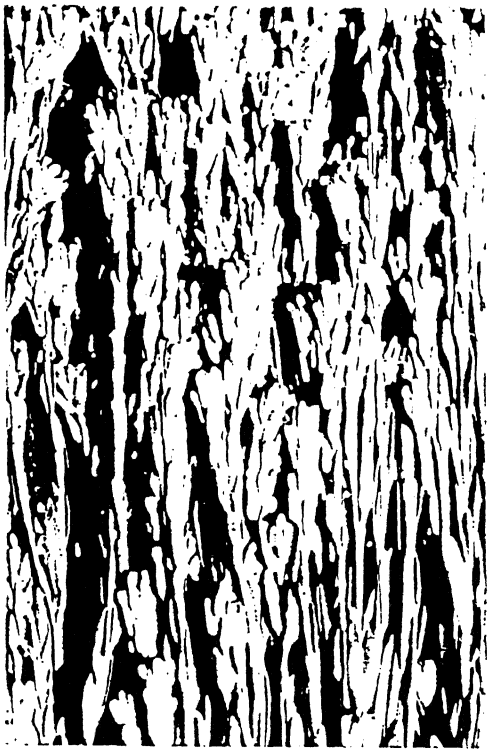
In Queensland, *Pimelea* poisoning is primarily caused by three herbaceous plants namely, *Pimelea simplex* (*P. simplex* comprises two subspecies - *simplex* and *continua*) *Pimelea trichostachya* and *Pimelea elongata*. These plants are native to Australia and are small annual herbs which usually grow less than 50 cm high at maturity. *P. simplex* is commonly known as the desert rice flower and *P. trichostachya* as flax weed or poverty weed (Anon, 1991. Figure. 1.1). These plants predominantly grow in the Longreach, Blackall, Charleville, Cunnamulla, Quilipie, Thargomindah, Mitchell, Roma and St George areas, in

Figure 1.1

Pimelea Species



Pimelea elongata (whole plant)



Pimelea elongata (seed heads)

Pimelea trichostachya (whole plant)

Pimelea trichostachya (seed heads)

an area below the Tropic of Capricorn and in inland areas of Queensland. In the Roma district of South Western Queensland, *P. trichostachya* commonly grows to 0.5 m in height and in some pastures contributes more than 30 % of the biomass (Pressland and Dadswell, 1992).

Germination

Pimelea generally thrives on light red, sandy, less fertile soils where pasture grasses are often severely over grazed, thus offering less competition. *Pimelea* species are hard-seeded. Evidence indicates that the seeds can remain viable in the soil for years. Thus it has high potential for survival even under very adverse conditions. Laboratory results from the Queenland Department of Primary Industries (QDPI) indicated that germination of *Pimelea* seeds occurred with night temperatures of 15° C to 20° C, and day temperatures of 20° C to 25° C. These optimum germination conditions coincide with the late summer/autumn period. Good rain in late summer/autumn stimulates the germination process. Hence establishment and survival of the plant is more likely in the south than the north, because of higher temperatures and lower probability of rain at the optimum time for germination in the north compared to southern districts (Pressland and Dadswell, 1992; Graham and Schefe, 1991).

Outbreaks of *Pimelea* poisoning generally occur towards the end of the year (August to December). Anecdotal evidence (Cunningham *et al.*, 1981) suggests that poisoning tends to be more common in years where a previous dry summer has left areas in the pasture bare of perennial grass due to overgrazing. A wet autumn or winter then stimulates germination of the *Pimelea* seed reserve. Thus, overgrazing and winter rain following a period of drought are conducive to outbreaks of *Pimelea* poisoning. Under these conditions, the density of *Pimelea* species in the pasture increases, leading to a higher

consumption of *Pimelea* by cattle and resultant poisoning (Pressland and Dadswell, 1992).

Susceptibility

All cattle are susceptible to *Pimelea* poisoning, regardless of genotype, sex and age. It seems that 18 month to 2 year old beasts are most commonly affected. The condition of the animal is not related to susceptibility, fat healthy cattle being equally likely to succumb to the disease as those in poor condition. Poisoning occurs in Brahmans as well as British breeds and crossbreds, and in home bred as well as introduced cattle; poisoning occurs less frequently in bullocks, steers and calves than in bulls, cows, heifers and weaners (Pressland and Dadswell, 1992).

Due to the unpalatable nature of *Pimelea* especially when green, native station cattle generally avoid grazing it. This explains the observation that stock introduced into *Pimelea* infested areas are more likely to develop signs of the disease than cattle native to these areas. *Pimelea* poisoning generally occurs when feed has become scarce and cattle are foraging less palatable feed; or when rainfall has produced a short stand of green feed, encouraging close grazing of associated green or dead flax weed (Trengove, 1982).

Confinement of affected animals with abundant good feed, such as wheaten or lucerne hay, supplementary vitamins and minerals and water usually overcomes the problem over a 3-4 week period, provided the condition is not too advanced. Farmers report that it is important to avoid stressing affected animals because this may result in sudden death. For example, badly affected animals need to be transported to the home paddock, as even the stress of walking can kill the animals.

Management

Producers find management of *Pimelea* difficult. Property sizes in the region often exceed 10,000 ha, making physical and/or chemical destruction of the plants prohibitive from an economic perspective. Some property owners have experimented with burning the plants, but available evidence seems to suggest that this practice encourages germination of seeds in the subsequent growing season. The seeds of *Pimelea* apparently remain viable in the soil for more than two years and these seeds are also light and fluffy and are likely to be easily carried by wind and dust. Farmers who attempt cultivation of the soil in badly affected areas find that this practice encourages growth of *Pimelea* .

The impact of *Pimelea* poisoning can be minimised by reserving an area of pasture with little or no *Pimelea* so that stock can be shifted from infested areas as soon as signs of the syndrome appears. *Pimelea* growth can be controlled by ensuring pasture seed (for example, buffel grass) is free of seeds of *Pimelea* and also by avoiding over-grazing of pasture land by cattle as *Pimelea* thrives when competition with other plants species is reduced. Grazing *Pimelea* infested areas when the plant is green is advocated by some producers since they believe the plant is less toxic at this stage. Since sheep are less sensitive to *Pimelea* toxicity than cattle, producers with mixed cattle/sheep operations tend to run their sheep in the paddocks most affected by *Pimelea* infestation (Pressland and Dadswell, 1992).

1.2 *Pimelea* TOXINS

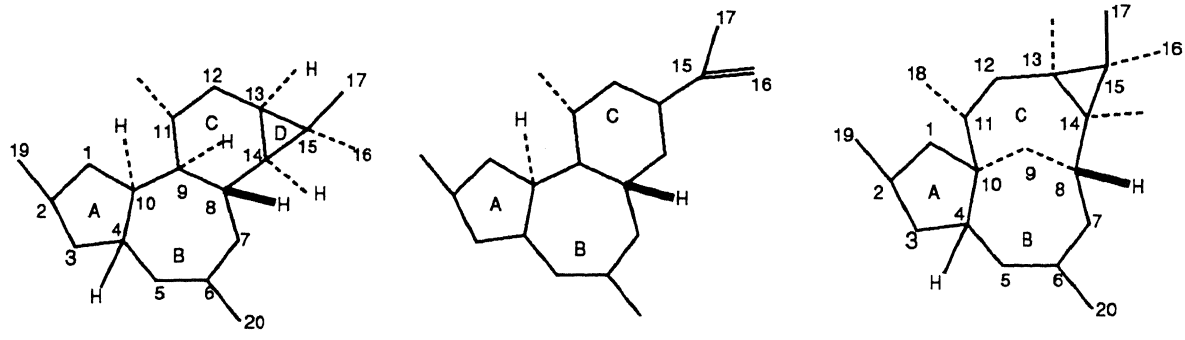
Representatives of the genus *Pimelea* (family Thymelaeaceae) occur in South Africa, New Zealand, Timor and New Guinea. Thymelaeaceae are a medium sized plant family of 650 species distributed among some 50 genera. Representatives of the Thymaleaceae family occur in South Africa, Australia,

New Zealand, the Mediterranean region, South America and the Steppes of Asia. Diterpene toxins have been isolated from several genera of Thymaleaceae (Borris *et al.*, 1988). Similar diterpene toxins have also been isolated from several genera of the closely related Euphorbiaceae family. Systemic toxicity symptoms resulting from the ingestion of plant materials is well established for humans as well as for several animal species (sheep, cattle, horse, goats etc) which is essentially constant throughout the Thymelaeaceae (Borris *et al.*, 1988).

The diterpene toxins of the Thymaleaceae are collectively known as tiglianes, daphnanes and ingenanes (Figure 1.2, adapted from Evan and Soper, 1978). These have been shown to occur in several genera of the families Euphorbiaceae and Thymaleaceae. These compounds are structurally related hydrocarbons consisting of tri- or tetracyclic rings which occur in higher plants as their O-acyl esters or more rarely as ortho-ester forms. Phytochemical evidence available indicates that these diterpene esters are responsible for toxic reactions and skin-irritant properties observed (Evans and Soper, 1978).

Figure 1.2

Structure of different Diterpenes found in Thymaleaceae species.



Interest in these compounds has centred upon two toxicological actions which are exhibited to a greater or lesser degree in test animals:

1. Intense inflammation produced on application to the skin (Kinghorn *et al.*, 1975).
2. Tumour-promoting activity arising from continued application to mice following a sub-threshold dose of a carcinogen (Berenblum *et al.*, 1947).

Two of the more conspicuous changes that occur in skin treated with diterpene esters, particularly those possessing tumour-promoting activity, are cell proliferation and hyperplasia. Some investigators have considered hyperplasia and proliferation to be critical events in tumour promotion (Frei *et al.*, 1968) but others have rejected this view (Raick *et al.*, 1972). Diterpene esters are able to modify the differentiation of epidermal cells, returning them to a less differentiated state. These ultrastructural changes induced by diterpene esters are, however, not permanent. For example, Raick and coworkers (1972), described histological changes that occur after a single application of 0.016 μg of a phorbol ester such as TPA to normal adult mouse epidermis skin. Within 48 hours, such TPA-stimulated cells acquire a secretory activity not evident in normal adult mouse epidermis. These TPA-stimulated dark epidermal cells are smaller, contain large mitochondria rich in cristae, and their nuclear and cytoplasmic matrices are highly electrodense compared to normal untreated mouse epidermal cells. It has been suggested that these less differentiated dark cells are precursors of neoplastic cells. These dark cells are reversed almost completely after four weeks (Evans and Soper, 1978).

Diterpene esters are amphipathic in nature. The tumour promoting (Baird *et al.*, 1971) and irritant effects are dependent upon the presence of a lipophilic side chain. There is much current interest in the chemical constituents of the Thymaleaceae as either potential therapeutic agents or new tools for cancer

research. Many members of the family have been used in traditional medicines and in hunting. Six of the genera belonging to Thymelaeaceae (eg. Daphne) contain about 40 species to have found extensive (China and India to Europe) application in the treatment of cancer. A substantial number of species are used in other primitive medical treatments (such as epilepsy, malaria, snake-bite, and certain viral infections) (Pettit *et al.*, 1983). Of clinical significance is the observation that certain of these esters have an anti-leukemic action and further structure activity studies are required to ascertain the structural features necessary for tumour promotion on one hand and anti-leukemic action on the other.

Toxins isolated from *Pimelea* species causing *Pimelea* poisoning

Simplexin is a diterpenoid orthoester which has been isolated from several *Pimelea* species and is known to be the primary cause of *Pimelea* poisoning in cattle in conjunction with other diterpenoid toxins present in *Pimelea* species. The first of the daphnane diterpenoid toxins isolated from *Pimelea simplex* (simplexin) was reported by Roberts *et al* (1975). Simplexin is a highly toxic compound, the LD_{50} for mice being 1 mg/kg. Simplexin is highly irritant in the mouse ear test and is moderately active as a co-carcinogen. In one instance intravenous injection of 9 mg simplexin into a calf (100 kg) caused death within 0.5 h. (Freeman *et al.*, 1979). Simplexin was identified from its similar spectral characteristics to the piscicide huratoxin (Sakata *et al.*, 1971).

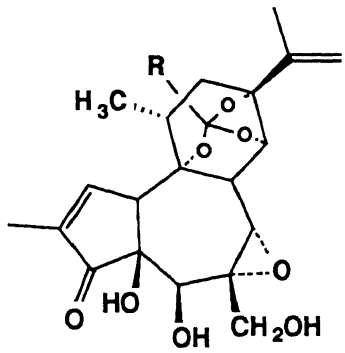
Robert *et al* (1975) reported that intravenous administration of simplexin produced a three fold increase in pulmonary arterial pressure within 100 secs at a dose of 400 μg (4 μg/kg) and oral dosing with simplexin produced the range of symptoms characteristic of *Pimelea* poisoning. Simplexin has been isolated from diverse *Pimelea* species such as *P. prostrata* (Pettit *et al.*, 1983); *P.*

trichostachya and *P. simplex* (Freeman *et al.*, 1979). The latter two species are associated with *Pimelea* poisoning of cattle in Queensland.

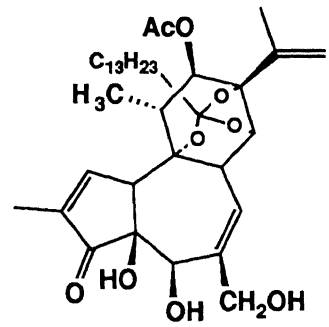
A family of daphnane diterpenes related to simplexin (Figure 1.3) was isolated from *P. simplex* and *P. trichostachya* and reported by Freeman *et al* ., (1979). Interestingly they reported 12β -acetoxyhuratoxin, tigliane derivatives related to mancinellin and loliolide in extracts of *P. simplex* and *P. trichostachya*. Further daphnane orthoesters were isolated from *Pimelea species* by Hafez *et al.* (1983) and Tyler and Howden (1985).

Figure 1.3

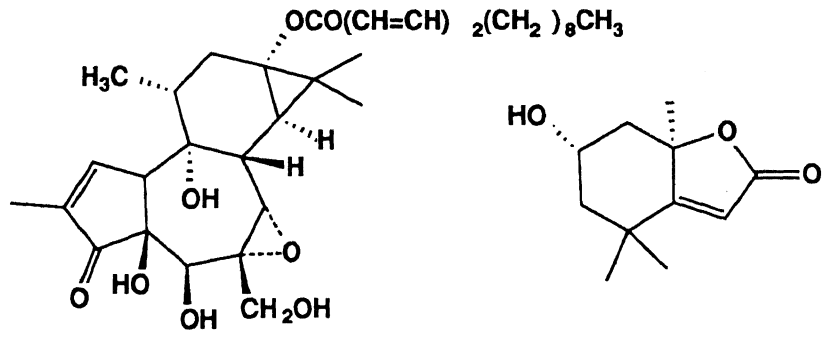
Various terpenoid compounds isolated from *P. trichostachya*.



SIMPLEXIN (R =C_9H_{19})
HURATOXIN (R= $C_{13}H_{23}$)



12-ACETOXY HURATOXIN



TIGLIANE ESTER (Mancinellin analogue) **LOLIOLIDE**

An interesting observation is that crude extracts of *P. simplex* (Horward and Howden, 1975) and *P. prostata* (Cashmore *et al.*, 1976) exhibited antileukaemic activity . These authors also report that extracts of *P. linifolia* and *P. ligustrina* possess antineoplastic activity.

1.3 SYMPTOMS OF *Pimelea* POISONING IN CATTLE

Correlation of the disease state with the occurrence of *Pimelea* species in the pasture was recognised in the late 1960's. Clark (1973) studied the pathogenesis of the condition through intoxication of cattle with dried, finely milled *Pimelea* plant material (introduced into the rumen by stomach tube). Animals receiving more than 50 mg kg^{-1}body weight day^{-1} of the dried material developed jugular distension, diarrhoea and mucous discharge from the eyes and nostrils within 3 days (Figure 1.4). Continuation of this dosage led to oedema of the neck and brisket and death of the animals within one week. Intoxication of animals with lower doses (25 mg kg^{-1} body weight day^{-1} and 15 mg kg^{-1}body weight day^{-1}) gave rise to recognisable signs of the disease state within 2 weeks and 28 weeks respectively.

The clinical and gross post-mortem findings in field cases vary considerably (Maunder 1947, Seawright and Francis 1971). Symptoms of *Pimelea* poisoning usually are diarrhoea, wasting, dependent oedema, reduced exercise tolerance, anaemia, hydrothorax, cardiac dilation, expanded hepatic sinusoids and portal venules (peliosis hepatis) and oedema of lymph nodes, depending upon the extent of the poisoning in each individual case.

One of the early symptoms (Clark, 1971; Roberts and Healy, 1971; McClure and Farrow, 1971) is profuse diarrhoea generally accompanied with loss of condition, roughening of the coat and jugular distension and pulsation. The animal becomes lethargic and has a peculiar, dejected walk, giving the

Figure 1.4

Cattle showing clinical signs of *Pimelea* poisoning: oedematous swelling of the head, jaws and brisket. Also note the prominent jugular vein.

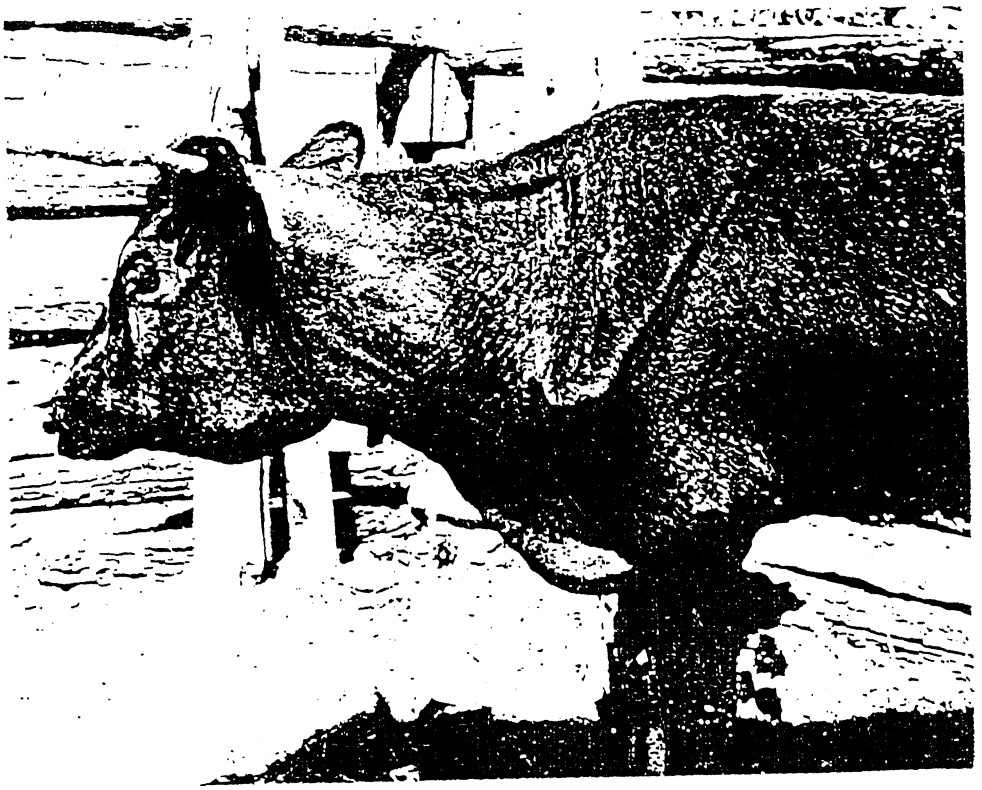
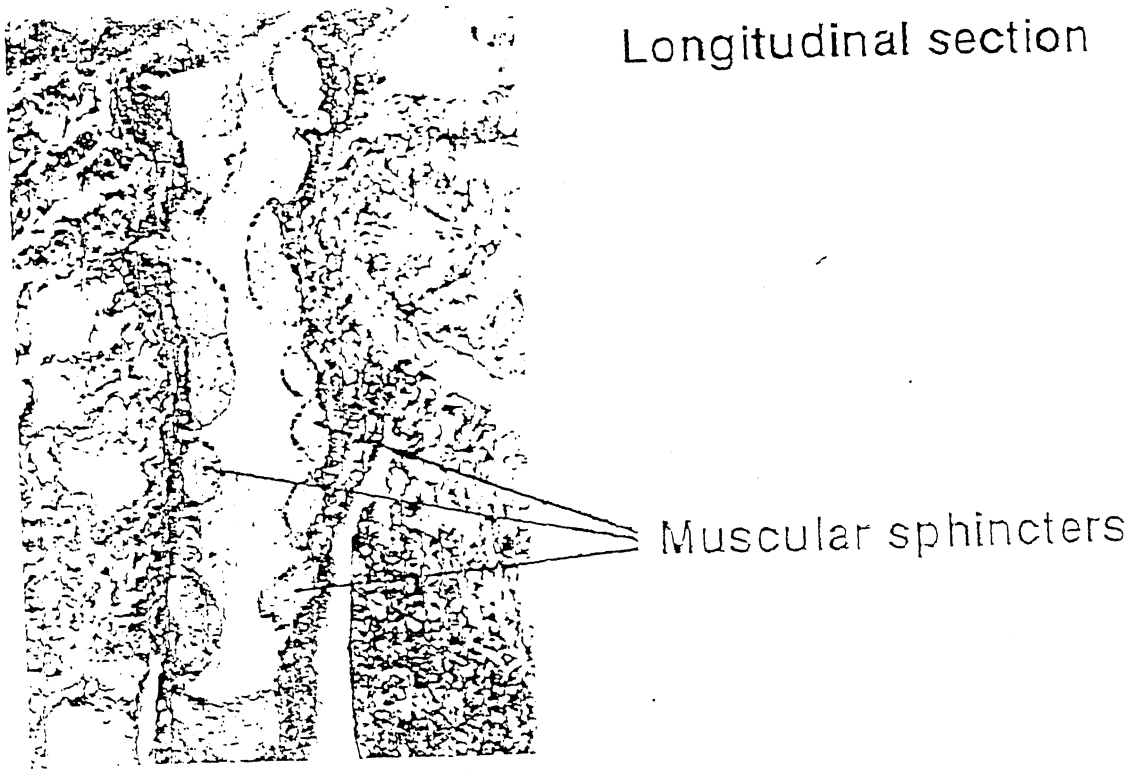


Figure 1.5

Longitudinal section of a normal bovine pulmonary venule (x 200).



impression of abdominal or thoracic pain. As the disease progresses, the oedema extends to the base of the neck, brisket and forelegs. Sometimes jugular distension and pulsation may occur before diarrhoea is evident. During oral dosage experiments, diarrhoea generally commences after 4-6 days. Diarrhoea persists with undigested blood appearing intermittently in the faeces. The severity and occurrence of diarrhoea and oedema are variable. Severe diarrhoea has been a feature of many of the outbreaks, although not every affected animal in the mob may necessarily scour. Generally, most of the affected cattle display varying degrees of a combination of diarrhoea and oedema rather than either extreme condition. The natural disease is caused by ingestion or inhalation of the poisonous plant material, or a combination of both. The extent and occurrence of the diarrhoea is dependent upon whether inhalation or ingestion predominates. Clark (1973) reported inhalation experiments where animals did not ingest the toxic plant material and these animals showed oedema without diarrhoea. The consistent trend is that diarrhoea is associated with ingestion of the toxic plant material. It is also possible to produce oedema with minimal diarrhoea through intravenous injection of the plant extract (Clark, 1973).

Cattle with *Pimelea* poisoning develop phlebectatic peliosis hepatitis (Seawright, 1984; Seawright and Francis, 1971). The term "peliosis" means blue-black and gross post-mortem findings in the advanced cases of the disease showed an enlarged swollen, bluish purple liver with liver lesions evident. This swelling of the liver is essentially due to massive dilation of the intrahepatic portal capillary bed. Distension was observed in most of the smallest branches of the portal vein, and the sinusoids into which they flowed were also dilated. Dilation of the portal venules caused formation of huge cavities lined by hepatocytes and Kuffer cells. Such distension of the sinusoids may lead to a breakdown of parenchymal structure and extreme atrophy of the

hepatocytes. The extent of these pathological symptoms were observed to vary from case to case.

As the disease progresses, the oedema extends to the base of the neck, brisket and forelegs. The dilated right heart, distended systemic veins and dependent oedema then leads to the right sided heart failure (Rogers and Roberts, 1976; Clark,1973; Kelly,1975a). Heart failure is the primary cause of death in poisoned animals due to increased pulmonary vascular resistance.

In the progressive stage of the disease Kelly, (1975b) noted a fall in haemoglobin concentration, red cell count, packed cell volume and plasma protein concentration resulting from haemodilution. The effect of *Pimelea* toxins on total plasma protein was more varied and Kelly concluded that hypoproteinaemia was not responsible for the subcutaneous oedema characteristic of *Pimelea* poisoning as suggested earlier by McClure and Farrow (1971).

Increased pulmonary resistance

Alexander and Jensen (1963) investigated the structure of the bovine pulmonary vasculature in normal cattle using serial histologic sections and corrosion cast preparations. They reported that the bovine pulmonary vasculature is unusual in that both the arteries and veins have a heavy muscular coat even down to a vascular diameter of 20μm . A distinct variation in venule and arteriole muscular media was observed in vessels of 300-400μm or less in diameter. The muscular media of veins less than approximately 300-400μm was characterised by abrupt disruptions in continuity (Figure 1.5). The thick interrupted muscular media were classified as sphincter-like structures. These were observed in veins down to a size of approximate 20 μm. In arteries these abrupt sphincter-like discontinuities in the media were not observed.

Cross sections of both arteries and arterioles revealed uniform wall thickness with no sphincter-like structures as observed in venules. Castigili (1958) referred to the thick muscular veins in the lungs of cattle as sphincter veins. Best and Heath (1961) also reported similar disruptions in the histological study of longitudinal sections of bovine pulmonary venules. These sections had a beaded appearance due to fibromuscular masses protruding into the lumen of the vessel. In transverse sections, the lumens of the smaller pulmonary veins appeared almost occluded by these muscular masses.

The distinct muscular sphincters present along the length of the pulmonary venules of cattle appear to be absent in sheep and horses (Alexander and Jensen, 1963). Sheep and horses can graze areas where *P. trichostachya* grows without developing dependent oedema. Therefore constriction of these sphincters in the bovine not only offers a pathogenesis for the condition but also an anatomical reason why only cattle are affected. Clark (1973) reported that intravenous injection of an ethanolic extract of the plant (at doses above a dried-plant equivalent of 130 mg kg^{-1}body weight threshold) caused rapid cardiovascular effects. Within 10 seconds of administration of the dose, systemic arterial pressure halved while right ventricular pressure doubled. These results were suggestive of immediate constriction of the pulmonary venous system. Autopsy examination of severely poisoned animals revealed hydrothorax and dilation of both the pulmonary artery and the right side of the heart.

Marked constriction of pulmonary venule sphincters occurs during *Pimelea* poisoning. This leads to an increase in the pressure of the pulmonary capillary bed, pulmonary arterial system and right ventricle, accompanied by variable pulmonary oedema. If the constriction continues, dilation of the right ventricle causes the right atrio-ventricular valve to close incompletely, allowing

regurgitation during ventricular systole. This increases the systemic venous pressure, and is expressed clinically first as distension and pulsation of the jugular veins and eventually as dependent oedema. This correlates well with the cardiovascular changes observed in a series of autopsies on natural and experimental *Pimelea* poisoning cases. After comparison of animals killed at various stages of the disease it appears likely that hydroperciardium and any pulmonary oedema develop when the hypertension is still contained within the pulmonary circulation, and there is as yet no change in heart sound or jugular vein appearance. Then after right atrio-ventricular valve insufficiency has developed, as evidence by heart sound changes, jugular distension and pulsation, dependent oedema and hydrothorax appear as a secondary systemic venous hypertension increases (Clark, 1973).

Mason (1976) showed that *in vitro* alcohol extracts of the plant caused contraction of the bovine venule tissue in organ bath studies which was in accordance with cardiovascular results obtained by Clark (1973) described above. These observations are discussed more fully in the introduction to Chapter 4.

1.4 PROTEIN KINASE C (PKC)

The primary cause of *Pimelea* poisoning is assumed to be prolonged activation of various isoenzymes of protein kinase C (PKC) by the slowly metabolised lipophilic diterpene toxins present in *Pimelea* plants. PKC plays a very important role in various physiological processes and the enzyme is normally transiently activated in the body by receptor mediated formation of diacylglycerol (DAG).

1.4.1 Receptor mediated activation of PKC

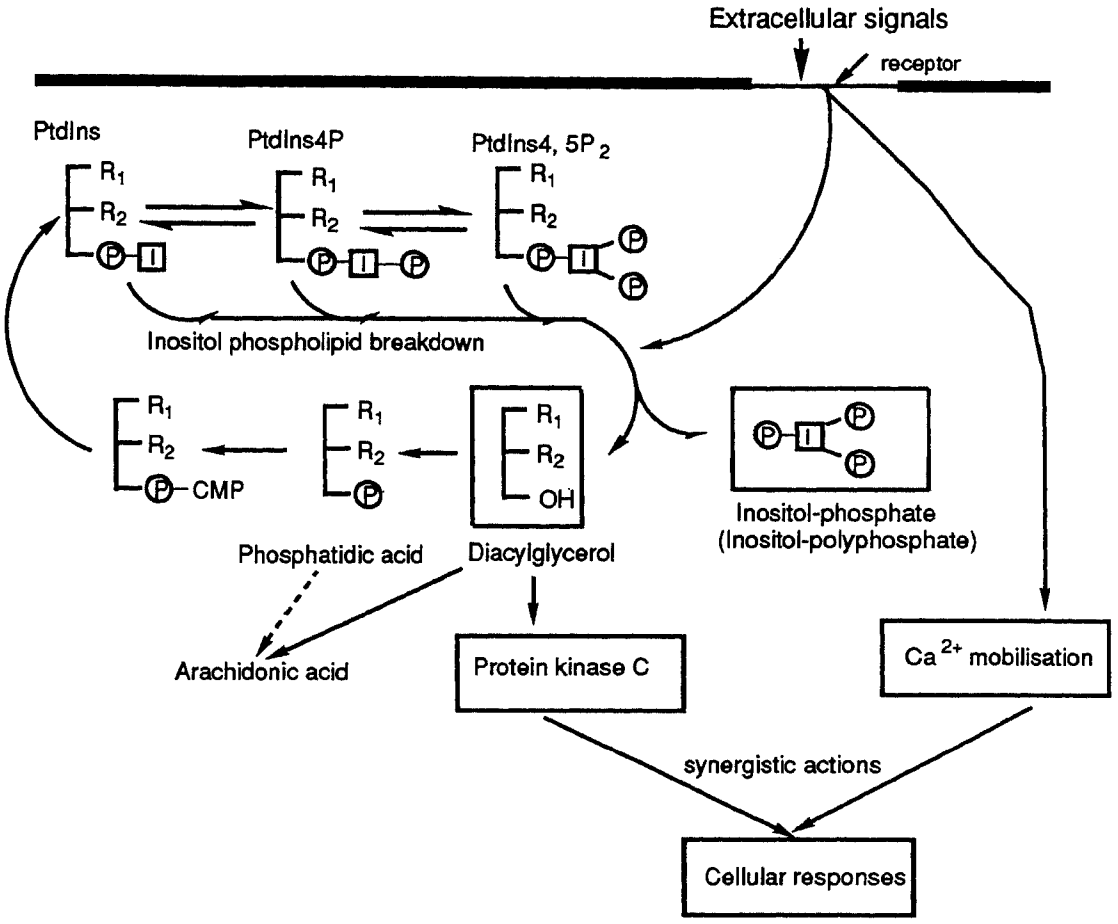
Stimulation of α_1-adrenergic receptors induces inositol phospholipid (PI) breakdown. Phosphatidylinositol-4-phosphate (PIP) and phosphatidylinositol-4,5-biphosphate (PIP2) are produced by sequential phosphorylation of the myo-inositol moiety (PI) initiated by extracellular signals such as certain hormones, neurotransmitters, antigens, growth factors and many biologically active substances. PIP2 is further hydrolysed to yield inositol-1,4,5-triphosphate and diacylglycerol (DAG) (Figure 1.6, adapted from Nishizuka, 1984). Inositol 1,4,5-triphosphate serves as mediator of Ca^{+2} mobilisation from an internal store, probably located in the endoplasmic reticulum. 1,2 - Diacylglycerol remains in the membrane and initiates the activation of PKC. Kinetic analysis indicates that a small amount of DAG dramatically increases the apparent affinity of PKC for Ca^{+2}. PKC activation and Ca^{+2} mobilisation play an important role (often synergistically) in control of various cellular functions and in cellular proliferation (Nishizuka, 1986; Berridge and Irvine, 1984).

