A PRELIMINARY INVESTIGATION: PLANT CYANOGENICITY AS A POSSIBLE CO-FACTOR IN A POSSUM SPECIFIC TOXIN

A thesis presented in partial fulfilment of the requirements
for the degree of
Master of Science in Biochemistry,
Massey University,
New Zealand

Elana Maree Gorrie
1998

ABSTRACT

Since the introduction of thirty Australian brushtail possums into New Zealand in 1858 to start a fur trade industry the possum population as grown considerably. New Zealand is now 'home' to approximately 70 million possums which wreak devastation on our native forests and wildlife.

Current effective strategies for the control of possums in uninhabited areas include the use of 1080, brodifacoum, cholecalciferol, cyanide, and trapping or shooting. However these strategies are relatively non-specific in their mode of action and as such cause non-target species, including native wildlife, to die. The use of non-specific toxins and traps is also inappropriate for situations where people, livestock, or pets are present. There is therefore a demand for alternative strategies of possum control that affect only the target species. Methods presently being researched include the development of contraceptive vaccines, possum repellents and possum specific bait stations.

This research investigates the feasibility of developing a cyanogenic bait that is activated by a co-factor within the possum diet. The fast acting hydrogen cyanide poison is present in some plants species in an inactive glycoside form. Upon tissue injury the inactive cyanogenic glycoside is exposed to and hydrolysed by catabolic enzymes within the plant thereby releasing the toxin, hydrogen cyanide, at potentially lethal levels for possums. Some plant varieties within cyanogenic species however, have evolved to be acyanogenic due to the absence of either the cyanogenic substrate, the enzyme, or both. The occurrence of these acyanogenic plants which contain either the substrate or the appropriate enzymes are the target of this research. It is these plants that may provide the necessary co-factor for a cyanogenic possum bait to become lethal.

Preliminary analyses involved measuring and maximising the cyanide release from plant species known to be highly cyanogenic. Clover leaves (*Trifolium repens*), cherries (*Prunus avium*), and almonds (*Prunus amygdalus*) were the plant tissues analysed to determine whether levels of cyanide toxic to possums could be liberated. All three plant varieties underwent *in vitro* analyses in which they were exposed to surplus substrate and/or enzymes at varied temperatures and acidities. The maximum cyanide release was determined for each plant variety and in the case of almonds (*Prunus amygdalus*) a further *in vivo* study was performed.

Although the clover, cherries and almonds all liberated cyanide after addition of either cyanogenic substrate or enzymes, the almonds were the only plant tissue to liberate sufficient levels of cyanide from the *in vitro* analyses to be considered toxic to possums. The almonds were found to contain high levels of active β-glucosidase enzymes which when incubated with the cyanogenic substrate, amygdalin, released high levels of cyanide. The *in vivo* analyses of almond macerates administered with amygdalin however were inconclusive in showing almonds as an effective co-factor for the hydrolysis of amygdalin. Nevertheless, two possums did die from cyanide poisoning after the administration of amygdalin with and without added enzymes. A third possum displayed signs of severe cyanide poisoning after it was gavaged with amygdalin and β-glucosidase enzymes but it later made a full recovery. As a result of the limitation imposed by the small size of the *in vivo* sample group further experimental trials are recommended to possibly obtain a more accurate set of results.

ACKNOWLEDGEMENTS

I would like to express my sincere appreciation to my supervisors Associate Professor David R.K. Harding, Professor Neville G. Gregory and Dr Kathryn Kitson for all their guidance, inspiration and encouragement during the research and completion of my Masterate.

My thanks also to the Separation Science Group, Simon Burton, Jennifer Cross, Louisa Fisher, Helen Guo, Yuming Li, Rekha Parshot and Dick Poll, for all their support and encouragement. Working in such a friendly environment made completing my thesis a whole lot easier.

Special acknowledgements to John Carradus and Derek Woodfield of Grasslands Research for supplying 'aran' clover, and to Jeremy Sandall of The Cherry Farm Orchard for providing the cherries for cyanogenic analysis.

Thanks to Kate Littin for procuring the possums for the *in vivo* analysis and for assisting in the gavage procedures. Another 'big' thank you to Eldon Ormsby and Debbie Chesterfield of the Massey Universtiy Small Animal Production Unit for their assistance in providing housing and care for the experimental possums. I am grateful also for the expert knowledge of Massey University veterinary anaesthetist Roz Machon and the veterinary pathologist involved in the autopsy of one of the trial possums. Thanks to Charles Eason of Landcare Research, Lincoln, for sharing his expertise on possum handling and methods of amygdalin administration in possums.

I would like to acknowledge Pak'n Save and Quality Bakers, Palmerston North for their generous donation of food for the possums (I know my possums appreciated it too!).

Lastly, and most importantly, I would like to thank my husband Keri for his endless encouragement, and my parents and siblings for their emotional and financial support throughout my studies at Massey University.

TABLE OF CONTENTS

Abstract ii

Acknowledgements iv

Table of Contents v

Figures viii

Tables x

Abbreviations and Terminology xi

CHAPTER ONE: INTRODUCTION 1

1.1 The Possum Peril 1

  1.1.1 Deforestation 1

  1.1.2 Bovine Tuberculosis 2

1.2 The Research Aim 2

1.3 Cyanogenic Plant Distribution 3

1.4 Cyanogenesis 4

  1.4.1 Chemical Hydrolysis 5

  1.4.2 Enzymatic Hydrolysis 7

1.5 Cyanide Toxicology and Detoxification 8

1.6 Signs of Cyanide Poisoning 10

1.7 Possum Control 11

1.8 Present Control Methods 12

  1.8.1 Cholecalciferol 12

  1.8.2 Cyanide 12

  1.8.3 Sodium Monofluoroacetate (1080) 13

  1.8.4 Brodifacoum 14

  1.8.5 Phosphorus 14

  1.8.6 Shooting 15

  1.8.7 Trapping 15

  1.8.8 Bio-dynamic repellents 15

1.9 Future Control Methods 15

CHAPTER TWO: MATERIALS AND METHODS 17

2.1 Reagents and Equipment 17

2.2 Sample Homogenisation 18

2.3 Extraction of Cyanide from Cyanogenic Plant Material 18

2.4 Quantitative Determination of Cyanide 19

2.5 *In vivo* Possum Study 20

CHAPTER THREE: WHITE CLOVER (*Trifolium repens*) 22

3.1 Introduction 22

3.2 Results and Discussion 24

3.2.1 Cyanide Release from Macerated and Intact Clover Tissue 24

3.2.2 Variation of Maceration Period 25

3.2.3 Testing the Microdiffusion Procedure 27

3.2.4 Rate of Cyanide Liberation 31

3.2.5 Effects of Added Substrate on Cyanide Liberation 32

3.2.6 Effects of Added Enzymes of Cyanide Liberation 33

3.2.7 The Cyanogenicity of 'Aran' Stem Tissue 37

3.3 Summary 38

CHAPTER FOUR: BLACK CHERRIES (*Prunus avium*) 39

4.1 Introduction 39

4.2 Results and Discussion 40

4.2.1 Cyanide Release from Sour Black Cherries 40

4.2.2 Cyanide Release from *Prunus avium* Cherries 41

4.2.3 Comparison of Amygdalin Hydrolysis by Cherry and Almond Macerates 45

4.3 Summary 46

CHAPTER FIVE: ALMONDS (*Prunus Amygdalus*) 48

5.1 Introduction 48

5.2 Results and Discussion 50

5.2.1 Amygdalin Hydrolysis using Commercial Emulsin 50

5.2.2 Amygdalin Hydrolysis using Macerated Almonds 52

5.2.3 Amygdalin Hydrolysis using Hydroxynitrile Lyase 53

5.2.4 Testing for the Presence of HNL's in Almonds 54

5.2.5 Analysis of Wild Almonds for Cyanogenicity 57

5.2.6 Analysis of the Flavour Enhancer for the *In vivo* Trials 58

5.3 Summary 59

CHAPTER SIX: *IN VIVO* POSSUM STUDY 61

6.1 Intoduction 61

6.1.1 Lethal Doses of HCN for Possums 61

6.1.2 *In vitro* HCN Recovery Rate at pH 4.0 62

6.2 Results and Discussion 63

6.2.1 *In vivo* Trial One 63

6.2.2 *In vivo* Trial Two 67

6.2.3 *In vivo* Trial Three 69

6.3 Summary 70

CHAPTER SEVEN: CONCLUSION AND FUTURE WORK 72

7.1 Conclusion 72

7.1.1 Clover 72

7.1.2 Cherries 73

7.1.3 Almonds 73

7.1.4 *In vivo* Possum Study 74

7.2 Future Work 75

REFERENCES 76

FIGURES

Figure 1.3.1 The known cyanogenic glycosides 4

Figure 1.4.1.1 Chemical degradation of cyanogenic glycosides 6

Figure 1.4.1.2 Cleavage point for the β-glycosidic bond of amygdalin 6

Figure 1.4.2.1 Structure of cyanogenic glycosides viacinin and lucumin 7

Figure 1.4.2.2 The hydrolytic release of HCN from cyanogenic glycosides 8

Figure 1.5.1 The metabolic disposal of inorganic cyanide 9

Figure 3.1.1 Structures of cyano-β-glucosides: linamarin and lotaustralin 22

Figure 3.1.2 Genetics of cyanogenesis 23

Figure 3.2.2.1 The effect of maceration duration on cyanide release from 'aran' clover 26

Figure 3.2.3.1 Linamarin hydrolysis by linamarase at pH 3.0 for varying temperatures 28

Figure 3.2.3.2 Linamarin hydrolysis by linamarase at pH 4.0 for varying temperatures 28

Figure 3.2.3.3 Linamarin hydrolysis by linamarase at pH 5.0 for varying temperatures 29

Figure 3.2.3.4 Linamarin hydrolysis by linamarase at pH 6.8 for varying temperatures 29

Figure 3.2.3.5 Linamarin hydrolysis by linamarase at pH 8.0 for varying temperatures 30

Figure 3.2.4.1 Time course hydrolysis of 'aran' clover 31

Figure 3.2.5.1 'Aran' incubated with surplus linamarin for 24 hours 32

Figure 3.2.6.1 'Aran' (pH 6.8) incubated with and without linamarase 33

Figure 3.2.6.2 'Aran' (pH 6.8) incubated with and without commercial emulsin 34

Figure 3.2.6.3 'Aran' (pH 6.8) incubated with and without linamarase and emulsin 35

Figure 3.2.6.4 'Aran' (pH 6.8) incubated with and without mandelonitrile lyase 36

Figure 3.2.6.5 'Aran' (pH 6.8) incubated with and without additional enzymes 36

Figure 3.2.7.1 'Aran' leaves and stems at pH 6.8 with no added enzymes........37

Figure 4.2.2.1 Cyanide release from 'avium' cherries incubated with amygdalinat 25°C (pH 3.0-8.0)........42

Figure 4.2.2.2 Cyanide release from 'avium' cherries incubated with amygdalin at 30°C (pH 3.0-8.0)........43

Figure 4.2.2.3 Cyanide release from 'avium' cherries incubated with amygdalin at 37°C (pH 3.0-8.0)........44

Figure 4.2.2.4 Cyanide release from 'avium' cherries incubated with amygdalinat 42°C (pH 3.0-8.0)........44

Figure 5.1.1 Catabolism of amygdalin to HCN........48

Figure 5.2.1.1 Time course analysis of amygdalin hydrolysis by emulsin........51

Figure 5.2.3.1 Amygdalin hydrolysis by emulsin, almond and MNL enzymes (pH 5.0)........53

Figure 5.2.3.2 Amygdalin hydrolysis by emulsin, almond and MNL enzymes (pH 6.8)........54

Figure 5.2.4.1 Percent liberation of HCN from amygdalin and MN (pH 4.0)....55

Figure 5.2.4.2 Percent liberation of HCN from amygdalin and MN (pH 5.0)....56

Figure 5.2.4.3 Percent liberation of HCN from amygdalin and MN (pH 6.8)....56

Figure 5.2.5.1 Sweet almonds incubated with 30 mg of amygdalin (pH 5.0)....58

TABLES

Table 3.2.1.1 Effect of pH on cyanide liberation from macerated and intact 'aran' clover leaves ... 25

Table 3.2.2.1 The relationship between the maceration period and HCN release ... 26

Table 4.2.1.1 The effect of enzymes on HCN release from sour black cherries at varied acidities ... 41

Table 4.2.1.2 The enzymatic hydrolysis of amygdalin using sour black and sweet black cherry macerates (37°C) ... 41

Table 4.2.3.1 HCN liberation from amygdalin hydrolysed with almond and cherry β-glucosidase enzymes ... 46

Table 5.2.1.1 Percentage of HCN liberation from amygdalin using emulsin ... 51

Table 5.2.1.2 Percent liberation of HCN from amygdalin using emulsin and almond enzymes (37°C) ... 52

Table 5.2.5.1 HCN release from wild almonds ... 57

Table 5.2.6.1 Effect of peanut butter and sugar on amygdalin hydrolysis (pH 4.0) ... 59

Table 6.1.1.1 The LD_{50} and estimated LD_{90} of HCN for each possum ... 61

Table 6.1.2.1 HCN recovery rate using the microdiffusion technique (pH 4.0) ... 62

Table 6.1.2.2 Estimated cyanide release within each possum ... 63

Table 6.2.1.1 Almonds and amygdalin consumed in trial one ... 65

Table 6.2.2.1 Substrate and enzyme consumed in trial two ... 68

Table 6.2.3.1 Substrate and enzyme consumed in trial three ... 70

ABBREVIATIONS AND TERMINOLOGY

AH	Amygdalin Hydrolase
DOC	Department of Conservation
Dyspnoea	Difficulty in, or laboured, breathing
EU	Enzyme Unit (1 linamarase EU hydrolyses 1 μmol of linamarin per minute at 30°C in phosphate buffer)
Gavage	Introduction of fluid into the stomach by an oesophageal tube passed orally
HCN	Hydrogen Cyanide
HNL	Hydroxynitrile Lyase
ID	Inner Diameter
i/m	Intra muscular
KCN	Potassium Cyanide
LD	Lethal Dose
LD_{50}	Lethal Dose for 50 % of the Test Animals
LD_{90}	Lethal Dose for 90 % of the Test Animals
MAF	Ministry of Agriculture and Fisheries
MN	Mandelonitrile
MNL	Mandelonitrile Lyase
NaCN	Sodium Cyanide
ND	Not Determined
PH	Prunasin Hydrolase
SAPU	Small Animal Production Unit
Tachycardia	Excessively rapid action of the heart
TCA Cycle	Tricarboxylic Acid Cycle

CHAPTER ONE

INTRODUCTION

1.1 The Possum Peril

Trichosurus vulpecula, the common brushtail possum, is responsible for widespread destruction to New Zealand's indigenous and exotic forests, the spread of bovine tuberculosis, and the disappearance of food for many unique native snails, birds and insects. The harmful effects of the estimated 70 million possums that inhabit New Zealand are of wide concern. This concern, as to the effect that possums have on New Zealand's forests and economy, has prompted research into new methods for controlling possum populations.

In 1858 the first thirty possums were successfully introduced to New Zealand from Australia to start a fur trade industry. Unlike Australia, New Zealand has few natural possum predators or competitors for food and nesting sites, hence the escalation in possum numbers to approximately 70 million in the mid 1990's (Glasgow, 1990). It is now estimated that 92% of New Zealand's land area is colonised by the Australian brushtail possum (Cuddihy, 1993).

1.1.1 Deforestation

Browsing by uncontrolled possum populations is a particular problem in native bush and shrub land areas in New Zealand where these marsupials first deplete the area of the most preferred species followed by less palatable species. The possums' systematic stripping of one tree of a particular species before moving onto another of the same species does not allow the tree time to regenerate, thus leading to a progressively degraded forest (Seitzer, 1992). Possums generally target the softer new growth on trees. After an initial browsing, a healthy tree will produce replacement growth. This inevitably attracts possums back and again the tree is heavily browsed of new leaves. The less palatable older leaves which by now are near the end of their average two year life span die thus leaving the tree with no leaves. Therefore a two year period of persistent defoliation by possums will cause the tree to die (Edwards, 1990). This situation is evident in the dieback of native mistletoe (*Tupeia antarctica*, *Peraxilla tetrapetalla* and *Ileostylus micranthus*), fuchsia (*Fuchsia excorticata*), titoki (*Alectryon excelsus*), kamahi (*Weinmannia racemosa*), pohutukawa and northern rata (*Metrosideros tomentosa* and *robusta*) which are all edible plant species highly favoured among possums (personal communication with DOC, Palmerston North).