Inositol-1,4,5 triphosphate once produced, disappears very rapidly, and a major mechanism for terminating this signal flow is thought to be removal of the 5-phosphate by the action of a specific phosphatase. Both Ca^{+2} transport adenosine triphosphatase (ATPase) and Na^{+}/Ca^{+2} exchange protein are known to be responsible for the extrusion of Ca^{+2} to maintain homeostasis (Nishizuka, 1984).

Under normal physiological conditions, free DAG is almost absent from membranes. Within a minute of formation it disappears, either for resynthesis of inositol phospholipid or becoming further degraded to acid for prostaglandin synthesis. Thus when cells are stimulated, PKC is only transiently activated by DAG. The active PKC in turn phosphorylates a range of cellular proteins involved in various cellular functions including

Figure 1.6

Inositol phospholipid turnover and signal transduction.



Abbreviations: PtdIns : phosphatidylinositol; PtIns4P : phospatidylinositol-4-phosphate; PtdIns4,5P2 : phosphatidylinositol-4,5-bisphosphate; R1 and R2 : fatty acyl groups; I : inositol; and P: phosphoryl group.

proliferation. The protein phosphorylation catalysed by PKC may exert profound modulation of various Ca^{+2} mediated processes, such as release reactions and exocytosis, cell proliferation and differentiation, membrane conductance and transport, potentiation and desensitisation of other receptor system, smooth muscle contraction and other metabolic processes. A summary of PKC associated cellular functions is given in Table 1.1 and Table 1.2 (information adapted from Nishizuka, 1986).

In the resting condition in most tissues, PKC is largely present in a soluble inactive form but when for example 12-O-tetradecanoylphorbol-13-acetate (TPA) or phorbol-12,13-dibutyrate (PDBu) is added to intact cells, PKC is recovered in a form tightly associated with the cell membrane (Nishizuka, 1984).

1.4.2 PKC Structure

PKC was identified in 1977 as a proteolytically activated protein kinase in many tissues (Inoue *et al.*, 1977). PKC exists as a single polypeptide (approximate molecular weight 77,000 g/mol) that appears to be composed of two functionally different domains namely; a regulatory domain (amino-terminal half) and a catalytic or protein kinase domain (carboxyl-terminal half). Molecular cloning and biochemical analysis have revealed the enzyme to exist as a family of multiple sub-species having closely related structures (Coussens *et al.*, 1986). The PKC sub-species thus identified show a slightly different mode of activation, kinetic properties and substrate specificities (Ono *et al.*, 1989[a]). Most cell types contain more than one subspecies of the enzyme. The various PKC subspecies can be classified into two major groups as described below (Figure 1.7, adapted from One *et al.* , 1988.)

Table 1.1
Possible roles of protein kinase C in cellular responses

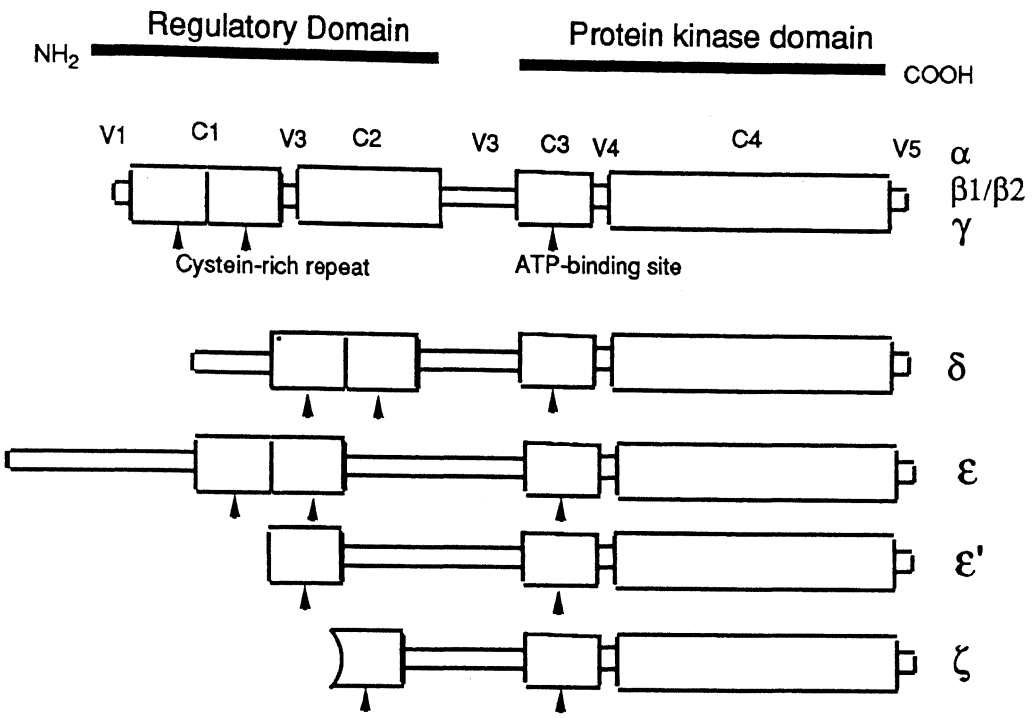
Tissues and cells	Responses
Endocrine systems:	
Adrenal medulla	Catecholamine secretion
Adrenal cortex	Aldosterone secretion
	Steroidogenesis
Pancreatic islets	Insulin release
Insuiinoma cells	Insulin release
Pituitary cells	Pituitary hormone release
	Growth hormone release
	Luteinizing hormone
	Prolactin release
	Thyrotropin release
Parathyroid cells	Parathyroid hormone release
Thyroid C cells	Calcitonin release
Leydig cells	Steroidogenesis
Exocrine system :	
Pancrease	Amylase secretion
Parotid cells	Amylase and mucin secretion
Submandibular gland	Mucin secretion
Gastric gland	Pepsinogen secretion
	Gastric acid secretion
Alveolar cells	Surfactant secretion
Nervous systems :	
Ileal nerve endings	Acetylcholine release
Neuromuscular junction	Transmitter release
Caudate nucleus	Acetylcholine release
PC 12 cells	Dopamine release
Neurons	Dopamine release
Muscular systems :	
Vascular smooth muscle	Muscle contraction
	Muscle relaxation
Inflammation and immune systems :	
Platelets	Serotonin release
	Lysosomal enzyme release
	Arachidonate release
	Thromboxane synthesis
Neutrophils	Superoxide generation
	Lysosomal enzyme release
	Hexose transport
Basophils	Histamine release
Mast cells	Histamine release
Lymphocytes	T-lymphocyte activation
	B-lymphocyte activation
Metabolic and other cell systems :	
Adipocytes	Lipogenesis
	Glucose transport
Hepatocytes	Glycogenolysis
Epidermal cells	Inhibition of gap junction
Fibroblasts	Inhibition of gap junction
Hepatocytes	Inhibition of gap junction

Table 1.2
Proposed substrate proteins of protein kinase C.

Receptor proteins
Epidermal growth factor receptor
Insulin receptor
Somatomedin C receptor
Transferrin receptor
Interleukin-2 receptor
Nicotinic acetylcholine receptor
Immunoglobulin E receptor
Membrane proteins :
Ca+2 transport ATPase
Na+/K+ ATPase
Na+ channel protein
Na+/H+ exchange protein
Glucose transporter
GTP-binding protein
HLA antigen
Chromaffin granule-binding protein
Synaptic B50 (F1) protein
Contractile and cytoskeletal proteins :
Myosin light chain
Troponin T and I
Vinculin
Filamin
Caldesmon
Cardiac C-protein
Microtubule-associated proteins
Enzymes :
Glycogen phosphorylase kinase
Glycogen synthase
Phosphofructokinase
beta -Hydroxy-beta-methylglutaryl-coenzyme A reductase
Tyrosine hydroxylase
NADPH oxidase
Cytochrome P450
Guanylate cyclase
DNA methylase
Myosin light chain kinase
Initiation factor 2
Other proteins :
Fibrinogen
Retinoid binding protein
Vitamin D binding protein
Ribosomal S6 protein
GABA modulin
Stress proteins
Myelin basic protein
High mobility group proteins
Middle T antigen

Figure 1.7

Schematic representation of the structures of the PKC family.



Abbreviations : Conserved regions, C1-C4 are homologues in PKC family. V1-V5 are variable regions. Cysteine-rich and ATP-binding sites are indicated by traingles.

1. Conventional PKC

PKC -α, -βI, -βII and -ζ isoforms belonging to the conventional PKC subclass have been identified. These are single polypeptides with four conserved (C_1-C_4) and five variable (V_1-V_5) regions. They are characterised enzymatically by their requirement for Ca^{+2}, phospholipid and diacylglycerol for activation (Parker *et al.*, 1986; Ono *et al.* , 1988).

Regulatory domain :

The amino-terminal half of each polypeptide, containing regions C1 and C2, is the regulatory domain . C1 is a phospholipid, diacylglycerol and phorbol ester binding domain, which contains a tandem repeat of cysteine-rich sequences. The C2 region is a Ca^{+2} binding domain (Gschwendt *et al.*, 1991).

Catalytic or Kinase Domain :

The carboxyl terminal half of polypeptide with conserved C3 and C4 regions constitutes the catalytic domain that resembles many other protein kinases. The conserved C3 region also has an ATP binding sequence. While the conserved region C4 contains a similar sequence to the ATP binding site, the significance of this repeat remains unknown (Kikkawa *et al.*, 1989). The catalytic moiety can be generated in *vitro* by limited trypsinolysis which generates a catalytically active fragment that is no longer dependent on Ca^{+2} and phospholipid.

2. Novel PKC

Isoforms of novel PKC that have been identified are PKC -δ, -ε, -ε' -ζ, -η, and -λ. PKC-η, and PKC-λ are predominantly observed in skin and lung, supporting the concept that different members of the PKC family might play different cellular roles. Novel PKC isoforms differ structurally from conventional PKC in that the regulatory domain of these isoforms lacks the C2 conserved region (i.e the Ca^{+2} binding site). All kinase assays with the novel isoforms PKC have shown them to be independent of Ca^{+2} giving the same kinetic results in the presence or absence of Ca^{+2}. Novel PKC isoforms also contain the characteristic region C1 of the conventional PKC encoded by α, -βI, -βII and -γ sequence (Nishizuka, 1986). Through a direct parallel comparison of several enzymes it rather appears that histone and other conventional PKC substrates are poor substrates for novel PKC's (Liyange *et al.*, 1992 ; Shin-ichi *et al* ., 1990).

It is important to note that the *in vitro* dependency of PKC on Ca^{+2}, phospholipid and DAG varies markedly with the phosphate acceptor protein. A typical example is protamine, the phosphorylation of which by PKC requires neither Ca^{+2}, phospholipid nor DAG. In fact, kinetic properties of PKC have

repeatedly been shown to vary greatly with the substrate used (Berridge *et al.*, 1984). PKC -γ sequence shows less activation by DAG, but is significantly activated by free arachidonic acid at micromolar concentration. Activation by arachidonic acid does not require Ca^{2+}, nor does it depend on phospholipid and DAG. Conventional PKC βI and βII show substantial activity without added Ca^{2+} in the presence of DAG and phospholipid, but respond much less to arachidonic acid. The PKC -α subform shows properties apparently similar to the γ-subspecies and responds to high concentrations of free arachidonic acid only when Ca^{2+} is increased. It is possible that some PKC subspecies may be activated successively by a series of phospholipid metabolites, such as DAG, arachidonic acid and lipoxin A, that successively appear subsequent to stimulation of the receptor (Kikkawa *et al.*, 1989).

The PKC Regulatory domain

The regulatory domain of all PKC isoenzymes contain a so-called pseudo-substrate motif and a tandem repeat of a cysteine-rich sequence in their regulatory domain. These cysteine rich motifs resemble, the "cysteine-zinc DNA binding finger" which are found in many metallo-proteins and DNA binding proteins (Berg, 1990). No evidence is currently available to suggest that any PKC isoforms bind to DNA under physiological conditions. Through mutagenesis antibody inhibition studies, the zinc finger structures in the N-terminal part of PKC molecules have been recognised as the sites of action of phospholipid and phorbol ester activators (Ono *et al.*, 1989[b]). PKC ζ is unique among the PKC enzymes in that it contains a single zinc finger-like structure and has been claimed not to bind phorbol ester tumour promoters.

The pseudo-substrate motif of PKC was first recognised by House and Kemp (1987) in isoenzymes α, β and γ. The pseudo-substrate motifs are polypeptide sequences which resemble a substrate phosphorylation site. It is believed that

the pseudo-substrate motif interacts with the active site and renders the enzyme inactive in the absence of activating factors. In all of the PKC forms, the pseudo-substrate motif and cysteine rich regions are separated by either 21 or 22 amino acids, whereas the region between the amino terminus and the cysteine-rich regions of the various isoforms are separated by distances ranging from 43-182 amino acids. The distance from the pseudosubstrate motif to the cysteine rich regions appears to be crucial for the inactivation of the enzyme (Gschwendt *et al.*, 1991).

1.4.3 Proposed mechanism of activation of PKC by DAG and Phorbol esters

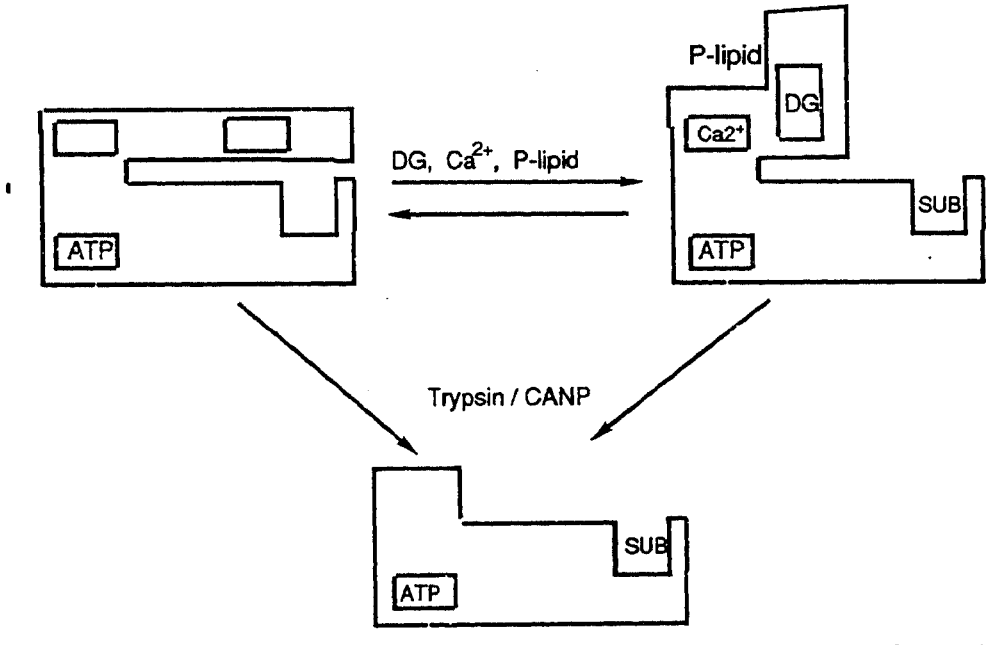
It has been suggested that the cysteine-rich regions of PKC are essential for phorbol-ester binding and that at least one of the cysteine rich finger-like sequences is needed for the tumour promoting phorbol ester to activate PKC (Ono *et al .*, 1989b). It seems likely that the phorbol esters and also DAG are hydrogen bonded to the thiol groups in these cysteine-rich regions. DAG dramatically increases the affinity of PKC for Ca^{2+} and thereby renders it fully active without a net increase in the Ca^{2+} concentration. DAG models having a 1,2,-Sn configuration with various fatty acids of different chain length, were capable of activating PKC. Analogues containing an unsaturated fatty acyl group were found to be most active. The hydrophobic domains of DAG (i.e. the acyl chains at C-1 and C-2) and of phorbol esters (acyl chains in C-12 and C-13) are believed to be required for non-specific interactions with the adjacent lipid microenvironment. In contrast, highly specific interactions involving the CH_2OH group (at C-3 of DAG and C-20 of phorbol esters) as well as additional residues in the case of phorbol esters (eg.3-keto, 4-OH) are essential for binding to the cysteine rich regulatory moiety of PKC (Castanga, 1987). Upon signal-receptor interaction, PKC tightly binds to the inner plasma membrane and becomes associated with DAG and phospholipid. The negatively charged

phospholipids are the most efficient in supporting PKC activation (Catagna, 1987).

The allosteric conformational change induced by the activator, together with membrane phospholipids, appears to be sufficient for removal of the pseudosubstrate motif from the catalytic centre of all PKC isoenzymes. Binding of calcium to a region located between the cysteine-rich region and the ATP-binding site of the conventional isoenzymes possibly increases the conformational change induced by DAG (Figure 1.8, adapted from Parker *et al.*, 1986). However, the isoenzymes that lack the calcium binding region are probably activated by DAG alone (Gschwendt *et al* ., 1991; Parker *et al.*, 1986).

Figure 1.8

Model for activation of PKC.



Abbreviations : DG : diacylgycerol, P-lipid: phospholipid, CANP : calpain, SUB: substrte binding site.

1.4.4 Prolonged activation of PKC by β-phorbol compared to DAG

While the natural activator of PKC is DAG, it is especially interesting that the enzyme also serves as the receptor for phorbol esters, a class of tumour

promoters. It is likely that inappropriate PKC activation induced by these compounds results in their tumour-promoting characteristics. Structural similarities exist between DAG and the 12,13-diesters of β -phorbol molecules in the region of the ester bonds. However, the conformer of 1,2-diacetyl-sn-glycerol that has a molecular geometry similar to the ester region of ß -phorbol is 3.2 kcal/mol above the lowest energy conformer and has the ester residues in a gauche conformation orientated at about -60 ° relative to one another (Leli *et al.*, 1989). This implies that substantial energy must be put into DAG for it to achieve the correct three dimensional structure for activation of PKC. This would be consistent with the much lower potency of DAG compared with phorbol esters. The half-life of phorbol esters is very long compared to DAG whereas the half life of DAG is less than one minute in platelets. Phorbol esters such as TPA are slowly metabolised and exert a sustained activation of PKC (Castanga, 1987). Autoradiographs of treated skin have demonstrated that TPA has a half-life of 23 hours in mouse skin (Witte and Hecker, 1971).

1.4.5 PKC activation and *Pimelea* poisoning

As evidenced by the data presented in Tables 1.1 and 1.2, PKC plays a very important role in controlling a plethora of cellular functions and is present in different tissues. Normally, the enzyme is transiently activated, and then immediately reverts to its inactive form. Phorbol esters and related daphnane diterpenes present in *Pimelea* toxins are likely to activate PKC for a long period of time (Witte and Hecker, 1971). This leads to abnormal activation of various cellular functions which are controlled by PKC, and precipitates as *Pimelea* poisoning symptoms in cattle. These toxins are lipophilic and metabolism of phorbol esters in the body is known to be slow. They may be metabolised by several enzymatic pathways including oxidation, reduction and hydrolysis (Segal *et al.*, 1975).

Phorbol esters increase phosphorylation of smooth muscle myosin heavy and light chains through activation of PKC (Kamm *et al.*, 1989). These compounds also potently down-regulate endothelin (ET-1) binding sites in vascular smooth muscle cells by a mechanism involving PKC (Roubert *et al.*, 1989). Phorbol ester-induced morphological changes in living cultured cells were accompanied by reorganisation of filamentous actin (Miyata *et al .*, 1988). The adherence of polymorphonuclear leucocytes to the pulmonary vascular endothelium may contribute to the acute lung injury (Gudewicz *et al .*, June 1989).

TPA has been shown to induce reorganisation of actin filaments and calspectin in 3T3 cells. Possible mechanisms for these cytoskeletal changes produced by TPA are discussed by Sobue *et al.* (1988). Stimulation of tyrosine phophorylation by phorbol diesters suggests that initial stimulation of PKC activates a tyrosine kinase cascade.

In vascular smooth muscle phorbol esters cause a slowly developing contraction and an associated transmembrane calcium flux, both of which are inhibited by dihydropyridine calcium antagonists (Fish *et al.*, 1988). There is a possible involvement of reorganisation of actin filaments induced by tumour promoting phorbol esters, in changes in colony shape and enhancement of proliferation of cultured epithelial cells. Related tumour promoters such as phorbol-12,13-didecanoate and mezerein caused effects similar to TPA (Sastrodihardjo *et al.*, 1987).

Lung injury induced by TPA is closely associated with toxic oxidants released from activated granulocytes. The available data indicates that the hydroxyl radical, a toxic oxidant derived from stimulated granulocytes, is deeply involved in the pathogenesis of TPA-induced lung injury (Kuroda *et al.*, 1987).

1.5 OUTLINE OF THE PRESENT STUDY

The aim of the research described in this thesis was to investigate the feasibility of raising antibodies in cattle and rabbits against diterpenoid toxins isolated from *Pimelea trichostachya*. The protective efficacy of any antibodies raised in cattle was to be determined through toxin challenge experiments and the effects of the toxins on bovine pulmonary venule preparations were to be examined *in vitro* using modern organ bath techniques. A major part of the study was to be devoted to establishing the organ bath methodology since it was anticipated that the technique would offer the opportunity to validate the efficacy of purified antibodies in protecting the bovine target tissue from the toxins and also allow studies of potential antagonists of the toxins.

There are two examples of Australian research which indicate that an immunogen approach to plant toxicity problems in livestock is worthy of investigation. A successful vaccine has been developed against lupinosis in sheep. This disease is caused by ingestion of infected lupin stubbles infected by the fungus *Phomopsis leptostromiformis*. This fungus produces mycotoxins (phomopsins) which are lethal to animals. Phopmopsin A is the most toxic compound produced from the fungus and is a potent inhibitor of tubulin polymerisation and microtubule formation.

Phomopsin A toxicity in animals is characterised by severe liver damage, jaundice, loss of appetite, emaciation and ultimate death. Edgar and co-workers have developed an effective anti-phomopsin vaccine for sheep which protects vaccinated animals against phomopsin toxicity. The exact details of their conjugation and vaccination methodology has not been disclosed because of its potential commercial value, however the general principles have been described by Ralph (1990). The effectiveness of the lupinosis vaccine was

discovered only by chance, since the aim of the initial research was to produce anti-phomopsin antibodies in sheep which could be purified for use in an ELISA method for detecting phomopsins. It was found that vaccinated animals were protected against lupinosis toxicity and antibody titres could be augmented in the following season through a single booster injection of antigen.

In the case of the lupinosis vaccine, successful protection against the toxin may be attributable to phomopsin A being a peptide and amenable to destruction by the enzymes released by the macrophages that bind to the toxin-antibody complex. Other (non-peptide) toxins, approached in a similar way, may only be held temporarily by the antibody; but may not be digested by the macrophage; thereby releasing the toxin back into the general circulation. This would mean that toxin specific antibodies may extend the half-life of the toxin, exacerbating the poisoning event (Cockrum and Edgar 1985; Edgar *et al.*, 1982).

The other example is perhaps more relevant to the present study since it involves a toxicity in cattle caused by triterpenoid toxins. Lantana poisoning is a disease that affects ruminant animals and is characterised by jaundice, cholestasis, liver and kidney damage, photo sensitisation, ruminal stasis, constipation, anorexia and in very acute cases death (Pass *et al.*, 1981; Pass, 1986).

Lantana poisoning is caused by pentacyclic triterpene toxins called lantadenes which are present in introduced *Lantana camara* plant species. Stewart and Pass (1988) vaccinated sheep with conjugates of lantadenes A and B isolated from *Lantana camara* . Because the lantadenes absorbed U.V. light very poorly, it was not possible for these workers to accurately quantify the degree of toxin

incorporation into the carrier protein. Despite the modest antibody titres obtained in the vaccinated animals, Stewart and Pass (1988) observed protective effects against hepatotoxicity when vaccinated and control animals were fed *Lantana camara* . Serum bilirubin levels were significantly lower in the vaccinated group.

In the case of *Pimelea* toxicity the toxins involved are tricyclic diterpenes related to phorbol esters which also contain an unusual orthoester functionality in the C - ring. These compounds are much more highly functionalised than the lantadenes and therefore possess potentially more antigenic determinants. The work of Tashjian *et al,* (1985) had shown that specific antibodies could be raised against phorbol 12,13 dibutyrate in mice and it therefore seemed reasonable to expect that specific immune responses to the structurally related daphnane toxins present in *Pimelea* could also be achieved. Whether these antibodies would offer any protection to *Pimelea* toxicity was a central question to be addressed through the present study.

CHAPTER TWO

TOXIN ISOLATION AND CONJUGATION

2.1 INTRODUCTION

The aims of the work described in this Chapter were: (i) to develop an efficient methodology for purification of the daphnane orthoester toxins from *Pimelea* ;and (ii) to conjugate these compounds to carrier proteins to enable preparation of experimental vaccines.

There had been a number of earlier reports describing isolation of daphnane and tigliane orthoesters from *Pimelea* species (Freeman *et al.*, 1979, Tyler and Howden 1985). In the present study, we sought to utilise preparative HPLC methodology for toxin isolation. The toxic fractions obtained from conventional column chromatography were to be identified using a fish toxicity assay and then these fractions further purified using preparative reverse phase HPLC techniques.

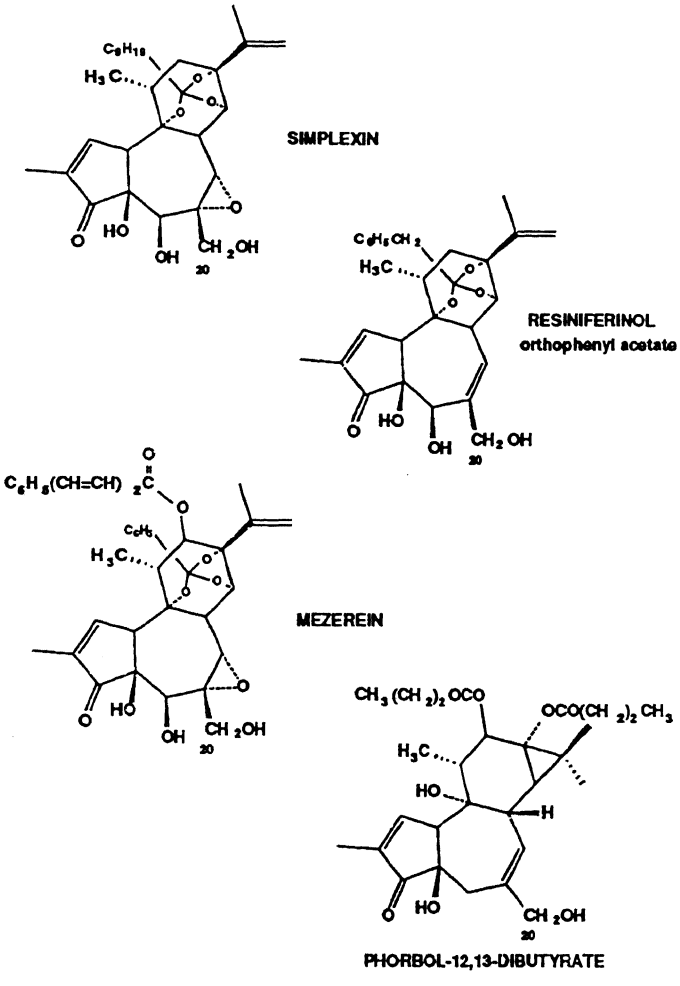
The *Pimelea* daphnane and tigliane toxins are relatively small molecules with molecular weights ranging from 500-700 mass units. To induce an immune response to the toxins in test animals, a toxin- protein conjugate needed to be prepared. Previous work in this laboratory had established some general procedures for the conjugation of daphnane type compounds to carrier proteins (Pegg, Hellqvist and D'Occhio, unpublished).

In the present study, conjugates of *Pimelea* A, B and C fractions, mezerein and resiniferinol were prepared using this methodology. As shown in Figure 2.1, the two commercially available daphnanes employed (namely mezerein and resiniferinol orthophenyl acetate) bear many structural and stereochemical similarities to simplexin and huratoxin. The rationale for the conjugation strategy was that the *Pimelea* daphnane and tigliane toxins and the two commercially available daphnanes all possessed a common C-20

hydroxymethyl grouping that was not sterically hindered and offered potential for chemical modification.

Figure 2.1

Structure of simplexin and three commercially available structural analogues.

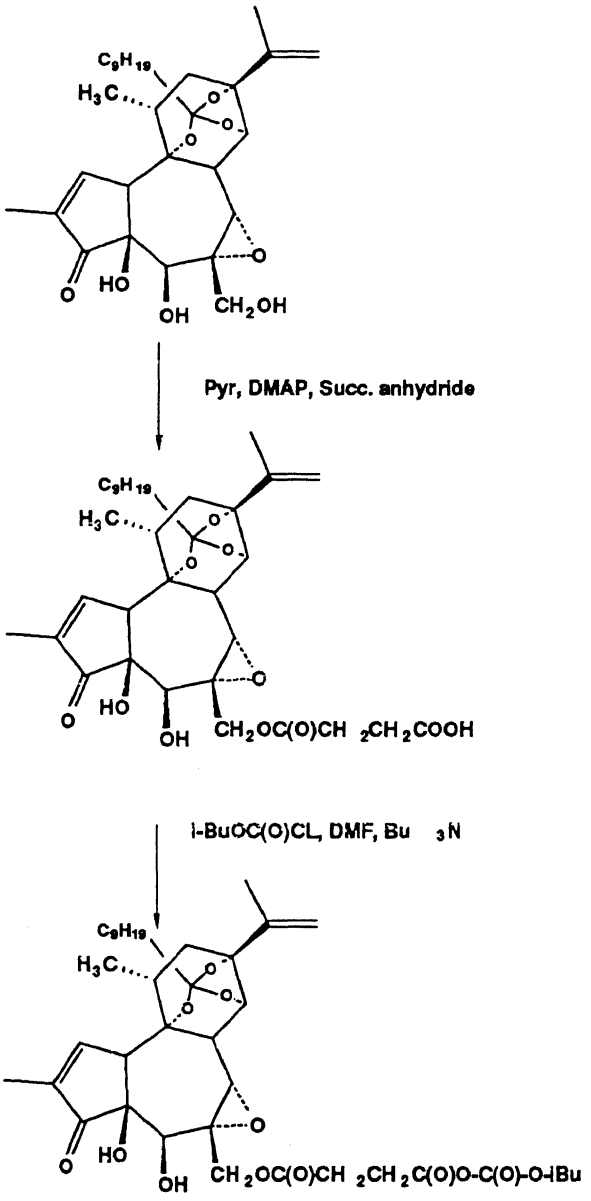


In preliminary work related to this project, antisera to phorbol-12,13-dibutyrate (PDBu) did not appear to recognise either pure simplexin (Dr W. Taylor, University of Sydney) or resiniferinol orthophenyl acetate. These findings suggested that the tricyclic ortho-ester functionality in the C-ring of simplexin and resiniferinol orthophenyl acetate (and absent in PDBu) was preventing molecular recognition by antibodies raised against PDBu. As a result of these experiments, all subsequent immunogen work directed at raising antibodies to

Pimelea toxins has been undertaken with analogues possessing the unusual ortho-ester function in the C-ring.

Figure 2.2

Chemical transformation of simplexin.



The strategy employed for the conjugation of simplexin is depicted in Figures 2.2 and 2.3. As shown in Figure 2.2, the daphnane ortho-ester was first reacted with succinic anhydride in pyridine at 80 °C, in the presence of a catalytic amount of dimethylaminopyridine (DMAP). This reaction was carried out in a

sealed glass ampoule containing an inert nitrogen atmosphere. It was presumed that most acylation would occur at the relatively unhindered C-20 hydroxymethyl grouping, although there also existed the possibility of some reaction occurring at the C-5 secondary hydroxyl grouping.

Earlier work showed that under these reaction conditions, simplexin was converted into a single product having a shorter retention-time on reverse phase HPLC (Pegg and Duivenvorden, unpublished). This increase in polarity was consistent with the incorporation of an additional polar free carboxyl functionality in the succinylated product. It was found that the reaction was sluggish in the absence of the DMAP acylation catalyst.

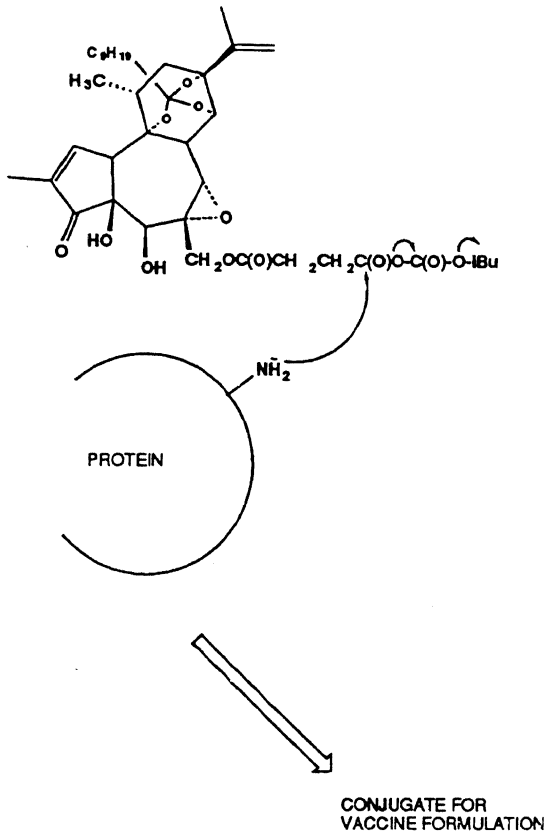
The next step in the procedure depicted in the lower portion of Figure 2.2 was the activation of the incorporated free carboxyl grouping by reaction with iso-butylchloroformate (IBCF) in anhydrous dimethylformamide (DMF). This reaction was carried out at 0° C in the presence of tributylamine to react with any hydrogen chloride liberated. HPLC analysis showed that complete conversion to a less polar mixed anhydride had occurred within 15 minutes. In this case, the mixed anhydride was very reactive and has been set up to liberate carbon dioxide and iso-butanol upon attack of a suitable nucleophile.

The final step of the procedure shown in Figure 2.3 requires the preparation of a solution of the carrier protein in 50:50 DMF : water, to which the "activated" toxin in DMF is added at 0° C. Free lysine amino groups in the carrier protein are sufficiently nucleophilic to effect a displacement reaction on the activated mixed anhydride intermediate, thereby linking the modified toxin to the protein via a new amide bond. The common observation in these reactions is evolution of carbon dioxide and a drop in pH of the reaction mixture. Particularly with ovalbumin, one must be careful to maintain the pH of the

reaction mixture near 8.0 to prevent precipitation of the carrier protein from the DMF/ water mixture.

Figure 2.3

Protein toxin conjugation



The same conjugation methodology was to be used in the present study except that a mixture of daphnane and tigliane toxins isolated from *Pimelea* was to be conjugated to the carrier protein rather than a single toxin. The rationale for this approach was to prepare a conjugate which possessed the full range of *Pimelea* toxin epitopes. The added advantage of using the mixture of toxins was that more material was available to work with, since even the major toxin simplexin makes up only 0.005% of the dry weight of *Pimelea simplex* (Freeman *et al.*, 1979).

2.2 METHODOLOGY

2.2.1 Toxin Extraction from *Pimelea trichostachya*

The technique employed for toxin isolation was adapted from a report by Tyler and Howden (1985) in which those authors described the isolation of toxins from *Pimelea simplex*. It has been reported by Freeman *et al.*, (1979) that dried *Pimelea* plant material retains toxicity for several years and that significant amounts of the active toxins can still be isolated from the plant after this time. It appears that the diterpenoid toxins are stable when protected from light and oxygen within the plant, however after isolation these compounds are readily degraded. Storage at -20°C in the dark under an inert nitrogen atmosphere is required. Schmidt and Hecker (1975) have described autoxidation breakdown products of phorbol esters stored in common solvents, such as dimethyl sulphoxide and chloroform and it was expected that the daphnane orthoesters isolated from *Pimelea* might show similar autoxidation tendencies.

Whole dry *Pimelea trichostachya* (100 g) was powdered using an electric hammer mill. The plant material used included stems, leaves, flowers, and roots. The powdered plant material was exhaustively extracted with methanol at room temperature (3 x 500 mL, 12 h) and the combined methanol filtrates evaporated to dryness using rotary evaporation. The resultant dark gum-like residue (5.4 g) was stored under nitrogen at -20°C in the dark.

The gummy residue was then partitioned between chloroform (150 mL) and water (300 mL) in a separatory funnel and the water layer then repeatedly extracted with chloroform (3 x 50 mL). Most piscicidal activity (*vide infra*) was found in the chloroform layer. The aqueous layer containing the water soluble non-toxic component was discarded. The combined chloroform extracts were dried over anhydrous sodium sulfate, filtered and then evaporated to dryness using a rotary evaporator. The resultant gum like residue (1.40 g) was stored

under nitrogen at -20°C in the dark. Piscicidal activity of the crude residue was tested using a fish bioassay technique.

2.2.2 Fish Bioassay

Sakata *et al.* (1971) have reported a fish bioassay technique to test huratoxin toxicity and these workers found that 0.003 ppm huratoxin concentration causes 100 % death in killie fish within 24 h. Since the daphnane toxins found in *Pimelea* spp. have very similar structures to huratoxin, it was decided to test the piscicidal activity of the crude chloroform extract directly using a fish bioassay.

Freeman, *et al.* (1979) reported that 0.005 % of the dry weight of *Pimelea simplex* is simplexin. On this basis it was assumed that the 1.40 g of residue obtained from 100 g of dry *Pimelea trichostachya* plant might contain 5 mg of simplexin. Since the diterpene toxins are immiscible in water but readily dissolve in acetone, a portion of the crude extract was dissolved in acetone by dissolving 0.14 mg of the crude extract (equivalent to 5 μg of simplexin) in 500 μL of acetone to approximate a 0.003 ppm simplexin concentration in 200 mL of water.

Six mosquito fish (*Gambusia* spp.) of similar size and weight (three test and three controls) were kept off food overnight and allowed to acclimatise in an eight chambered perspex tank. Each chamber contained 200 mL of continuously aerated water. Test fish were challenged with the test solution (500 μL of the toxin solution in acetone) while control fish were challenged with an equivalent volume of pure acetone. The fish were then observed for a period of 24 h.

2.2.3 Silica gel column chromatography

The crude extract (1.4 g) was further purified using vacuum assisted silica gel column chromatography. The toxic material was dissolved in 2 mL of benzene and chromatographed on silica gel (Merck Kieselgel G type 60) packed in a Buchner funnel. The column was eluted under reduced pressure with 100 mL aliquots of the following solvents :

1. Benzene
2. Benzene - ethyl acetate (9:1)
3. Benzene - ethyl acetate (6: 4)
4. Ethyl acetate
5. Methanol

TLC analysis

Thirty five fractions (each approximately 15 mL) were collected from the chromatography and small samples were spotted on silica TLC plates (Merck DE-Plasticfolien Kieselgel 60 F_{254}). Separation of components was achieved using benzene : ethyl acetate (6:4) as the mobile phase. The plates were first observed under short wave U.V. light (Extech Equipment Pty. Ltd.) and later were sprayed with a solution of concentrated sulphuric acid/vanillin. Colour development was achieved by heating the plates for three minutes at 100°C.

2.2.4 Modification of the silica gel column chromatography solvent system

Since benzene is a hazardous solvent, and that scale-up of the procedure described above would require handling a large quantity of benzene (3 L) over 6-7 h, we sought to substitute petroleum spirit for benzene in the chromatography.

To determine suitable mixtures of petroleum spirit/ethyl acetate, the following procedure was adopted.

1. A series of five control plates were spotted with the crude extract dissolved in chloroform. Each control plate was run against one of the series of eluting solvents used for the initial silica gel column chromatography described above. Separated compounds were observed by UV fluorescence and then each plate was sprayed with the sulphuric acid/vanillin spray.

2. Test silica plates were spotted with the crude extract in chloroform. Each test plate was then run in a petroleum spirit : ethyl acetate solvent of known ratio and each test plate was observed for UV fluorescence and sprayed with the concentrated sulphuric acid/vanillin spray for visualisation of components. Each test plate was compared with the series of control plates. This procedure was then repeated, each time with a fresh test plate and a new solvent mixture until the separation of the components on the test plate was almost identical with the corresponding control plate.

2.2.5 HPLC Purification

Sample preparation

C-18 reverse phase cartridges (Waters Associates) were used routinely for sample clean-up prior to HPLC analysis. Since the diterpene toxins from *Pimelea* have some polarity, a methanol solution of the toxins was eluted through C-18 sep-pak cartridges to remove highly hydrophobic impurities present in the sample. The recovered solution was then evaporated to dryness using a rotatory evaporator and the residue thus obtained was stored under nitrogen at -20°C in the dark. Using the fish bioassay technique described above, this material was found to be highly toxic to fish.

HPLC Separation

The *Pimelea* A,B fraction was then further purified using reverse phase HPLC. The components were separated using a methanol-water gradient on a C-18 reverse phase column with UV detection at 240 nm wavelength. The HPLC system used comprised a Waters 600E quaternary pumping system, a U6K injector and a Waters Model 486 Tunable Absorbance Detector. Chromatographic data were recorded via Baseline 8 software (Waters) on an IBM compatible 486 computer.

After preliminary sep-pak purification, *Pimelea* A,B fraction was dissolved in methanol before injecting into column. Good separation of components was achieved on analytical, semi-preparative and preparative scale C-18 reverse phase columns. For each of these columns, the optimum methanol-water gradient was established by trial and error application of different methanol-water gradients, until acceptable separation of components was obtained. HPLC grade methanol and Millipore filtered water were used for all separations. All solvents were filtered through a 0.45 μm filter before use and de-gassed by intermittent sparging with helium gas (10 mL/minute). Optimum conditions for effecting separation on a analytical column was first established, and then equivalent separation was successively achieved on semi-preparative scale followed by larger scale preparative column HPLC.

Details of the columns and guard columns used, the methanol-water gradients employed and the quantity of *Pimelea* A,B sample that could be adequately separated by each technique is given below.

(a). Analytical scale reverse phase HPLC :

Analytical column :

Waters Nova-pak C18 reverse phase analytical column

Size : 3.9 mm x 150 mm

Packing Material : dimethyloctadecylsilyl bonded amorphous silica, pore size- 60 Å, particle size - 4μm

Guard column :

Nova-pak C18 cartridge type

Sample quantity used :

Approximately 3-5 mg of *Pimelea* A,B fraction was dissolved in 50 μL of methanol

Methanol gradient :

The methanol/water solvent gradient employed is detailed in Table 2.1

Table 2.1

Methanol : Water gradient for HPLC separation of *Pimelea* toxins on analytical scale.

Time (min.)	Flow rate (mL/min.)	Methanol (%)	Water (%)
0	2.0	60	40
10	2.0	65	35
15	2.0	70	30
20	2.5	75	25
30	2.5	85	15
35	2.5	90	10
80	2.5	90	10

(b) Semi-preparative scale reverse phase HPLC :

Semi preparative column :

Activon Gold Pak C18 reverse phase column.

Size : 25 x 1 cm

Packing material : Exil 100/5 Octadecylsilica

Guard column :

Activon Gold Pak C18 cartridge.

Sample quantity used :

Approximately 15-25 mg of *Pimelea* A,B fraction was dissolved in 50 µL of methanol.

Methanol Water Gradient used :

The methanol/water solvent gradient that was employed is detailed in Table 2.2.

Table 2.2

Methanol : Water gradient for separation of *Pimelea* toxins on a semi-preparative scale.

Time (min)	Flow rate (mL/min)	Methanol (%)	Water (%)
0	3.5	60	40
10	3.5	65	35
15	3.0	70	30
20	3.0	75	25
30	3.0	85	15
35	3.0	90	10
80	3.0	90	10

(c) Preparative scale reverse phase HPLC :

Preparative column :

Waters Prep-pak cartridge reverse phase preparative column.

Size : 25 X 100 mm

Packing material : Prep Nova Pak HR C18.

Pore size - 60 Å, particle size : 6 µm.

Guard Column :

Guard Pak TM Cartridge Prep Nova-Pak HR C18

Size : 25 x 10 mm

Packing Material : Prep Nova Pak HR C18.

pore size- 60 Å, particle size - 6μm.

Sample quantity used :

Approximately 30-40 mgs of *Pimelea* A,B fraction was dissolved in 200 μL of methanol.

Solvent Gradient :

The solvent gradient employed was dependent on the sample size. For separation of up to 40 mg, the gradient detailed in Table 2.3 was employed.

Table 2.3

Methanol : Water gradient used for preparative separation of *Pimelea* toxins (up to 40 mg sample size).

Time (min.)	Flow rate (mL/min.)	Methanol (%)	Water (%)
0	4.0	60	40
13	4.0	65	35
21	4.0	70	30
29	4.0	75	25
45	4.0	85	15
53	3.5	90	10
83	3.5	90	10

Sample quantity used :

Approximately 40 mg of *Pimelea* A,B fraction was dissolved in 500 μL of methanol.

Table 2.4 below details the solvent gradient employed for preparative scale separation of sample sizes of 40-150 mg of *Pimelea* A,B toxic fraction.

Table 2.4

Methanol : Water gradient used for separation of *Pimelea* toxins on preparative scale (40-150 mg sample size).

Time (min)	Flow rate (mL/min)	Methanol (%)	Water (%)
0	7.0	60	40
3	7.0	65	35
7	7.0	70	30
10	7.0	75	25
13	7.0	80	20
19	7.0	85	15
23	7.0	90	10
60	7.0	90	10

Sample quantity used :

Approximately 150 mg of *Pimelea* A,B fraction was dissolved in 700µL of methanol

As the preparative run took approximately 80 minutes, fractions obtained each minute were collected using a Gilson Model 203 Micro Fraction Collector.

2.2.6 Conjugation Methodology

General Mezerein and resiniferinol orthophenyl acetate were purchased from LC Services Corporation, Woburn, MA, USA. The best analytical or HPLC grade solvents available in the laboratory were used routinely. Pyridine (A.R.) was dried over potassium hydroxide pellets and distilled just prior to use. The dried distilled pyridine was stored over 3A molecular sieves. DMF (A.R.) was distilled from calcium hydride and stored over 3A molecular sieves. Solvent and liquid chemical transfers were generally accomplished with oven-

dried glass syringes. Where required, a positive pressure of dry nitrogen gas was maintained in reaction flasks from nitrogen-filled balloons connected through Suba-Seal rubber septa.

Conjugates

For the present study, the following conjugates were prepared for immunisation experiments in rabbits and cattle :

Pimelea A - human serum albumin

Pimelea B - human serum albumin

Pimelea C - ovalbumin

Mezerein - ovalbumin

Resiniferinol - ovalbumin

Methodology

The conjugation methodology is described in detail for preparation of the *Pimelea* A-HSA conjugate. The same methodology was then applied for preparation of the other four conjugates. *Pimelea* A and B toxic fractions were obtained after silica gel column chromatography as described previously (Section 2.2.4). *Pimelea* C fraction was obtained after preparative HPLC purification of *Pimelea* A and B fraction as explained earlier (Section 2.2.5). Because the *Pimelea* toxic fractions are a cocktail of different toxins, an average molecular weight of toxin molecules was assumed to be 500 g/mol for calculation purposes. The molecular weights of the *Pimelea* toxins range from 500-700 g/mol, depending on the substituent groupings on the daphnane or tigliane molecular backbone (Freeman *et al.*, 1979).

Pimelea A fraction (350 mg, 0.7 mmol) obtained from silica column chromatography was dissolved in 2 mL of dry pyridine and succinic anhydride

(0.15 g, 1.5 mmol) and a catalytic amount of DMAP was then added. The reaction mixture was transferred to a pyrex ampoule and the contents sparged with nitrogen and sealed. The reaction ampoule was then heated overnight (15 h) at 80°C.

After cooling the sample to room temperature, the pyridine solvent was removed using a vacuum pump and dry ice/acetone trap. TLC (silica) comparison of the starting material and crude product from the reaction was undertaken to establish that succinylation of the toxin had occurred.

A sample for TLC analysis was prepared by removing the pyridine from a 0.3 mL aliquot of the reaction mixture. The residue thus obtained was partitioned between saturated ammonium chloride (1 mL) and ethyl acetate (1 mL) in a small glass tube. The ethyl acetate layer was removed by aspiration. The aqueous layer then was extracted a further 2-3 times with ethyl acetate (1 mL) washes. The combined ethyl acetate layers were then evaporated to a small volume (0.25 mL) and small aliquots spotted on the plate. Elution with benzene : ethyl acetate (1:1) showed that the reaction products (Rf approximately 0.3) were more polar than the starting material (Rf approximately 0.5).

The gummy residue (0.50 g) obtained from the succinylation reaction was dissolved in dry DMF (5 mL) and the solution cooled to 4° C. To the cooled solution, tributylamine (500 µL, 0.02 mmol) followed by isobutylchloroformate (300 µL, 0.023 mmol) was added. The reaction generated HCl which was neutralised by the tributylamine base. The activation reaction was normally run for 40 minutes at 4°C, although previous work in this laboratory has shown that these reactions are often complete within 5 minutes.

The activated mixed anhydride product was not isolated and immediately was reacted with HSA at 4° C to form the toxin-HSA conjugate. Firstly, HSA (2.5 g) was dissolved in distilled water (200 mL). The solution was cooled in an ice-bath and DMF was added in small portions until a 50:50 DMF : water mixture was achieved. The activated toxin was added dropwise to the protein solution with constant stirring at 4°C. Carbon dioxide was evolved, resulting in a fall in pH. The pH of the reaction mixture was maintained at 8-9 through the dropwise addition of 2 M NaOH as required. The conjugation reaction mixture was then stirred for 15 hours at 4° C before being dialysed.

The crude conjugate in DMF/water solvent was transferred into dialysis tubing and then dialysed at 4°C over a period of 48 hours against 5 litres of chilled 50 mM $NaHCO_3$. The bicarbonate solution was replaced 5 times during this period. After dialysis, the final volume of the protein solution was 1.37 L. Before freeze drying, the U.V. spectrum was recorded to establish that toxin molecules had been incorporated into HSA molecules.

The same methodology was used for preparation of *Pimelea* -B-HSA; *Pimelea*- C-ovalbumin; mezerein-ovalbumin and resiniferinol ortho phenylacetate-ovalbumin conjugates. The amounts of reagents used in each case are detailed in Table 2.5.

2.2.7 Estimation of Toxin Incorporation

A solution of HSA for U.V spectral analysis was made by dissolving 4 mg of HSA in 100 mL of 50 mM $NaHCO_3$. Correspondingly, a 4 mg protein/100 mL solution of the dialysed conjugate was prepared by appropriate dilution in 50 mM $NaHCO_3$. U.V. spectra were recorded over the range 220-330 nm and the extent of toxin incorporation estimated by spectral difference measurements at 244 nm.

Table 2.5

Preparation of other toxin-protein conjugates.

Toxin (mg)	Succinic anhydride (mg)	isobutyl-chloro-formate (µL)	tributyl-amine (µL)	protein (mg)
Pimelea B (450) 0.9 mmol	(2000.0) 2.0 mmol	(300.0) 0.23 mmol	(500.0) 0.2 mmol	(2000.0) BSA 30.3 µmol
Pimelea C (25) 50 µmol	(6.0) 60 µmol	(8.0) 62 µmol	(18.3) 77 µmol	(90.0) OVAL 2 µmol
Mezerein (20) 30 µmol	(5.0) 50 µmol	(5.0) 36 µmol	(10.7) 44.8 µmol	(44.0) OVAL 0.98 µmol
Resiniferinol (20) (43 µmol)	(5.0) 50 µmol	(7.0) 53.19 µmol	(15.0) 66 µmol	(63.0) OVAL 1.4 µmol

2.2.8 Estimation of protein recovery for the HSA conjugates

As expected, the weights of the freeze-dried conjugate material recovered exceeded the combined weights of the protein, toxic fraction and succinic anhydride used, due to incorporation of sodium bicarbonate during dialysis. A precipitation method was used to estimate the amount of protein in the freeze dried material recovered from the *Pimelea* A-HSA and *Pimelea* B-HSA conjugates.

Method

(i) The *Pimelea*-HSA conjugate (100 mg) was dissolved in 10 mL of distilled water.

(ii) To this solution 10 mL of 20 % trichloroacetic acid (TCA) was added and the mixture allowed to stand 20 minutes before it was centrifuged (5000 g, 5 minutes, 5°C).

(iii) The pellet was resuspended in 10 % TCA and the precipitate recovered by centrifugation as above.

(iv) The protein precipitate was washed three times with warm distilled water.

(v) The pellet thus obtained was freeze-dried and weighed.

The other conjugates prepared for this study involved lesser amounts of material than the *Pimelea* A and B conjugates. For this reason, protein recovery by the TCA precipitation method was not undertaken.

2.3 RESULTS AND DISCUSSION

2.3.1 Identification of Toxic Components by the Fish Bioassay Technique

After initial solvent extraction and chloroform /water partitioning, 1.40 g of a green gummy residue was obtained from 100g of dried *Pimelea trichostachya*. This material was stored under nitrogen at -20°C in the dark. Piscicidal activity of the crude residue was tested using the fish bioassay technique described in Section 2.2.2.

It was found that all three test fish were dead within 30 minutes of adding the crude toxin extract (at an estimated 0.003 ppm simplexin concentration), whereas the three control fish remained healthy after 24 hours. The fish bioassay experiment confirmed that piscicidal activity was concentrated in the crude organic extract from *Pimelea trichostachya* . The gummy residue was found to be freely soluble in both methanol and chloroform.

After conventional silica column chromatography as decribed in Section 2.2.3, the 35 fractions obtained were analysed by T.L.C and then grouped into five major fractions corresponding to the five solvent systems used. Each of the combined fractions was evaporated to dryness using a rotary evaporator and the residues thus obtained were stored under nitrogen at -20°C in the dark. Piscicidal activity of each fraction was determined using the fish bioassay system.

It was observed that piscicidal activity was greatest in fractions eluted with benzene - ethyl acetate (6:4) solvent, followed by the fraction collected from ethyl acetate. No piscicidal activity was observed in the other fractions. This activity pattern correlated with the UV absorption observations on the TLC analysis. For ease of identification within the laboratory, the toxic residues from the benzene - ethyl acetate and ethyl acetate fractions were named as *Pimelea* A toxic fraction and *Pimelea* B toxic fraction, respectively. These data are summarised in Table 2.6.

Table 2.6

Recovery, piscicidal activity and U.V. activity of fractions eluted from silica gel chromatography of the crude chloroform *Pimelea* extract.

SOLVENTS	WEIGHT (g)	FISH BIOASSAY	U.V. ABSORPTION
Benzene	0.89	Not Active	minimal
Benzene - ethyl acetate (9:1)	0.39	Not Active	minimal
Benzene - ethyl acetate (6:4)	0.05	Active	strong
Ethyl acetate	0.10	Active	strong
Methanol	0.40	Not Active	minimal

2.3.2 Modification of the Solvent System Employed for Silica Column Chromatography.

The original solvent system used for the silica column chromatography required the use of large amounts of benzene. Because of the hazardous nature of this solvent, its replacement with appropriate mixtures of petroleum spirit and ethyl acetate were investigated as described in Section 2.2.4. The data given in Table 2.7 shows the solvent mixtures identified which have equivalent eluting characteristics for separation of *Pimelea* toxins on silica TLC plates.

The chromatographic purification of the crude toxin extract was repeated using the petroleum spirit : ethyl acetate solvent mixtures specified in Table 2.7 below. The five solvent fractions obtained from the column were evaporated to dryness using rotatory evaporation. The residues thus obtained were stored under nitrogen at -20°C in the dark.

Table 2.7
Equivalent solvent systems for separation of *Pimelea* toxins by TLC.

Benzene : ethyl acetate	Petroleum : ethyl spirit : acetate
90 : 10	100 : 0
75 : 25	90 : 10
50 : 50	60 : 40
0 : 100	0 : 100

Piscicidal activity was found to be greatest in the fraction eluted with petroleum spirit - ethyl acetate (50:50) solvent, followed by the fraction eluted with ethyl acetate solvent. The residues eluted from petroleum spirit - ethyl

acetate (50 : 50) and ethyl acetate were named as *Pimelea* A and B fractions respectively. The three other non-toxic fractions obtained were discarded.

2.3.3 HPLC Purification

For subsequent purification of the *Pimelea* toxins by preparative HPLC, the *Pimelea* A & B fractions were combined. The combined material was called *Pimelea* A,B toxic fraction. The various toxic and non-toxic components in the combined fraction were then separated by reverse phase HPLC using a methanol/water gradient. Appropriate solvent regimes for analytical, semi-preparative and preparative scale separations were developed as described in Section 2.2.5 and representative chromatograms are given in Figures 2.4 - 2.7.

From the preparative scale HPLC separation of 150 mg of *Pimelea* A,B toxic fraction as shown in Figure 2.4, 80 fractions were collected and these fractions were grouped into 9 major fractions as described in Table 2.8. Each of the 9 fractions were evaporated to dryness and the residues tested for piscicidal activity using the fish bioassay technique. It was observed that fractions 1-3 obtained from the first 45 minutes of the run were non-toxic to fish. All test fish remained alive after 24 hours.

Residues from fraction 4-9, i.e. fractions eluted between 55-80 minutes were toxic to fish. Residues derived from fractions 6 and 7 were found to be most toxic to fish , causing the death of the test fish within 15 minutes. The toxic fractions were combined together and evaporated. The residue thus obtained was called *Pimelea* C toxic fraction. *Pimelea* C fraction was stored under nitrogen at -20° C in the dark until required for the conjugation procedure.

Figure 2.4

A representative chromatogram for separation of *Pimelea* A,B Fraction by analytical scale reverse phase HPLC

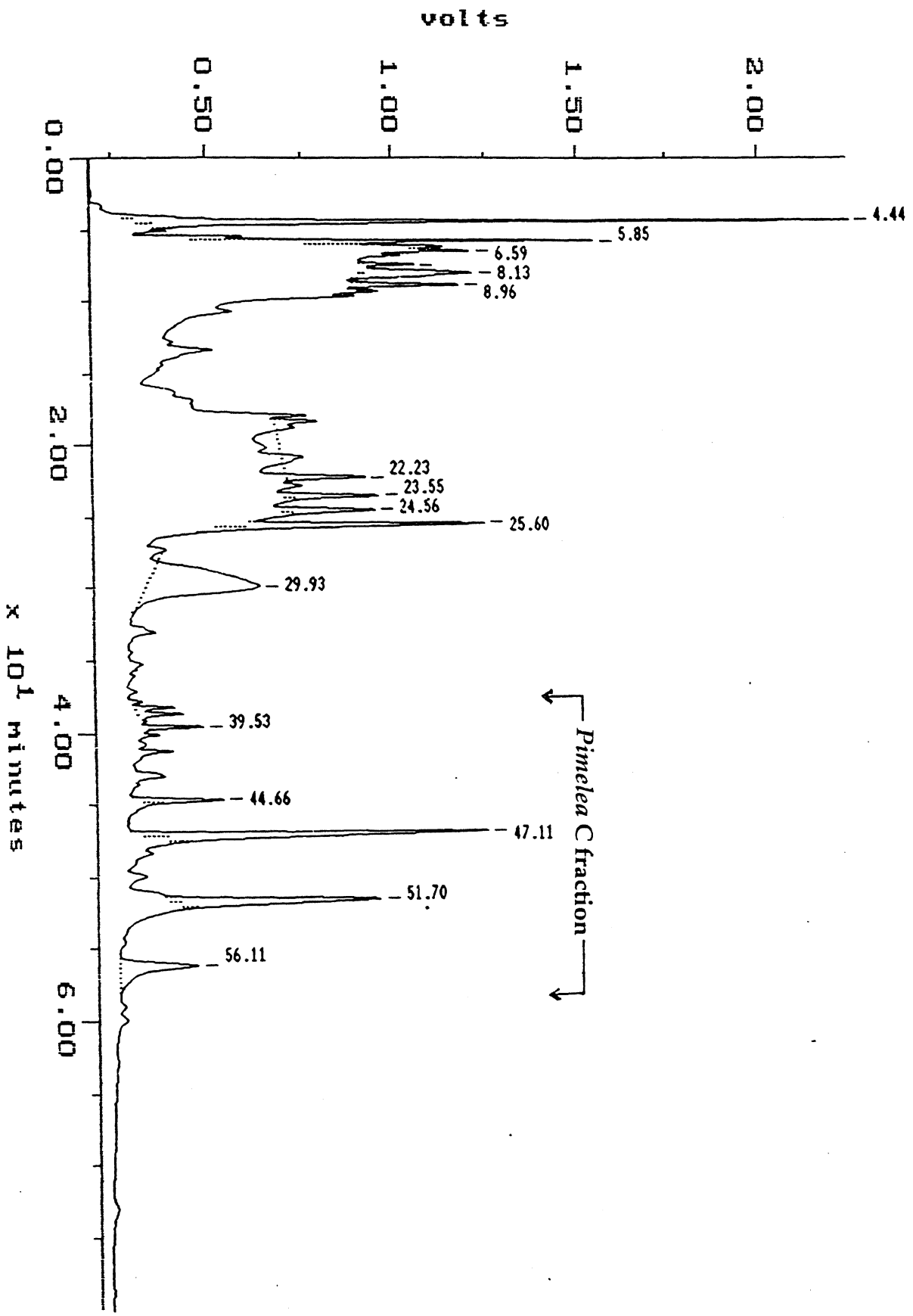


Figure 2.5

A representative chromatogram for separation of *Pimelea* A,B Fraction by semi-preparative scale reverse phase HPLC

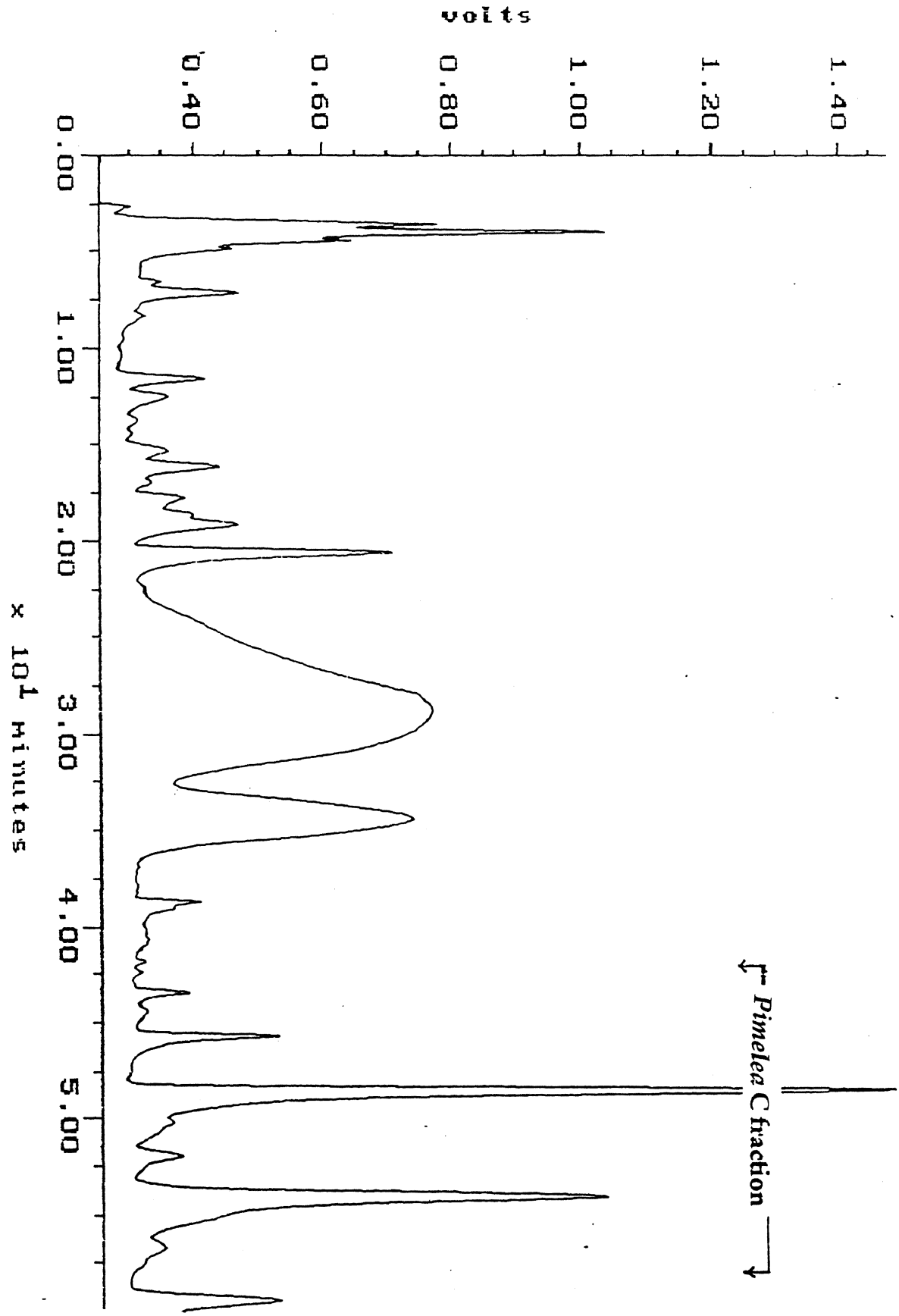


Figure 2.6

A representative chromatogram for separation of *Pimelea* A,B Fraction by preparative scale (40 mg) reverse phase HPLC