The damage caused by wild possums is not only restricted to native fauna, but also affects native wildlife, insects and introduced plants. Possums deprive birds of berries, nectar and insects, compete for nest sites in hollow trees, and on occasion they will devour eggs and young birds (Seitzer, 1992). By stripping tree foliage, possums considerably reduce the numbers of insects living in the canopy along with a vast amount of organisms inhabiting leaf litter on the forest floor. Commercial pine plantations, orchards, wind breaks and erosion control plantings also undergo heavy browsing. In pine plantations possums not only eat young shoots and catkins, but also cause damage to the tops of the trees as they jump from plant to plant, so browsing is not the only peril to the growing pines (Seitzer, 1992).

1.1.2 Bovine Tuberculosis

Aside from weather extremes New Zealand's farming industry has the added dilemma of the spread of bovine tuberculosis (TB) by the brushtail possum. Bovine TB is a bacterial disease carried by cattle, and to a lesser extent sheep, which is able to infect humans who consume contaminated meat or dairy products. Possums, with an immune system of low resistance, act as an ideal vector for this very easily spread disease. Marsupials acquiring TB suffer extensive lesions in their lungs, gut and lymph nodes. When infected possums graze on farmland these bacteria-filled lesions release bacteria onto pasture through respiration, droppings, urine and weeping wounds thereby providing the contact between possums and livestock (Kelly, 1990).

1.2 The Research Aim

The overall aim of this research is to develop a species-specific cyanogenic toxin for possum control which utilises naturally occurring cyanogenic glycosides or their catabolic enzymes within the possum diet. The specific scientific aim is to provide preliminary information as to whether cyanogenic plant material will release enough cyanide at the required rate to cause possums to die. This will be achieved by testing the cyanogenicity of selected plant materials (clover, almonds and cherries) *in vitro* and where possible *in vivo* in possums.

This research was planned with the view that future work in this area may lead to the development of a cyanogenic possum bait that is activated by an enzyme or substrate present in the possum diet therefore conferring species specificity. The bait should present greater protection for plant species heavily browsed by possums and therefore increase the chance of survival for the targeted vegetation.

1.3 Cyanogenic Plant Distribution

The ability to make cyanogenic glycosides which liberate hydrogen cyanide on hydrolysis is widespread among plants. At least 2050 species from 110 plant families are able to do this. Although the number of cyanogenic plant species is large, the number of known cyanogenic glycosides and lipids is relatively small, with only 27 having been identified so far, 23 of which are cyanogenic glycosides (Conn, 1980). The remaining few are cyanogenic lipids or pseudocyanogenic glycosides.

Due to the difficulty in isolating and characterising cyanogenic precursors in the field, very few species have had the structures of their cyanogenic glycosides determined. However, the simple qualitative picrate test has enabled many species having a cyanogenic potential to be identified. Plant families noted for being cyanogenic include the Rosaceae (150), Leguminosae (125), Gramineae (100), Araceae (50), Compositae (50), Euphorbiaceae (50) and Passifloraceae (30) (Gibbs, 1974). Because the cyanogenic material belonging to a genus or family usually consists of the same compound or group of compounds, discovery of new cyanogenic glycosides or lipids would be most likely to occur in unstudied families and genera.

Prunasin and amygdalin are two common cyanogenic glycosides generally associated with the Rosaceae family, while Dhurrin is the glycoside commonly associated with grasses (the gramineae). Linamarin and lotaustralin are present in numerous and unrelated families and coexist with their hydrolytic enzyme, linamarase (**Figure 1.3.1**).

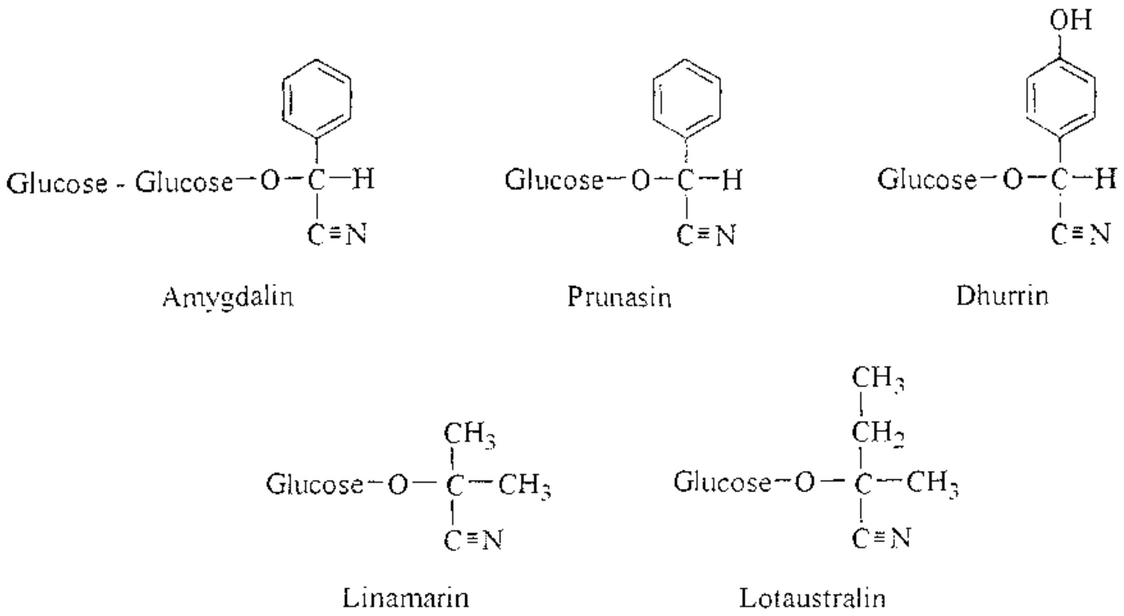


Figure 1.3.1 The Known Cyanogenic Glycosides
(adapted from Table 1., Eyjolfsson, 1970)

A combination of the linamarase and almond emulsin enzymes is capable of cleaving many different cyanogenic glycosides. This ability of a few enzymes to hydrolyse many different glycosides is thought to be an advantage in this research. It may mean that a variety of plant species in the possum diet should have the ability to release cyanide from a synthetic cyanogenic bait.

White clover (*Trifolium repens*), sweet almonds (*Prunus amygdalus*) and sweet black cherry (*Prunus avium*) were the three plant species examined in this research. They were chosen because they are species known to have cyanogenic properties.

1.4 Cyanogenesis

The process by which hydrogen cyanide (HCN) is released from plants containing cyanogenic glycosides or cyanogenic lipids is known as cyanogenesis. Under normal growth conditions the tissues of a cyanogenic plant do not contain detectable HCN and are therefore not toxic (Conn, 1979). The toxicity of the cyanogenic plant is directly attributable to the liberation of HCN which is effected by mild chemical hydrolysis, or by the action of catabolic enzymes present in the plant which react when the plant tissue is damaged. Damage resulting in HCN liberation may be as a result of maceration, wilting, frosting or stunting of the plant (DOC, 1997).

It has been suggested that the cyanogenic glycosides and the hydrolytic enzymes capable of liberating the cyanide are located in separate compartments, "*The lack of large scale hydrolysis of cyanogenic glycosides in cyanogenic plant tissue until that tissue is crushed or otherwise disrupted is usually attributed to physical separation of the glycoside from its catabolic enzymes,*" (Conn, 1980). Conn describes three models proposed to explain the lack of hydrolysis in intact plants. They include the occurrence of glycoside and catabolic enzyme:

- in separate cells or tissues
- in different subcellular compartments within the same cell
- within the same compartment but with inhibitors present to prevent cyanogenesis while the plant remains undisturbed.

All three models give valid explanations for the release of HCN only upon tissue disruption.

1.4.1 Chemical Hydrolysis

Summarised in **Figure 1.4.1.1** are the reactions of several chemical hydrolysing agents on cyanogenic plant materials where the RR^1C(CN)-O- moiety is known as an aglycone.

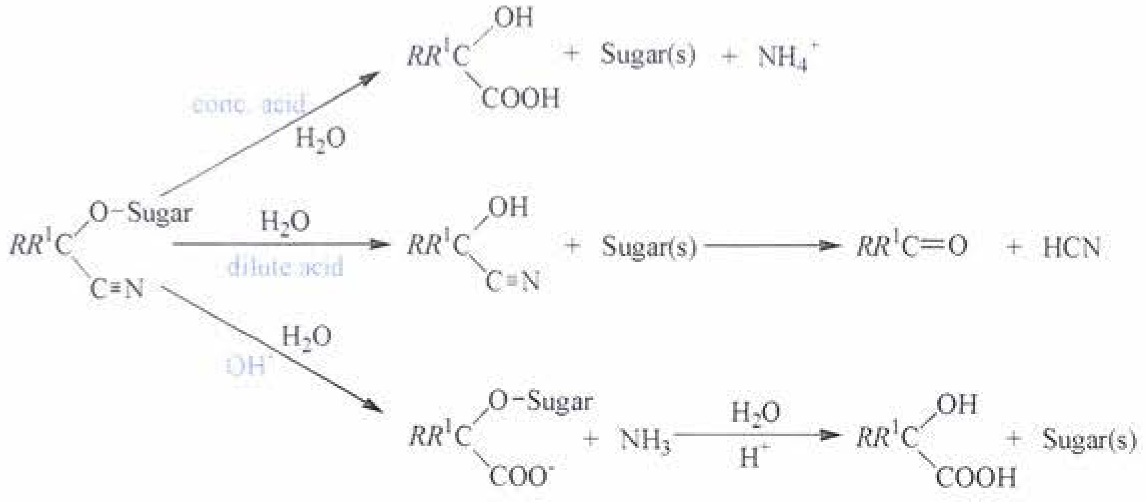


Figure 1.4.1.1 Chemical degradation of cyanogenic glycosides

(Eyjolfsson, 1970)

At elevated temperatures, dilute acid will cleave the β-glycosidic bond between the sugar and aglycone (Conn, 1978) (**Figure 1.4.1.2**). The released aglycone intermediate may then dissociate either spontaneously, or enzymatically. Non-enzymatic dissociation of the aglycone proceeds at a negligible rate below pH 5.5, but with increasing alkalinity it increases its rate of dissociation.

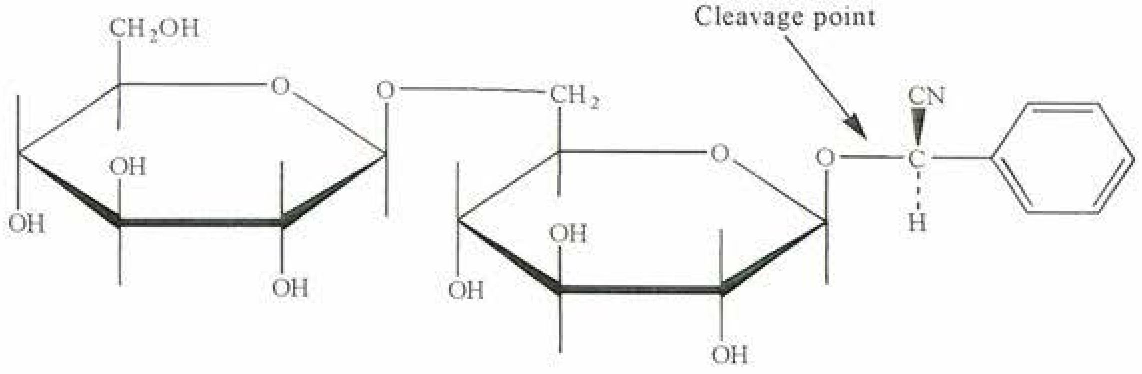
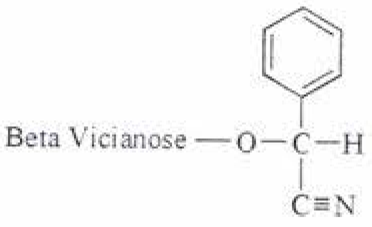


Figure 1.4.1.2 Cleavage point for the β-glycosidic bond of Amygdalin

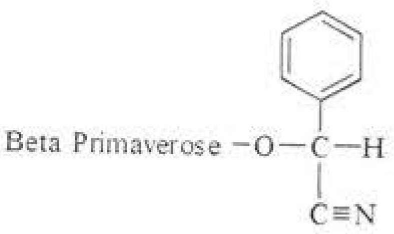
Concentrated acid hydrolyses the cyanogenic glycoside to its corresponding 2-hydroxy acid, sugar and ammonium ion. Like concentrated acid, mild alkaline conditions also result in the production of the corresponding 2-hydroxy acid, sugar and ammonia (**Figure 1.4.1.1**).

1.4.2 Enzymatic Hydrolysis

Cyanogenic glycosides generally undergo sequential hydrolysis to liberate cyanide. The initial enzymatic reaction in plants involves hydrolysis by β-glucosidases of the β-glycosidic bond joining the aglycone to the sugar group. The sugar moiety of most cyanogenic glycosides is a monosaccharide therefore only the β-glucosidase specific to that particular sugar-aglycone bond is required. In the case of amydalin (**Figure 1.3.1**), vicianin and lucumin (**Figure 1.4.2.1**) however, there is a disaccharide attached to the aglycone (Conn, 1980). These disaccharides require the action of two β-glucosidases to produce the intermediate aglycone. More than one type of cyanogenic glycoside may be present in any one species of cyanogenic plant therefore there is often a requirement for more than one type of β-glucosidase to be present within the plant.



Vicianin



Lucumin

Figure 1.4.2.1 Structure of cyanogenic glycosides vicianin and lucumin
(Conn, 1978)

Upon tissue injury in plants, vacuolar acids and cytoplasmic components mix generating a slightly acidic plant macerate. It is in these slightly acidic conditions (pH 4.0 to 6.2), that the β-glucosidases of most cyanogenic plants have optimal activity for the hydrolytic cleavage of the β-glucosidic bond (Poulton, 1990). The aglycone produced is however relatively stable under these pH conditions and therefore has a negligible rate of non-enzymatic decomposition.

Although the aglycones (α-hydroxynitriles or cyanohydrins) will dissociate non-enzymatically due to their instability (particularly at alkaline pH), the presence of plant hydroxynitrile lyases will catalyse the more rapid dissociation of the aglycone to HCN and its product ketone, or aldehyde (**Figure 1.4.2.2**).

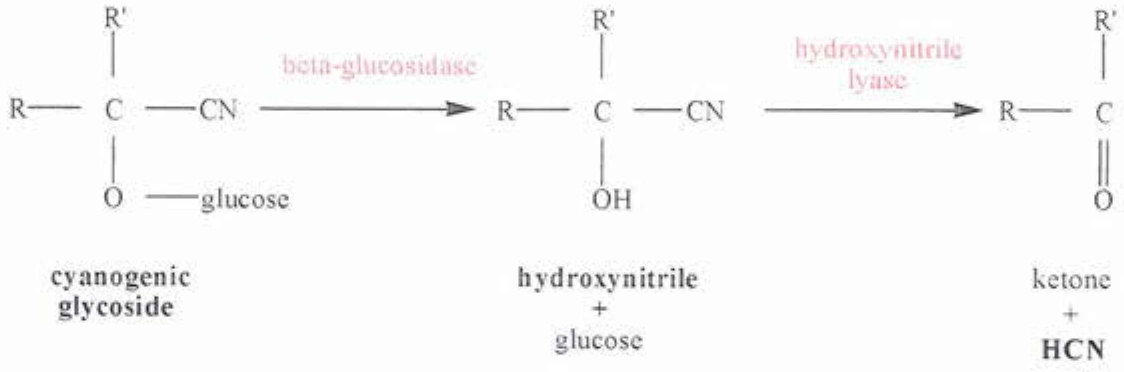


Figure 1.4.2.2 The hydrolytic release of HCN from cyanogenic glycosides

A study by Selmar *et al.* in 1989 used the hydroxynitrile lyase (HNL) of *Hevea brasiliensis* on the cyanogenic glycoside, linamarin, and the aglycone, mandelonitrile. The study showed that addition of HNL increased the rate of HCN liberation up to 20-fold therefore demonstrating the significance of HNL for rapid cyanogenesis. Its physiological importance is shown by the fact that only plants possessing high hydroxynitrile lyase activity are able to liberate HCN efficiently.