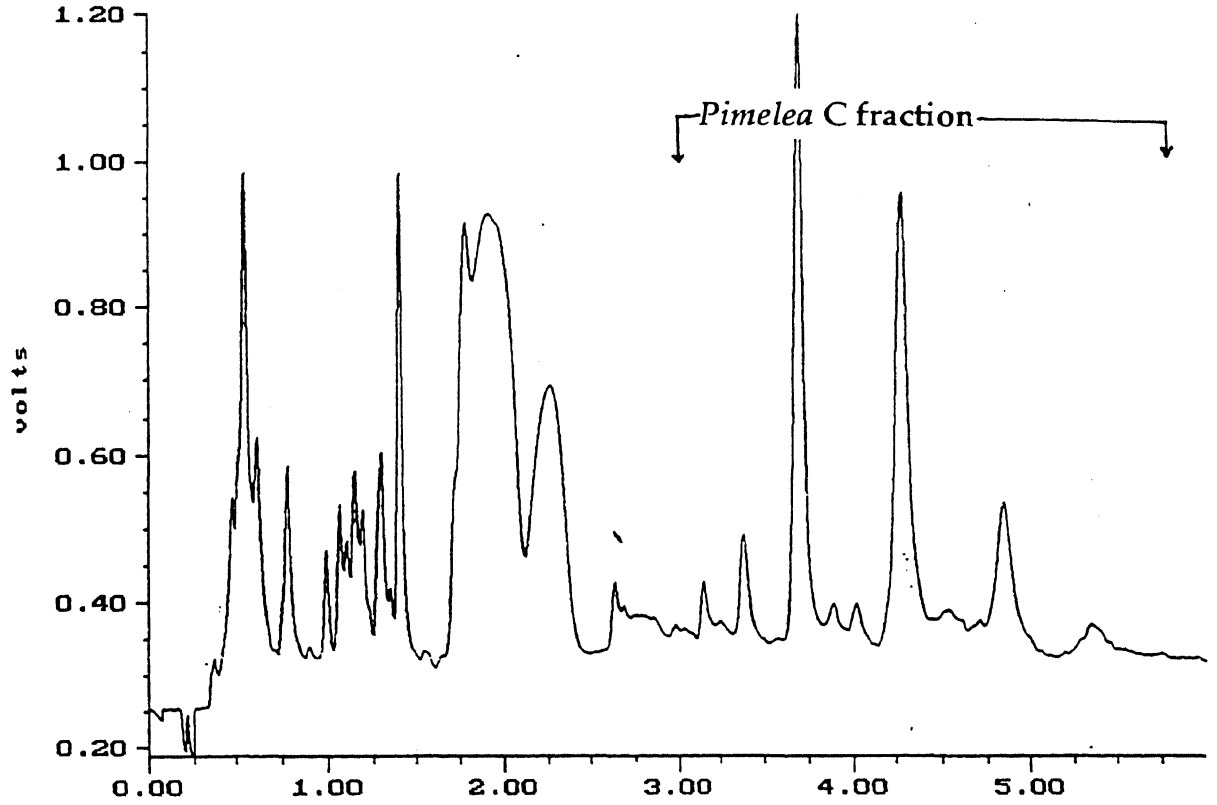


Figure 2.7

A representative chromatogram for separation of *Pimelea* A,B Fraction by preparative scale (150 mg) reverse phase HPLC

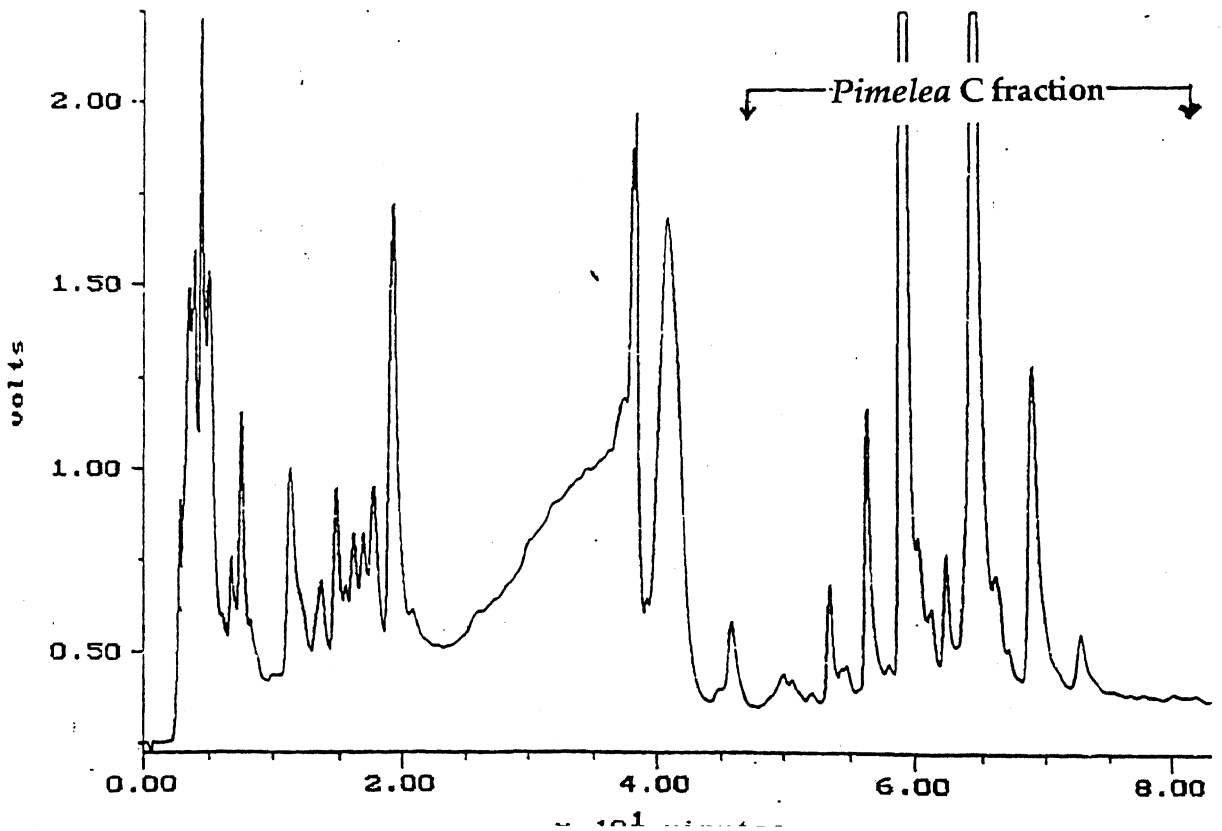


Table 2.8

Grouping of fractions obtained from preparative HPLC purification of *Pimelea* toxins.

Time (min)	Fraction No.	Toxicity
0-19	1	non toxic
20-40	2	non toxic
41-45	3	non toxic
46-54	4	toxic
55-59	5	toxic
60-63	6	highly toxic
64-68	7	highly toxic
69-73	8	toxic
74-80	9	toxic

2.3.4 Toxin : Protein Molar Incorporation Ratios

Freeman *et al.*, (1975) reported molar absorbtivity coefficients at 244 nm for the main daphnane toxins in *Pimelea simplex* , namely: simplexin (8100); simplexin diacetate (5900); and 12-acetoxy huratoxin (18,600). For the purposes of calculating molar incorporation ratios after completing the conjugation procedure, the molar absorbtivity coefficient for simplexin was used and the absorbance contributed by the 4 mg/100 mL protein component at 244 nm subtracted as described in Section 2.2.7. Molar incorporation ratios ranging from approximately 7.0 to 17.0 were obtained and these data are detailed in Table 2.9.

Table 2.9

Determination of toxin/protein incorporation ratios by U.V. analysis at 244 nm. for 4 mg/100 mL protein solutions.

Conjugate	Δ Abs. between conjugate and protein solutions.	Protein (μM)	Toxin (μM)	Incorporation ratio.
A-HSA[a]	0.083	0.580	10.2	17.6
B-HSA[a]	0.033	0.580	4.07	7.0
C-OVAL[b]	0.14	0.89	12.4	13.9

[a]HSA molecular weight 69,000. [b]OVAL molecular weight 45,000.

2.3.5 Protein Recovery

The protein recovery for the freeze dried *Pimelea* A -HSA and *Pimelea* B - HSA conjugates was determined using a TCA precipitation method as described in Section 2.2.8. The data presented in Table 2.10 indicates a high level of $NaHCO_3$ incorporation during the dialysis step. These data were used for determining the amount of conjugate required for the vaccination experiments described in Chapter 3.

Table 2.10

Protein recovery in freeze dried conjugates of *Pimelea* A and B fractions.

Pimelea -HSA conjugate	Wt of freeze dried conjugate (mg)	Wt of protein (mg)	Wt. of salt (mg)
A	100	27	72
B	100	35	65

The *Pimelea* C-, mezerein- and resiniferinol ortho phenyl acetate -ovalbumin conjugates were prepared on a much smaller scale than the corresponding HSA conjugates of the *Pimelea* A and B fractions. TCA precipitation was not attempted for these smaller scale preparations and for the purposes of vaccine preparation described in Chapter 3, the amount of protein employed initially in each case was used to estimate the amounts of freeze dried conjugate required. The recovery of each of the ovalbumin conjugates obtained is given in Table 2.11 below.

Table 2.11

Recovery of freeze-dried conjugates

Phorbol	wt of protein conjugate (mg)	Toxin (mg)	wt. of freeze dried (mg)
Pimelea C	67.5	30	161
Mezerein	45	20	250
Resiniferinol	45	20	216

2.4 CONCLUSIONS

Pimelea toxins were isolated successfully from *Pimelea trichostachya* whole dry plant through modification of the methodology described by Tyler and Howden (1979). Significant improvements were made to the silica column chromatography step by replacing benzene/ethyl acetate mixtures with equivalent polarity petroleum spirit/ ethyl acetate solvent mixtures. The fish bioassy technique used by Sakata *et al.*, (1971) to establish the toxicity of huratoxin like compounds was applied to test the piscicidal activity of fractions isolated from *Pimelea*. It was reasoned that the daphnane and tigliane toxins present in *Pimelea* should also be potent piscicides and this was confirmed experimentally.

Preparative scale HPLC methodology for separation of components in toxic fractions obtained from silica column chromatography was developed. This approach could be further scaled up if required for future work. The method developed allows preparative separation of each of the major toxin components. Toxic fractions (A and B) obtained from silica gel chromatography and toxic fraction C obtained from preparative HPLC were conjugated either to HSA or ovalbumin. U.V analysis of the dialysed conjugates showed molar incorporation ratios of toxins to protein ranging from 7 to 17.0.

CHAPTER THREE

IMMUNOLOGICAL STUDIES

3.1 INTRODUCTION

The isolation of *Pimelea* toxins was described in the previous chapter, along with the conjugation chemistry used for linking the toxins to immunogenic carrier proteins. This Chapter describes the preparation of prototype vaccines derived from these conjugates, and presents the results of vaccination trials in rabbits and cattle. The aims were first to confirm the presence of specific antibodies against *Pimelea* toxins in rabbits and cattle and then to evaluate the potential of vaccinated cattle to combat *Pimelea* poisoning in toxin challenge experiments. In addition, we wished to purify total serum IgG from vaccinated cattle and rabbits for use in organ bath experiments utilising bovine pulmonary venule preparations (see Chapter 4). The first laboratory task to initiate these studies was to prepare water-in-oil emulsions of the freeze-dried toxin-protein conjugates. These preparations also contained adjuvants to promote the immune response in test animals (Harlow and Lane, 1988).

Adjuvants are non-specific stimulators of the immune response. Most adjuvants incorporate two components. One is a substance designed to form a deposit protecting the antigen from rapid catabolic breakdown. The second component needed for an effective adjuvant is a substance that will stimulate the immune response non-specifically. These substances act by raising the level of a large set of soluble peptide growth factors known as lymphokines. Lymphokines stimulate the activity of antigen processing cells directly and cause a local inflammatory reaction at the site of injection. In the present study, Freund's adjuvant was used to prepare vaccines for rabbits, whilst a commercial vaccine formulation was used for cattle (Hoskinson, 1990).

Freund's adjuvant is a water-in-oil emulsion prepared with non-metabolisable oils. If the mixture contains killed *M. tuberculosis*, it is referred to as complete Freund's adjuvant (CFA), whereas without the bacterial component it is known

as incomplete Freund's adjuvant (IFA). CFA is most often used for the primary vaccination only, while IFA is employed for secondary and any subsequent vaccinations. Freund's adjuvant has proven to be one of the best adjuvants available for stimulating strong and prolonged responses in experimental animals. Major disadvantages of Freund's adjuvant is that it often invokes very aggressive and persistent granulomas and also, it cannot be used for commercial livestock applications. For these reasons, the commercial formulation was used for the cattle experiments.

In the present study, two groups of rabbits were to be vaccinated with either the *Pimelea* A-HSA or *Pimelea* B-HSA conjugate. Three groups of cattle were to be vaccinated with either *Pimelea* C-ovalbumin, mezerein-ovalbumin or resiniferinol-ovalbumin conjugates. Vaccination of cattle with the mezerein and resiniferinol conjugates would determine whether the antibodies raised against these two commercially available daphnane orthoester compounds would cross react with toxins present in the *Pimelea* C fraction. The rationale for this approach was to establish whether the commercially available compounds might form the basis of a vaccine to combat *Pimelea* toxicity in cattle.

An additional requirement of this part of the project would be the development of an enzyme linked immunosorbent assay (ELISA) to detect specific antibodies against *Pimelea* (or commercially available) toxins from vaccinated animals.

3.2 METHODOLOGY

3.2.1 Vaccination of Rabbits with *Pimelea* A-HSA and *Pimelea* B-HSA Conjugates

This section describes: the formulation of vaccines derived from freeze-dried *Pimelea* toxin-protein conjugates; the vaccination schedule for primary and

subsequent booster vaccinations; injection and bleeding protocols; and the collection of serum for ELISA studies. Rabbits A-C were vaccinated with the *Pimelea* -A conjugate, while rabbits D-F were vaccinated with *Pimelea* -B conjugate.

Vaccine Preparation :

Oil-in-water emulsion vaccines were formulated from the *Pimelea* toxin-HSA conjugates as follows :

(i) Freeze-dried material equivalent to 250 µg of conjugate (*Pimelea* A-HSA or *Pimelea* B-HSA) was used per vaccine per animal.

(ii) The weighed freeze dried conjugate (9.24 mg of *Pimelea* A-HSA conjugate and 7.14 mg of *Pimelea* B conjugate) was dissolved in 5 mL of saline. This preparation represents the aqueous phase.

(iii) CFA (Sigma Chemical Co.) was used as the adjuvant for primary vaccination and IFA (Sigma Chemical Co.) was used for subsequent booster injections.

(iv) To 5 mL of the appropriate adjuvant solution, the aqueous phase prepared in (ii) above was added dropwise. An emulsion was formed by homogenisation of the mixture with an Ultra-Turrax homogeniser employing a 527 N head attachment at 135000 rpm. Care was taken to avoid denaturing the protein due to heat of mixing. A drop of the emulsion thus formed was tested on water to establish that it did not disperse.

(v) Each rabbit was vaccinated with a total of 1 mL of the emulsified preparation, usually via multisite intra dermal (I.D.) injection.

Vaccination Schedule

The following vaccination schedule was employed:

Day	Vaccine formulation
0	250 μg conjugate, CFA adjuvant, multisite I.D.
27	250 μg conjugate, IFA adjuvant, multisite I.D.
118	250 μg conjugate, IFA adjuvant, multisite I.D.
140	1 mg conjugate, 1 mL saline containing 100 μg commercial adjuvant, I.P.
176	1 mg conjugate, 1mL saline containing 100 μg commercial adjuvant, I.P.

Mode of Injection

Two 5.0 cm x 5.0 cm sections on the lower back of the rabbit were shaved. Then given multiple site intradermal injections up to 10-12 sites (0.1 mL/site) on the rabbit's back in an effort to limit granuloma formation. The injected material was expected to drain into the local lymphatic system and become concentrated in the lymph nodes closest to the sites of injection. In an effort to boost the antibody responses in animals late in the experiment, two intraperitoneal injections (I.P.) of the conjugates in saline (containing the commercial adjuvant) were administered on days 140 and 176. Antigens injected into the peritoneum drain into the thoracic lymphatic system.

Bleeding Animals via the Marginal Ear Vein

Samples of blood from vaccinated animals were usually taken 7-14 days after injection. This timing corresponds with the peak antibody response for most injection routes. The blood is usually collected from the ear vein of rabbits since this site is easily accessible and does not have high numbers of nerve endings. Between 5-10 mL of blood was collected from each animal using the procedure described below.

The rabbit was wrapped in a towel and the ear shaved about two-thirds of the distance from the head to the tip of the ear around the marginal ear vein . The marginal vein was clearly visible on the inner edge of the ear. The easiest method found for collecting blood from the ear was to make a transverse cut at a 45° angle to the vein with a sterile scalpel and then collect the blood by allowing it to drip into a clean glass test tube. After collecting 5-10 mL, the blood flow was stopped by gentle application of pressure to the cut with a sterile piece of gauze or bandage for 10-20 seconds. It is important that the rabbit is kept warm and relaxed throughout this procedure. If the rabbit is stressed, the fright response is to constrict the ear artery.

Bleeding schedule

Animals were bled according to the following schedule:

(i) pre-immunisation bleed;

(ii) 28 days post-2° vaccination;

(iii) 10 days post-3° vaccination;

(iv) 10 days post-4° vaccination;

(v) 7 days post-5° vaccination.

Isolation of serum from blood

Blood was allowed to clot in the collection container at room temperature for 2-3 h. Serum was then removed by pipette and the clot was then centrifuged to allow collection of residual serum. Serum aliquots were stored in plastic tubes at -20° C for future use.

3.2.2 Immunisation of Cattle with *Pimelea* C - Ovalbumin, Mezerein -Ovalbumin and Resiniferinol - Ovalbumin Conjugates

Three experimental groups comprising six cattle (3 males and 3 females) each were vaccinated with either *Pimelea* C-ovalbumin, mezerein-ovalbumin or resiniferinol-ovalbumin conjugates. A group of six control cattle were also vaccinated with the carrier protein (ovalbumin). As the conjugates were all dialysed for several days against 50 mM $NaHCO_3$, the freeze dried material also incorporated a significant amount of sodium bicarbonate along with the toxin-protein component. For each vaccination, an amount of the freeze-dried conjugate material approximating 2 mg of conjugate was used per animal. The original weight of protein used in the conjugation and recovery of freeze-dried material is given in Table 3.1 below. Estimation of the amount of freeze-dried conjugate needed to prepare each dose of the vaccine was based on the amount of protein originally used to prepare the conjugate. (The rationale for this simple approach was that while a 20-fold incorporation of toxin molecules per protein molecule would raise the molecular weight of the protein conjugate by approximately 10,000 mass units to 55,000, this increase in molecular weight would be almost exactly compensated for if only 80% of the protein fraction was recovered.)

Table 3.1

Recovery of freeze-dried toxin-ovalbumin conjugates used for vaccination of cattle

Toxin	wt of protein (mg)	freeze-dried recovery (mg)	amount of freeze-dried material for 7 vaccines (mg)
Pimelea C	67.5	161.0	38
Mezerein	45.0	250.0	81
Resiniferinol	45.0	216.0	70

Vaccine Preparation

(i) A mixture of ondina 15 oil (94.5 mL, Shell company of Australia Ltd.) and emulsifier, arlacel A (10.5 mL, Sigma) was stirred vigorously on a magnetic stirrer.

(ii) The amount of freeze-dried conjugate required (see Table 3.1) was dissolved in a mixture of saline (5 mL) and then made up to 10.5 mL with 15 % DEAE-Dextran solution with pH between 6.5-7.5. (The pH of the 15 % DEAE-Dextran solution was adjusted with conc TRIS). Hereafter this solution is called the aqueous phase.

(iii) The aqueous phase (10.5 mL) was added slowly via a 10 mL syringe to a vigorously stirred 24.5 mL aliquot of the oil/arlacel A solution prepared in (i) above. (The mixture turned milky at this stage.)

(iv) The loose emulsion thus formed was then further emulsified using an Ultra-Turrax homogeniser T25 (527 N head attachment at 13500 to 20500 rpm). This gave a creamy emulsion which did not disperse when tested on water.

(v) Animals were given subcutaneous vaccinations on both sides of the shoulder with 5 mL of the emulsion.

(vi) The same protocol as described in (i)-(v) above was used for formulation and administration of vaccines for secondary and subsequent vaccinations. The only modification being that half the amount of conjugate was used (approximately 1 mg/ vaccination) for 3° and 4° vaccinations.

Vaccination Schedule

The vaccination schedule employed is given below.

Day	Vaccination
0	1°
27	2°
55	3°
85	4°

Bleeding schedule

The following bleeding schedule was used to collect serum from immunised cattle for ELISA studies. Blood (20 ml) was collected from the jugular vein using disposable plastic (25 mL) syringes and sterile needles.

Day	Collection
0	1°
27	2°
42	2 weeks post 2°
55	3°
70	2 weeks post 3°
85	4°
99	2 weeks post 4°
104	post toxin challenge experiment

3.2.3 Enzyme-Linked Immunosorbent Assay (ELISA) Analysis of Antibody Responses

ELISA is a powerful technique applied routinely for the detection and quantitation of a wide variety of analytes. For determining the presence and amount of antibody, a diluted serum sample is incubated with a solid phase (ELISA plate) coated with excess antigen (i.e. more than enough antigen to bind all of the antibody present in a given sample). Any antigen-specific antibodies present bind to the antigen coated on the solid phase. After washing, bound antibody is detected by an enzyme-labelled anti-immunoglobulin- conjugate. Unbound conjugate is washed away, and an enzyme substrate added to yield a coloured product. The amount of colour developed is proportional to the amount of antibody in the sample.

In the present study, the daphnane and tigliane diterpene toxins being investigated had very limited water solubility so a toxin-protein conjugate was required to coat the plate with antigen. The protein used for coating should be

unrelated to the carrier protein used in the vaccine. Hence the ELISA plate would need to be coated with a toxin-ovalbumin conjugate in the case where animals had been vaccinated with a toxin-HSA conjugate.

Antigen attachment and the stability of that attachment to the solid phase is important in ELISA. Experience with a number of monoclonal antibodies, polyclonal antibodies and other proteins has shown that 1-10 μg/mL is usually the optimum range for coating of antigens. It is important not to coat the plates with highly concentrated antigen since high levels of coating protein usually leaves behind a significant amount of loosely adsorbed material which desorbs during subsequent assay steps and interferes in the assay. The conformation of the antigen also may be altered at high binding densities and antibody binding sites may be sterically obscured because of multi-layered packing. The most widely used coating buffers are 50 mM carbonate at pH 9.6, 20 mM Tris-HCl at pH 8.5 and 10 mM PBS at pH 7.2. When coating proteins, it is best to use buffers with a pH value 1-2 units above the pI of the protein. Wash solutions are required to remove all extraneous and loosely bound material from the surface of the solid phase. Commonly used wash solutions are PBS or TBS containing 0.05 % Tween 20 (to lower surface tension and assist the flushing process).

After the plate has been coated with antigen, a blocking step is used to minimise non-specific binding of antibody to the solid phase support. Casein or non-fat dry milk, which contains a large proportion of casein, are most effective blocking agents. The rationale proposed for the efficacy of casein is that it is a heterogeneous mixture of large protein complexes (casein micelles), individual casein molecules and smaller peptide fragments of various charges and hydrophobicity. It is believed that this mixture of proteinaceous material

inhibits both hydrophilic and hydrophobic binding of antibody molecules which would otherwise cause background problems.

In the present work, an antibovine IgG-HRP (horse radish peroxidase) conjugate was to be used for cattle antibody detection, while an analogous antirabbit-HRP conjugate would be required for the rabbit ELISA. Normally, a 1:4000 dilution of the commercially available antibody-HRP conjugate in assay buffer is a useful starting concentration , although experiments to determine the optimum dilution of the second antibody need to be undertaken for each batch.

On an antigen coated microtitre plate, different concentrations of sample are added to each row of wells. After incubation and washing, different concentrations of anti-IgG-HRP conjugate are added to each column of wells. The plate is incubated and washed, then enzyme substrate is added to facilitate colour development. The results allow the selection of the conditions which provide optimum substrate colour development in the desired concentration range of the analyte. Diluents for horseradish peroxidase conjugates should never contain sodium azide, since azides are powerful peroxidase inhibitors.

A number of different HRP substrates are routinely used in ELISA. TMB was chosen for this study as it is known to be efficient for the detection of low levels of enzyme (approximately 4 times more sensitive than o-toluidine and ABTS and 2 times more sensitive than OPD) and also has a higher absorbance plateau than the other substrates. The incubation time required for colour development to be complete is 25 minutes. For most ELISA assays, phosphate or Tris buffered saline of pH 7.0 - 7.5 is a suitable buffer. The buffer should be supplemented with some type of non-interfering protein, to help block non-specific binding. Commonly used proteins are BSA, SMP, gelatine or normal

animal sera. (If an assay only uses antibodies from one species only, then non-immune serum from the same species would be the best one to use.)

Reagents and Materials

Immunol 4 (Flat bottom) 96 well polystyrene microtiter immunoassay plates (Dynatech Laboratories, USA), were used in all experiments. The coating buffer used to dissolve the antigen was 50 mM carbonate buffer (pH 9.6) which was prepared by dissolving 4.2 g of $NaHCO_3$ in 1 litre of distilled water and the pH was then adjusted by the addition of 2M NaOH. Stock solutions (0.5 mg/mL) of the freeze-dried conjugate materials (including salts) were prepared in coating buffer .

Working solutions for coating plates were prepared by diluting 100 µL of the 0.5 mg/mL stock solutions in 10 mL of coating buffer which gives 0.5 µg/mL actual conjugate concentration. The wash solution (0.01 M PBS/0.05 % Tween 20) was prepared by dissolving Na_2HPO_4 (7.1 g), EDTA (1.9 g), NaCl (40.9 g) in 2 L of distilled water. The pH of the mixture was adjusted to 7.5; 2.5 mL of Tween 20 added and then the final volume made up to 5 L. The blocking solution was prepared by dissolving 1 g of skim milk powder in 100 mL of assay buffer. The blocking solution was then filtered and the pH adjusted to 7.5. The colour reagents used was either TMB or ABTS (Kikegard and Perry laboratories Inc.).

A stock solution of TMB (0.5 mg/mL) in DMSO was stored at 4 ^{o}C. A working TMB solution was prepared just before use by mixing 2 mL of the stock solution and 1.5µL of 30 % H_2O_2 in 8 mL of 0.1 M acetate buffer (pH 5.6; 100 µL required per well). The acetate buffer was prepared by dissolving sodium acetate (13.6 g) in 1 L of double glass distilled water and the pH adjusted to 5.6 by the addition of glacial acetic acid. The stop solution employed for the TMB

colour reagent system was 0.5 M H_2SO_4 (100 µL added per well at the completion of the substrate incubation period). For ABTS colour reagent the stop solution used was 1 % SDS (100 µL per well). The assay buffer (0.01 M PBS /0.2 % BSA, pH 7.5) was prepared by dissolving Na_2HPO_4 (1.42 g), EDTA (0.37 g) , NaCl (8.17 g) and BSA (2.0 g) in approximately 500 mL of distilled water. The pH of the mixture was adjusted to 7.5 and the volume then made up to 1L. The assay buffer was filtered before use. Unless otherwise specified, assay plates were sealed with sticky plate sealer after each step in the procedure to prevent evaporation of solutions from the wells.

Coating Plates with Antigen

(i) Immunol 4 microtiter immunoassay plates were coated overnight at 4°C with 50 µL per well of the conjugate coating solutions. (Care was taken throughout the procedure to ensure that all samples were incubated for the same length of time.)

(ii) The plates were washed 5 times on an automatic washer (Titrek's Microplate Washer 120) with wash solution to remove the unbound antigen. Plates were then flicked dry on a blotting paper to ensure that the wells were as dry as possible.

Blocking

After coating the wells with antigen, non-specific binding sites in the wells were blocked with 300 µL of blocking solution added via a multi-channel pipette. The plate was then incubated for 1h at room temperature before being washed 5 times using the automatic washer. Plates were then flicked dry to ensure that the wells were as dry as possible.

Sample Addition and Incubation

Appropriate dilutions of serum in assay buffer (50 µL/well) in duplicate or triplicate were added to the wells according to a pre-determined layout arrangement. The well contents were mixed using a automatic shaker (IKA-Schuttler MTSG shaker, Flow Laboratories), the plates covered with aluminium foil and then incubated at room temperature for two hours. After incubation, the plates were washed 5 times using the automatic washer and then flick-dried as previously described. Serum samples were added quickly to the wells to minimise the variations in incubation times.

Addition of the second antibody

Fresh antibody-HRP conjugate (Silenus Laboratories, diluted 1:4000) was prepared in assay buffer and 50 µL of this solution then added to each well. (Anti-bovine IgG-HRP was used for analysis of cattle sera while anti-rabbit-HRP conjugate was used for detecting antibodies in the rabbit ELISA.) The solutions in the wells were mixed and the plates then incubated at room temperature for 1 hour. As the HRP conjugate is light sensitive, plates were always covered with aluminium foil. After incubation, the plate was washed 5 times using the automatic washer and then flicked-dry as previously described.

Colour development

The TMB working solution (100 µL/well) was added via a multi-channel pipette. The plate was then covered with aluminium foil and incubated for 30 minutes at room temperature. Colour development was stopped by adding 100 µL of the stop solution to each well using a multi-channel pipette. This changes the colour from blue to yellow and this absorbance was read at 450 nm using a Biorad 3550 Microplate Reader via Microplate Manager software on an IBM compatible 386 microcomputer.

3.2.4 *Pimelea* Challenge Experiment with the *Pimelea* C -Ovalbumin Vaccinated Cattle

Control cattle (n=6) and cattle vaccinated against *Pimelea* C fraction (n=6) received an oral drench daily of milled *Pimelea* plant material based on body weight (Table 3.2). The dose of milled dried plant material was mixed in water (500 mL) and the slurry put directly into the rumen using a stomach tube and funnel. Each animal had to be restrained in a crush and the tube inserted carefully through the mouth until the rumen was reached (as evidenced by the odour of ruminal fluid).

Table 3.2

Body weights and sex of cattle used in the vaccination trial.

Control Cattle			*Pimelea*-C -OVAL vaccinated cattle		
CattleNo.	Sex	Weight (kg)	CattleNo.	Sex	Weight (kg)
1001	F	200	1008	F	190
1002	F	222	1010	F	166
1006	F	190	1011	F	200
1016	M	174	1015	M	186
1019	M	188	1021	M	212
1023	M	246	1022	M	190

Doses were administered on a body weight basis as follows :

50 mg/kg live weight	Day 0 to 9
100 mg/kg live weight	Day 10 to 14
250 mg/kg live weight	Day 15 to 17

Blood samples were collected each day and the physical appearance and pathology of each animal monitored. Blood parameters such as haemoglobin levels, haematocrit, leucocytes, neutrophils, cortisol, transaminases, total protein, urea and red cell count were measured at Dr T. B. Lynch Pathology Laboratory, Rockhampton.

3.2.5 Purification of IgG from Vaccinated Cattle and Rabbits

General

Econo-Pac Serum IgG purification columns (3 mL serum capacity; Bio-Rad Laboratories, Richmond, CA., USA) were used for purification of IgG from rabbit and cattle serum. Serum was collected from two of the vaccinated cattle (1008 and 1016) showing high antibody responses on ELISA and also from rabbit F vaccinated with the *Pimelea* B- HSA conjugate. Econo-Pac 10 DG desalting columns were used for sample preparation. The purified serum sample was chromatographed on pre-packed DEAE Affi-Gel Blue agarose gel columns (Econo-Pac serum IgG purification columns) to produce a purified IgG fraction expected to be free from other serum proteins and plasminogen with only residual contamination from transferrin.

Reagents

Application and regeneration buffers were supplied as premixed solids. Buffer solids were stored at room temperature. To prepare the application buffer (0.02 M Tris-HCl, pH 8.0, 0.028 M NaCl), 1.3 g of the supplied buffer solids were dissolved in 300 mL of Millipore filtered water and the solution then filtered through a 0.45 μm filter. The pH of the buffer was adjusted to 8.0 ± 0.2 with either NaOH or HCl as required and the solution stored at 4° C.

The regeneration buffer (2M guanidine.HCl in application buffer) was prepared by dissolving 12.2 g of the supplied buffer solids in 100 mL of Millipore filtered

water and the solution filtered through a 0.45 µm filter. No pH adjustment was necessary and the buffer was stored at 4° C until use.

Chromatography

For preparation of 3 mL of serum for purification on serum IgG purification columns the following procedure was used. (If the serum sample was less than 3 mL, application buffer was added to achieve a starting volume of 3 mL) The buffer above the top frit of Econo Pac 10 DG column was discarded and the bottom tip of the column was snapped off. Application buffer (20 mL) was added to column and the buffer allowed to drain to the top frit. The serum sample (3 mL) was added to the column and the first 3 mL eluted was discarded. A further 4 mL of application buffer was used to elute the serum from the column. The desalting columns were washed with 20 mL of application buffer before re-use. The desalted serum sample was then further purified as described below.

The buffer above the top frit of the Econo-Pac serum IgG purification column was discarded and the bottom tip of the column snapped off. Each column was then prewashed with 40 mL of regeneration buffer, followed by 40 mL of application buffer. (This initial wash removes residual blue dye in the columns which otherwise might be eluted with serum proteins.) The 4 mL fraction eluted from the desalting column was applied to the Econo-Pac serum IgG purification column in one portion. After allowing the sample to pass into the column, the IgG fraction was eluted with 20 mL of application buffer. Albumins were then eluted by washing the column with application buffer containing 1.4 M NaCl. Columns were then regenerated with 20 mL of regeneration buffer to ensure removal of bound proteins and to prevent cross-over contamination from one run to the next.

Spectrophotometric absorbance at 280 nm was measured for each IgG fraction so that the actual freeze dried recovery could be compared with that expected on the basis of absorbance. To remove salts, the IgG fractions were dialysed against distilled water for 24 h at 4°C. After dialysis, the liquid was freeze dried and the lyophilised material used for *in vitro* experiments described in Chapter 4.

Calculations of expected IgG recovery based on absorbance at 280 nm were performed as detailed below.

Rabbit :

Volume of rabbit serum used = 5 mL

Volume of the IgG fraction obtained = 25 mL

Absorbance at 280 nm = 0.812

Freeze dried IgG obtained = 29.7 mg

Standard absorbance of IgG is 1.35/1 mg

Therefore, calculated IgG present in IgG fraction (25 mL) = 33 mg which compares favourably with the amount actually recovered.

Cattle :

Volume of serum used = 150 mL

Volume of IgG fraction obtained = 850 mL

Absorbance at 280 nm = 1.920

Freeze dried IgG material = 1.4 g

Standard absorbance of IgG is 1.35/1mg

Therefore, calculated IgG present in IgG fraction (850 mL) = 1.1 g which approximates the amount actually recovered.

The Effect of Freeze Drying Purified IgG from Rabbits and Cattle.

To investigate the possibility for loss of antibody binding as a result of IgG purification and freeze drying, ELISA analysis was conducted with fresh serum

and an equivalent amount of the lyophilised IgG fraction. Serum dilutions of 1:500 for vaccinated rabbit, vaccinated cattle and normal rabbit serum (unvaccinated) were tested for antibody binding. These were compared with freeze dried IgG material obtained from the same quantity of serum in each case. Quantities of freeze dried material required were calculated as described below.

Cattle :

1.4 g of purified IgG was obtained from 150 mL of vaccinated cattle serum. Therefore, 128 μL of cattle serum is equivalent to 1.2 mg of IgG and this amount of the solid was dissolved in 128 μL of assay buffer. A 2.5 μL aliquot of each solution was further diluted in 5 mL of assay buffer to give equivalent 1: 500 dilutions.

Rabbit :

30 mg of purified IgG was obtained from 5 mL of vaccinated rabbit serum. Therefore, 167 μL of rabbit serum is equivalent to 1 mg of IgG and this amount of the solid was dissolved in 167 μL assay buffer. A 2.5 μL aliquot of each solution was further diluted in 5 mL of assay buffer to give equivalent 1: 500 dilutions.

3.3 RESULTS AND DISCUSSION

3.3.1 Antibody Responses in Rabbits Vaccinated with *Pimelea* A-HSA and *Pimelea* B-HSA Conjugates

Generally, an increase in B-cells bearing surface antibodies specific for the inoculated antigen should be detectable 5-6 days after the primary vaccination. Antibody is usually detected in the serum from around 7 days and persists at a low level for a few days, typically reaching a peak around day 10. Rabbits should remain effectively primed for at least a year after receiving the first vaccination. The response to a second injection of the same antigen given after

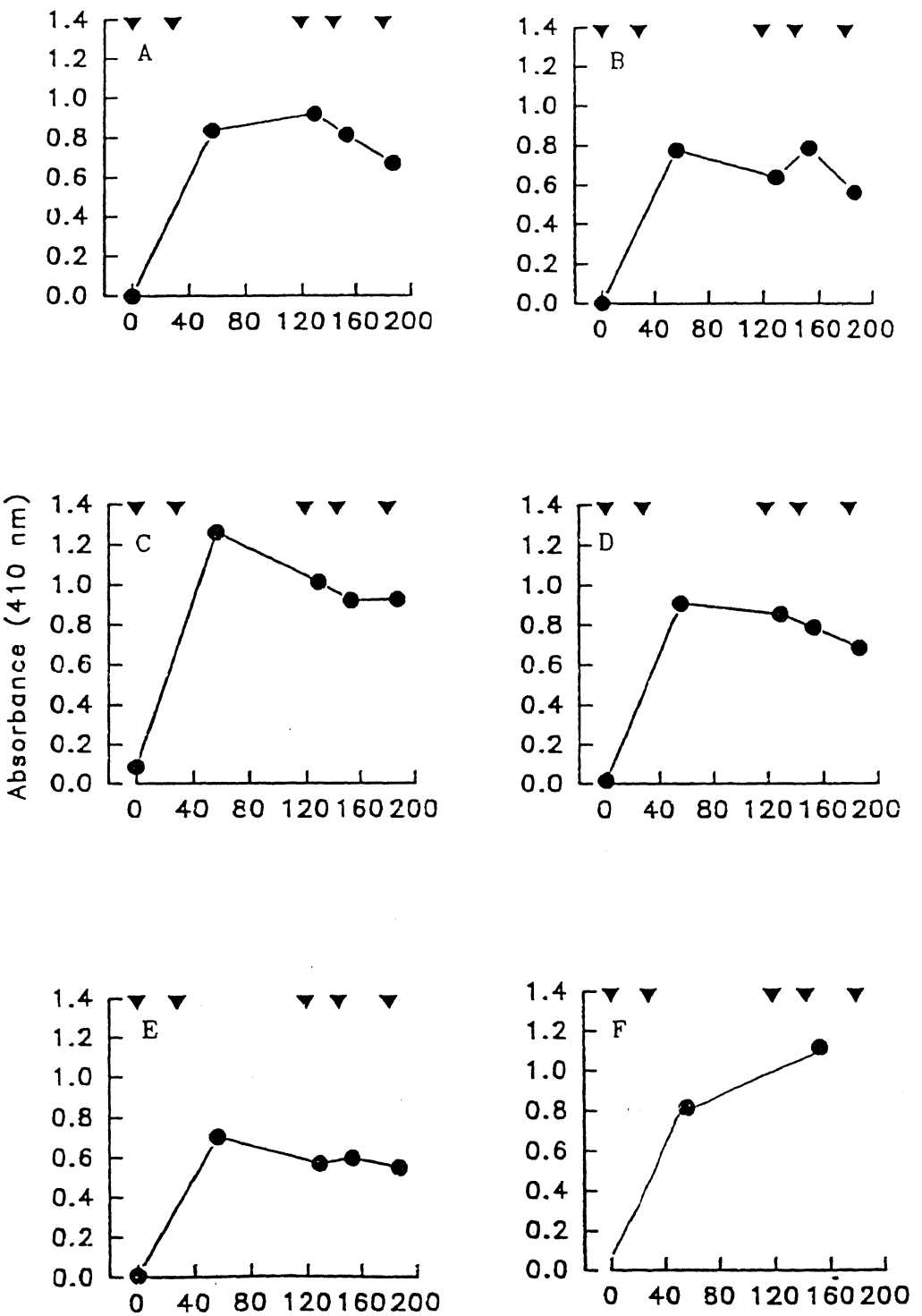
3-4 weeks is dramatically different. The number of B cells bearing antigen-specific cell-surface antibodies increases exponentially after the secondary vaccination, usually peaking between days 3 and 4. Antibodies in the serum are also detectable at this time but peak levels are usually observed in the second post-secondary week. High antibody levels persist for about 2-4 weeks after the secondary vaccination and then gradually diminish over time.

Rabbits A- C and rabbits D-F were vaccinated with the *Pimelea* A-HSA conjugate and the *Pimelea* B-HSA conjugate respectively, five times over a period of 129 days. Serum was collected as detailed in the methodology section and ELISA analysis was undertaken to monitor the time course of antibody responses for each rabbit. Serum dilutions of 1:500, 1:1000 and 1:5000 were analysed and the serum of an unvaccinated rabbit was used as a control blank. The absorbance of the control was subtracted from the test data.

Results for individual rabbits are given in Figure 3.1 and Table 3.3 and the group data is summarised in Table 3.4. The control serum and serum from test animals prior to immunisation (Day 0) showed no specific IgG which recognised the *Pimelea*- C -BSA conjugate coated on the plate. In contrast, significant antibody responses were detected 28 days post 2° vaccination for all six experimental animals at 1/5000 serum dilution. The data shows that the antibody responses remained high for the duration of the experiment. Subsequent booster vaccinations did not appear to cause further peaks in antibody responses. (The data presented for rabbit F is incomplete due to a shortage of serum.)

Figure 3.1

Time course of antibody responses in rabbits vaccinated with *Pimelea* A-HSA (animals A-C) or *Pimelea* B-HSA (animals D-F)



Abbreviation : ▼ indicates the day of vaccination.

Table 3.3

ELISA absorbance results[a] for rabbits A-F against a *Pimelea* C-BSA conjugate plate coating.

Rabbit	Serum dilution	Day 0	28 days post 2°	10 days post 3°	10 days post 4°	7 days post 5°
Control	1:500	0.127				
	1:1000	0.076				
	1:5000	0.008				
A	1:500	0.115	1.384	1.447	1.374	1.324
	1:1000	0.045	1.291	1.368	1.230	1.194
	1:5000	0.001	0.839	0.922	0.817	0.674
B	1:500	0.038	1.369	1.286	1.362	1.247
	1:1000	0.013	1.253	1.139	1.220	1.106
	1:5000	0.002	0.779	0.643	0.789	0.563
C	1:500	0.267	1.517	1.501	1.470	1.488
	1:1000	0.086	1.555	1.442	1.333	1.369
	1:5000	0.018	1.262	1.014	0.920	0.925
D	1:500	0.115	1.387	1.422	1.377	1.304
	1:1000	0.038	1.323	1.276	1.206	1.160
	1:5000	0.017	0.909	0.854	0.788	0.687
E	1:500	0.056	1.236	1.253	1.187	1.199
	1:1000	0.018	1.170	1.105	1.055	1.072
	1:5000	0.011	0.704	0.569	0.595	1.550
F	1:500		1.426		1.446	
	1:1000		1.269		1.415	
	1:5000		0.814		1.114	

[a] average of duplicate determinations

The grouped data presented in Table 3.4 reinforces the observation that the antibodies developed against the less pure *Pimelea* fractions, A and B conjugates gave good recognition to the more highly purified daphnane and tigliane fraction C conjugate coated on the plate. Moreover, these data vindicated our first approach to immunise animals with a conjugate derived from a mixture of compounds. It was reasoned that only those compounds containing a relatively unencumbered hydroxyl grouping would covalently

link to the protein. Fortunately, all of the main toxic components in *Pimelea* have a free hydroxymethyl grouping in the B-ring and the results suggest that these compounds were selectively conjugated from the mixture.

Table 3.4

Grouped ELISA data[a] for rabbits A-C and D-F [b]

Group	Serum dilution	Day 0	28 days post 2°	10 days post 3°	10 days post 4°	7 days post 5°
Cont-	1:500	0.127				
rol	1:1000	0.076				
	1:5000	0.008				
A -C	1:500	0.14±0.063	1.42 ± 0.004	1.41 ± 0.06	1.40 ± 0.028	1.35 ± 0.06
	1:1000	0.02±0.005	1.36 ± 0.090	1.31 ± 0.08	1.26 ± 0.034	1.22 ± 0.07
	1:5000	0.29±0.023	0.96 ± 0.150	0.85 ± 0.10	0.84 ± 0.034	0.72 ± 0.10
D-F	1:500	0.08 ±0.028	1.34 ± 0.050	1.33 ± 0.063	1.33 ± 0.075	1.25 ± 0.049
	1:1000	0.028±0.007	1.25 ± 0.040	1.19 ± 0.069	1.22 ± 0.10	1.11 ± 0.042
	1:5000	0.014 ± 0.002	0.80 ± 0.050	0.71 ± 0.11	0.83 ± 0.15	1.11 ± 0.43

[a] Results are presented as means ± SEM.

[b] Data compiled from Table 3.3

3.3.2 Antibody Responses in Cattle Immunised with *Pimelea* C - Ovalbumin, Mezerein -Ovalbumin and Resiniferinol - Ovalbumin Conjugates

Three groups of cattle (n=6) were vaccinated with ovalbumin conjugates prepared from *Pimelea* C fraction, resiniferinol and mezerein. Control cattle were vaccinated only with the carrier protein ovalbumin. The purposes of this experiment were to determine if cattle would mount a specific immune response to the daphnane-protein conjugates and also to gain preliminary

information on the degree of cross-reactivity of the antibody response. A central question was whether antibodies raised against resiniferinol or mezerein would cross react with daphnanes isolated from the plant.

ELISA analysis of serum samples collected one week post secondary vaccination was undertaken with plates coated with BSA conjugates of *Pimelea* C, mezerein and resiniferinol, and also with BSA alone. (The cross reactivity tests with *Pimelea* C-BSA toxin coated plates were carried out subsequent to the other analyses but care was taken to ensure that all analytical conditions were identical so that the data obtained could be compared.) The average absorbance derived from control serum (cattle vaccinated with ovalbumin only) was subtracted from all test absorbance values. In general, the cattle responded less strongly than the rabbits and the data given below are for 1/500 dilution of serum samples.

Binding of anti-ovalbumin antisera to BSA

All of the cattle groups had been immunised with ovalbumin conjugates, whereas the plate coating chosen to detect specific antibody responses was a BSA conjugate. The first task was to demonstrate that antisera from vaccinated animals did not bind to the wells when BSA alone was coated on the plate. Seven samples were analysed as detailed in Table 3.5. These data demonstrated very low binding levels to the BSA coating and confirmed that anti-ovalbumin antibodies did not significantly cross react with BSA. (Note that individual animals were numbered from 1001 - 1024 for the purposes of the experiment.)

Table 3.5

ELISA data for sera from animals immunised with ovalbumin carrier protein against a BSA plate coating.

Control group	Abs.	"Mez"- Oval group	Abs.	"Res" - Oval group	Abs.	*"Pim* - C" group	Abs.
1001	0.032	1003	0.043	1007	0.003	1008	
1002		1004		1009		1010	
1006		1005	0.028	1012	0.034	1011	
1016		1013		1014		1015	
1019		1017		1020		1021	
1023	0.019	1018	0.016	1024		1022	

Cross Reactivity

The data presented in Table 3.6 shows that all experimental groups raised antibodies which recognised a mezerein-BSA plate coating, indicating a considerable degree of cross - reactivity.

In an analogous experiment, plates were coated with a resiniferinol ortho phenyl acetate-BSA conjugate and cross-reactivity of antibody responses between the experimental groups established. The data obtained (Table 3.7) confirmed that antibodies raised in all three experimental groups did bind to the resiniferinol conjugate.

Table 3.6

ELISA analysis (means ± s.e.m.) of sera from vaccinated test animals with a mezerein-BSA conjugate plate coating (Average absorbance of controls has been subtracted.)

Control group	Abs.	"Mez"- Oval group	Abs.	"Res"- Oval group	Abs.	*"Pim* - C" group	Abs.
1001	0.156	1003	0.695	1007	0.452	1008	0.975
1002	0.106	1004	1.115	1009	0.533	1010	0.728
1006	0.118	1005	0.844	1012	1.024	1011	0.551
1016	0.121	1013	0.833	1014	0.476	1015	0.930
1019	0.116	1017	1.101	1020	0.486	1021	0.942
1023	0.144	1018	1.66	1024	0.889	1022	0.599

Table 3.7

ELISA analysis (means ± s.e.m.) of sera from vaccinated test animals with a resiniferinol-BSA conjugate plate coating.[a]

Control group	Abs.	"Mez" - Oval group	Abs.	"Res" -Oval group	Abs.	*"Pim* - C" group	Abs.
1001	0.148	1003	0.400	1007	0.353	1008	0.775
1002	0.103	1004	0.865	1009	0.302	1010	0.473
1006	0.11	1005	0.534	1012	0.814	1011	0.354
1016	0.110	1013	0.512	1014	0.343	1015	0.724
1019	0.102	1017	0.762	1020	0.270	1021	0.718
1023	0.148	1018	0.836	1024	0.641	1022	0.382

[a]Average absorbance of the control group has been subtracted from values given for experimental test groups.

The cross-reactivity results presented in Tables 3.6 and 3.7 for individual animals 28 days post 2° vaccination is summarised in Table 3.8 for each experimental group .

Table 3.8

Summary of ELISA absorbance data[a] for 1/500 serum dilution in cross-reactivity assays

Vaccination group (n=6)	Mezerein -BSA plate coating	Resiniferinol-BSA plate coating
Pimelea C-Oval	0.79 ± 0.07	0.57± 0.07
Mezerein - Oval	1.04 ± 0.13	0.65 ± 0.07
Resiniferinol-Oval	0.65 ± 0.09	0.45 ± 0.08
Control	0.12 ± 0.007	0.12 ± 0.009

[a] Average absorbance of the control group has been subtracted from values given for experimental test groups. Values are given as means ± SEM.

This data supported our initial hypothesis that antibodies developed to commercially available daphnane orthoesters such as resiniferinol orthophenyl acetate and mezerein should also recognise the native daphnane toxins from *Pimelea* species. Of the two compounds, the data suggests that mezerein would be the preferred candidate for further studies since it gives a stronger antibody response and also provides better recognition of anti -*Pimelea* toxin antibodies than resiniferinol. A further advantage is that mezerein is the less expensive of the two compounds. [Unfortunately the *Pimelea* C - BSA conjugate (see Section 3.3.3) was not available at the time these antibody cross-reactivity assays were being conducted.]

In performing these cross-reactivity experiments, it would have been preferable to coat the toxin directly to ELISA plates by first dissolving the toxin in an organic solvent. Some preliminary experiments were conducted but coating these small organic toxin molecules in this manner proved capricious. There was a great deal of variability in the data, suggesting non-uniform

retention of the toxin on the plate during the ELISA procedure. For this reason the BSA -toxin conjugates were used and reproducible results were obtained.

3.3.3 Time course of Antibody Responses in *Pimelea* C- Ovalbumin vaccinated Cattle.

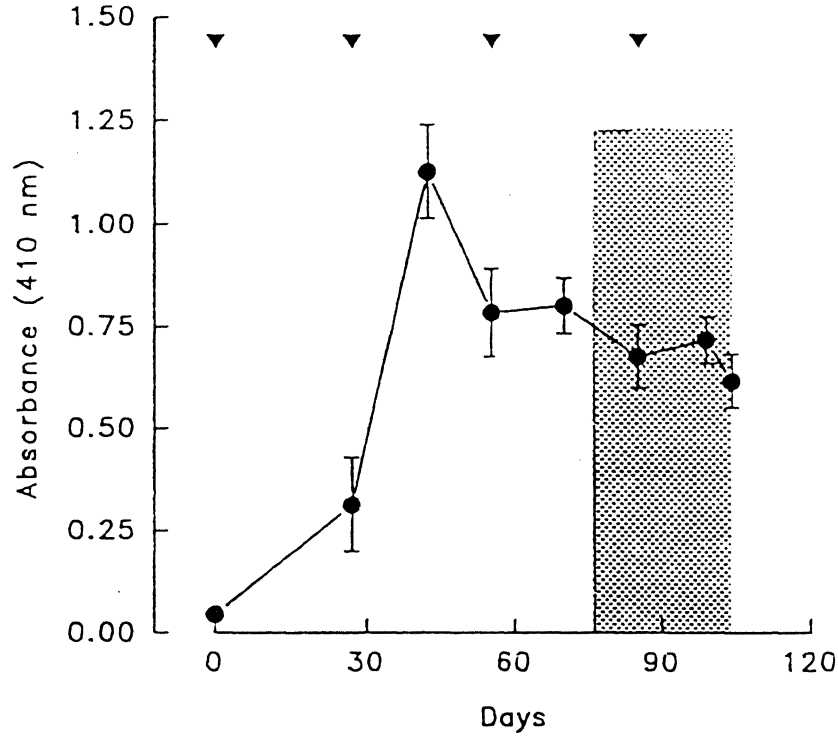
Since animals were vaccinated with the *Pimelea* C -ovalbumin conjugate, ELISA analysis of the antisera was conducted with a *Pimelea* C - BSA plate coating. It had already been established that bovine anti-ovalbumin antibodies did not bind significantly to BSA.

In an effort to boost antibody responses in preparation for the toxin challenge experiment, the *Pimelea* C group (n=6) were given several booster injections and the time course of the antibody response over 104 days followed by ELISA analysis. The vaccination schedule for this experiment has been described in section 3.2.2 .

The data given in Figure 3.2 and Table 3.9 shows that all animals responded to the vaccination and that the highest antibody levels occurred after the second vaccination and then declined gradually despite additional booster vaccinations. Antibody titres were sufficiently strong to be detectable at 1:5000 serum dilution. Average absorbance of control animals (ovalbumin immunised) was subtracted from all test data. All analyses were conducted simultaneously using an identical protocol.

Figure 3.2

Time course of antibody responses in cattle vaccinated with *Pimelea* C-ovalbumin conjugate.



▼ : Day of vaccination.

Shaded area : Period during which cattle were intoxicated with *Pimelea* material.

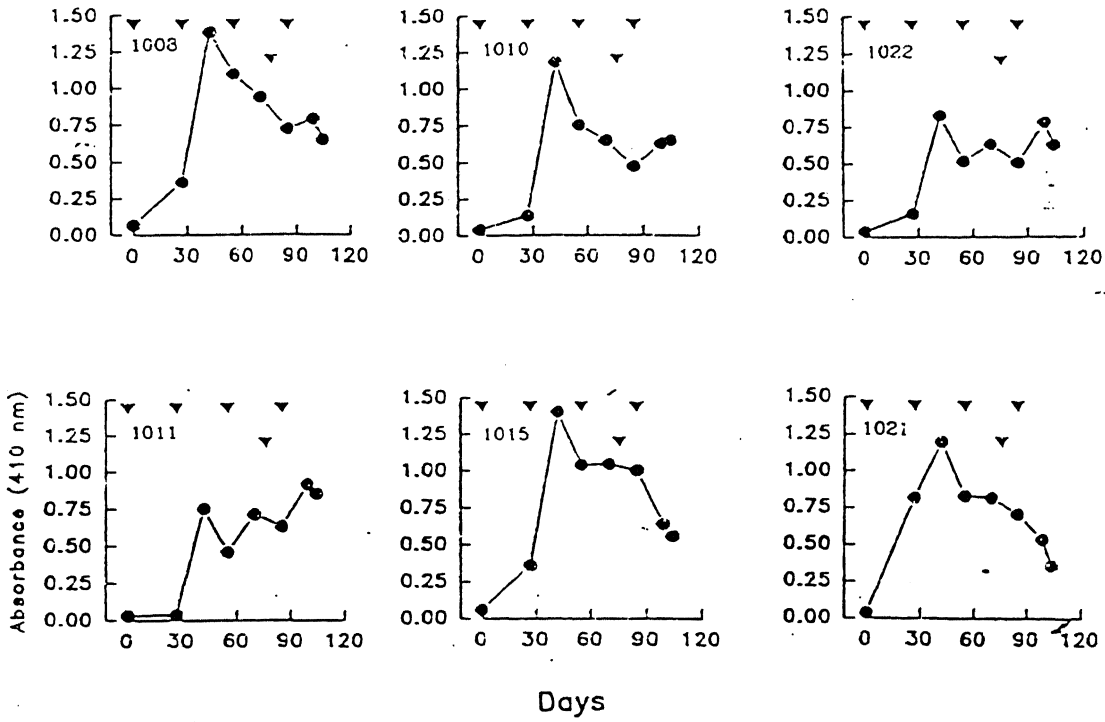


Table 3.9

Time course of antibody responses in *Pimelea* C- Ovalbumin vaccinated cattle.

Cattle no.	Serum dilution	Day 0	Day 27 (2^o)	Day 42	Day 55 (3^o)	Day 70	Day 85 (4^o)	Day 99	Day 104
1008	1:500	0.067	0.365	1.388	1.098	0.723	0.941	0.789	0.649
	1:1000	0.031	0.124	0.999	0.727	0.385	0.541	0.351	0.366
	1:5000	0.014	0.025	0.233	0.123	0.023	0.042	0.033	0.029
1010	1:500	0.040	0.134	1.183	0.752	0.473	0.647	0.625	0.644
	1:1000	-	0.025	0.782	0.447	0.181	0.305	0.251	0.351
	1:5000	-		0.157	0.036	-	-	0.020	0.018
1011	1:500	0.031	0.039	0.753	0.458	0.634	0.717	0.920	0.855
	1:1000			0.376	0.186	0.298	0.412	0.566	0.544
	1:5000			0.027	0.006	-	0.017	0.072	0.064
1015	1:500	0.061	0.361	1.403	1.041	1.005	1.047	0.638	0.558
	1:1000		0.153	0.952	0.633	0.576	0.670	0.273	0.275
	1:5000			0.186	0.090	0.051	0.075	0.019	0.018
1021	1:500	0.037	0.820	1.201	0.834	0.708	0.818	0.538	0.355
	1:1000	0.081	0.334	0.790	0.501	0.272	0.431	0.223	0.129
	1:5000	0.121		0.121	0.045	0.004	0.031	0.019	0.004
1022	1:500	0.038	0.160	0.830	0.517	0.508	0.633	0.780	0.624
	1:1000		0.025	0.485	0.254	0.207	0.308	0.345	0.323
	1:5000		0.071	0.072	0.028	0.003	0.047	0.054	0.021

3.3.4 *Pimelea* Challenge Experiment with the *Pimelea* C -Ovalbumin Vaccinated Cattle

Control cattle (n=6) and cattle vaccinated against *Pimelea* C fraction (n=6) received an oral drench daily of milled *Pimelea* plant material as described in

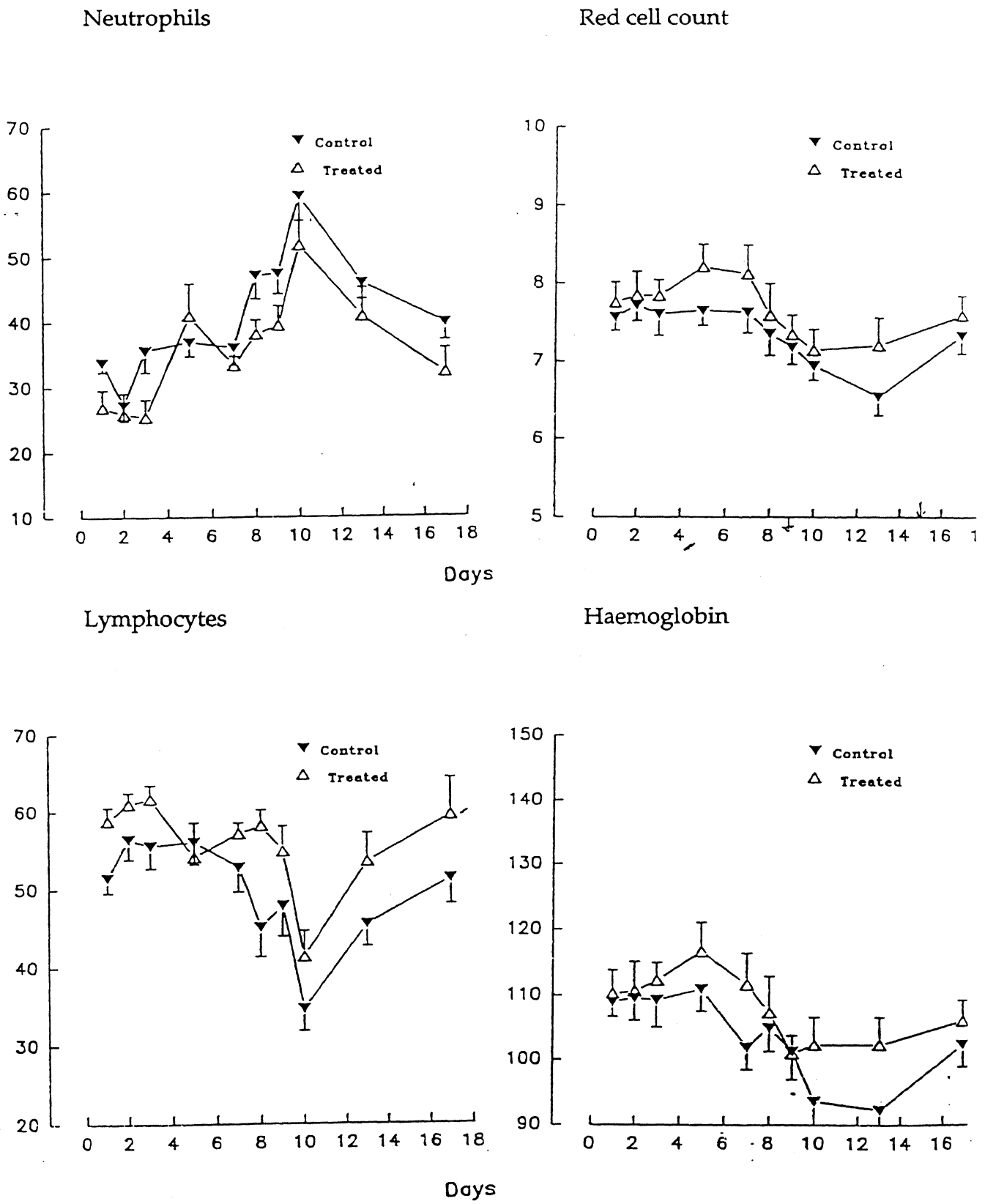
section 3.2.4. The dose was progressively increased after day 10 since the physical and clinical signs reported by Clark (1973) were not observed within the anticipated time. At the higher doses, both groups of cattle developed varying degrees of diarrhoea during the experiment. Several animals from both groups showed oedema of the lower jaw and upper brisket typical of *Pimelea* poisoning. By the end of experiment all the animals were severely emaciated, lethargic, had stopped eating and exhibited variable diarrhoea.