Because cyanide is not a cumulative poison (refer to section 1.5) it is the activity of the cyanide-liberating enzymes upon an appropriately toxic quantity of substrate within the cyanogenic plant that is the major determining factor in the toxicity of the plant.

1.5 Cyanide Toxicology and Detoxification

Hydrogen cyanide is a respiratory poison that has a capacity to form a reversible complex with the terminal oxidase of the mitochondrial electron transport pathway. Cyanide is not a cumulative poison and as such it is only toxic when the rate of absorption exceeds that of detoxification and elimination. The danger of cyanide exposure derives from its rapid absorption which overwhelms the natural defensive detoxification mechanisms of the body.

In cases of acute cyanide poisoning, the inhibition of cytochrome aa_3, the terminal oxidase, results in death. With a sub-lethal cyanide intake, HCN removal can be achieved by elimination or by detoxification (**Figure 1.5.1**).

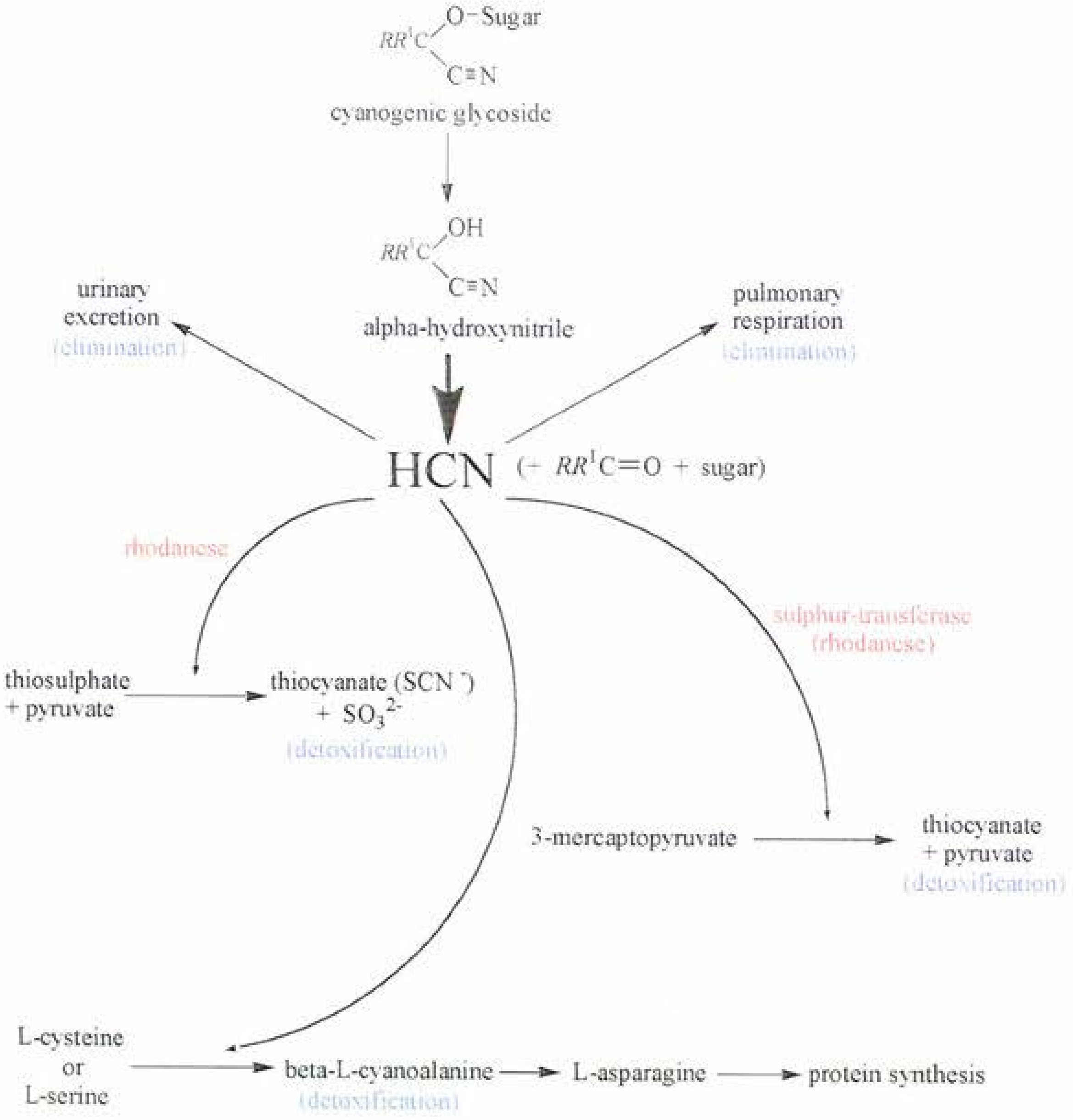


Figure 1.5.1 The metabolic disposal of inorganic cyanide

(adapted from Montgomery, 1969)

A small amount of cyanide can be eliminated by pulmonary respiration or by excretion in urine. Further removal of cyanide is achieved by its incorporation into vitamin B_{12} or cysteine, or by its oxidation to formate and carbon dioxide (Salkowski and Penney, 1994).

The body's principle metabolic pathway for detoxification is by reaction with thiosulphate to form thiocyanate (SCN) and sulphite, the reaction being catalysed by rhodanese (sulphur-transferase) (**Figure 1.5.1**). The SCN produced, although less toxic than cyanide, is still a harmful substance that inhibits the organic binding of iodine in tissues of the thyroid gland (Greer *et al*, 1966). The rhodanese enzyme is widespread in living tissues, reaching its highest concentrations in the liver, kidney, thyroid, adrenal, and pancreas. The thiocyanate produced is excreted in the urine. The presence of sulphur in the blood facilitates cyanide detoxification.

The dose of cyanide, along with the speed of ingestion, is of importance in ensuring that the amount of cyanide taken is fatal. To be most effective, the cyanide release from cyanogenic glycosides needs to occur between mastication of the plant material and its subsequent delivery to the stomach. This is because the acidity of the monogastric stomach is not favourable for the reaction of the HNL catalysing the formation of HCN (and aldehyde or ketone) from the intermediate aglycone. The pH of the duodenum is more favourable for the HNL's which have a slightly alkaline pH optimum (Conn,1979). Further factors affecting the toxicity of cyanogenic plant material to animals includes the age of the plant material (younger growth is generally more cyanogenic), degree of mastication, rate of digestion, size and type of animal and its ability to detoxify cyanide.

As with the present use of cyanide paste, this project aims to produce a product that would release a high dose of cyanide rapidly, to ensure a quick and humane death. The LD_{50} for hydrogen cyanide in possums is approximately 11 mg/kg. With the average possum weighing around 2.5 kg, approximately 28 mg of cyanide is required to kill 50 percent of the possums.

1.6 Signs of Cyanide Poisoning

The effect of cyanide poisoning in animals is dependent upon the dose taken. Literature on the sub-lethal consumption of cyanide in possums is rare. However studies on beagles have recorded a period of dyspnoea and tachycardia occurring in the animals before complete recovery (Salkowski and Penney, 1994). A recent publication by Gregory *et al*, (1998) describes the death process from lethal potassium cyanide (KCN) ingestion in possums. Signs that were noted in the trial included a short period (approximately one minute) of impaired balance and co-ordination about 2¼ minutes after cyanide consumption, followed by a phase where the possums were prostrate and experienced short periods of dyspnoea or hyperpnoea. Convulsions presented on average three minutes and 40 seconds into the experiment and lasted in general 19 seconds for 73% of the test animals. Limb movements and spasms were also observed. In the final and longest phase of cyanide poisoning the possums were relatively inactive in a prostrate position. Respiration ceased on average 14 minutes and 8 seconds after cyanide (19 mg/kg) consumption.

The salivation, nausea, vomiting and anxiety associated with low doses of cyanide poisoning in humans were not apparent in possums (Gregory *et al*, 1998).

1.7 Possum Control

For nearly five decades *Trichosurus vulpecula* have resisted sporadic pest control measures in New Zealand. A variety of toxins have been used alongside the conventional pest culling methods of shooting and trapping but to no avail as possum numbers are still escalating. An important point in the inability to bring the possum population under control is the lack of a suitably toxic and environmentally friendly species specific bait.

With the present possum toxins, the only realistic strategy for combating the possum onslaught in New Zealand is through sustained control. Irrespective of how successful an initial kill is, it must be followed up with maintenance control measures to prevent a new possum population from establishing itself. Eradication, although the most desirable and cost-effective option in the long term, is restricted to islands and peninsulas where reinvasion from neighbouring areas can be prevented. Successful eradication of *Trichosurus vulpecula* has been achieved for Kapiti island where the benefits to native vegetation and wildlife are now beginning to show (James, 1990).

Although all of the current culling methods are effective in reducing possum numbers, they all have the unfortunate disadvantage of inadvertently killing and maiming native wildlife, farm animals and pets. For the toxins this can be through non-targeted species directly ingesting poisoned bait or by secondary poisoning. The latter occurs when a non-target species consumes a poisoned insect or carcass.

With increasing public concern over the impact of pesticides on the environment there is a real need to develop more environmentally friendly and humane methods of pest control.

1.8 Present Control Methods

The Department of Conservation (DOC) presently use the acute poisons cholecalciferol, cyanide and sodium monofluoracetate (1080), and the anticoagulant brodifacoum in their fight against the possum invasion. These toxins are used along side non-chemical control measures such as shooting and trapping.

1.8.1 Cholecalciferol

Cholecalciferol (vitamin D3) is a naturally synthesised compound in animal skin and is also present in egg yolks, fish liver, fish oils and milk fat. Its action, after its conversion in the body to 25-hydroxycholecalciferol, is to mobilise calcium from bone into the bloodstream. Excessive cholecalciferol levels in the body cause hypercalcaemia and calcification of blood vessels. In possums this is believed to lead to heart failure within 4-7 days of a lethal dose being consumed. Signs of cholecalciferol poisoning in possums include loss of appetite, constipation and lethargy followed by death (Jolly *et al.* 1993, as referenced in DOC, 1997).

Although cholecalciferol is not designed as a species-specific bait it is distributed in specially made bait stations that limit accessibility by non-target species. Cinnamon flavouring and green colouring to deter birds further reduces the risk of non-targeted species consuming the bait. This bait does have a low risk of primary poisoning from loose bait and from secondary poisoning via consumption of poisoned carcasses (DOC, 1997).

1.8.2 Cyanide

Cyanide paste and 1080 are the possum poisons most commonly used in New Zealand. The ability of cyanide to kill quickly via its interference with the mitochondrial electron transport chain makes this form of pest control ideal for possum skin recoverers as the killed possums are found within a short distance of the bait station.

The conventional cyanide bait does have a high risk of killing non-target species, but careful placement can minimise accidental poisoning of livestock, wildlife and humans. Unfortunately cyanide paste is among the possum baits that can induce possum shyness if a sub-lethal dose is taken.

Feratox , a new cyanide pellet recently released on the market, has been designed to overcome the learned aversion problem. Between 85 and 90 mg of KCN is encapsulated in a hard pellet and is released rapidly into the mouth when the pellet is crushed upon chewing (Feratox, 1997). Possums are rendered unconscious within one minute from when the bait is crushed, with time to death only three minutes. Trials using this bait show it to have an approximate 90% kill rate. Further success in the use of this bait has been due to a specially designed self feeding bait station in which the pellets are housed, which restricts access to the bait to possums.

There is a minimal risk of secondary poisoning with cyanide bait due to its rapid degradation to less toxic substances which is an advantage that cyanide has over 1080.

1.8.3 Sodium Monofluoroacetate (1080*)*

Since 1955, 1080 has been used extensively as a pest control in New Zealand. It contains a synthetically made and chemically identical monofluoroacetate to that occurring naturally in plants such as gifblaar (*Dichapetalum cymosum*), rat weed (*Palicourea margravii*), ratsbane (*Dichapetalum toxicarium*) and some 40 other plant species in Australia (DOC, 1997).

The metabolic conversion of monofluoroacetate to fluorocitrate in animals results in the inhibition of the energy producing tricarboxylic acid cycle (TCA cycle). Specifically, fluorocitrate interferes with the conversion of citrate to isocitrate with a resultant build up of citrate in the body. These high citrate levels can inhibit the phosphofructokinase enzyme of the glycolytic pathway. As a consequence of the TCA cycle disruption, the animal suffers from energy deprivation prior to death. Signs of poisoning in possums first begin about 30 minutes after consumption and may include vomiting, cyanosis, tremors, drowsiness, staggering, respiratory and cardiac failure (DOC, 1997). Time to death is generally between 8 and 48 hours from time of ingestion. It therefore has a much longer kill time than that of cyanide.

Unlike cyanide paste, the bait is extremely safe to handle and at the spread rate of 5 kg a hectare, any poison entering the waterways would be sufficiently diluted so as not to harm humans or animals drinking it (51,000 litres would contain a fatal dose for a human). Sub-lethal doses tend not to result in learned aversion as cyanide does (Agricultural Pest Destruction Council, 1971). A major disadvantage of the bait is that it is not species specific.

1.8.4 Brodifacoum

Brodifacoum is an anticoagulant and as such disrupts the normal synthesis of vitamin K-dependent clotting factors in the liver. Poisoning commonly results in anaemia, weakness and haemorrhaging from the orifices. The effects of this bait may take several weeks to develop and time to death varies among possums according to the major haemorrhage site (DOC, 1997).

The potency and persistence of this second generation anticoagulant results in a high risk for primary and secondary poisoning in both targeted and non-targeted species.

1.8.5 Phosphorus

Phosphorus is no longer used by DOC, but regional councils use it in areas where 1080 is a risk to domestic animals. Phosphorus does not have a high risk of secondary poisoning as the toxin is not absorbed into muscle tissue like 1080. Death from phosphorus poisoning can occur by either cardiac failure or at a later stage by hepatic failure.

There are three phases in the action of phosphorus. The first is the acute gastrointestinal, abdominal and circulatory phase where vomiting, diarrhoea, shock, cyanosis and coma may occur. This is followed by a dormant phase whereby the possum stabilises or may even recover slightly. In the third and final stage liver failure appears. Time to death may vary from 1 day to several weeks (DOC, 1997).

1.8.6 Shooting

Shooting, other than the new Feratox bait, is at present the only other pest control method that is species specific. This form of control is, however, time consuming and restricted to easily accessible parts of the New Zealand native bush. Many areas are not accessible by road or foot, so this method can really only be effective in reducing possum numbers New Zealand-wide if used in conjunction with an aerial bait.

1.8.7 Trapping

Like shooting, trapping is restricted to those areas in New Zealand that are accessible by foot. Many trapping devices, in particular the Lane's Ace trap (more commonly known as a gin trap), are controversial because of the inhumane way in which the animal suffers and often mutilates itself. An animal caught in the gin trap is held until the hunter kills it which legally has to be within 24 hours from the time of capture. Kill traps in which the animal dies instantly, although more humane, are impractical to use in many areas because of their large size and weight (Allen, 1989).

1.8.8 Bio-dynamic Repellents

A bio-dynamic technique using burnt possum pelts and testes was tested as a form of possum repellent by Forest Research scientists. This trial was unsuccessful however, with the material having no effect on the test possums (Atkinson, 1991).

1.9 Future Control Methods

The main requirements of any new pest control are that it be effective, humane in use, safe for non-target species, environmentally friendly and safe to handle.

Biological control using contraceptive vaccines to cause sterility in female possums is a definite possibility for a species-specific control. This form of pest control is, however, reliant on natural mortality to reduce possum numbers, and as such will take a long time period to produce a noticeable decline in the population.

Research currently undertaken at Lincoln University, New Zealand, includes an immunocontraception method which will cause the female's immune system to attack the sperm. Reproductive physiologist, Dr Janine Duckworth, is working at reducing the reproductive rate of possums by injecting a vaccine into female possums that produces antibodies against one or more proteins in possum sperm. Preliminary studies have shown the vaccine to cause 80 per cent of the sample group of female possums to cease reproducing. Further research to find a way of spreading the biological control agent is presently being investigated. The present method of subcutaneous injection is not a practical method for spreading this or any form of similar pest control. Identification of a possum-specific pathogen or parasite would be the most effective mode of spreading a vaccine such as this (Gee, 1995).