Overall, it would appear that vaccination against purified *Pimelea* C toxin fraction did not afford protection from ingested plant material at the doses administered. However, the response in vaccinated cattle requires further testing, since the 100 and 200 mg/kg live weight doses of *Pimelea* plant material were very high. According to Clark (1973), this dosage killed the cattle within 9 days. Although the ELISA results showed that specific antibodies against *Pimelea* toxins were present, the animals were probably overdosed compared to the levels of toxin they are likely to experience in the field. Therefore, any protective effects of the vaccines should be tested under field conditions.

Figure 3.3 compares the variations in red cell count RCC, haemoglobin Hb, lymphocytes and neutrophils for both the control and treated cattle group over the time course of the experiment. In earlier work, Kelly (1975) had reported falls in total leucocytes, lymphocytes, neutrophils and haemoglobin levels arising from the oral intoxication of cattle with *Pimelea* . Although the differences in these parameters between the control and vaccinated group were

Figure 3.3

Neutrophil, red cell count, lymphocytes and haemoglobin levels in vaccinated and control animals during the *Pimelea* challenge experiment



not significantly different at high toxin dosage, a consistent trend over the early part of the experiment (while toxin dosage was low) was that vaccinated cattle performed better than controls. This experiment needs to be repeated with animals having a higher antibody response and with lower toxin dosage before any definite conclusions can be made.

As an adjunct to the toxin challenge experiment, the antibody response in the vaccinated group was compared before and after challenge with the toxin. Although it was considered unlikely that challenge with the plant would augment the antibody response, this had not been previously verified. The ELISA data given in Table 3.10 for 1:500 diluted serum shows that there was little difference in antibody binding for serum collected two weeks post secondary vaccination and serum collected after the toxin challenge experiment. It is therefore concluded that free *Pimelea* toxins are not immunogenic and do not invoke any further immune response in cattle previously vaccinated with protein-toxin antigen.

Table 3.10

Difference in IgG absorbances pre and post *Pimelea* challenge treatment.

Controls	Abs[a.] (2 wk post 2° vac)	Abs[a.] (Post treatment with *Pimelea* material)
0.237	1.169	1.168
0.167	1.443	0.991
0.312	1.113	1.530
0.252	1.726	1.388
0.418	1.531	0.904
0.174	1.239	1.282
0.26 ± 0.094	1.45 ± 0.10	1.21 ± 0.10

[a]Control average absorbance was subtracted from each of tested serums (from vaccinated cattle). Values are means ± s.e.m.

3.3.5 The Effect of Freeze Drying Purified IgG from Rabbits and Cattle

As explained earlier in this chapter, IgG was purified from serum collected from vaccinated cattle and rabbits and then freeze dried for use in the *in vitro* organ bath studies (Chapter 4). We were interested in comparing ELISA binding data for serum and an equivalent amount of the freeze dried IgG fraction. As shown in the data given in Table 3.11, antibody binding in fresh cattle serum was much higher than an equivalent amount of purified freeze dried IgG material. In contrast, there was minimal loss of specific binding observed for the purified freeze dried rabbit IgG. These findings may help to explain the results of *in vitro* studies reported in Chapter 4, where the rabbit IgG was shown to be protective against the toxins, whereas the bovine IgG lacked any efficacy.

Table 3.11

Comparision of ELISA absorbance data for serum and freeze dried IgG.

	Cattle IgG absorbance[a]	Rabbit IgG absorbance[a]
Purified freeze dried IgG	0.623	1.325
Serum	1.670	1.502

[a]average absorbance normal rabbit serum (0.259) has been subtracted from each of the tested serum.

3.4 CONCLUSIONS

ELISA results for serum obtained from vaccinated cattle and rabbits showed that specific antibodies were raised against *Pimelea* toxin- protein conjugates. There was a significant rise in IgG titres after secondary vaccination compared with primary vaccination. The data given in Figure 3.1 and 3.2 shows that all animals responded to the vaccination and that the highest antibody levels occurred after the second vaccination and then declined gradually despite

additional booster vaccinations. Antibody responses were sufficiently strong to be detectable at 1:5000 serum dilution. Attempts were made to prove that antibody binding could be displaced with free toxin. In general, these experiments achieved 20 -50% displacement and while this might reflect methodological difficulties (the toxins being insoluble in aqueous media), it might also help explain the lack of protection in cattle exposed to plant material.

The data given in Table 3.11 shows that antibody titres in fresh cattle serum are higher compared to purified freeze dried IgG material. There was a significant loss of antibody binding associated with purification of the IgG fraction from serum. In contrast, there was no apparent loss of binding observed for the purified rabbit IgG fraction. This difference may either have been due to unknown problems associated with the purification procedure or perhaps reflects a species difference in the immune responses.

Control cattle (n=6) and cattle vaccinated against *Pimelea* C fraction (n=6) received an oral drench daily of milled *Pimelea* plant material as described in section 3.2.4. Taken overall, it appeared that vaccination against purified *Pimelea* C toxin fraction did not afford protection from ingested plant material at the doses administered. However, the response in vaccinated cattle requires further testing, since the 100 and 500 mg/kg live weight doses of *Pimelea* plant material were very high. According to Clark (1973), this dosage should kill the cattle within 9 days. Although the ELISA results showed that specific antibodies against *Pimelea* toxins were present, the animals were probably overdosed compared to the levels of toxin they are likely to experience in the field.

CHAPTER FOUR

IN VITRO STUDIES OF THE EFFECTS OF *PIMELEA* TOXINS ON THE BOVINE PULMONARY VENULE

4.1 INTRODUCTION

As discussed in the introductory chapter, the primary mode of action of *Pimelea* toxins in cattle is sustained contraction of the pulmonary venule system. Clark (1973) showed that intravenous injection of an ethanolic extract derived from *Pimelea trichostachya* dried plant material (at doses above a dried-plant equivalent of 130 mg kg^{-1} bodyweight threshold) caused rapid cardiovascular effects in cattle. Within 10 seconds of intravenous administration of the dose, systemic arterial pressure halved while right ventricular pressure doubled. These results were suggestive of constriction of the pulmonary venous system.

An increase in right ventricular pressure and dilatation of the right side of the heart follows as a natural consequence of chronic *Pimelea* poisoning in cattle grazing in the field. Following logically from the *in vivo* cardiovascular observations of Clark (1973), Mason (1976) reasoned that constriction of the pulmonary venule system was the most immediate effect of the toxins. Kelly and Bick (1976) and Mason (1976) performed *in vitro* studies of bovine pulmonary venule preparations using rudimentary organ bath techniques available at the time and showed that an alcoholic extract derived from *Pimelea trichostachya* caused contraction of the venule tissue. These studies showed that the effects of the toxins were essentially irreversible, and the contraction could not be washed out by replacing the organ bath buffer solution. These observations are consistent with highly lipophilic toxins such as the daphnane orthoesters now known to be present in the plant.

In the studies described in this Chapter, physiologically viable pulmonary venule preparations dissected from fresh lung tissue were used for organ bath studies. The first aim of these *in vitro* studies was to establish that purified *Pimelea* toxins (ie. Fraction C comprising simplexin and other structurally

similar daphnanes) did cause contraction of pulmonary venule preparations. This had not previously been established in the literature since the only previous work had used a large amount of a crude alcoholic extract of the plant. Once the efficacy of Fraction C had been confirmed, a dose-response curve would need to be determined in order to establish the EC_{50} concentration of the toxins that caused half maximal contraction. The toxin concentration in the bath would then be fixed at the EC_{50} level and the efficacy of purified antibodies (from *Pimelea* vaccinated cattle and rabbits) to attenuate the contractile response would be investigated. It was also planned to use the organ bath technology to explore the potential of possible inhibitors of *Pimelea* toxicity such as PKC inhibitors and calcium channel blockers.

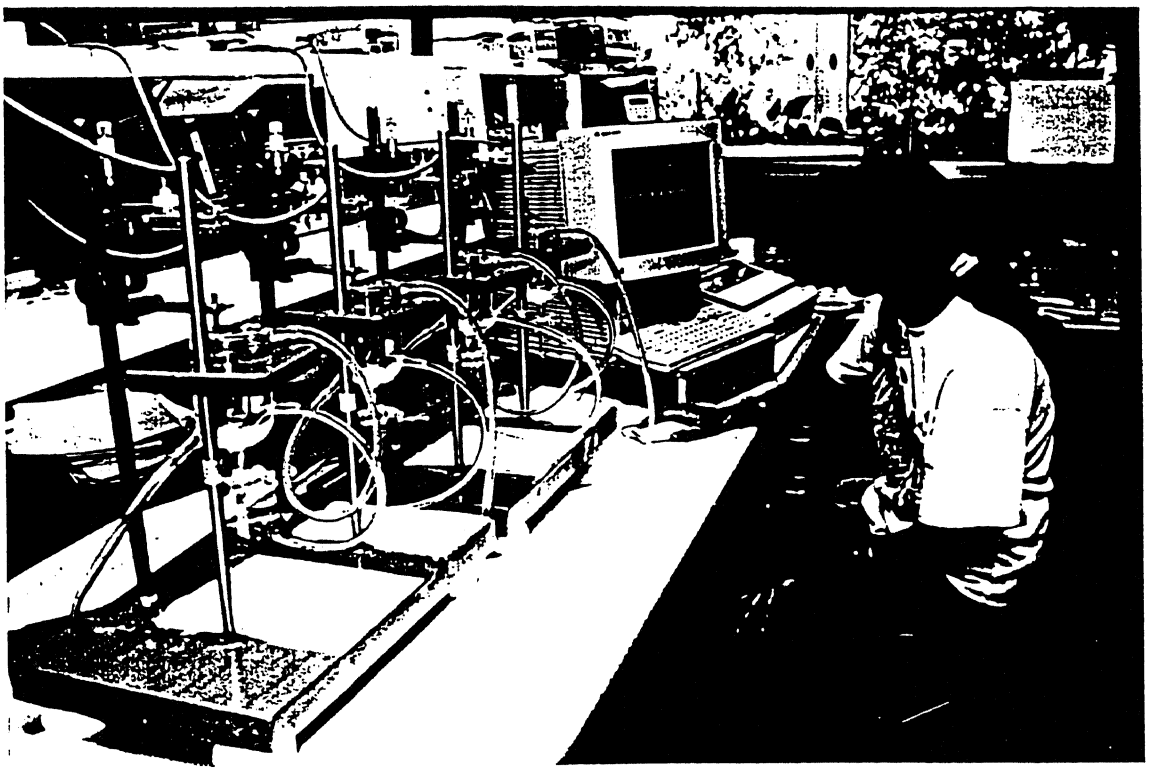
4.2 METHODOLOGY

4.2.1 General

In all experiments, the force of contraction was measured using Grass FT03 Force Transducers connected to a Transbridge TBM4 amplifier manifold (World Precision Instruments Ltd) and recorded for subsequent analysis via a MacLab 8 using Chart software (AD Instruments Ltd) running on a Macintosh II CX microcomputer. The layout of the four channel system and the 25 mL organ baths employed (manufactured by Mr Tom Jeston of James Cook University, Queensland, Australia) is shown in Figure 4.1. A schematic layout of the system is given in Figure 4.2. The design of the baths was such that bubbling of carbogen in the side chamber caused a continuous circulation of the buffer in the larger sample chamber.

Figure 4.1

The four channel computerised organ bath system



Organ bath (25 mL)

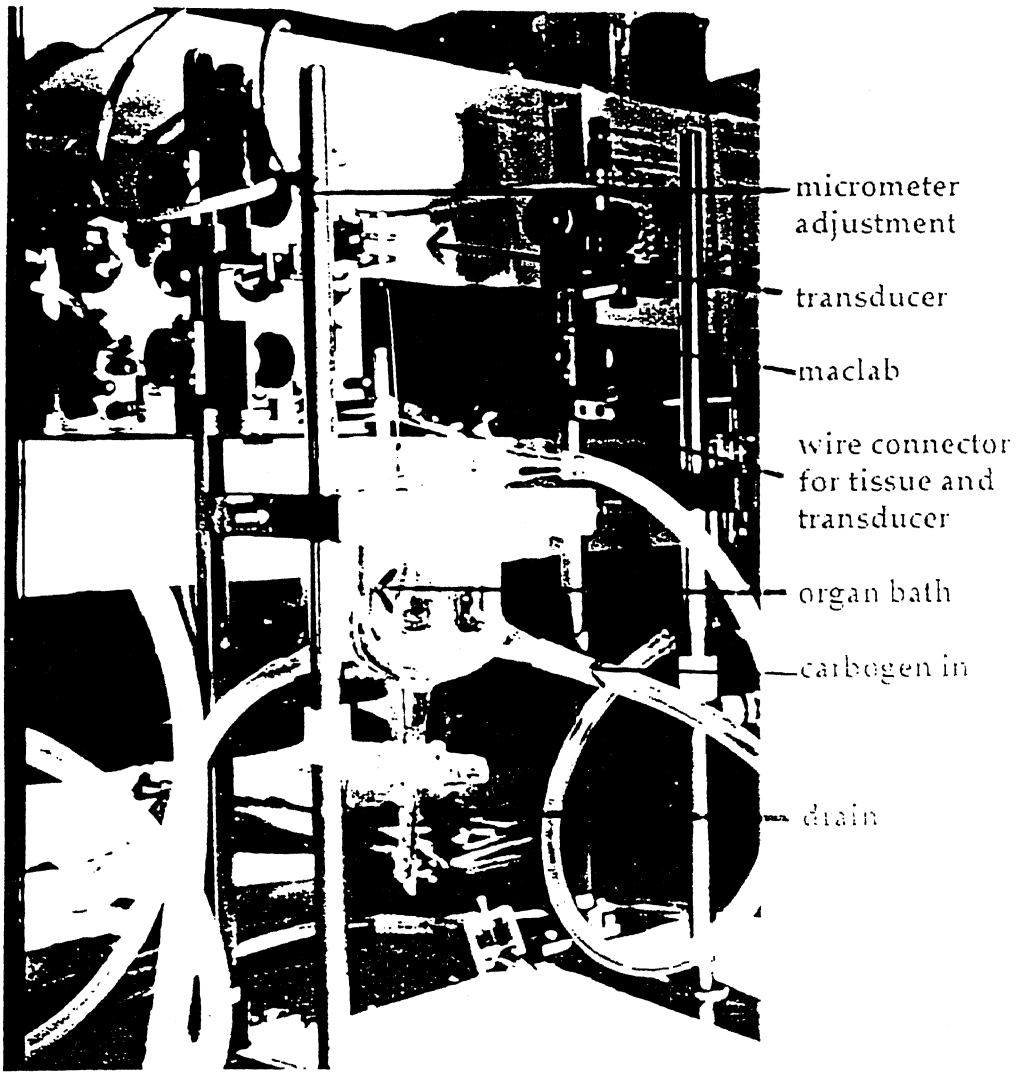
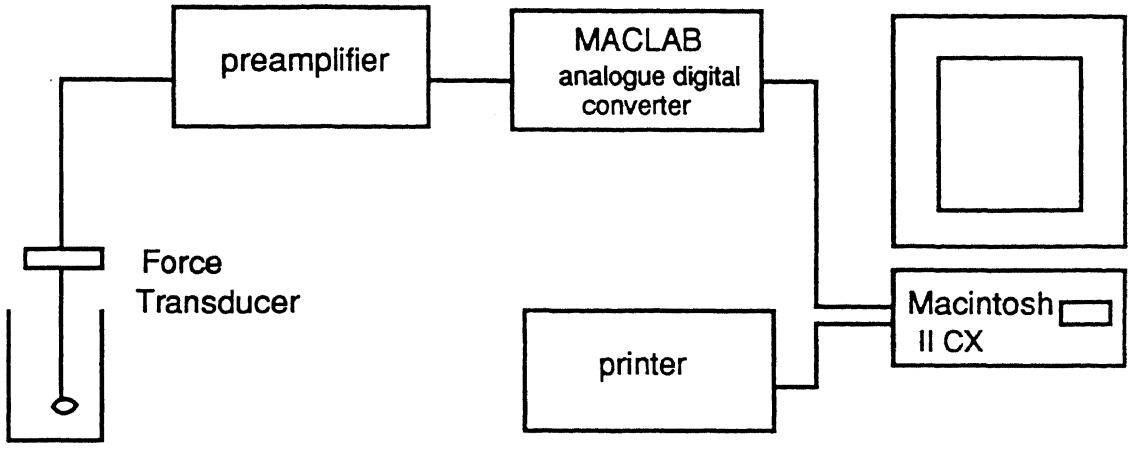


Figure 4.2

The layout of the four channel system.



Preparation of Normal Tyrodes Solution and Tyrodes Solution Containing 100 mM KCl

For preparation of Tyrodes solution and Tyrodes solution containing 100 mM KCl, Stock A, Stock B and Stock C solutions were prepared and then stored at 4° C. The same B and C stock solutions were used for both forms of the buffer. Fresh Tyrodes solutions were prepared each day and remaining stock solutions were always discarded after two weeks.

Stock A for Tyrodes solution containing 100 mM KCl :

The chemical composition for 2 L of modified Stock A solution is given below. The solution was prepared using demineralised water.

Chemical	Weight (g)	Concentration
NaCl	98.9	0.85 M
KCl	300	2.0 M
$MgCl_2.H_2O$	8.6	38 mM
$NaH_2PO_4.2H_2O$	2.6	8.3 mM

Normal Stock A solution

Chemical composition and final component concentrations for 2 L of normal Stock A solution is given below. Solutions were prepared using demineralised water.

Chemical	Weight (g)	Concentration
NaCl	320	2.74 M
KCl	16.2	108 mM
$MgCl_2.H_2O$	8.6	38.0 mM
$NaH_2PO_4.2H_2O$	2.6	8.3 mM

Stock B

Stock B solution was 0.45 M sodium bicarbonate and was prepared by dissolving 76.0 g (0.45 mM) of $NaHCO_3$ in 2 L of demineralised water.

Stock C :

Stock C solution was prepared by dissolving 10.6 g (36 mM) of $CaCl_2.2H_2O$ in 2 L of demineralised water.

Working 100mM KCl Tyrodes Solution

To prepare a working 100 mM KCl Tyrodes solution, Stock A (100 mL), Stock B (100 mL) and Stock C (100 mL) and 2 g of glucose were made up to a 2 L final volume with demineralised water in a volumetric flask. (Final concentration of different component in 100 mM KCl tyrodes solution in mM; NaCl 42.3, KCl 100, $MgCl_2.H_2O$ 1.90, $NaH_2PO_4.2H_2O$ 0.42, $NaHCO_3$ 22.6, $CaCl_2.2H_2O$ 1.8)

Working Tyrodes Physiological Solution

The working Tyrodes (physiological) solution was prepared exactly as described above for the 100 mM KCl Tyrodes except that normal Stock A solution was used. The pH of this solution is normally 5.4 although after bubbling carbogen through the chilled solution for 30 minutes, the pH rises to approximately 7 because of dissolved CO_2. (Final concentration of different component in tyrodes solution in mM; NaCl 136.9, KCl 5.4, $MgCl_2.H_2O$ 1.90, $NaH_2PO_4.2H_2O$ 0.42, $NaHCO_3$ 22.6, $CaCl_2.2H_2O$ 1.8)

4.2.2 Dissection of pulmonary venules from bovine lung tissue

The tips of bovine lung lobes (approx 150 g) were removed from whole lungs within 30 minutes of slaughter of the animals at the abbatoir and after inspection by AQIS Officers. The lung tissue was then immersed in chilled, pre-carbogenated Tyrode solution and transported to the laboratory on ice. Small pulmonary venules were dissected free from the branches of bronchioles and cleared of fat and connective tissue.

4.2.3 Mounting tissue preparations in the organ baths

After dissection, the venules were cut into ring sections of approximately 3 mm in length and diameter, mounted on support posts and loosely connected to the Force Transducers via a wire connector. The tissue preparation needs to be thin enough to allow oxygenation of the innermost layers of the tissue and for adequate removal of waste products by diffusion. Rings of between 0.5 to 5 mm thickness generally meet these requirements. The preparations were then suspended in 25 mL water jacketed organ baths (35° C) circulated with Tyrode solution and bubbled with carbogen (95% O_2 and 4% CO_2). The baths were designed so that carbogen was not bubbled directly on the tissue to avoid mechanical damage to the preparation. Gas flow was set at the beginning of the experiment and was maintained at the same level throughout, thereby

preventing variations in gas tension which may effect the metabolism of the tissue.

After allowing the tissues to equilibrate in the baths, the preparations were gradually brought up to pre-load tension. Preload tension was applied using micro and macro adjustment mechanisms on the support posts. The precise engineering of the stands allowed adjustment of tension down to 0.1 mN increments. Care was taken to avoid damaging the tissue by applying excessive force at any time during the mounting procedure or during the pre-load adjustment phase. Tissues were equilibrated for 60-70 minutes, with exchange of the bath buffer every 20 minutes. Over this time the pre-load tension on each preparation was adjusted to the optimum 25 mN.

4.2.4. Determination of Optimum Pre-load Tension

Optimum pre-load tension to obtain maximum contractile response to 100 mM KCl for the preparations was determined by repeatedly challenging three pulmonary venule rings with 100 mM KCl Tyrodes solution at various pre-load tensions (1, 5, 10, 20 and 30 mN) and the maximum contraction produced in each case was measured. The weights of the tissue preparations used in these initial experiments ranged from 2.0 - 12.6 mg. (Once the techniques had been established, most tissue preparations used were in the 2-3 mg range.)

4.2.5 Contractile Responses to 5-HT (1μM and 3μM) and 100 mM KCl

The maximal contraction of the venule preparations in response to 5HT (5-hydroxytryptamine) and Tyrodes solution containing 100 mM KCl was determined as follows. Venule ring preparations were set to approximately 25 mN pre-load tension in normal Tyrodes buffer and the tissue then exposed to Tyrodes solution containing 100 mM KCl. The maximum tension produced in response to KCl was measured when the contraction of the tissue had

stabilised. The maximum contraction was calculated by subtracting the pre-load tension from the maximum tension produced in response to 100 mM KCl.

As it took more than one hour for the maximal response to 100 mM KCl to be achieved in most cases, the maximal contractile response of the venule preparations to (5-HT) (Sigma) was also investigated. Mason (1976) had also used 5-HT in earlier experiments to demonstrate normal function and sensitivity of the preparation before experimentation. Both 1 μM and 3 μM 5-HT concentrations in the bath were trialed and it was found that maximal response was achieved with the lower concentration. The contractile response to 5-HT was readily reversible by wash-out and replacement of the chamber buffer with fresh Tyrodes solution. It was observed that preparations maintained for over 6 hours in Tyrodes solution at 37° C remained relaxed throughout this time but would still then respond normally to challenge with 1.0 μM 5-HT.

4.2.6 Preparation of *Pimelea* C fraction

Despite having been purified by solvent partition, silica column chromatography and preparative reverse phase HPLC, the *Pimelea* C toxin fraction (see Chapter 2) remained a complex mixture of four major components. For the purposes of the organ bath studies, it was assumed that the average molecular weight of the toxin components was 500 g/mol. The daphnane and tigliane components of *Pimelea* which have been described by other workers generally have molecular weights ranging over 500-700 mass units. A stock solution of the *Pimelea* C fraction (1 mM) was prepared by dissolving 70 mg in 1.4 mL of HPLC grade methanol and this solution was stored at -20°C in darkness. From this stock solution, fresh serial dilutions were prepared every two weeks and stored in freezer and protected from sunlight. Based on the assumed molecular weight of 500, aliquots of the stock solution were added to

the organ bath directly in order to achieve the required concentration in the 25 mL total volume (given that the average molecular weight of the toxins in the *Pimelea* C fraction was expected to be greater than 500 mass units and that it was unlikely that all components in the mixture would cause contraction of the venules, the calculated molar concentrations used in these experiments are likely to be over-estimates).

4.2.7 Experimental Protocol

Preparations were equilibrated to 25 mN as described above and thereafter the Tyrodes solution was exchanged at approximately 20 minute intervals. After 75 minutes, the preparations were subjected to 1μM 5-HT to determine maximum contractile response for each individual tissue. The response usually stabilised after 30 minutes and the 5-HT was then washed away by exchanging the Tyrodes solution three times at 15 minute intervals, thereby allowing complete relaxation of the tissue. The preparations were then re-equilibrated to approximately 25 mN. Once the preparations were stable, a small volume of a methanolic solution of the toxin was added and the effect on the contraction of the venule was observed until the tissue was stabilised. The *Pimelea* toxins cause a slowly developing contraction after a latent period of 15-30 minutes and it usually took 300 minutes for maximum contraction to be achieved.

The concentration of methanol in the bath never exceeded 0.3% (v/v). Preliminary experiments had shown that methanol concentrations up to 1.0% (v/v) had no effect on the contractile response. Each tissue preparation was subjected to only one concentration of toxin. The experimental apparatus available allowed four tissue preparations to be studied simultaneously. Any tissue which responded abnormally in the pre-load sequence or to 5-HT was rejected. Occassionally, some tissues gave normal responses in the set-up phase but then gave a spasmodic contractile response to the toxin such that waves of

contraction and then partial relaxation were observed. These preparations were also rejected.

Experiments involving different toxin concentrations were assigned randomly to the baths so that results from all baths contributed to the replicates for any one concentration. Usually, the response to a particular toxin concentration was measured 4-6 times. The baths were cleaned at the completion of each experiment and then filled with absolute alcohol and flushed with demineralised water. The baths were subsequently cleaned with nitric acid and flushed repeatedly with demineralised water so as to avoid cumulative contamination with toxins.

4.2.8 Experiments with Antibodies and Inhibitors

To test the attenuating effect of purified antibodies from vaccinated rabbit or cattle serum on the contraction produced by *Pimelea* toxins, venule preparations were incubated with the freeze dried IgG dissolved in water (200 µL) for 30 minutes before adding the EC_{50} concentration of *Pimelea* toxin. The contractile response was then compared to the response obtained from one or more control preparations exposed only to the EC_{50} toxin concentration. Wherever possible, the control and "treated" preparations were dissected from the same venule.

Determination of EC_{50} required for the contraction of Bovine Pulmonary Venules by Mezerein

Mezerein (a daphnane orthoester) is a tumour promoter similar in structure to the *Pimelea* toxins and is known to activate PKC both *in vitro* and *in vivo* (Miyake *et al.*, 1984). The EC_{50} Mezerein concentration required for contraction of bovine pulmonary venules was determined and then the effects of known PKC inhibitors in attenuating the contraction produced by mezerein (EC_{50})

was investigated. As with the *Pimelea* toxins, mezerein was dissolved in methanol for these experiments.

Preliminary experiments with the PKC inhibitors, D-*erythro*--sphingosine derived from bovine brain (200 μM dissolved in 200 μL ethanol) and polymyxin B sulfate (250 μM dissolved in 200 μL water), (Calibochem Corporation), were conducted in a similar manner to the antibody experiments. After wash out of the 5-HT response, the preparations were equilibrated for 30 minutes in the presence of the PKC inhibitor (D-*erythro*--sphingosine or polymyxin B sulfate) prior to adding the mezerein (EC_{50}).

To test the attenuating effect of *Nux vomica* CM tincture (containing icajine, novacine, vomicine, pseudostrychnine, pseudobrucine) (Bratati and Bisset,1991) on the contraction produced by the EC_{50} concentration of *Pimelea* toxins, preparations were exposed to an aliquot of *Nux vomica* CM tincture (Homeopathic medicine, Brauer Biotherapies Pty Ltd) immediately followed by addition of the EC_{50} *Pimelea* toxin concentration. Control preparations were exposed only to the EC_{50} *Pimelea* toxin concentration. The contraction was monitored over 200-300 minutes as previously described. Experiments were also conducted to establish the response of the preparations to 200 μL of *Nux vomica* CM tincture in the absence of added toxin. After 300 minutes exposure, the response of the tissue preparations to 1.0 μM 5-HT was examined both before and after wash-out of the *Nux vomica* .

4.3 RESULTS AND DISCUSSION

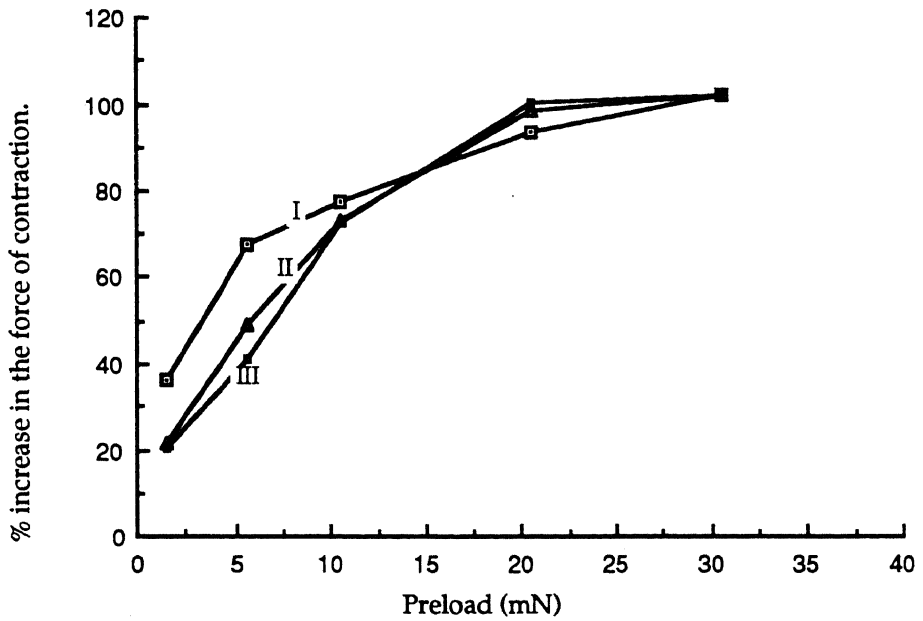
4.3.1 Pre-load determination

Pre-load is the tension applied to the preparation before testing it with the contractile agent. The force of contraction produced usually increases as the

pre-load tension is increased until the optimum conditions are reached. Thus, optimum pre-load is the tension applied to the vessel at which maximum contraction is produced. At this tension, the tissue preparation is stretched so that myosin and actin filaments are at the optimum angle for initiation of the contractile force. The data given in Figure 4.3 and Table 4.1 are the results obtained from testing the responses of three separate tissue preparations to 100 mM KCl.

Figure 4.3

Determination of optimum preload.



Abbreviations : I : Preparation no. 1; II : Preparation no. 2; III : Preparation no. 3.

As there was only a slight increase in maximum response obtained for all tissues between the 20-30 mN pre-load range, the optimum pre-load tension was taken to be 25 mN. These results were obtained with tissue preparations ranging in weight from 2-12 mg so the physical size of the preparation did not appear to have a major effect on the optimum pre-load tension.

Table 4.1

Determination of optimum preload for contraction of bovine pulmonary venule preparations

	Preparation - 1		Preparation - 2		Preparation - 3	
Pre-load mN [a]	Δ mN [b]	% cont-raction [c].	Δ mN	% cont-raction.	Δ mN	% cont-raction.
1	34	34 %	14	19 %	29	18 %
5	67	65 %	35	47 %	64	39 %
10	77	75 %	53	71 %	115	70 %
20	94	91 %	72	96 %	160	98 %
30	103	100 %	75	100 %	164	100 %

[a] Preload mN : different tensions at which vessels were stretched before inducing contraction with 100 mM KCl .