The use of repellents to suppress possum browsing is another area in which there is current interest. Although this method does not set out to kill possums, the ability to repel possums from targeted areas would be of great benefit in forestry and related industries where possums cause a lot of seedling damage each year. Orchards, commercial and private, could also benefit from this sort of pest control. The ability to use repellents to shepherd possums to more accessible areas for shooting or trapping would also be of benefit. Research into the use of sulphur-rich formulations have had positive repellency results with rabbits and hares being repelled for up to two months and possums refusing to eat treated apples (Woolhouse and Morgan, 1995). Further studies include the use of biological fluids from carnivorous predators of herbivores.

Development of a cyanogenic bait that is activated by an enzyme or substrate common to the possum diet is another new pest control concept for the fight against possums. The ability to produce a bait that is non-toxic unless coupled with particular plant species would be a novel way to produce a species specific toxin. This preliminary study investigates whether such a toxin is feasible.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Reagents and Equipment

'Aran' clover plants (*Trifolium repens*) of the seed line C13336 were borrowed from Dr John Caradus, Grasslands Research Centre, AgReseach, Palmerston North, raw sweet almonds (*Prunus amygdalus*) were purchased from Binn Inn, Palmerston North and sweet black cherries (*Prunus avium*) were gifted from The Cherry Farm, Blenheim, New Zealand. Possums (*Trichosurus vulpecula*) were caught by local possum trapper Kate Littin and housed individually at and in cages provided by the Small Animal Production Unit (SAPU), Massey University.

Sigma Chemical Co., St Louis, MO, USA supplied linamarin, *D*-amygdalin, *D*-mandelonitrile *β*-*D*-glucoside (Prunasin), and mandelonitrile lyase. Succinimide and *N*-chlorosuccinimide used in the colourimetric assay procedure of Lambert *et al.* (1975) were also obtained from the Sigma Chemical Co. Linamarase, potassium cyanide and barbituric acid were purchased from BDH Chemicals Ltd, Poole, England. Emulsin enzyme *β*-glucosidase was supplied by Worthington Biomedical Corporation, Lakewood, New Jersey, USA. Water was MilliQ grade.

Linamarase was made up in sodium phosphate buffer pH 6.8 to a capacity of 20 μmol HCN-liberating activity per 24 hours. . A 50 EU vial of linamarase was diluted to 1 EU/ml by addition of 50ml of sodium phosphate buffer (pH 6.8). 1 EU hydrolyses 1 μmol of linamarin per min at 30°C, phosphate buffer pH 6.0.

The anaesthetic medetomidine and reversal agent atipamazole were supplied by the Massey Veterinary Clinic for use in the possum trials. The anaesthetic carbon dioxide was of food grade and provided by SAPU.

Incubations were performed in a Julabo SW-20C shaking water bath. A PYE UNICAM PU8610 UV/VIS kinetics spectrophotometer and a Hitachi U-1100 Spectrophotometer were used for determining absorbances. A Waring blender was used for almond paste preparation.

2.2 Sample Homogenisation

Where possible fresh plant material was used for the samples. Aran clover leaves were harvested a few minutes prior to weighing and subsequent homogenisation. Raw almonds were purchased from a local bulk food market. Their time from harvesting was not able to be determined. Cherries were harvested from early December through to and including January. Fresh samples for analysis were packaged and couriered under refrigeration to the lab for analysis. Between experiments the cherries were kept under refrigeration.

Plant material was tested either intact, or ground to a fine powder in liquid air or liquid nitrogen with a pestle and mortar. Maceration of plant tissue was performed in a fume hood.

2.3 Extraction of Cyanide from Cyanogenic Plant Material

The procedure for cyanide concentration determination involves the diffusion of hydrogen cyanide into NaOH thereby forming NaCN. The Miller and Conn microdiffusion technique (1980) was the cyanide extraction method used for this project.

Either 0.5g or 1 g samples of macerated or intact plant material were placed in the outer well of chilled 50 ml Erlenmeyer flasks, each fitted with a centre well (ID 13 mm, depth 22 mm). 3 ml of sodium acetate buffer (pH 4.0 or 5.0), or 3 ml of sodium phosphate buffer (pH 6.8 or 8.0), were added to the outer well of each flask. The enzymes linamarase (14 or 30 μl), *β*-glucosidase (1.5 mg) and mandelonitrile lyase (5 μl), and substrates amygdalin (5 mg), linamarin (2.5 mg) and mandelonitrile (20 μl), were added either individually, in various combinations, or not at all to the outer chamber. 1 ml of 1M NaOH was placed in the centre well of each flask before being stoppered and incubated at 37°C in a shaking water bath for periods between 10 minutes and 48 hours.

This allowed hydrogen cyanide to diffuse into the centre well to produce NaCN. After incubation the NaCN was removed and diluted 1/10 in 0.1 M NaOH before further dilutions, if necessary, were made. This procedure was carried out in a fume hood. The cyanide concentration of the diluted samples was determined by a modification of the procedure of Lambert *et al.* (1975).

Controls contained 1 ml 1M NaOH in the centre well and 3 ml of buffer with and without addition of the enzymes or substrates to the outer well. Controls were performed for each buffer pH.

The effectiveness of this procedure for trapping liberated cyanide was tested with two separate cyanogenic substrates and enzymes. The hydrolysis of a known quantity of linamarin by a known quantity linamarase was performed and the percent recovery calculated. This experiment was repeated using amygdalin and emulsin β-glucosidase enzymes. A paper by Torres *et al.* (1988) obtained a recovery rate of 90 percent from this method when the cyanogenic glycoside linamarin and the linamarase enzymes were analysed.

2.4 Quantitative Determination of Cyanide

As per the procedure of Lambert *et al.* (1975), 0.5 ml of 1 M acetic acid was added to 1 ml of suitably diluted sample solution followed by 5 ml of a solution containing succinimide (2.5 g), *N*-chlorosuccinimide (0.25 g) and water (1 L). A further 1 ml of a freshly made solution containing 6 g barbituric acid, 30 ml pyridine and 70 ml water was added to each test tube. The samples were then mixed vigorously before being left at room temperature for 20 minutes to allow colour development. Absorbance was determined at 580 nm against a reagent blank containing 1 ml water and the reagents.

A standard curve of cyanide (0-50 nmol KCN) was prepared daily following the same procedures and the cyanide content of each sample was measured against this. All assays were performed in duplicate or triplicate.

2.5 *In vivo* Possum Study

Cage traps were used to procure 4 possums (2 male and 2 female) for the trial from land near Massey University. The possums were caged individually under laboratory conditions at SAPU for the period up to and including the experiment. They were fed daily on a diet containing fresh fruit (apples, bananas, kiwifruit, oranges, raisins, tamirillos), bread and raw almonds during the period of adaptation to the experimental facilities. Water was available *ad libitum* throughout the entire study.

There were three trials; the first trial involved the voluntary consumption of a peanut butter, amygdalin and sugar paste, and natural almonds. For the first seven days in captivity the possums were fed a normal diet. On day eight all the possums were fed a small portion of a peanut butter (10 g), amygdalin (0.5 g) and sugar (6 g) paste along with small portions of their normal meals before they were observed and their actions recorded for a period of four hours. The normal diet resumed on the ninth day. On the tenth day, all of the possums were fed 80 grams of raw almonds (which contain *β*-glucosidase enzymes).

Trial one began the following day. Three of the possums were fed between 17 and 17.3 g of the peanut butter/sugar/amygdalin paste (containing either 1 or 1.3 grams amygdalin) along with 60 grams of raw almonds. The fourth possum, which was the control, was fed only with the paste containing amygdalin. All the possums were observed and their behaviour recorded for four hours post feeding. At the end of the observation period quarter of an orange was fed to each possum. No other food was made available to the possums on this day.

In both the second and third trials the possums were gavaged as insufficient substrate and enzymes were voluntarily ingested in the previous trail. Carbon dioxide and medetomidine (up to 360 µg/kg i/m) were the two anaesthetics used. Upon the jaw tone becoming sufficiently relaxed the possums were gavaged. Atepamazole (up to 1.44 mg/kg) was the anaesthetic reversal agent for medetomidine. Removal of carbon dioxide once the gavage procedure was complete enabled a normal level of consciousness to resume almost immediately.

Two possums were used for the second trial of which possum A was pre-fed 40 grams of almonds in the sixteen hour period prior to the gavage. This possum was orally dosed with 1 gram of amygdalin (dissolved in 15 ml of MilliQ water) followed immediately by 50 ml of a ground natural almond and water paste (37.8% almond dry weight). Possum B in this trial was pre-fed a small portion of its normal diet and gavaged with the amygdalin solution. No almonds were fed to possum B. Both possums were monitored closely for the first four hours and then checked regularly over the following 24 hours.

The third trial involved the gavaging of the three surviving possums with amygdalin (1 g/15 ml of water) and emulsin β-glucosidase enzymes (100 mg/1 ml water) which were dissolved in MilliQ water. Possum A was gavaged with 1500 mg of amygdalin (526 mg/kg) dissolved in water followed by 450 mg of emulsin dissolved in water. Possum B was gavaged with 1000 mg amygdalin (400 mg/kg) and 300 mg of emulsin and possum D was orally dosed with 1250 mg amygdalin (781 mg/kg) and 375 mg emulsin (Possum C had died in the first trial). The possums were observed and their actions recorded for the first four hours after the gavage procedure and then checked regularly for the next four days.

The normal diet was fed in between trials. Smaller meals were provided on the days prior to gavaging.

CHAPTER THREE

WHITE CLOVER (*Trifolium repens*)

3.1 Introduction

The polymorphic nature of white clover (*Trifolium repens*) is indicated by the presence of both cyanogenic and acyanogenic individuals within the same species. The white clover 'aran', which is studied in this research, is one of the most cyanogenic morphs of clover in New Zealand.

Cyanogenesis in white clover is determined by two independent genes designated *Ac* and *Li*. Alleles of the *Ac* gene are responsible for the production of the two most commonly encountered cyanogenic glycosides, linamarin and lotaustralin (**Figure 3.1.1**). These glycosides are always present in the same plant, although their respective ratios may differ.

Linamarin

Lotaustralin

Figure 3.1.1 Structures of cyano-β-glucosides: linamarin and lotaustralin
(Conn, 1979)

Linamarin and lotaustralin may be hydrolysed by the non-specific *β*-glucosidase, linamarase, the production of which is governed by a dominant allele of the gene *Li* (**Figure 3.1.2**). HCN, glucose and water are liberated from the hydrolysis of both these cyanogenic glycosides along with acetone (from linamarin), and methylethyl ketone (from lotaustralin).

Figure 3.1.2 Genetics of cyanogenesis

(Conn, 1979)

All cyanogenic clover plants contain *Li* and *Ac* genes, while acyanogenic plants may contain one or neither of the dominant genes *Li* and *Ac* (Paim and Dean, 1976). Individual plants may be classified into four distinct homozygous genotypes in regard to cyanogenic properties: (Corkill, 1940).

- Plants containing both the cyanogenic glucoside and the appropriate hydrolytic enzymes (*AcAcLiLi*).
- Plants which contain the glucoside but not the enzyme (*AcAclili*).
- Plants which contain the enzyme but not the glucoside (*acacLiLi*).
- Plants which contain neither the glucoside nor the appropriate enzyme (*acaclili*).

Heterozygous genotypes have intermediate levels of cyanogenic glycosides and enzymes in relation to the homozygous individuals (Conn, 1979).

There is considerable variation in the expression of cyanogenesis in white clover. A single white clover plant at any one time may contain both cyanogenic and acyanogenic leaves. Furthermore, Till (1987), showed that the phenotype of a single white clover plant is not homogeneous. External factors including temperature and browsing by herbivores may influence the proportion of cyanogenic to acyanogenic leaves at any one time. An example of this is the stimulation in synthesis of cyanogenic plant material when a plant undergoes heavy browsing.

A 1992 paper by Till-Bouttraud and Gouyon discusses an optimum frequency theory for the production of cyanogenic leaves in an individual plant. The theory is based on the cost to the plant (in terms of growth) in producing cyanogenic glycosides versus the cost to the plant from destruction by herbivores. Variation in the production of cyanogenic plant material within a species may at times be related to the population of plants of the same species in an area. With white clover, an individual (or genet) in a field is indistinguishable to a herbivore therefore a cyanogenic genet within the species is able to confer herbivory protection for the acyanogenic genets of that same species. As a result of this protection, a higher proportion of plants within the field may be the less energy consuming acyanogenic variety.

To date there is no experimental procedure in which cyanogenic glycosides can be measured directly. The conversion of cyanogenic glycosides to the measurable HCN however allows a means for calculating cyanogenic glycoside concentration in plant tissues.

In plant tissue it is believed that the substrate and enzyme are found in separate locations (Conn, 1980; Poulton, 1990), this geographical separation allowing the plant to grow without unnecessary production and hydrolysis of the energy consuming cyanogenic glycosides. Maceration of the plant tissue therefore provides a means for exposing the catabolic enzymes to the cyanogenic substrate.

Determination of HCN release from cyanogenic plants requires the complete exposure of substrate and enzymes to each other with subsequent liberation of HCN. The HCN is trapped and an effective quantitative procedure is performed to detect the level of HCN liberated. There is considerable scope for error in this procedure as a result of the multiple manual steps in the liberation and subsequent measurement of the HCN. For this reason individual experiments were performed in triplicate and where the triplicates varied more than 10% of the mean value the analysis was repeated.

3.2 Results and Discussion

3.2.1 Cyanide Release from Macerated and Intact Clover Tissue

A preliminary analysis to determine the extent of HCN liberation from macerated versus intact clover at various acidity levels identified an appropriate pH to use for the 'duration of maceration' experiments that followed **(Table 3.2.1.1)**. A standard maceration period of 30 seconds was used for each of the macerated samples. All samples were incubated in a 37°C shaking water bath for 24 hours.

Buffer pH	Cyanide (μg) (Intact)	Cyanide (mg) (Macerated)
3.0	0.268	0.024
4.0	2.39	0.211
5.0	3.71	0.320
6.8	4.87	0.390
8.0	3.52	0.283

Table 3.2.1.1 Effect of pH on cyanide liberation for macerated and intact 'aran' clover leaves

0.5 g samples of either intact or macerated 'aran' leaf tissue were incubated with 3 ml of 0.1 M sodium acetate buffer (pH 3.0, 4.0, 5.0) or 0.1 M sodium phosphate buffer (pH 6.8 and 8.0) in the outer chamber of modified Erlenmeyer flasks. The centre wells contained 1 ml of 1 M NaOH. All samples were incubated in a 37°C shaking water bath for 24 hours before undergoing the quantitative cyanide analysis procedure of Lambert *et al.* (1975).

As indicated in the above table, cyanide release was the greatest at pH 6.8 for the 30 second macerated 'aran' samples.

3.2.2 Variation of Maceration Period

Maceration tests were performed to minimise any error related to the incomplete hydrolysis of the substrate due to inadequate exposure to all the catalytic enzymes. Analysis of a series of tests in which plant material was ground in liquid air for varying lengths of time before incubation, allowed the determination of the most effective duration of time to macerate plant tissue to maximise HCN release yet minimise possible enzyme denaturation **(Table 3.2.2.1, Figure 3.2.2.1)**. The pH optimum of 6.8 determined from the previous experiment was used. All the samples were incubated at 37°C for 24 hours.

Time (seconds)	Average Cyanide Liberated (mg)
0	0.004
5	0.010
10	0.167
15	0.328
20	0.376
30	0.360
45	0.289
60	0.232

Table 3.2.2.1 The relationship between the maceration period and HCN release

0.5 g plant tissue samples were macerated for between 10 and 60 seconds in liquid air before incubation with 3 ml of 0.1 M sodium phosphate buffer (pH 6.8) in the outer chambers of modified Erlenmeyer flasks. The centre wells contained 1 ml of 1 M NaOH. All samples were incubated in a 37°C shaking water bath for 24 hours before undergoing the quantitative cyanide analysis procedure of Lambert *et al.* (1975).

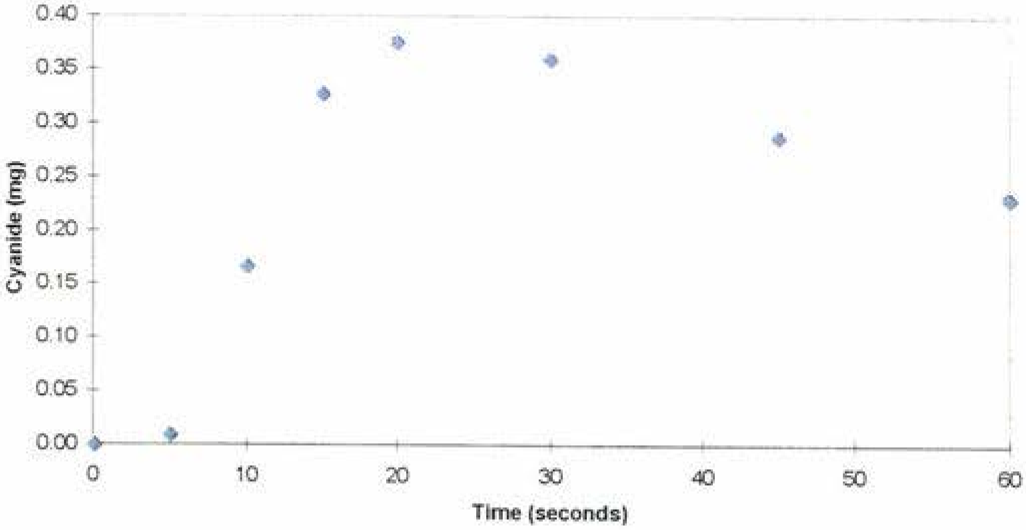


Figure 3.2.2.1 The Effect of Maceration Duration on Cyanide Release from 'Aran' Clover

0.5 g clover leaf samples were macerated for between 10 and 60 seconds in liquid air before incubation with 3 ml of 0.1 M sodium phosphate buffer (pH 6.8) in the outer chamber of modified Erlenmeyer flasks. The centre wells contained 1 ml of 1 M NaOH. All samples were incubated in a 37°C shaking water bath for 24 hours before undergoing the quantitative cyanide analysis procedure of Lambert *et al.* (1975).

It followed that with insufficient maceration of plant tissue, the substrate and enzyme interaction was not maximised leading to low cyanide release. Likewise, over-maceration also resulted in a decreased cyanide release, this was probably due to enzyme denaturation and/or loss of the cyanide gas to atmosphere (the use of liquid air during maceration halts the enzyme activity thereby retarding the ability of the cyanogenic glycosides to be hydrolysed at this temperature). As the data indicates, a maceration time of between 20 and 30 seconds gave the best results for cyanide release from 'aran' clover. In all subsequent analyses, clover was macerated for 20 seconds before incubation.

3.2.3 Testing the Microdiffusion Procedure

The efficiency of the microdiffusion procedure in liberating HCN was determined by the hydrolysis of a known quantity of substrate with a known quantity of enzyme. Pure samples of the substrate linamarin (2.5 mg) and catalytic enzyme linamarase (14 μl), were tested at pH 3.0, 4.0, 5.0, 6.8 and 8.0 for maximal cyanide release. The activity of linamarase (in relation to cyanide release) over a range of temperatures (25, 30, 37 and 42°C) was also assessed. Once the effectiveness of this procedure was determined, analysis of the clover plant tissue commenced.

Initial results showed that there was minimal cyanide release (up to 0.015 mg) in very acidic conditions (pH 3.0 and pH 4.0) over the entire temperature range (**Figures 3.2.3.1 and 3.2.3.2**). As the pH of the macerates tended towards neutral (pH 6.8, 37°C) the cyanide release increased to as high as 0.29 mg (**Figure 3.2.3.4**). Further increase in alkalinity to pH 8.0 resulted in a decline in the cyanide release to about 0.12 mg (**Figure 3.2.3.5**). Linamarase was the most active between pH 5.0 and 6.8 and at the possum body temperature of 37°C (**Figures 3.2.3.3 and 3.2.3.4**).

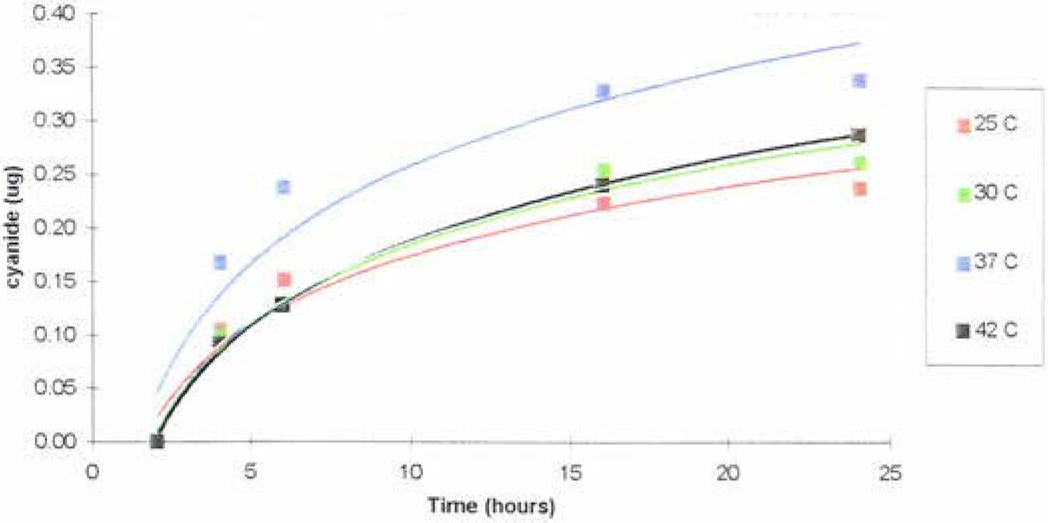


Figure 3.2.3.1 Linamarin hydrolysis by linamarase at pH 3.0 for varying temperatures

Samples containing 2.5 mg of linamarin (Sigma) and 14 µl of linamarase (BDH Chemicals) were incubated with 3 ml of 0.1 M sodium acetate buffer (pH 3.0) in the outer chamber of modified Erlenmeyer flasks. The centre wells contained 1 ml of 1 M NaOH. All samples were incubated for between 2 and 24 hours at either 25°C, 30°C, 37°C or 42°C before undergoing the quantitative cyanide analysis procedure of Lambert *et al.* (1975).

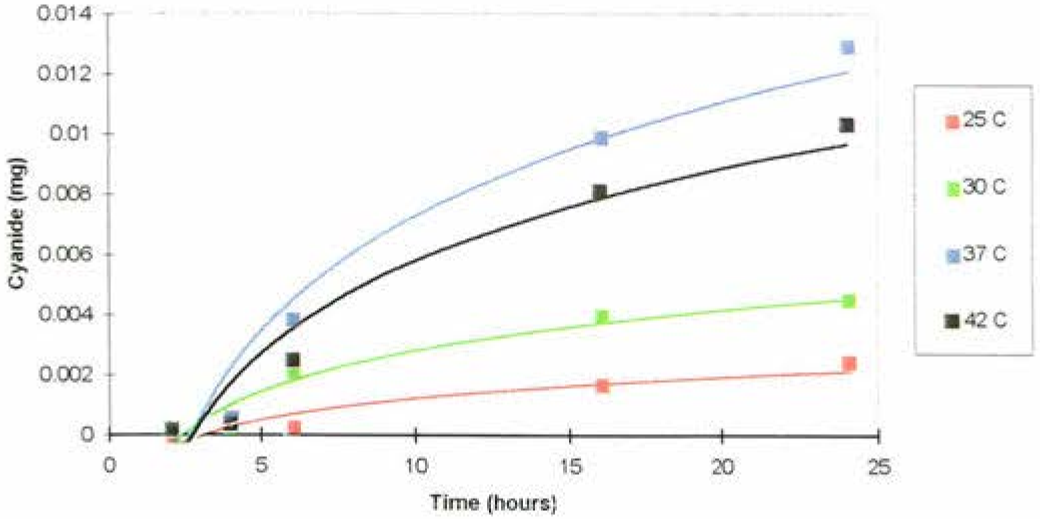


Figure 3.2.3.2 Linamarin hydrolysis by linamarase at pH 4.0 for varying temperatures

Samples containing 2.5 mg of linamarin (Sigma) and 14 µl of linamarase (BDH Chemicals) were incubated with 3 ml of 0.1 M sodium acetate buffer (pH 4.0) in the outer chamber of modified Erlenmeyer flasks. The centre wells contained 1 ml of 1 M NaOH. All samples were incubated for between 2 and 24 hours at either 25°C, 30°C, 37°C or 42°C before undergoing the quantitative cyanide analysis procedure of Lambert *et al.* (1975).

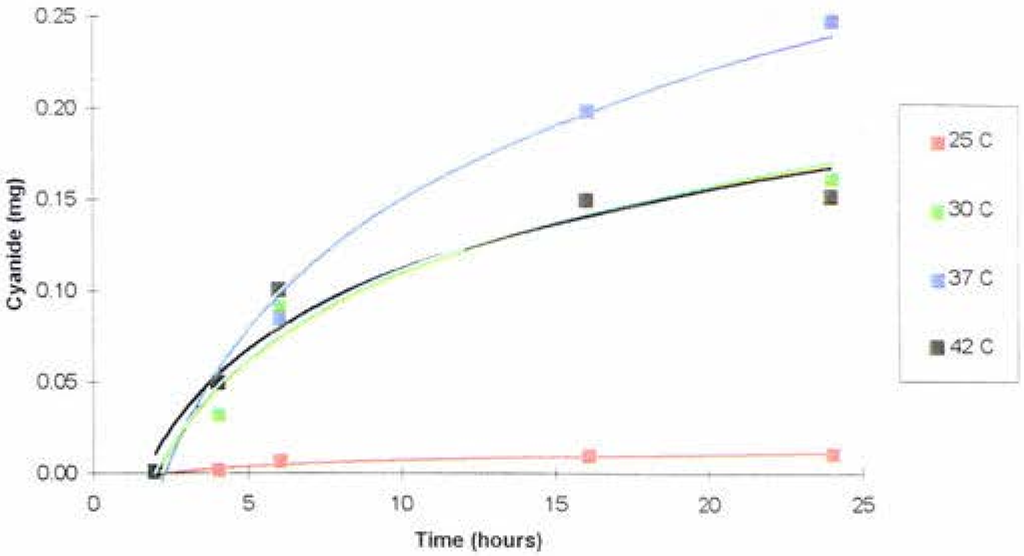


Figure 3.2.3.3 Llinamarin hydrolysis by linamarase at pH 5.0 for varying temperatures

Samples containing 2.5 mg of linamarin (Sigma) and 14 µl of linamarase (BDH Chemicals) were incubated with 3 ml of 0.1 M sodium acetate buffer (pH 5.0) in the outer chamber of modified Erlenmeyer flasks. The centre wells contained 1 ml of 1 M NaOH. All samples were incubated for between 2 and 24 hours at either 25°C, 30°C, 37°C or 42°C before undergoing the quantitative cyanide analysis procedure of Lambert *et al.* (1975).

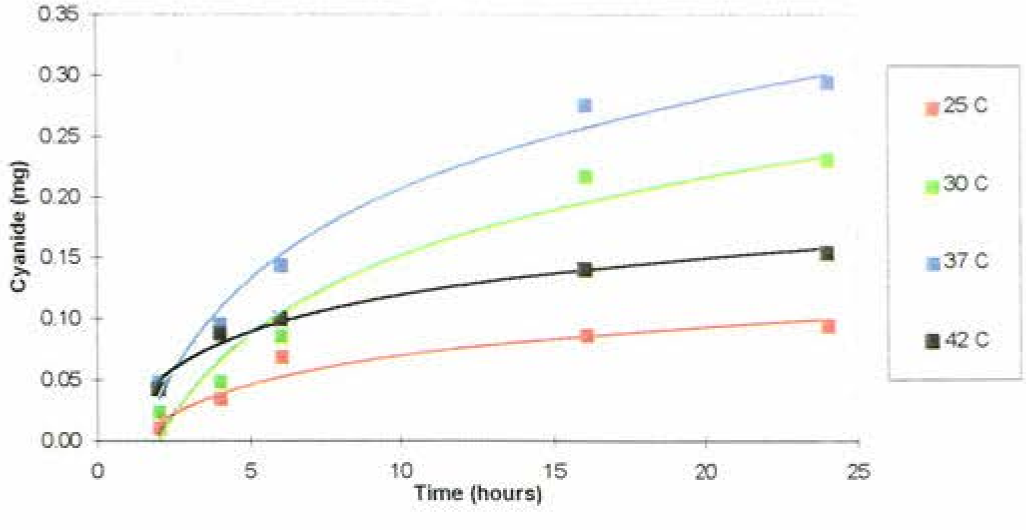


Figure 3.2.3.4 Linamarin hydrolysis by linamarase at pH 6.8 for varying temperatures

Samples containing 2.5 mg of linamarin (Sigma) and 14 µl of linamarase (BDH Chemicals) were incubated with 3 ml of 0.1 M sodium phosphate buffer (pH 6.8) in the outer chamber of modified Erlenmeyer flasks. The centre wells contained 1 ml of 1 M NaOH. All samples were incubated for between 2 and 24 hours at either 25°C, 30°C, 37°C or 42°C before undergoing the quantitative cyanide analysis procedure of Lambert *et al.* (1975).

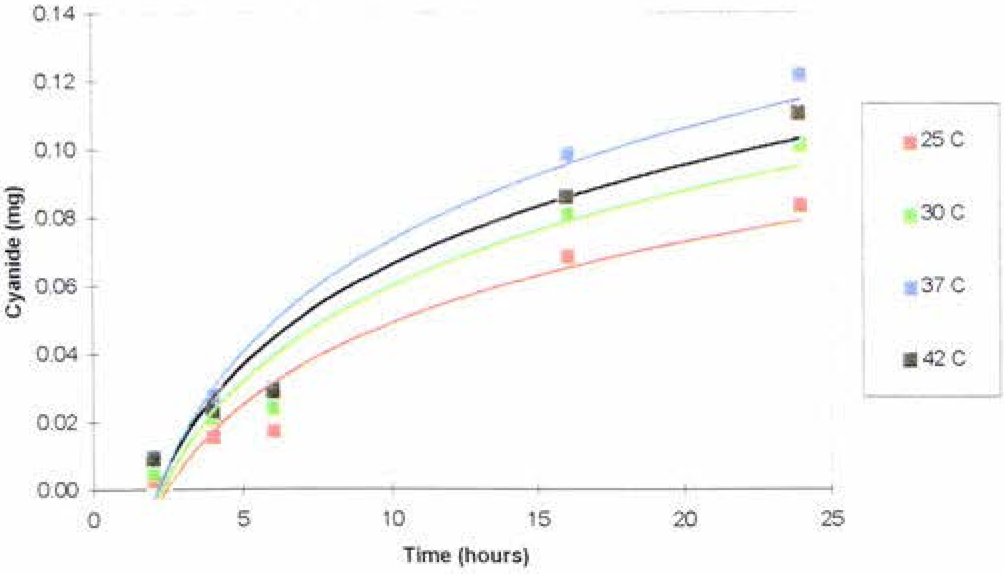


Figure 3.2.3.5 Linamarin hydrolysis by linamarase at pH 8.0 for varying temperatures

Samples containing 2.5 mg of linamarin (Sigma) and 14 μl of linamarase (BDH Chemicals) were incubated with 3 ml of 0.1 M sodium phosphate buffer (pH 8.0) in the outer chamber of modified Erlenmeyer flasks. The centre wells contained 1 ml of 1 M NaOH. All samples were incubated for between 2 and 24 hours at either 25°C, 30°C, 37°C or 42°C before undergoing the quantitative cyanide analysis procedure of Lambert *et al.* (1975).

Theoretically, 1 μmol of the cyanogenic glycoside should yield 1 μmol of HCN if the reaction goes to completion. Recoveries from the linamarin/linamarase standards however, showed that a maximum of only 37.8 % of the total cyanide theoretically available was recovered from linamarin over the 24 hour incubation at pH 6.8. These results were in contrast to results recorded in a research paper by Torres *et al.* (1988) in which a recovery rate of 90 per cent was achieved using the same substrate and enzymes. Further investigation of the microdiffusion technique analysing the release of HCN from the cyanogenic glycoside amygdalin and the catabolic enzyme emulsin, demonstrated that the recovery rates at pH 4.0 and 37°C were as high as 92% after only 6 hours incubation (**Table 4.2.1.1**). This result indicated that the low cyanide release with the linamarin and linamarase was not due to loss through the procedure, but probably due to the early expiry of some of the linamarase enzymes. Subsequent experiments in which linamarase was used contained 30 μl of the enzyme solution. The amygdalin/*β*-glucosidase results indicate that the microdiffusion technique is effective in liberating and trapping HCN.