[b] Δ mN : maximum force of contraction induced by 100 mM KCl calculated by subtracting the Pre-load applied from the measured maximum contraction.

[c] % contraction : For each preparation force of contraction produced at different preloads were calculated as a % of the maximum contraction produced at 30 mN pre-load.

4.3.2 Maximum Contractile Responses to 5-HT (1 μM and 3 μM) and 100 mM KCl

The contractile behaviour of each bovine pulmonary preparation was expected to be dependent on numerous factors such as the size of the tissue ring, the ratio of collagen and muscle present, genetic variations between animal breeds, the age of the animal, receptor densities, time variations from the death of the animal and dissecting out the tissue, and damage to the tissue during preparation. Hence to allow comparison between tissue preparations. the maximum contractile response for each individual tissue was determined at the commencement of the experiment and then any contraction measured during the experimental phase expressed as a percentage of this maximum response.

A requirement for the determination of maximal contractile response was that the agent used should be fast acting and also be conveniently washed out by replacement of Tyrodes solution. The tissue should then rapidly re-equilibrate to the pre-load tension prior to being contracted with the test agent. Tyrodes solution containing 100 mM KCl was first investigated as it is often used for this purpose in organ bath experiments. With bovine pulmonary venule preparations however, maximal response to 100 mM KCl was never achieved in less than 1 hour and this was considered too time consuming for our purposes. The data obtained from these preliminary experiments are given in Figure 4.4 and Table 4.2.

Table 4.2

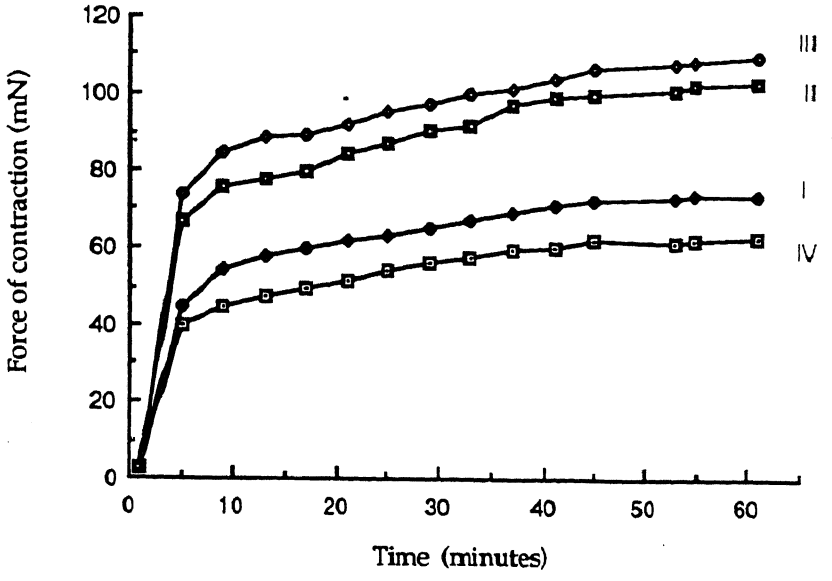
Contractile responses of bovine pulmonary venule preparations to 100 mM KCl

Preparation No.	Preload (mN)	Δ mN [a]	Weight of tissue (mg)
1	24.5	72	2.8
2	23.9	101.5	2.9
3.	25.4	108.5	7.4
4	23.1	61.5	4.7

[a] Maximum contractile response minus pre-load tension.

Figure 4.4

Determintion of maximum contractile response to 100 mM KCl.



Abbreviations : I : Preparation no. 1; II : Preparation no. 2;

III : Preparation no. 3 ; IV : Preparation no 4

The maximal contractile response of the preparations to 1μM and 3μM concentrations of 5-hydroxytryptamine (5-HT) were determined. It was found that maximal response was achieved even with the lower concentration after 30 minutes (see Figure 4.5 and Table 4.3). The contractile response to 5-HT was observed to be readily reversible by wash-out and replacement of the chamber buffer with fresh Tyrodes solution. Tissue preparations which had been contracted with 1 μM 5-HT and then washed out remained relaxed over 6 hours in Tyrodes solution at 37° C, but then still gave the maximum response to further challenge with 1.0 μM 5-HT.

Table 4.3

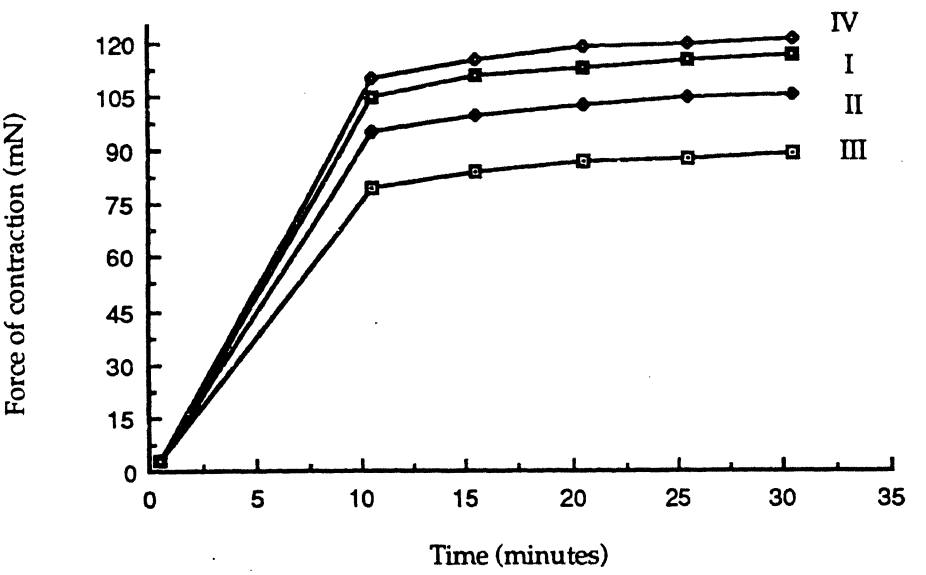
Contractile responses of bovine pulmonary venules to 5-HT

Preparation No.	Preload (mN)	Δ mN [a] (1μM 5HT)	Δ mN [a] (3 μM 5HT)	weight of tissue (mg)
1	22.1	114.2	111.7	2.8
2	23.4	102.7	101.1	2.9
3	23.9	86.5	74.1	7.4
4	23.7	118	109.8	4.7

[a] Maximum contractile response minus pre-load tension.

Figure 4.5

Determination of maximum contractile force in response to 1μM 5HT.



Abbreviations : I : Preparation No. 1; II : Preparation No. 2;

III : Preparation No. 3 ; IV : Preparation No 4

4.3.3 Concentration / Response Curve for the Contraction of Bovine Pulmonary Venules by *Pimelea* Toxins.

Mason (1976) reported an *in vitro* contraction of bovine pulmonary venules in response to 10 mg of a crude alcoholic extract of *Pimelea trichostachya* dissolved in 40 μL of ethyl alcohol. In the present study, the *Pimelea* -C toxic fraction employed had been purified from the crude alcoholic extract by solvent partition, silica column chromatography and preparative reverse phase HPLC. It was expected that the *Pimelea* -C fraction would prove much more efficacious in the *in vitro* studies compared with the crude extract used by Mason (1976). Figure 4.6 shows typical slow developing contraction over a period of 300 minutes in bovine pulmonary venule in response to *Pimelea* toxins.

Repeated experiments were undertaken in order to establish the concentration-response relationship for the contraction of bovine pulmonary venules by the *Pimelea* -C toxic fraction. This data is presented in Table 4.4 and from the plot of the averaged results (Figure 4.7), the EC_{50} concentration was determined. The EC_{50} value was taken as the apparent toxin concentration required to produce half the maximal response produced by 1 μM 5-HT.

Figure 4.6

Slow developing contraction in bovine pulmonary venule in response to *Pimelea* toxins.

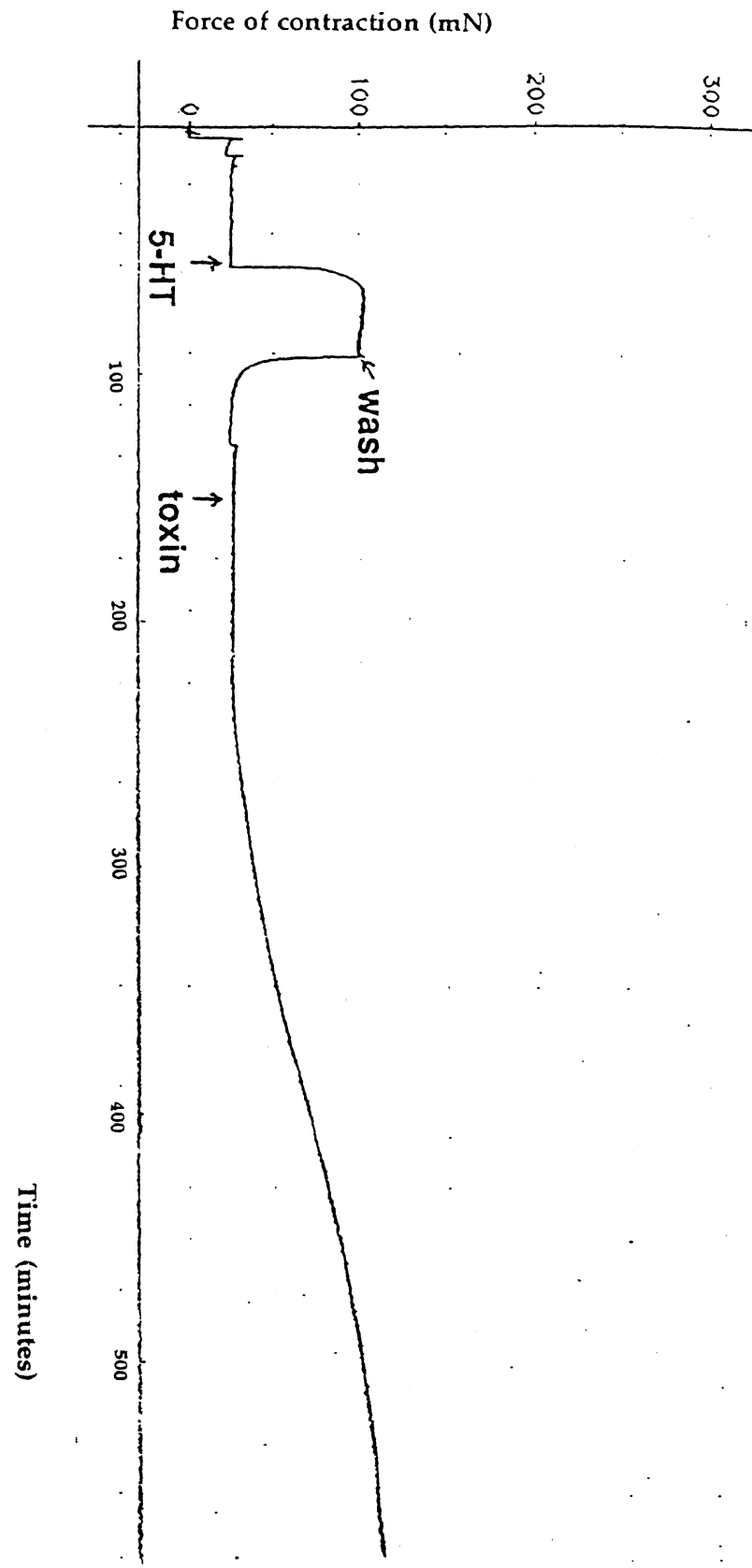


Table 4.4

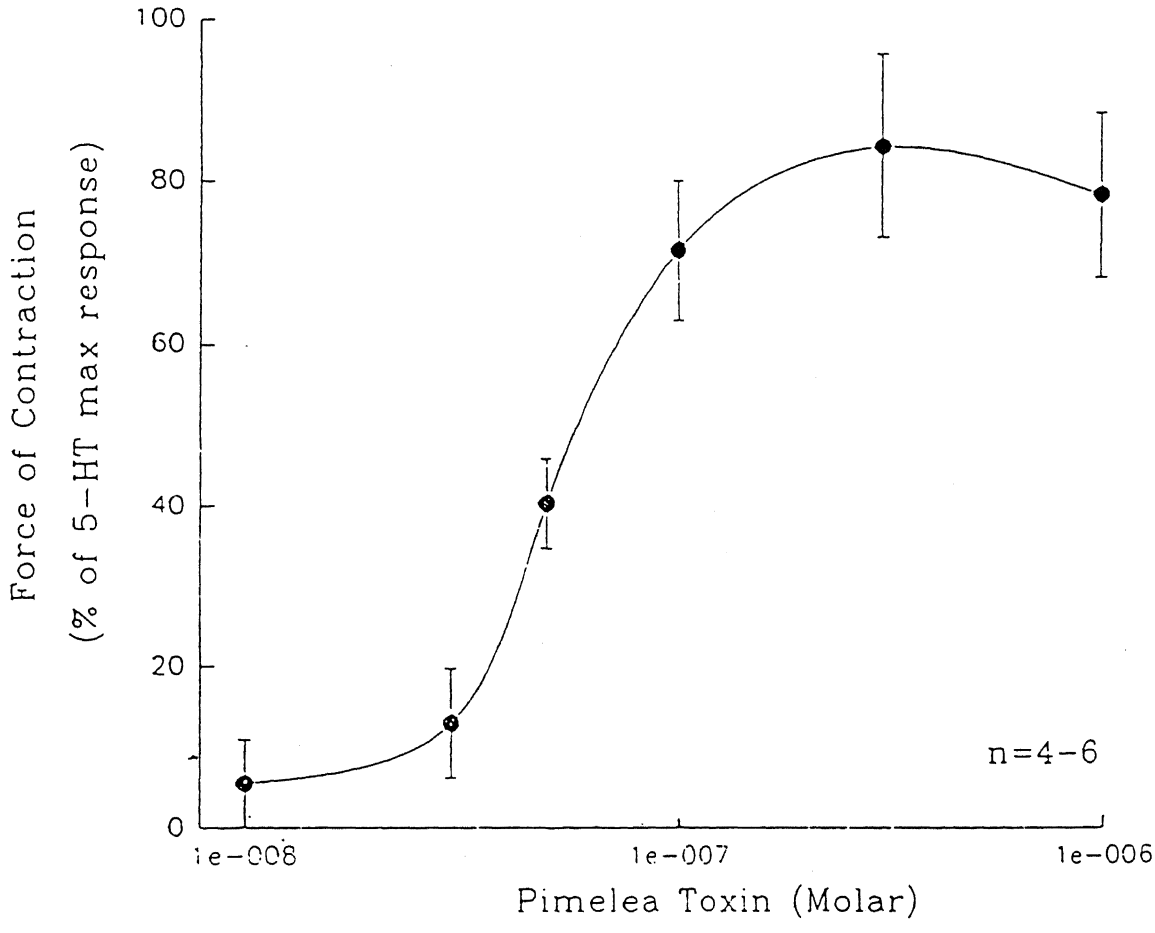
In vitro contractile responses of bovine pulmonary venules to *Pimelea* -C fraction.

[*Pimelea* -C] µM [a]	Maximum contraction (Δ mN) [b]	*Pim.elea* contraction (Δ mN) [c]	% Contraction by toxin [d]	Weight of tissue (mg).
0.01	180.7	0	0	6.3
	192.6	78.3	40.7	6.3
	162	0	0	6.5
	108.6	0	0	6.9
0.03	192.3	57.7	30	6
	99.3	18.2	18.3	7
	108.5	3.8	4	5.7
	108	0	0	6.2
0.05	127	57.7	45.5	6.2
	179.3	89.6	51	6.0
	172	43.9	25.5	3.4
	132.8	53.4	40.4	5.4
0.10	155.2	78.9	50.83	8.3
	169.8	163.0	96	–
	129.9	80.9	62.3	7.6
	81.9	95.9	117	3.7
	150.3	139.8	93	4.4
	156.8	120.7	77	7.4
0.30	128.3	77.4	60.3	–
	150.8	149.3	99	5.7
	78	79.6	102	3
	112.7	142	126	6.2
	192.2	102.8	53.5	8
	95	66	69.5	5
1.0	144.2	103.8	72	7
	110.8	100.6	90.8	4.6
	145	162.4	112	4.6
	126.6	49	70	7.3
	221	112.7	51	7

[a] Based on assigned molecular weight of 500 g $mole^{-1}$. [b] Maximum contraction to 1.0 µM 5 -HT minus pre-load tension. [c] Maximum contraction in response to *Pimelea* toxin minus the pre-load tension. [d] Expressed as a % of the 5-HT maximal response.

Figure 4.7

Concentration response data for *Pimelea* toxins on bovine pulmonary venule preparations. (Values are means ± s.e.m.)



From the concentration-response curve (Figure 4.7), the EC_{50} concentration was calculated to be 5.28×10^{-8} M (0.053 μM). The bovine pulmonary venule preparations did not respond to 0.01 μM toxin concentration (10.17 ± 10.17 %, n=4, mean ± s.e.m.) and minimum concentration required to produce maximum contraction was 0.3 μM (85.05 ± 11.55 %, n=6, mean ± s.e.m.). Although there is considerable variation in the results, it must be remembered that this data was derived over many weeks from tissue preparations of differing size from a large number of animals. For each toxin concentration increment, pulmonary venule tissue from at least four separate animals was investigated. The data is presented in summary form in Table 4.5.

Table 4.5

Summary of contractile responses of bovine pulmonary venules in response to *Pimelea* C fraction. (Values are means ± s.e.m.)

Pimelea -C μM	% Contraction relative to 1.0 μM 5-HT	Weight of tissue (mg).
0.01	10.2 ± 10.2, n=4.	6.50 ± 0.14
0.03	13.1 ± 6.9, n=4	6.22 ± 0.27
0.05	40.6 ± 4.9, n=5	5.25 ± 0.56
0.10	82.7 ± 9.9, n=6	6.28 ± 2.47
0.3	85.0 ± 11.5, n=6	5.93 ± 0.73
1.0	80.0 ± 10.3, n=5	6.1 ± 0.61

4.3.4 *In Vitro* Effects of Purified Rabbit Antibodies

In the previous chapter, ELISA data was presented which suggested that a specific antibody response had been developed in both rabbits and cattle after vaccination with *Pimelea* toxin -protein conjugates. To further confirm the presence of specific IgG against *Pimelea* toxins, it was proposed to examine the attenuating effect of different concentrations of purified rabbit IgG using the *in vitro* bovine pulmonary venule preparation.

For this purpose, a minimum of four pulmonary venules from four different animals were tested for each dose of IgG in the presence of the EC_{50} *Pimelea* C fraction concentration. Control tissue preparations from the same animal as the test preparations were incubated with the EC_{50} *Pimelea* C concentration only. The contractile response of the antibody-treated preparations and control tissues were compared to establish the efficacy of rabbit IgG in preventing the contraction response to *Pimelea* toxins (Table 4.6, Figure 4.8).

The results of these experiments are summarised in Figure 4.9. Contraction of bovine control pulmonary vein tissue incubated with EC_{50} concentration of *Pimelea* toxin (shaded region; mean ± s.e.m., n = 13) and attenuation of this contraction (mean ± sem, n = 4 to 6) with increasing amounts of purified rabbit anti-toxin IgG antibodies are shown. From these results it is clear that the purified rabbit IgG attenuates the toxin-induced contraction in a dose dependent manner. Mean contractions (± s.e.m.) produced by treated bovine pulmonary venules in the presence of 5 mg of rabbit IgG and EC_{50} *Pimelea* C toxic fraction was only 9.3 ± 11.2 %, n=6 compared to average contraction of control tissues, 42.0 ± 21.8 %, n=13.

These results (summarised in Table 4.7) suggest that a specific antibody-toxin association either in the organ bath or on the surface of the tissue prevents the action of the toxin. Control experiments conducted with equivalent amounts of non-specific rabbit IgG showed no attenuation of the contractile response (results not shown).

Table 4.6

Contractile responses of bovine pulmonary venule preparations in the presence of IgG and EC_{50} *Pimelea* toxin concentration.

Treated preparations (preparations exposed to IgG and *Pimelea* toxin (EC_{50})					Control preparations (preparations exposed to *Pimelea* toxin (EC_{50}) only)			
IgG (mg) [a]	Δ mN 1μM 5HT [b]	Δ mN *Pim.* [c]	% *Pim.* Con. [d]	Tissue (mg).	Δ mN 1μM 5HT	Δ mN *Pim.* [c]	%*Pim.* Con.[d]	Tissue (mg).
0.1	81.7	34.3	42	3.1	69	15.9	23	1.7
0.1	98	43.1	44	3.5	156	113.9	73	3.4
0.1	70	48.3	69	–	58	25	43	–
0.1	73.3	38.1	52	–	91	4.6	5	–
0.3	117.5	64.6	55	2.9	112	73.9	66	3
0.3	63.2	32.9	52	1.8	93	4.6	69	2.7
0.3	72	39.6	55	1.6	57.5	64.2	43	–
0.3	122.6	67.4	55	–	118.2	17.8	31	3.8
0.3	57.8	41.6	72	1.7	70.9	36.6	51	1.2
0.3	113.2	83.8	74	–	74.1	36.2	55	3
1	127	5.7	4.5	3.1	60.3	40.8	55	–
1	92.2	64.5	70	1.4	76.5	33.2	111	3.2
1	76.1	42.6	56	2.1	67.6	84.9	54	2
1	68	28.6	42	–	76.5	36.5	41	3.6
2	54	6.2	11.5	1.7	57.2	16.6	29	1.7
2	75.8	136.4	18	1.2				
2	71	39	55	2.2				
2	68	12.9	19	–				
5	99	5	5	2.7				
5	107	0	0	4				
5	126.4	10.1	8	3				
5	87.3	0	0	4.1				
5	86	13.8	16	2.6				
5	71	20.6	29	2.6				

[a] weight of the freeze dried rabbit IgG used in organ bath. [b] Maximum contraction to 1.0 μM 5 -HT minus pre-load tension. [c] Maximum contraction in response to *Pimelea* toxin minus the pre-load tension. [d] Expressed as a % of the 5-HT maximal response.

Figure 4.8
Attenuating effects of rabbit IgG (5mg) on contraction of bovine pulmonary venules in response to *Pimelea* toxin (EC_{50})

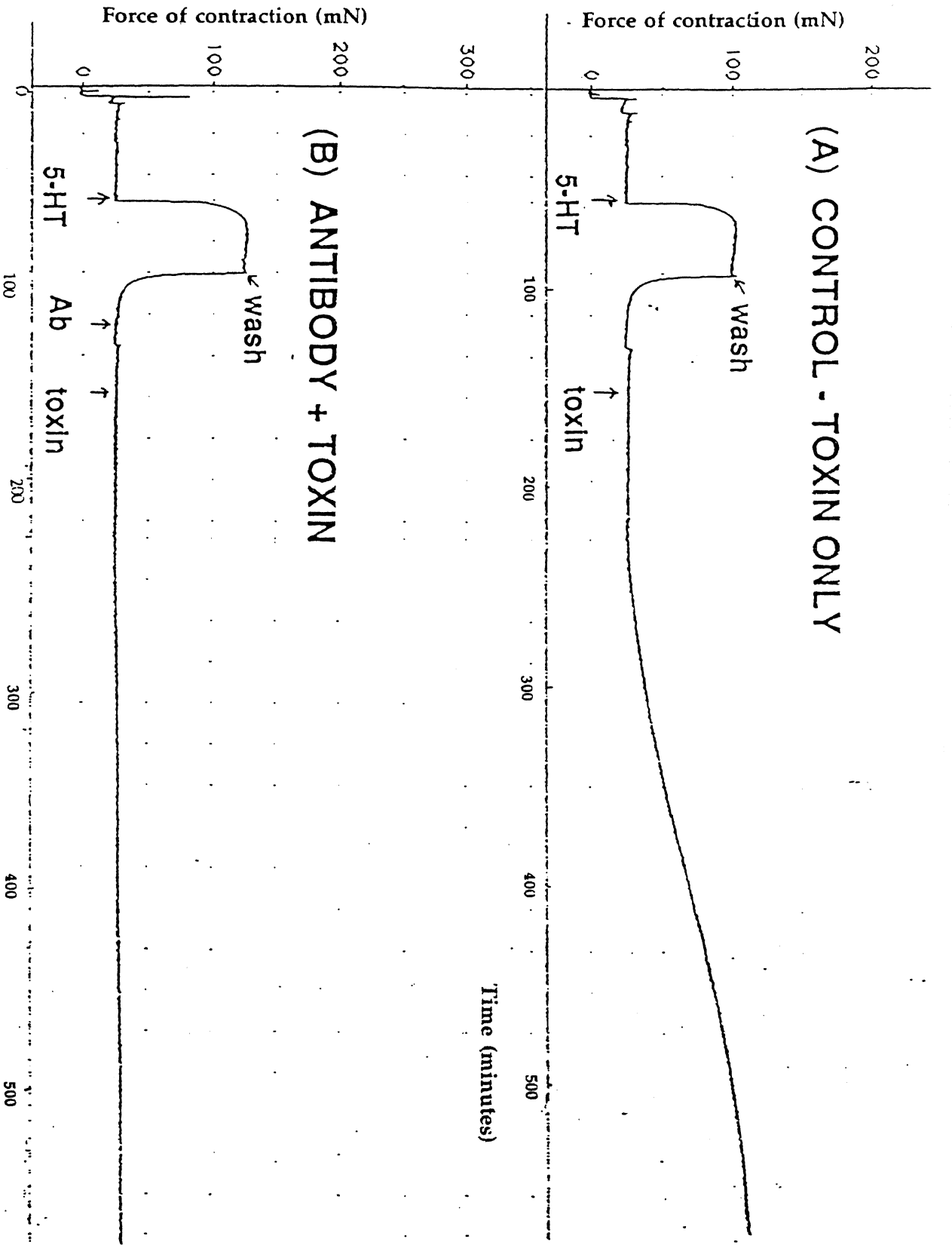


Figure 4.9

Purified rabbit IgG attenuates the contractile response of bovine pulmonary venules to *Pimelea* toxin (EC_{50}) in a dose-dependent manner. (The shaded region represents contraction with EC_{50} toxin concentration.)

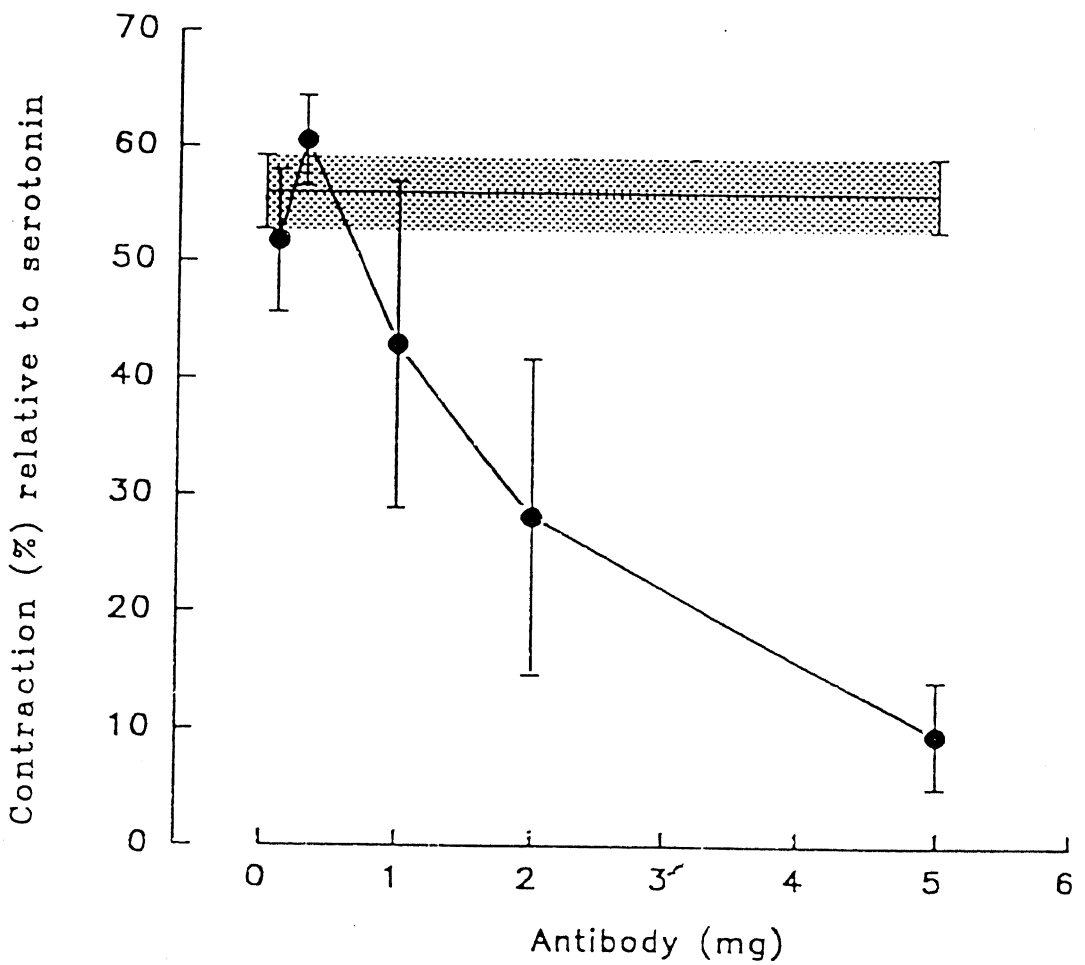


Table 4.7

Average contraction (± s.e.m.) of bovine pulmonary venules in response to purified rabbit IgG in the presence of EC_{50} *Pimelea* C concentration.[a]

Antibody added(mg)	% of 1.0 μM 5-HT contraction.
0.1	51.7 ± 12.2, n=4
0.3	60.5 ± 9.7, n=6
1.0	42.0 ± 28.0, n=4
2.0	25.8 ± 19.6, n=4
5.0	9.6 ± 11.1, n=6

[a] Average control contraction (n=13) was 47.1 ± 21.7 %

4.3.5 *In Vitro* Effects of Purified Cattle Antibodies

ELISA analysis revealed the presence of a specific immune response in cattle against *Pimelea* toxins; however, purified IgG from the vaccinated cattle had no neutralising action in the organ bath even at a 100 mg dose (treated tissue : 63.0 ± 14.7 %, n=4 and control tissue, 55.0 ± 13.2%, n=4). With such a large dosage of IgG in the bath, frothing was a significant experimental problem. The data for the 50 and 100 mg antibody dosage experiments are given in Table 4.8. Much lower concentrations of antibody (comparable with the rabbit data) were trialed initially but no effect was observed.

Table 4.8

Contractile responses of bovine pulmonary venule preparations in the presence of bovine IgG and EC_{50} *Pimelea* toxin concentration.

Treated preparations (preparations exposed to IgG and *Pimelea* toxin EC_{50})					Control preparations (preparations exposed to *Pimelea* toxin EC_{50} only)			
IgG (mg)	Δ mN 1μM 5HT[b]	Δ mN *Pim.*[c]	% PIm Con.[d]	Tissue (mg).	Δ mN 1μM 5HT	Δ mN *Pim.*	% *Pim.* Con.	Tissue (mg).
50	95	65.6	69	2.6	70	43.4	62	1.5
50	88.7	40	45	–	75	39	52	2
50	93.3	43	46	4.6	120	36	30	4.6
100	116	70.8	61	3.1	93.1	54.9	59	4.8
100	129	104.5	81	5.5	117.8	69.5	59	4.1
100	156	101.4	65	2.8	155.1	105.4	68	5.2
100	112.9	50.8	45	5.2				

[a] weight of the freeze dried cattle IgG used in organ bath. [b] Maximum contraction to 1.0 μM 5 -HT minus pre-load tension. [c] Maximum contraction in response to *Pimelea* toxin minus the pre-load tension. [d] Expressed as a % of the 5-HT maximal response.

It is possible that the purified IgG antibody fraction prepared from cattle serum does not contain any neutralising anti-toxin antibodies, which may be present in another antibody subclass such as IgM. As reported in Chapter 3, the effect of freeze-drying the purified IgG was also investigated using ELISA analysis. There was the possibility that structural change or loss of IgG activity had occurred during purification and freeze drying of the bovine samples. The results indicated that there was substantial loss of binding as a result of freeze drying and that this effect was much more dramatic for bovine IgG compared with the purified rabbit IgG. However, even allowing for the loss of IgG

activity with freeze drying, some response would have been expected at the very high doses used. Given that the purified rabbit IgG had shown neutralising actions in the organ bath work, the cattle IgG results were disappointing given that this was the target species.