3.2.4 Rate of Cyanide Liberation

The ability of possums to detoxify cyanide is a factor that needed to be taken into account in this research. The rate and time at which cyanide is released after maceration is of importance as the rate of liberation needs to be greater than the rate of detoxification in order for death to occur. A time course analysis for cyanide release from 'aran' clover at pH 6.8 and 37°C is shown below. Random pickings from eight clover genets were used in each of the six individual trials used for this experiment. The data at each time interval were then averaged. Cyanide was liberated the fastest in the first two hours of incubation. Over the next 22 hours cyanide was liberated at an increasingly slower rate until at about 24 hours incubation (1440 minutes) the cyanide level peaked and began to taper off (**Figure 3.2.4.1**). The decrease in the cyanide release after 24 hours may be as a result of a slight leakage from the Erlenmeyer flasks.

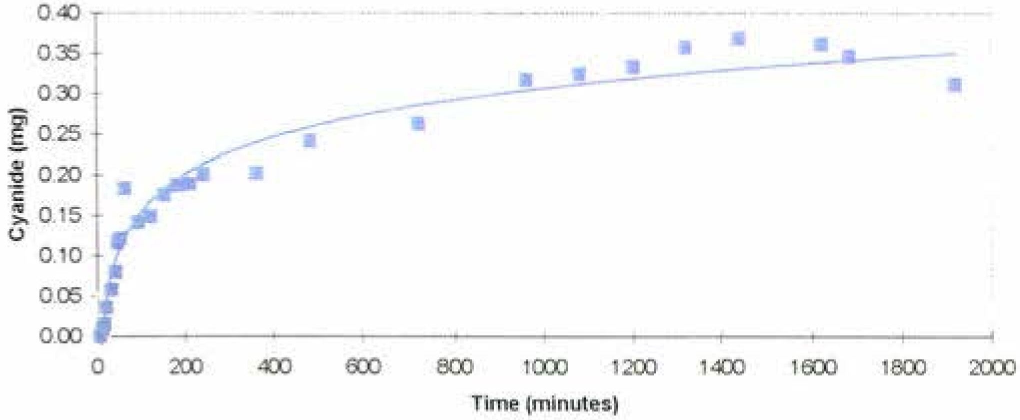


Figure 3.2.4.1 Time course hydrolysis of 'aran' clover
0.5 g macerated 'aran' leaves were incubated with 3 ml of pH 6.8 sodium phosphate buffer in the outer well of a modified Erlenmeyer flask without the addition of any enzymes or substrate. The centre wells contained 1 ml of 1 M NaOH. All samples were incubated at 37°C in a shaking water bath for between 0.5 to 32 hours before undergoing the quantitative cyanide analysis procedure of Lambert *et al.* (1975).

The maximal cyanide release from the clover with no extra enzymes or substrate added was 0.371 mg of cyanide per gram of plant tissue. At this level of cyanide liberation a 2.5 kg possum would have to consume about 120 grams of clover leaves within a period of about 30 minutes to even have a possibility of succumbing to death by cyanide poisoning (this would be highly unlikely since possums are sample feeders and would generally take a six or more hours to eat between 100 and 200 grams of plant material from many different plant species).

3.2.5 Effects of Added Substrate on Cyanide Liberation

Methods for increasing the level of cyanide release in 'aran' clover were investigated. Linamarin and lotaustralin are the most abundant cyanogenic glycosides in clover and are both hydrolysed to HCN by the catabolic enzyme linamarase. Whether 'aran' clover is saturated with substrate was unknown so a series of tests were made in which extra substrate (linamarin) was added to the clover macerates (**Figure 3.2.5.1**).

The results indicated that clover tissue incubated with additional linamarin released more cyanide than the samples with no extra substrate added. This could indicate that the clover's endogenous enzymes are not saturated with substrate. However, cyanogenic glycosides are known to spontaneously dissociate under certain conditions. For this reason controls containing solely the linamarin and buffer were run in parallel.

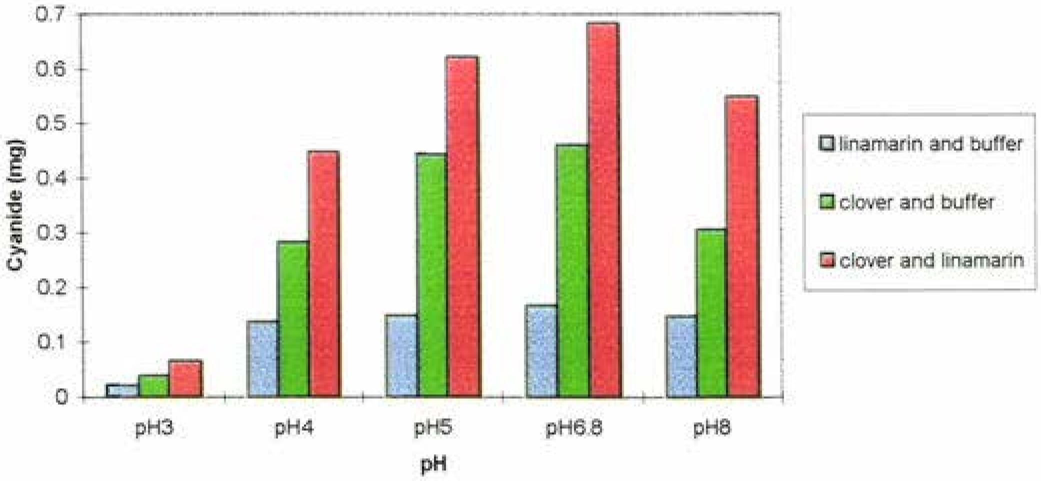


Figure 3.2.5.1 'Aran' incubated with surplus linamarin for 24 hours
0.5 g of macerated 'aran' clover leaves were incubated in 3 ml of pH 3.0, 4.0, and 5.0 sodium acetate buffer, and pH 6.8 and 8.0 sodium phosphate buffer with and without the addition of 2.5 mg linamarin. Controls containing 2.5 mg linamarin in 3 ml of each buffer were run in parallel. 1 ml of 0.1 M NaOH was added to the centre well of each flask. All samples were incubated in a 37°C shaking water bath for 24 hours before undergoing the quantitative cyanide analysis procedure of Lambert *et al.* (1975).

The results indicated that to some extent linamarin did spontaneously hydrolyse to HCN, therefore the increase in cyanide released when 'aran' and linamarin were incubated together is likely to be the effect of the spontaneous hydrolysis of linamarin and not surplus cyanide liberating enzymes within the plant tissues (**Figure 3.2.5.1**). Due to the spontaneous hydrolysis of the substrate these results were not conclusive in showing whether or not the plant enzymes were saturated with substrate.

3.2.6 Effects of Added Enzymes on Cyanide Liberation

Further experiments with 'aran' to determine if enzymes were present in excess included incubating the macerated clover at pH 6.8 (37°C) with the addition of either the enzyme linamarase (**Figure 3.2.6.1**), commercial emulsin (**Figure 3.2.6.2**), a mixture of emulsin and linamarase (**Figure 3.2.6.3**), or mandelonitrile lyase (**Figure 3.2.6.4**).

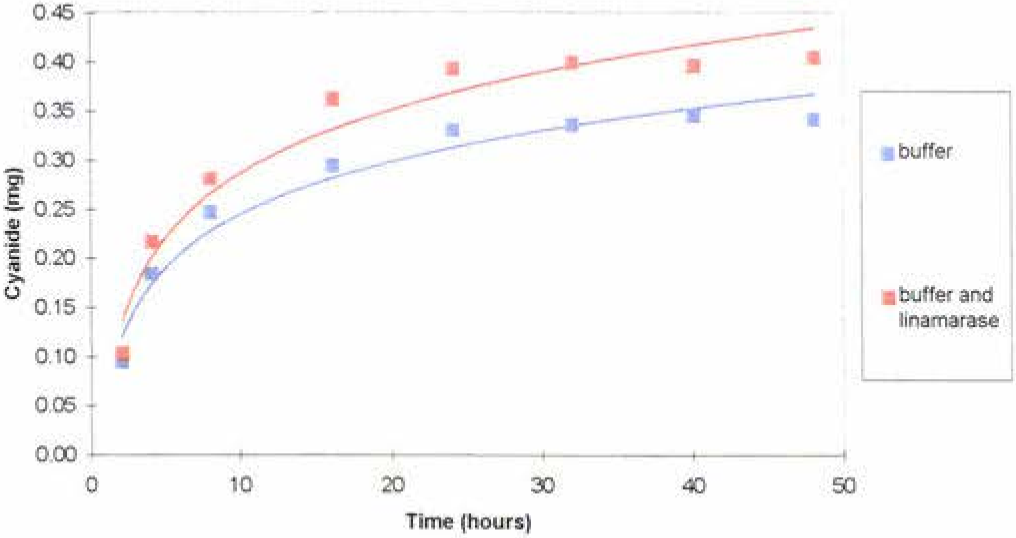


Figure 3.2.6.1 'Aran' (pH 6.8) with and without linamarase
0.5 g samples of macerated clover leaves were incubated in 3 ml of pH 6.8 sodium phosphate buffer with or without the addition of 14 μl linamarase (BDH Chemicals). 1 ml of 1 M NaOH was added to the centre well of each flask. All samples were incubated in a 37°C shaking water bath for between 2 to 48 hours before undergoing the quantitative cyanide analysis procedure of Lambert *et al.* (1975).

Addition of linamarase led to an 18 percent increase in cyanide liberation over the 24 hour period. This would suggest that the activity of the enzyme was limiting in the production of cyanide from endogenous substrate. As the previous data, in relation to cyanide liberation from linamarin using the linamarase enzyme, were lower than expected it was inconclusive whether this 18 percent increase in liberation with addition of the same linamarase indicated the total liberation of naturally available linamarin in the clover had occurred.

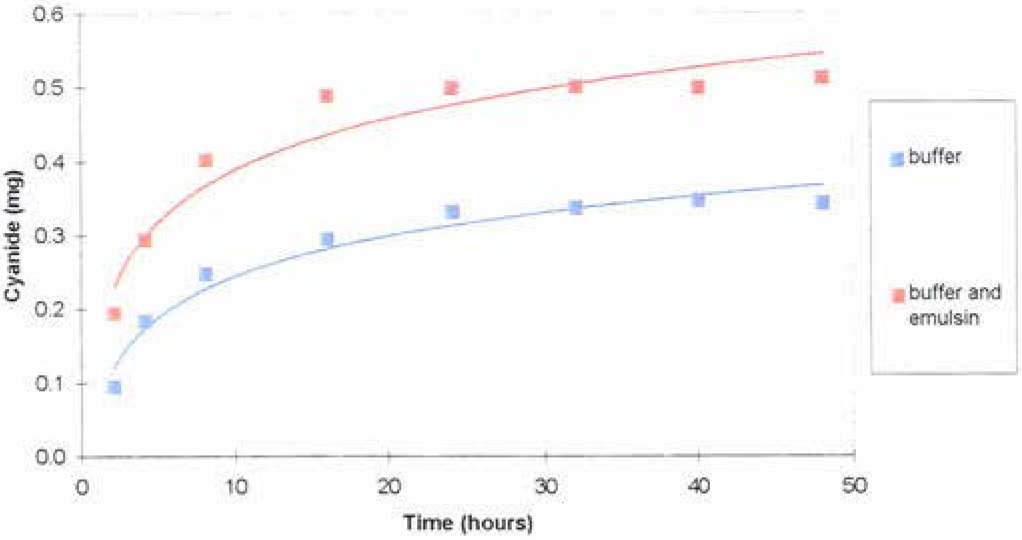


Figure 3.2.6.2 Aran (pH 6.8) with and without commercial emulsin
0.5 g samples of macerated clover leaves were incubated in 3 ml of pH 6.8 sodium phosphate buffer with or without the addition of 1.5 mg of commercial emulsin (Worthington). 1 ml of 1 M NaOH was added to the centre well of each flask. All samples were incubated in a 37°C shaking water bath for between 2 to 48 hours before undergoing the quantitative cyanide analysis procedure of Lambert *et al.* (1975).

Addition of commercial emulsin resulted in a 40 percent increase in the cyanide liberated. Commercial emulsin is a mix of several catabolic enzymes that will act on not only linamarin and lotaustralin but also most other cyanogenic glycosides. The increased HCN release may be as a result of cyanogenic glycosides other than linamarin and lotaustralin being present or it may be due to all the naturally occurring linamarin and lotaustralin in the plant being hydrolysed by the active emulsin enzymes.

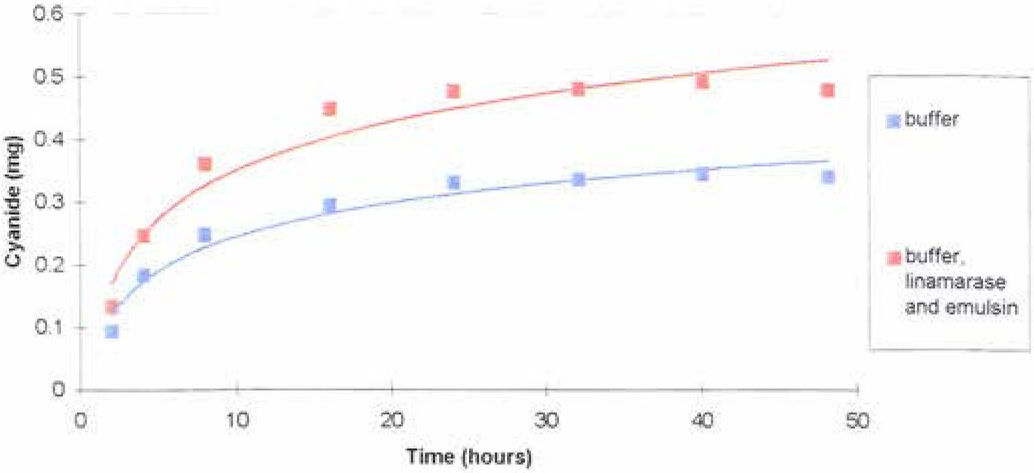


Figure 3.2.6.3 'Aran' (pH 6.8) with and without linamarase and emulsin
0.5 g samples of macerated clover leaves were incubated in 3 ml of pH 6.8 sodium phosphate buffer with or without the addition of 1.5 mg of commercial emulsin (Worthington) and 14 μl linamarase (BDH Chemicals). 1 ml of 1 M NaOH was added to the centre well of each flask. All samples were incubated in a 37°C shaking water bath for between 2 to 48 hours before undergoing the quantitative cyanide analysis procedure of Lambert *et al.* (1975).

The results for the experiments in which both linamarase and emulsin were added to 'aran' were very similar to those of the analysis of 'aran' incubated with extra emulsin. Both trials producing about a 44 percent increase in HCN release compared to the control clover incubations with no enzymes added. Again these results may indicate that the linamarase has hydrolysed all the available linamarin, and the emulsin has hydrolysed any other cyanogenic glycosides present, or that the linamarase enzyme is not totally active and that the emulsin is liberating all the available cyanogenic material including linamarin and lotaustralin.

Addition of mandelonitrile lyase, an enzyme that acts generally on the cyanogenic glycoside amygdalin or its intermediate mandelonitrile, gave only a slight 10 percent increase in the cyanide liberated. This suggests that linamarin and linamarase are not the only cyanogenic glycosides present and that there is a small amount of amygdalin in the 'aran' clover.

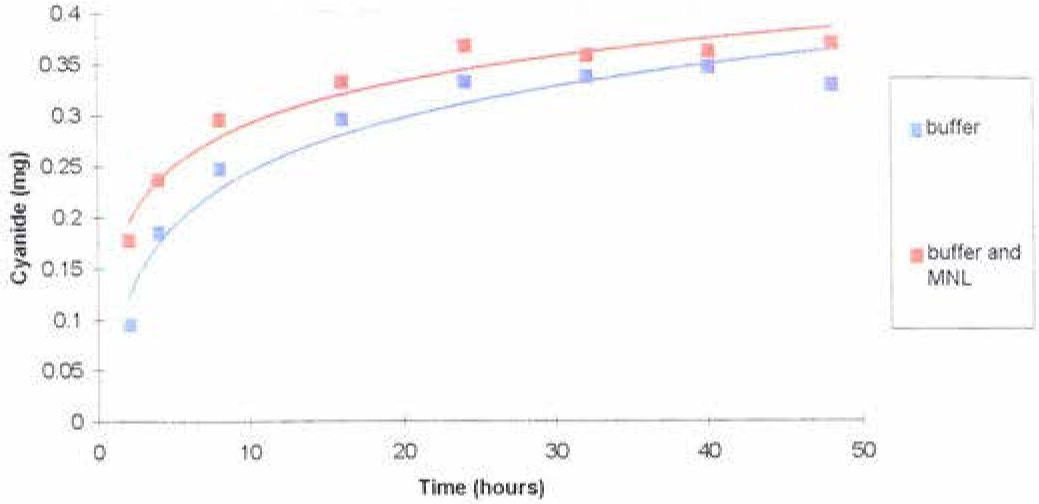


Figure 3.2.6.4 'Aran' (pH 6.8) with and without mandelonitrile lyase
0.5 g samples of macerated clover leaves were incubated in 3 ml of pH 6.8 sodium phosphate buffer with or without the addition of 5 µl mandelonitrile lyase (Sigma). 1 ml of 1 M NaOH was added to the centre well of each flask. All samples were incubated in a 37°C shaking water bath for between 2 to 48 hours before undergoing the quantitative cyanide analysis procedure of Lambert *et al.* (1975).

A summary of the action of each of the enzymes added to the clover is represented below (**Figure 3.2.6.5**). All the data indicates that there is an excess of substrate rather than cyanogenic liberating enzymes in the clover tissue as indicated by the increase in cyanide liberation on the addition of each of the catabolic enzymes.

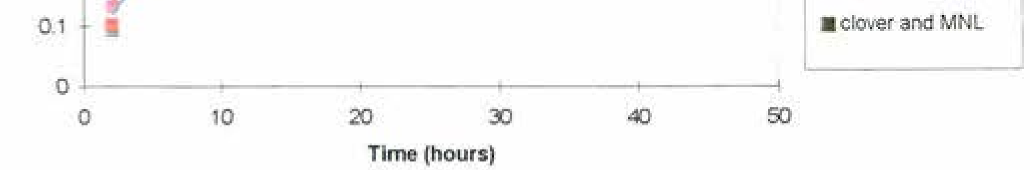


Figure 3.2.6.5 'Aran' (pH 6.8) with and without additional enzymes
0.5 g samples of macerated clover leaves were incubated in 3 ml of pH 6.8 sodium phosphate buffer with or without the addition of the following enzymes; 14 µl linamarase, 1.5mg commercial emulsin, 14 µl linamarase and 1.5 mg emulsin, or 5 µl mandelonitrile lyase. 1 ml of 1 M NaOH was added to the centre well of each flask. All samples were incubated in a 37°C shaking water bath for between 2 to 48 hours before undergoing the quantitative cyanide analysis procedure of Lambert *et al.* (1975).

The maximum cyanide release with the inclusion of added enzymes was 0.50 mg per gram of clover leaves, still requiring an average 2.5 kg possum to consume approximately 90 grams of the plant material within a relatively short time (30 minutes). The rate of cyanide liberation even with addition of the enzymes was not increased enough to counter the rate at which a possum would be able to detoxify the cyanide. For this reason analysis of cyanide liberation from clover was not investigated *in vivo*.

3.2.7 The Cyanogenicity of 'Aran' Stem Tissue

Cyanogenic plants are also known to release cyanide from other tissue. Plant stems are often cyanogenic along with roots (as in root vegetables, i.e. cassava). Present literature generally notes cyanogenic plant stems as being less toxic when damaged. Clover stems were tested for their cyanide liberating ability, but as the data shows (**Figure 3.2.7.1**), there was about one third less cyanide liberated from the stems than from the leaves.

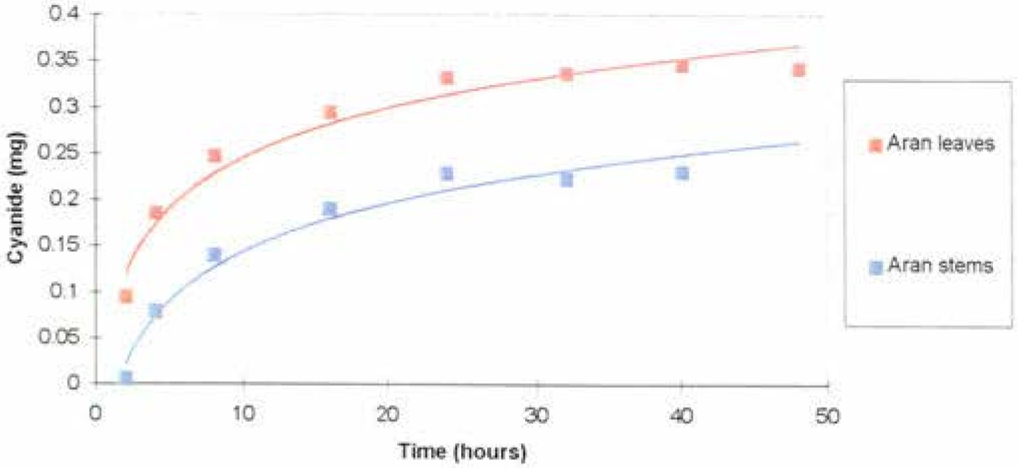


Figure 3.2.7.1 'Aran' leaves and stems at pH 6.8 with no added enzymes
0.5 g samples of macerated 'aran' leaf and stem tissue were incubated in 3 ml of pH 6.8 sodium phosphate buffer without the addition of any enzymes. 1 ml of 1 M NaOH was added to the centre well of each flask. All samples were incubated in a 37°C shaking water bath for between 2 to 48 hours before undergoing the quantitative cyanide analysis procedure of Lambert *et al.* (1975).

No further analyses of the cyanide liberating abilities of the clover stems were performed. As clover roots are not generally consumed by animals the roots were not analysed in this study.

3.3 Summary

The microdiffusion technique used to liberate and capture HCN from cyanogenic glycosides was initially analysed for its effectiveness using the substrate linamarin and catabolic enzyme linamarase. The maximum recovery of HCN from linamarin was very low (37.8% recovery at pH 6.8 and 37°C). Because this technique had been reported as being capable of recovering 90% of available cyanide (Torres *et al*, 1988) from linamarin, a further set of tests was performed in which a new combination of cyanogenic substrate and enzyme (amygdalin and emulsin) were analysed. These tests, performed at 37°C and pH 4.0, showed the microdiffusion technique to be very effective with 92% HCN liberation in six hours. From comparing this data with the initial linamarin/linamarase data it appeared that the low HCN liberation from linamarin must have been a result of low linamarase activity.

The results of the clover leaf maceration tests indicated that a 20 second period of maceration provided sufficient maceration of plant tissue to maximise enzyme/substrate exposure whilst avoiding possible denaturation of the enzymes through the crushing procedure.

The release of HCN from clover leaves compared to stem tissue showed the leaf tissue to liberate about one third more HCN than the stem samples. All further analyses of the clover were therefore performed on the leaves. The time course analyses indicated that cyanide was released the fastest in the first two hours of incubation and then slowly tapered off for the next 22 hours. After this the HCN levels in the flasks decreased, presumably due to loss through the seals. The maximum cyanide release of 0.371 mg per gram of plant tissue (for a 24 hour period) was too low to affect possums.

Addition of surplus substrate (linamarin) to the clover incubations had little effect on the cyanide released. However, the addition of extra enzymes, did increase the levels of HCN liberated. Emulsin had the largest affect on the HCN liberated (40% increase) followed by linamarase (18% increase) and then MNL (10% increase). Even with these increases there was still insufficient HCN released to be toxic to possums. Therefore further cyanogenic analyses of clover tissue were halted and analysis of black cherries (*Prunus avium*) commenced.

CHAPTER FOUR

BLACK CHERRIES (*Prunus avium*)

4.1 Introduction

Rosaceous stone fruits (e.g. almonds, apricots, cherries and peaches) contain the cyanogenic disaccharide amygdalin and its associated catabolic enzymes amygdalin hydrolase, prunasin hydrolase and mandelonitrile lyase within the seed (**Figure 5.1.1**). While the seeds and leaves of the *Prunus* species are generally highly cyanogenic, the fruits' pericarp are normally acyanogenic because they lack either the glycoside, the associated catabolic enzymes, or both.

Black cherry (*Prunus serotina* Ehrh.) is known to contain the cyanogenic glycoside amygdalin within its pericarp, however the catabolic enzymes are missing hence its acyanogenic nature (Swain *et al*, 1992a). Swain, Li and Poulton's (1992) research followed the changing concentrations of the cyanogenic glycosides prunasin and amygdalin, and the activities of the associated catabolic enzymes (AH, PH, and MNL) during black cherry fruit maturation. Their research showed that early in fruit development there was an accumulation of prunasin. At this stage the whole fruit was acyanogenic due to the lack of any appropriate catabolic enzymes. In the mid-growth phase the seeds produced both cyanogenic glycoside and enzymes. It was also at this stage that the pericarp produced increasing volumes of amygdalin with a concomitant decrease in prunasin content.

The sour black cherry (*Prunus serotina* Ehrh.) is not commonly available in New Zealand, however other varieties of sour black cherries are grown within New Zealand. The bitterness of the *Prunus serotina* variety is thought to be due to the presence of amygdalin therefore it would follow that other sour black cherries may also contain this same cyanogenic glycoside. On this assumption a cherry company in Blenheim, New Zealand was contacted and some sour black cherries (unknown variety) from trees that formed a shelter belt in the orchard were analysed. Fresh samples of edible sweet black cherries (*Prunus avium*) from the same company were also analysed for their cyanide producing ability.

It is believed that the bitterness of sour cherries contributes to the lack of predation of the immature fruit, but as the cherry matures its sugar content increases with the resultant sweetening of the cherry. It is at this stage that they become attractive to birds, other wildlife and humans.

This chapter reviews the results of incubating both the sweet 'avium' cherry and sour black cherry of unknown variety with a melange of cyanogenic enzymes and substrates to determine the fruits' maximal cyanogenicity and thus their potential as cofactors in a cyanogenic possum pesticide.

4.2 Results and Discussion

4.2.1 Cyanide Release from Sour Black Cherries

Initial analysis for cyanide release started with the sour cherries. Fresh samples of macerated cherry pericarp were incubated for 24 hours with addition of either emulsin, emulsin and MNL, linamarase enzymes or no added enzymes.

During the course of the analysis it was discovered that the bitterness of the sour black cherry was not related to the presence of amygdalin or other cyanogenic glycosides. Results for the 'no added enzymes' and 'extra linamarase' incubations as expected showed no significant difference in cyanide release (linamarase enzymes catalyse the dissociation of the cyanogenic glycosides linamarin and lotaustralin, not amygdalin). The data for the cherry samples incubated with emulsin, or emulsin and MNL, however showed a small increase in cyanide release (**Table 4.2.1.1**). Even in the presence of excess enzymes, all the cherry samples released only small amounts of cyanide (in the order of a few micrograms).

My theory that the bitterness of other sour black cherries may be related to the presence of cyanogenic glycosides was disproved by these incubations as commercial emulsin and linamarase enzymes cover a broad spectrum in their ability to hydrolyse cyanogenic glycosides.

	pH 4.0 (μg cyanide)	pH 5.0 (μg cyanide)	pH 6.8 (μg cyanide)
No added Enzymes	0.196	1.23	1.26
Linamarase	0.204	1.20	1.35
Emulsin	2.97	5.77	6.46
Emulsin and MNL	2.86	6.08	7.09

Table 4.2.1.1 The effect of enzymes on HCN release from sour black cherries at varied acidities.

1 g samples of macerated sour black cherries were incubated in the outer well of modified Erlenmeyer flasks with 3 ml of buffer. To this either no enzymes, linamarase (BDH Chemicals), commercial emulsin (Worthington), or emulsin and MNL (Sigma) enzymes were added. The above combinations were performed at pH 4.0 and 5.0 (0.1 M sodium acetate buffer), and pH 6.8 (0.1 M sodium phosphate buffer). To the inner well of each flask 1 ml of 1 M NaOH was added. All flasks were incubated at 37°C for 24 hours before undergoing the quantitative cyanide analysis procedure of Lambert *et al.* (1975).

Further incubations were performed in which the cyanogenic substrate amygdalin was added to both the sour black and sweet black cherries. This time both cherry types had positive results for cyanide release. Interestingly both the sweet and sour varieties released similar quantities of cyanide (**Table 4.2.1.2**) indicating that both types of cherry contained *β*-glucosidases capable of liberating cyanide from the cyanogenic substrate amygdalin. From here research continued only on the sweet varieties as these cherries were more readily accessible.

	Sour Black Cherry (μg cyanide)	Sweet Black Cherry (μg cyanide)
No substrate added	1.26	0.24
Amygdalin added	166	176

Table 4.2.1.2 The enzymatic hydrolysis of amygdalin using sour black and sweet black cherry macerates (37°C).

1 g samples of sour black cherries and 'avium' cherries were incubated in the outer well of modified Erlenmeyer flasks with 3 ml of pH 6.8, 0.1 M sodium phosphate buffer. To one set of each variety of cherries 5 mg of amygdalin (Sigma) was added. 1 ml of 1 M NaOH was placed in the inner well of each flask. All flasks were incubated for 24 hours in a 37°C shaking water bath before undergoing the quantitative cyanide analysis procedure of Lambert *et al.* (1975).

4.2.2 Cyanide Release from *Prunus avium* Cherries.

Sweet cherry macerates were incubated with 5 mg of amygdalin at varying temperatures to find the effect of temperature on enzyme activity and subsequent cyanide release.

The pH of the incubations ranged from 3.0 to 8.0. At 25°C there was very little cyanide release over the 24 hour period for most of the samples (about 4 μg), although at pH 5.0 the cherries displayed a significantly higher cyanide production (about 30 μg) (**Figure 4.2.2.1**).

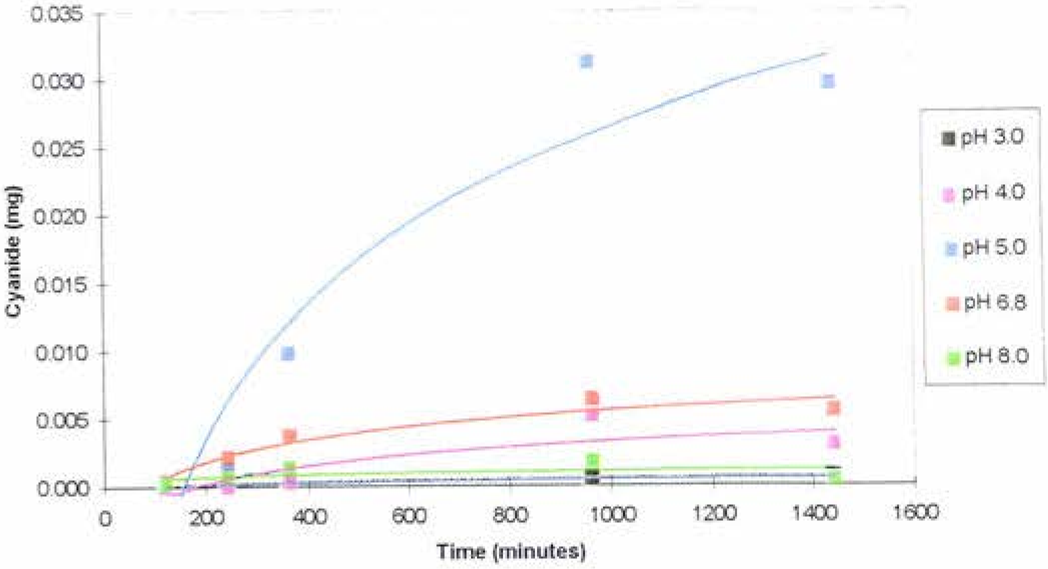


Figure 4.2.2.1 Cyanide release from 'avium'cherries incubated with amygdalin at 25°C (pH 3.0 - 8.0).

1 g samples of macerated 'avium' cherries were incubated in the outer well of modified Erlenmeyer flasks with 5 mg of amygdalin (Sigma) and 3 ml of either 0.1 M sodium acetate buffer (pH 3.0, 4.0, 5.0) or 0.1 M sodium phosphate buffer (pH 6.8 or 8.0). 1 ml of 1 M NaOH was placed in the inner well of each flask. All flasks were incubated for up to 24 hours in a 25°C shaking water bath before undergoing the quantitative cyanide analysis procedure of Lambert *et al.* (1975).