4.3.6 Preliminary Investigations with *Nux vomica* CM Tincture

Nux vomica CM tincture tincture is an extract obtained from the nut of *Strychnos nux vomica* which is a moderate sized tree native of the Cormandel coast and Cochin China. The fruit is very like an orange in appearance and contains numerous flattened circular seeds, each about the size of a 10 cent piece, ash grey in colour and covered with fine silky hair. The seeds are intensely bitter but the pulp is innocuous and is said to be eaten by birds. If nitric acid is added to the seeds, a deep orange yellow colour is produced. In India, tinctures of *Nux vomica* are administered in cases of intermittent fever and also for snake bite. A leaf extract of the plant is used externally in rheumatism. Dried seeds of *Strychnos nux vomica* L. (Loganiaceae) have been used in traditional Chinese medicine for treatment of blood circulation problems and relieving pain (Cai *et al.*, 1990).

In India *Nux vomica* CM tincture is used as an oral drench for treatment of animals suffering certain plant toxicities. Preliminary experiments were therefore carried out to examine whether *Nux vomica* may show promise as an antidote for *Pimelea* poisoning.

The experimental design was similar to that used in the antibody experiments. The maximum contraction of the venules was measured in response to 5-HT, preparations (n= 10) were then exposed to 200 µL, *Nux vomica* CM potency tincture followed immediately by addition of EC_{50} *Pimelea* toxin. Control preparations were exposed to EC_{50} *Pimelea* toxin only. Contraction was

recorded for 200-300 minutes. It was observed that treated tissue remained totally relaxed even after 300 minutes (0.70 ± 0.5 %, n= 10) whereas control tissues always contracted (50 ± 10 %, n=7) in the normal way (Figure 4.10).

There was some concern that the *Nux vomica* extract might have adverse effects on the physiological viability of the tissue preparations. However, further experimentation showed that preparations which had remained relaxed after being exposed to *Nux vomica* and *Pimelea* toxin for 300 minutes could be washed out by changing the Tyrode solution three times at intervals of 10 minutes, and these preparations still gave the normal rapidly developing contraction to 1μM 5-HT. After washing away the 5-HT in the usual way, these preparations were re-equilibrated to 25 mN pre-load and then exposed to EC_{50} *Pimelea* toxin concentration. The tissues showed a slow developing contraction (39.0 ± 12.1, n=3) over a further 300 minutes (Figure 4.11). It was concluded that the *Nux vomica* extract had not significantly affected the viability of the tissues since such significant contraction had been achieved in preparations which had been mounted in the organ baths for more than 12 hours.

In another set of experiments, tissues which had remained relaxed after being exposed to *Nux vomica* and *Pimelea* toxin for 300 minutes also gave normal contractile responses to 1.0 μM 5-HT without any wash-out of the bath buffer (Figure 4.12). These results suggested that*Nux vomica* tincture can inhibit the normal contraction response to *Pimelea* toxin (EC_{50}). The mechanism of inhibition remains unresolved; however, it is possible that *Nux vomica* may inhibit PKC activity or it may block the action of the toxins by affecting calcium mobilisation within cells (O'Neil and Bolger, 1990).

Figure 4.10

Attenuating effect of *Nux vomica* tincture (200 µL) on contraction of bovine pulmonary venule in response to *Pimelea toxin* (EC_{50}).

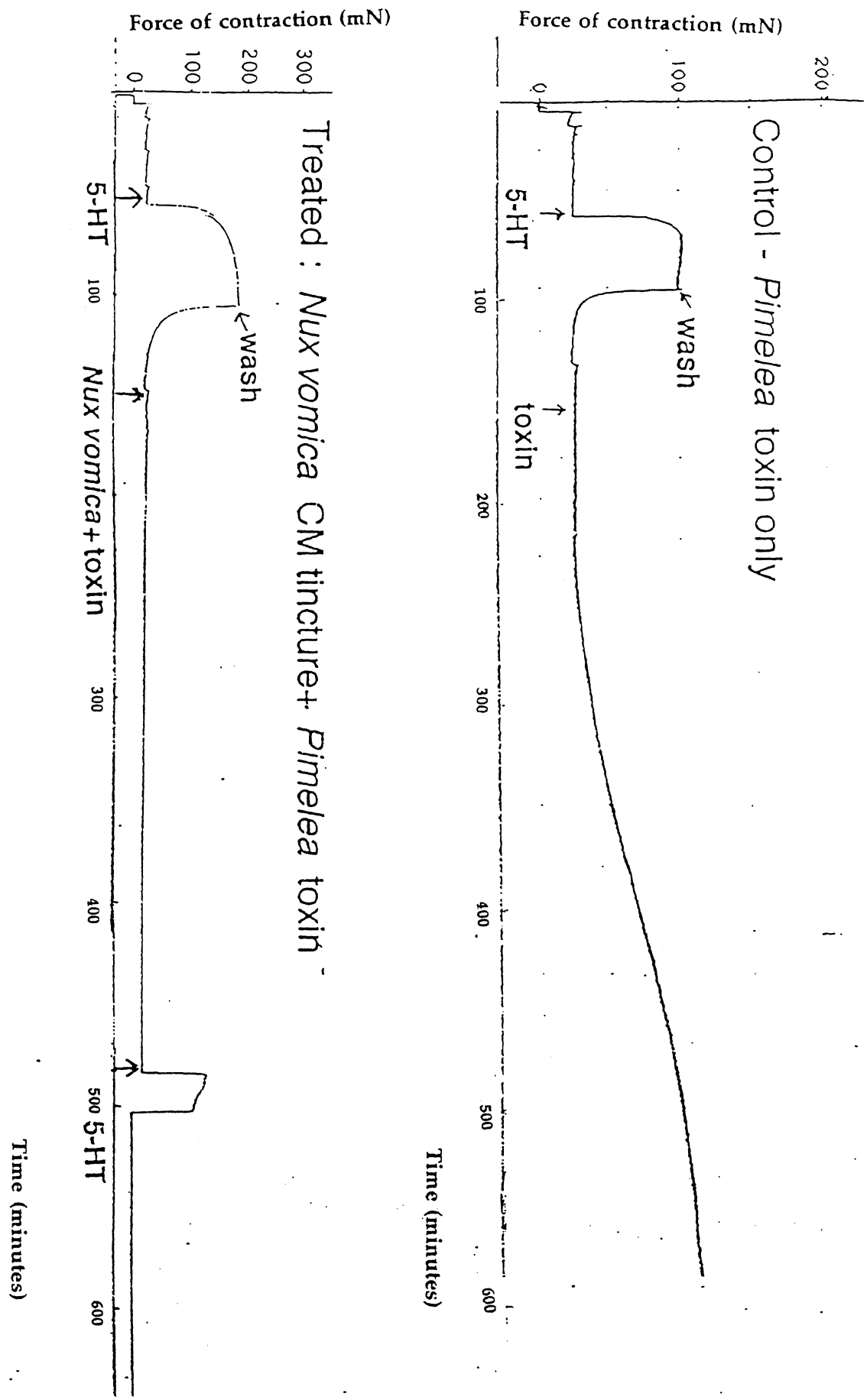


Figure 4.11
Attenuating effect of *Nux vomica* is reversible.

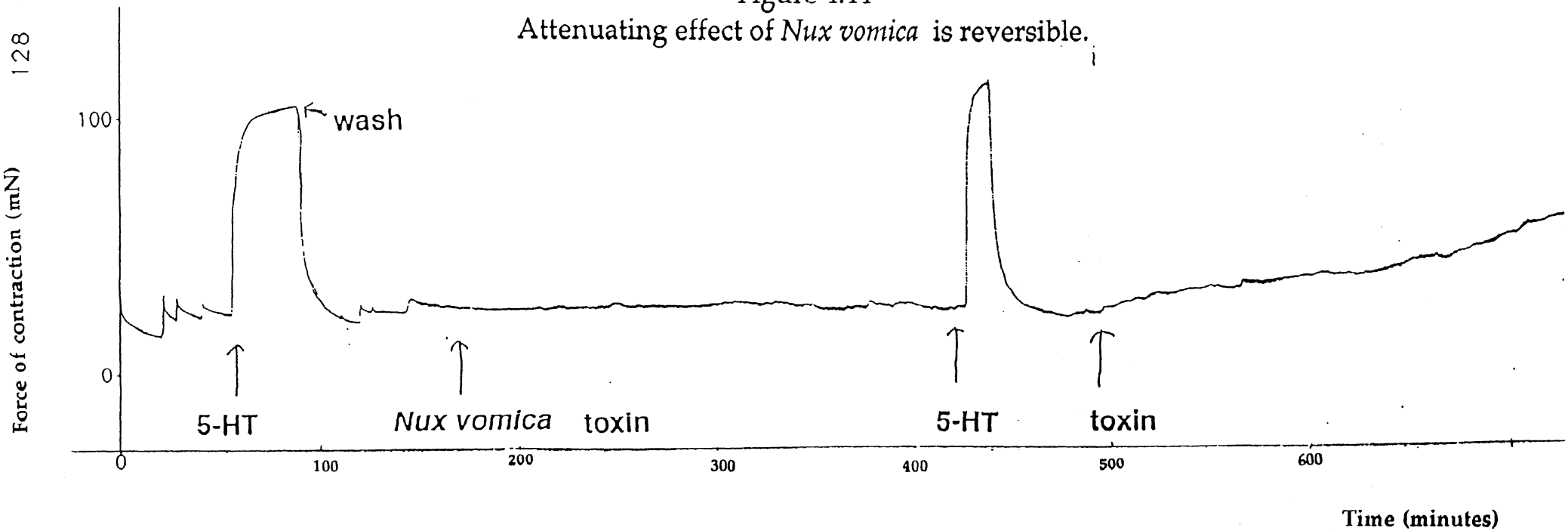
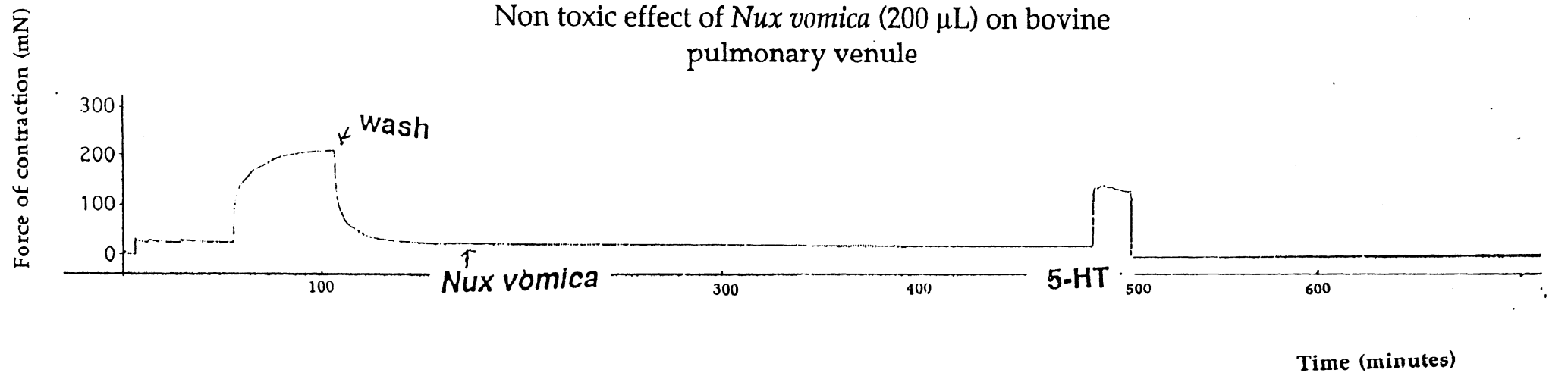


Figure 4.12
Non toxic effect of *Nux vomica* (200 µL) on bovine pulmonary venule



4.3.7 Preliminary Experiments with the Tumour Promoter Mezerein

Miyake *et al.*, (1984) report that mezerein activates PKC *in vivo* (platelets) and *in vitro* (rat brain tissue). Mezerein is found in *Daphne mezereum L* plant species which belong to the same family (Thymelaeaceae) as *Pimelea* species (Borris *et al*,, 1988).

It is presumed that the mode of action of the *Pimelea* toxins on the bovine pulmonary venule system is by way of activation of PKC. The organ bath technique therefore provided a useful experimental tool to verify that a known PKC activator having close structural similarity to the *Pimelea* toxins also caused contraction of bovine pulmonary venule preparations.

Table 4.9

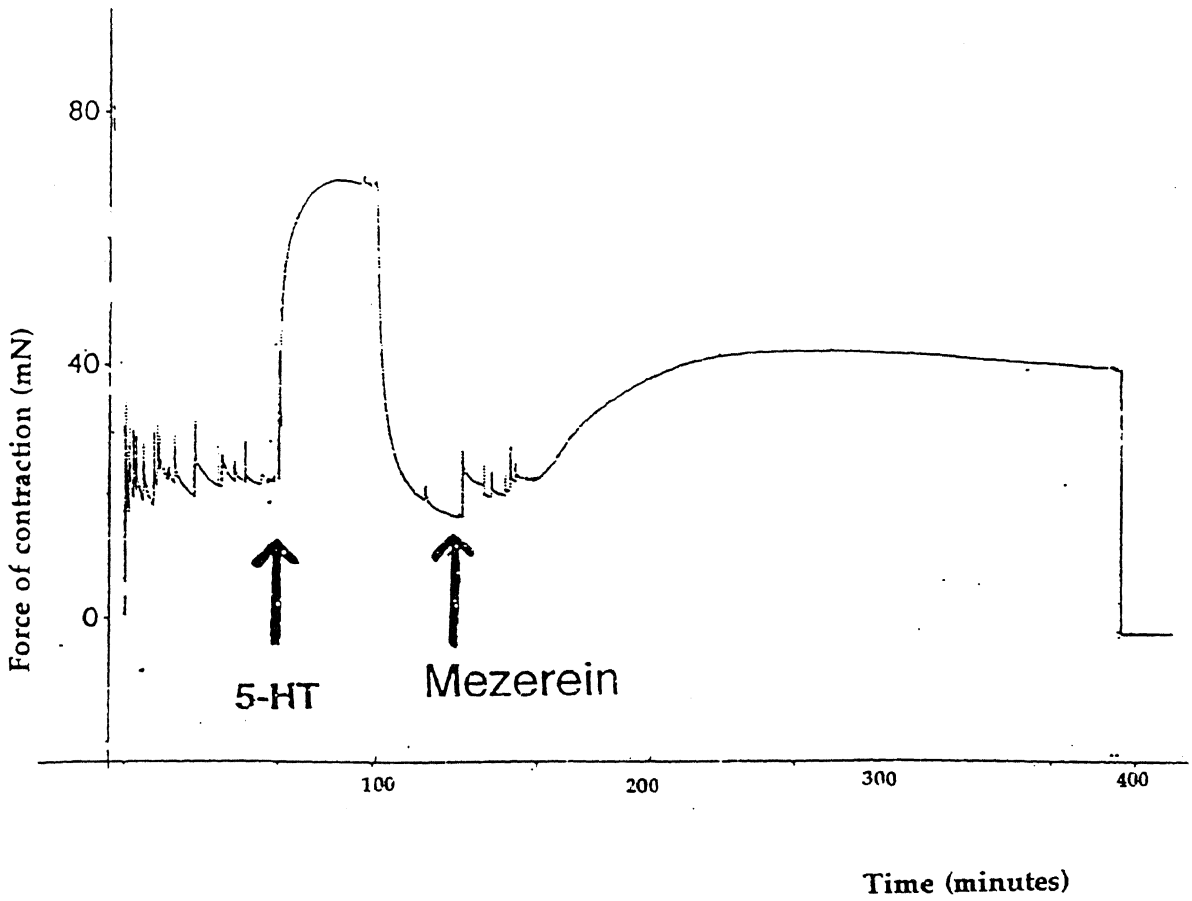
Responses of bovine pulmonary venule preparations to mezerein

Preparation no.	1.0 µM 5-HT (mN)	% of 5-HT contraction to 1µM mezerein.	weight of tissue (mg)
1	94	42	2
2	60	48	1.6
3	141	49	2.4
4	63	39	3.6

In vitro studies showed that mezerein (LC Services, USA) causes a slow developing contraction in bovine pulmonary venule preparations, similar to the contraction induced by *Pimelea* C fraction (Figure 4.13). The EC_{50} concentration for Mezerein was found to be approximately 1 µM (average contraction 44.5 ± 4.7 % relative to 1.0 µM 5-HT; n= 4, Table 4.9). These findings are consistent with the suggestion that contraction of the bovine pulmonary venule system by *Pimelea* toxins may be mediated by PKC activation.

Figure 4.13

Slow developing contraction of bovine pulmonary venule induced by Mezerein



Further to the exploratory experiments with mezerein, some preliminary experiments were also undertaken to observe whether two commercially available PKC inhibitors, polymyxin B sulfate and D-*erythro*- sphingosine (Calbiochem), and also *Nux vomica* (200 µL), attenuated the contraction response to Mezerein (EC_{50}). Hannun *et al.* (1986) reported inhibition of PKC activity and of phorbol dibutyrate binding *in vitro* and in human platelets by sphingosine. Muller *et al.*, (1991), reported prevention of glucose induced insulin receptor tyrosine kinase resistance in rat fat cells by PKC inhibitors. Oishi *et al.*, (1988) have also shown *in vitro* inhibition of PKC activity by polymyxin B and sphingosine.

Control tissue preparations were exposed to mezerein (1µM) only, whereas test preparations were exposed to mezerein (1µM) in addition to either sphingosine (200 µM), polymyxin B (250 µM), or *Nux vomica* (200 µL). The average contraction of control preparations exposed to mezerein (EC_{50}) only was 36.5%, (n=2). The average contraction of treated preparations was : sphingosine 24.5%, (n=2); polymyxin B 22.5%, (n=2); *Nux vomica* 12%, (n= 1). Although the data is very preliminary, it was suprising that the PKC inhibitors were not more efficacious given other literature reports of K_D values.

4.4 CONCLUSIONS

It was shown that the contractibility of bovine pulmonary venule preparations in response to *Pimelea* toxins was concentration dependent. From the concentration response data, the EC_{50} concentration was determined to be 5.3×10^{-8} M (0.053 µM).

Experiments with purified rabbit anti-toxin IgG showed that these antibodies were able to neutralise the effects of *Pimelea* toxins on bovine pulmonary

venules in the *in vitro* system. While these results were highly encouraging, the corresponding bovine anti-toxin IgG appeared to lack any measurable efficacy even at very high dosage. This probably reflects a lack of high affinity antibodies in the cattle IgG preparation and in part may be due to denaturation of the bovine antibodies during freeze drying. Further work needs to be done on improving the experimental vaccine.

The results obtained with *Nux vomica* suggest that this material may provide an antidote strategy to combat *Pimelea* poisoning, however this needs testing in live animals before any firm conclusions can be made. The organ bath results for mezerein show that this compound causes contraction of the pulmonary venule tissue, consistent with its known PKC activation characteristics.

CHAPTER FIVE

CONLUSIONS AND DIRECTIONS FOR FURTHER WORK

5.1 Achievement of Aims

A major aim of this project was to examine the feasibility of inducing protective immunity against *Pimelea* toxicity in cattle. This required isolation of the toxins, conjugation of these compounds to suitable carrier proteins and vaccine formulation. The experimental problem was that the lipophilic daphnane toxins in *Pimelea* species were low molecular weight diterpenes (molecular weight range 500-700 mass units) and in their native form were not immunogenic. The toxins were also present in very low levels in the plant, therefore the extraction procedure had to be highly efficient. For example, even the major toxin component (simplexin) made up only 0.005% of the dry weight of *Pimelea trichostachya* . The first experimental task was to separate out sufficient quantities of the toxic components from the myriad of other organic components present in methanol extracts of dried plant material. There was some information on suitable chromatographic methods in the literature, but most of these studies were undertaken prior to HPLC methodology becoming routine laboratory practice.

The chromatographic approach developed for this project involved a combination of solvent partition, silica column chromatography and preparative HPLC. Identification of toxic fractions was accomplished with a fish bioassay technique. It was reasoned that since the huratoxins were known piscicidal agents and that *Pimelea* daphnane toxins were structurally similar to huratoxin, then these toxins should also be active piscicides. This assumption proved correct and it was found that the *Pimelea* toxins were extremely potent in the bioassay. The HPLC methodology was scaled up from analytical through to semi-preparative and finally preparative scale to enable isolation of sufficient quantities of *Pimelea* toxins to conduct the vaccination trials in both experimental animals and cattle. The entire procedure could be scaled up further for commercial scale separation of *Pimelea* toxins.

The next task was to link the isolated toxins covalently to immunogenic carrier proteins so that experimental vaccines could be prepared. In previous work (G. Pegg and L. Hellqvist, unpublished) resiniferinol orthophenyl acetate and mezerein had been linked to HSA and BSA. The strategy involved succinylation of a C-20 hydroxymethyl group common to these compounds by reaction with succinic anhydride in pyridine solvent. The free carboxyl grouping was then activated by reaction with iso-butylchloroformate and the resultant mixed anyhydride then reacted with free lysine residues on the carrier protein. The initial activation chemistry was conducted in DMF solvent and the protein coupling reaction then accomplished in a 50:50 DMF: water mixture.

It was reasoned that since almost all of the published *Pimelea* daphnane and tigliane toxins also had a free C-20 hydroxymethyl group then this same chemistry could be applied in the present project to a mixture of *Pimelea* toxins isolated from the plant by chromatographic methods. The rationale for working with the natural mixture of toxins was that antibodies might then be developed to a range of toxin epitopes rather than to a single compound. Conjugation of toxin mixtures to ovalbumin and BSA proved successful. UV spectrophotometric analysis of solutions of the conjugates clearly showed that toxins had been linked to the carrier proteins in each case.

One concerning outcome of the conjugation chemistry was the apparent cross-linking of carrier protein molecules. This was a result of traces of unreacted *iso*-butylchloroformate activating free aspartate and glutamate sidechains in the carrier protein and these activated groups then cross linking with free lysines on adjacent protein molecules. Advice from immunologists associated with the group was that any supramolecular protein complexes would still be processed

by macrophages and could even prove advantageous for development of an immune response.

Oil-in-water emulsions of the conjugates were prepared using Freund's adjuvant for the rabbit studies and a commercial formulation for cattle. The rabbit experiments were conducted first to establish that the conjugates were immunogenic. ELISA analyses to recognise anti-toxin antibodies had to be conducted with conjugates of the toxins rather than free toxin coated to the plate. This approach raised some concerns about measuring the absolute specificity of the immune response but was unavoidable because the toxins were not water soluble and these molecules would not bind in their native form to the plate. Displacement of bound antibodies with free toxin was equivocal, since only 20-50% of the binding could be displaced. This may well reflect the insolubility of the toxins in aqueous media. While the inclusion of organic solvents such as DMSO in the ELISA improved the percent displacement, total displacement was never achieved (L. Hellqvist, unpublished).

The ELISA data consistently provided strong evidence that immune responses to the toxins had been obtained in both the rabbits and cattle. In rabbits, antibody levels rose dramatically after the secondary vaccination and were maintained by subsequent booster vaccinations. In cattle, a similar rise in antibody levels were observed after secondary vaccination, but the titre then continued to decline slowly, despite booster vaccinations. IgG was purified from the serum of immune animals for *in vitro* studies of the efficacy of the antibodies raised to the toxins.

The vaccinated cattle group and an equivalent number of control animals were challenged with a slurry of dried *Pimelea* plant material, administered directly into the rumen of each animal via a stomach tube. The dosage rate

administered was based on literature reports and the experiment was initially conducted for more than a week using a dosage that should have affected the animals within a few days. Because of the lack of response, the dosage was increased in two stages over the second week. Unfortunately, the animals appeared to have been severely over-dosed by the completion of the experiment and both the control and vaccinated group had developed clear signs of *Pimelea* poisoning such as variable diarrhoea, distension of the jugular vein and oedema of the neck and brisket. Blood parameters were monitored daily and for a number of these parameters such as total haemoglobin and red cell volume, the vaccinated group seemed to be less affected than controls over the first week and half of the experiment. The result of the intoxication experiment was that vaccination did not appear to counteract *Pimelea* toxicity at the doses tested.

It might be argued that the highly lipophilic character of the daphnane toxins may prove the downfall of the immunological strategy to combat *Pimelea* poisoning. Antibodies may not have the opportunity to bind and neutralise the toxins in the circulatory system if these small lipophilic molecules are rapidly taken up into membranes and fat reserves. However, steroids are similar in size and hydrophobicity to *Pimelea* toxins and these molecules are found in the circulatory system in the free form and also associated with binding proteins. It has proven possible to vaccinate successfully against steroids, through inducing highly specific antibodies. For this reason, the experimental *Pimelea* vaccine formulation developed in this study is worthy of further research and development.

The primary site of action of *Pimelea* toxins in cattle is sustained constriction of the pulmonary venules in the bovine lung due to peculiar sphincter like structures present in the walls of these vessels. This constriction impedes the

returning flow of blood from the lungs and also results in right-sided heart failure. There had been a single report of an *in vitro* study of bovine pulmonary venules which demonstrated that a 10 mg quantity of the tarry residue obtained by extracting the plant with alcohol caused contraction of ring preparations (Mason, 1976). We were interested in conducting more refined *in vitro* studies aimed first at establishing the efficacy of the toxin fractions obtained from extensive chromatographic purification of plant extracts. The EC_{50} concentration for the purified toxin fraction was found to be in the sub-micromolar range. It was found that while 1.0 μM 5-HT caused rapid maximal contraction of the venule preparations within 30 minutes, a slow developing response to the toxins required 300 minutes to reach equilibrium.

The *in vitro* technique provided the opportunity to assess whether purified rabbit and cattle IgG resulting from the immunisation experiments had any protecting effect on the target tissue in cattle. It was observed that the rabbit IgG gave dose-dependent protection from the toxins, whereas the cattle IgG lacked efficacy. The rabbit IgG data showed that antibodies raised to the toxin-protein conjugates are capable of both toxin recognition and target tissue protection at least in the *in vitro* system.

There may be several reasons for the lack of efficacy observed for the purified bovine IgG in the organ bath experiments. It was considered that there might have been loss or structural change of toxin specific IgG during purification of the serum and in the freeze-drying process. ELISA binding data for reconstituted freeze-dried IgG was compared with an equivalent amount of serum for both rabbits and cattle. It was observed that there had been a significant loss of binding for the freeze dried bovine IgG material compared to the native serum, whereas the rabbit serum sample and the freeze dried IgG equivalent gave similar binding. However, such high concentrations of the

purified cattle IgG were tested that attenuating effects should have been observed if the bovine IgG fraction contained even low levels of specific anti-toxin antibodies. It is also possible that the purified IgG antibody fraction prepared from cattle does not contain the toxin specific antibodies present in the serum and these may be present in another antibody subclass.

The finding that *Nux Vomica* CM tincture was effective in preventing contraction of bovine pulmonary venules in the organ experiments was an interesting observation. *Nux Vomica* may prevent PKC activation of muscle contraction by impeding calcium mobilisation (O'Neill and Bolger, 1990).

DIRECTIONS FOR FURTHER WORK

The problem of cross-linking of protein molecules during the conjugation of toxin to carrier protein may have compromised the efficacy of the prototype vaccines. Further development of the conjugation chemistry needs to be conducted. One possibility might be to activate the succinylated toxin by reaction with N-hydroxysuccinimide, rather than with *iso*- butylchloroformate. The resultant N-hydroxysuccinimide ester toxin derivative should be stable enough to tolerate chromatographic purification prior to the final cross-linking step and this approach should eliminate the possibility of protein aggregation.

Another aspect of the conjugation chemistry that requires investigation is to determine the optimum ratio of toxin to protein required to illicit a highly specific immune response. The present study has shown that incorporation of up to 20 molecules of toxin per protein molecule can be achieved. However, this incorporation ratio may have been too high as this raises the possibility of deleterious steric interactions between toxin molecules which might compromise the specificity of the antibody response. McAdam *et al.*, (1992) reported that in development of an immunoassay for pyrethroids, the optimum

incorporation ratio of pyrethroid was 10. Higher incorporation ratios caused more antibody production, but these antibodies were less specific.

The extraction and isolation of *Pimelea* toxins from dried plant material is a time consuming and expensive process. A logical substitute compound for further work is mezerein, since this daphnane orthoester is commercially available and is a close structural homologue of simplexin and huratoxin. It was shown in the present work that cattle vaccinated with a mezerein conjugate developed an immune response and these antibodies also cross-reacted with *Pimelea* toxin-protein conjugates.

While this study has shown that it is possible to raise antibodies to *Pimelea* daphnane orthoester toxins and that the purified rabbit anti-*Pimelea* IgG has demonstrable efficacy at protecting the bovine target tissue in *in vitro* studies, the likelihood that the immunogen approach might result in effective protection of cattle against *Pimelea* toxicity remains uncertain. The study has produced some interesting results and further work aimed at improving the vaccine formulation should be undertaken.

An alternative approach may be to investigate the modulation of PKC activity by generation of sphingosine intracellularly by breakdown of sphingomyelin and related molecules by drugs or antibodies. Hannun *et al* ., (1986) reported inhibition of PKC activity by sphingosine in *in vitro* preparations and in human platelets. Sphingosine inhibition was modulated by Ca^{2+} and by the molar ratios of diacylglycerol and phospholipid present. Sphingosine prevents the formation of an active lipid-PKC complex by displacement of the activator (DAG or phorbol ester) from the complex. Sphingosine levels may be regulated in response to either intra or extracellular signals. Sphingomyelin was observed to undergo rapid N-acyl exchange when L-929 fibroblasts were stimulated with

specific antibodies (Ulrich *et. al.*, 1984.). The deacylation of sphingosyl phosphorylcholine also leads to the generation of sphingosine through hydrolysis of the phosphorylcholine head group. Further literature investigation is required to determine if these catabolic pathways offer any opportunities for modulating PKC activity in the bovine lung.

Validation of an assay for measuring PKC activation by *Pimelea* toxins is a worthwhile future research endeavour. The PKC assay technique might prove the most efficient and cost effective approach to assaying toxin levels in plant and soil extracts. The present study has shown that direct HPLC assay is too insensitive and the ELISA analysis would likely be problematic because the toxins are not water soluble. It would be useful to be able to conveniently analyse toxin levels from different parts of the plant (roots, stems, leaves, flowers) as there is no information in the literature regarding the time course and location of toxin levels in the plant. Producers believe that the plant is more toxic as it flowers and dries; however, it could be that pasture grasses also become limiting at this time and foraging animals therefore consume more of the plant under these conditions.

Another potential future research direction is to investigate detoxification/ chemical transformation of the daphnane orthoesters by rumen microflora. For this work it would be advantageous to have a radiolabelled toxin component (perhaps derived from mezerein in the first instance) where the label was likely to be retained in the metabolites. For example, the methylene hydrogens on the C-20 hydroxymethyl group could be replaced by tritium through selective oxidation followed by reduction. Experiments could be conducted both with live animals and rumen broth fermentation studies in the laboratory. The labelled toxin would also be useful for determining the residence time of

Pimelea toxins in the live animal and the uptake of toxins into the animal's organs and lipid reserves.

With regard to structure-activity studies of the requirements for PKC activation, there is information in the literature suggesting that reduction of the A ring 5-membered enone moeity might lessen the efficacy of the toxins towards PKC (Jeffery *et al.*, 1985). This presents a research opportunity to specifically reduce the enone (and perhaps other reducible moieties) using modern organic chemistry methodologies, verify the structures by high field NMR and mass spectrometry, and then determine the ED_{50} of the reduced toxins in the organ bath studies and in the PKC assay system. The results from these experiments might then shed new light on what types of anaerobic microflora could be advantageous for detoxification of *Pimelea* toxins in the rumen and perhaps offer some hope for solving the problem of *Pimelea* toxicity to cattle by either rumen modification or introduction of new rumen microbes.

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