Increase in temperature to 30°C (approximate temperature of macerated food in the oral cavity) also resulted in low cyanide liberation for the pH 3.0, 4.0 and 8.0 samples although cyanide liberation was higher than the 25°C incubations. Both the pH 5.0 and 6.8 samples increased cyanide liberation by about 40%, although the pH 5.0 incubations were still significantly higher (**Figure 4.2.2.2**).

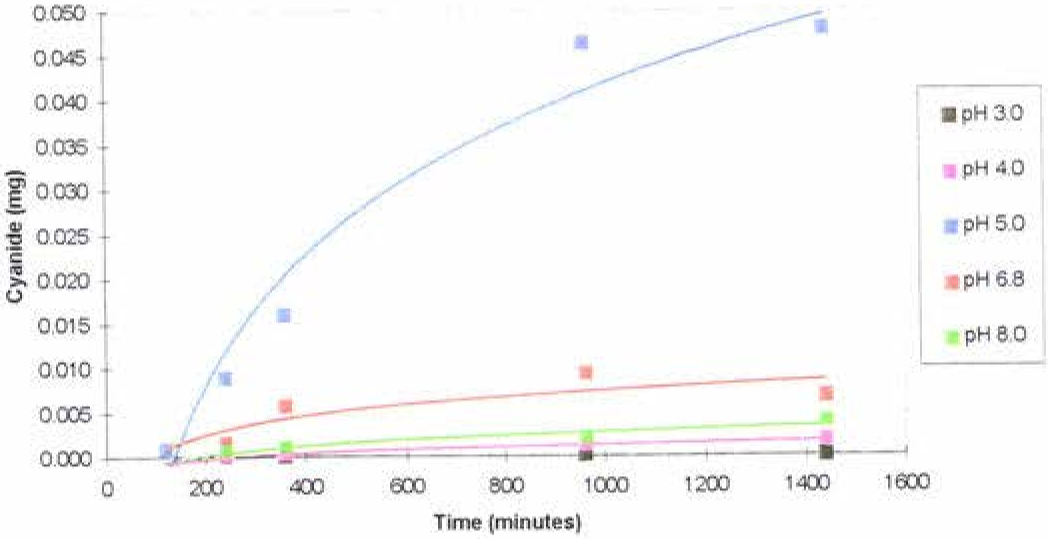


Figure 4.2.2.2 Cyanide release from 'avium' cherries incubated with amygdalin at 30° C (pH 3.0 - 8.0).

1 g samples of macerated 'avium' cherries were incubated in the outer well of modified Erlenmeyer flasks with 5 mg of amygdalin (Sigma) and 3 ml of either 0.1 M sodium acetate buffer (pH 3.0, 4.0, 5.0) or 0.1 M sodium phosphate buffer (pH 6.8 or 8.0). 1 ml of 1 M NaOH was placed in the inner well of each flask. All flasks were incubated for up to 24 hours in a 30°C shaking water bath before undergoing the quantitative cyanide analysis procedure of Lambert *et al.* (1975).

A further increase in incubation temperature to normal body temperature (37°C), doubled the cyanide release over 24 hours for the pH 5.0 samples to approximately 80 µg. The pH 6.8 samples also showed a huge increase in cyanide release from just 6 µg at 30°C to 50 µg at 37°C (**Figures 4.2.2.2 and 4.2.2.3**). Again the hydrolysis of amygdalin was relatively slow for the pH 3.0, 4.0 and 8.0 cherry macerates.

The highest incubation temperature was 42°C at which there was a significant decrease in the liberation of cyanide for the pH 5.0 cherry macerates from 80 µg over a 24 hour period to just 16 µg over the same time frame. Although the pH 6.8 samples also decreased in their ability to hydrolyse cyanogenic substrate, the drop was not as significant (50 µg to 18 µg) with the overall result that the pH 6.8 macerate had a slightly higher release of cyanide (**Figure 4.2.2.4**). The remaining pH samples released negligible amounts of cyanide.

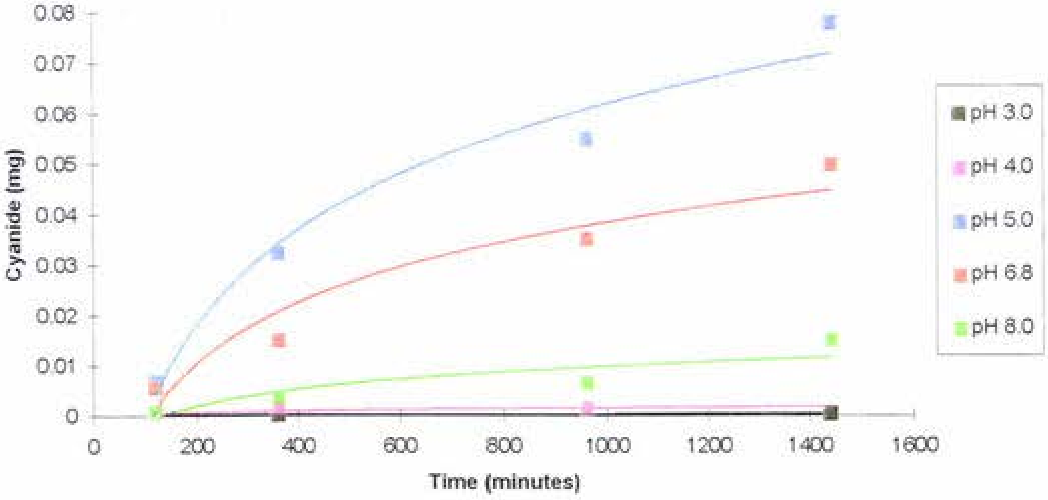


Figure 4.2.2.3 Cyanide release from 'avium' cherries incubated with amygdalin at 37°C (pH 3.0 - 8.0).

1 g samples of sweet black cherries were incubated in the outer well of modified Erlenmeyer flasks with 5 mg of amygdalin (Sigma) and 3 ml of either 0.1 M sodium acetate buffer (pH 3.0, 4.0, 5.0) or 0.1 M sodium phosphate buffer (pH 6.8 or 8.0). 1 ml of 1 M NaOH was placed in the inner well of each flask. All flasks were incubated for up to 24 hours in a 37°C shaking water bath before undergoing the quantitative cyanide analysis procedure of Lambert *et al.* (1975).

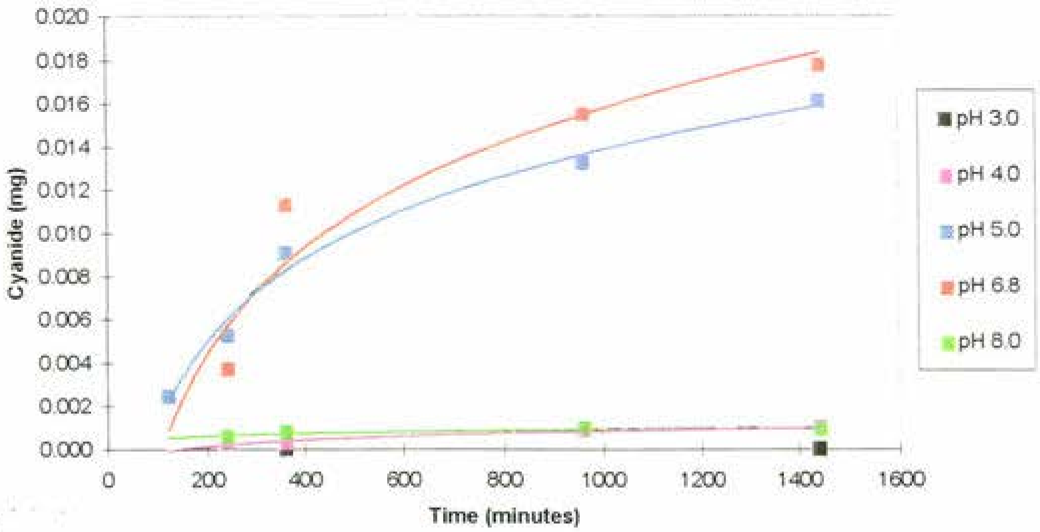


Figure 4.2.2.4 Cyanide release from 'avium' cherries incubated with amygdalin at 42°C (pH 3.0 - 8.0).

1 g samples of sweet black cherries were incubated in the outer well of modified Erlenmeyer flasks with 5.0 mg of amygdalin (Sigma) and 3 ml of either 0.1 M sodium acetate buffer (pH 3.0, 4.0, 5.0) or 0.1 M sodium phosphate buffer (pH 6.8 or 8.0). 1 ml of 1 M NaOH was placed in the inner well of each flask. All flasks were incubated for up to 24 hours in a 42°C shaking water bath before undergoing the quantitative cyanide analysis procedure of Lambert *et al.* (1975).

The general trend indicated that as the temperature of the incubations increased the pH at which the most cyanide was released changed (pH 5.0 macerates liberated more cyanide until 42°C at which time the pH 6.8 macerates hydrolysed more amygdalin). Whether these results related to the presence of two different β-glycosidases, (AH and PH) with different ideal activity conditions was not investigated. The results however do show that at the lower temperatures (25°C to 37°C) the catabolic enzymes appeared to function better at pH 5.0 (more cyanide release), and as the temperature increased to 42°C the pH 6.8 cherry macerates showed more cyanide release than the pH 5.0.

Although the pericarp of these sweet and sour cherries has been shown to contain enzymes capable of hydrolysing amygdalin, their cyanide liberating abilities are not fast enough to liberate the amount of cyanide required for use in possum control. The LD_{50} of HCN for a 2.5 kg possum is a straight dose of around 18 mg. The cherry macerates liberate only 2-3 percent of available cyanide per gram of cherry in two hours therefore an unreasonable quantity of substrate and cherry pericarp would need to be consumed for death to occur.

4.2.3 Comparison of Amygdalin Hydrolysis by Cherry and Almond Macerates

Table 4.2.3.1 shows figures for percentage liberation of cyanide from 5 mg of amygdalin using 1 g of either almond or cherry macerates. The data clearly indicates that amgdalin hydrolysis is more prevalent in the almond macerates.

Time (minutes)	Cherries pH 5.0 (% liberation)	Almonds pH 5.0 (% liberation)	Cherries pH 6.8 (% liberation)	Almonds pH 6.8 (% liberation)
120	3	55	2	56
360	12	84	5	88
960	20	93	13	98
1440	29	96	18	100

Table 4.2.3.1 HCN liberation from amygdalin hydrolysed with almond and cherry β-glucosidase enzymes.
1 g samples of sweet black cherries and almonds were incubated in the outer well of modified Erlenmeyer flasks with 5 mg of amygdalin (Sigma) and 3 ml of either 0.1 M sodium acetate buffer (pH 5.0) or 0.1 M sodium phosphate buffer (pH 6.8). 1 ml of 1 M NaOH was placed in the inner well of each flask. All flasks were incubated for up to 24 hours in a 37°C shaking water bath before undergoing the quantitative cyanide analysis procedure of Lambert *et al.* (1975).

4.3 Summary

The low HCN release (0.204 to 7.09 μg HCN) from incubation of sour cherry pericarps with a variety of cyanogenic enzymes showed that the fruit did not contain the high levels of cyanogenic glycosides that were expected. Substitution of sweet cherry pericarps for the sour cherry pericarps also resulted in a negligible HCN release.

Further analysis of both cherry varieties for enzyme content involved incubation of the pericarps with 5 mg of amygdalin for 24 hours at 37°C. These experiments resulted in a sizeable increase in the HCN liberated (176 μg HCN) compared to the cherry samples incubated with additional enzymes (7.09 μg HCN). As there was little difference in the HCN release between the sweet and the sour cherry varieties all further analyses were performed on the more accessible sweet 'avium' cherries.

The pH studies on the 'avium' cherries incubated with 5 mg of amygdalin showed that HCN liberation was the highest at pH 5.0 for all temperatures, with the exception of the 42°C incubations. Although at 42°C less HCN was liberated overall, the pH 6.8 cherries liberated more HCN than the pH 5.0 samples. Nevertheless the maximum cyanide liberated was still too low (about 80 μg/5 mg amygdalin) to be useful as a co-factor in a cyanogenic bait for possum control. All further analyses of amygdalin hydrolysis were therefore performed using natural almonds as the plant source of cyanogenic enzymes.

CHAPTER FIVE

ALMONDS (*Prunus amygdalus*)

5.1 Introduction

Bitter almonds are an abundant source of the cyanide-containing compound (*R*)-amygdalin, its catabolic *β*-glucosidases and mandelonitrile lyase. Upon tissue disruption, amygdalin undergoes sequential hydrolysis to release its two glucose sugars, firstly by the action of amygdalin hydrolase (AH) and secondly by the action of prunasin hydrolase (PH) to form the hydroxynitrile *D*-mandelonitrile (Swain *et al.*, 1992b). This intermediate hydroxynitrile is further catabolised by the plant's mandelonitrile lyase (MNL) enzymes to produce hydrogen cyanide (HCN) and benzaldehyde (**Figure 5.1.1**).

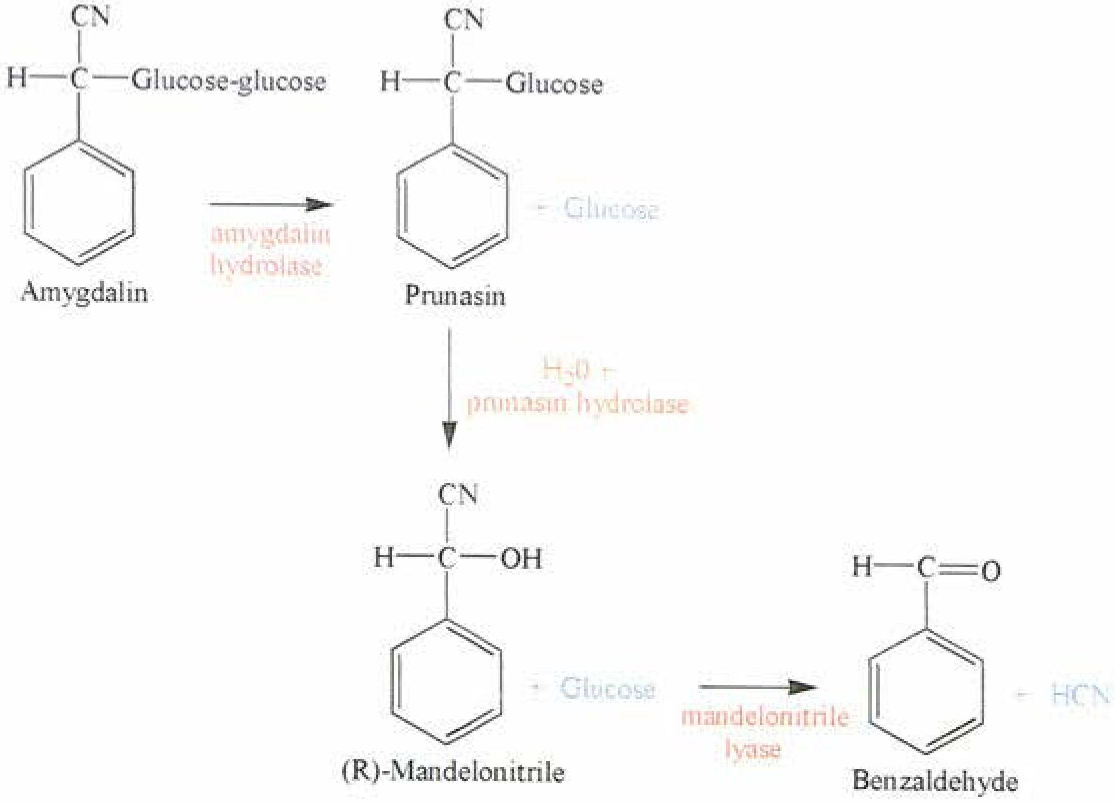


Figure 5.1.1 Catabolism of amygdalin to HCN

Since the overall aim of this research is to locate a source of a cyanogenic substrate separate from its catabolic enzyme, the use of bitter almonds which contain both substrate and enzyme would be inappropriate. However, the knowledge that bitter almonds are highly cyanogenic and that cyanogenic polymorphism occurs in several plant species, led to the analysis of sweet almonds for the presence of either the substrate or the enzymes.

Sweet almonds are commonly regarded as acyanogenic hence natural untreated almonds can be consumed by humans without any resultant ill effects. Yet research by Moertel *et al*, 1981, studying the pharmacological and toxicological effects of amygdalin in cancer patients, showed that patients treated orally with amygdalin and raw almonds exhibited escalated blood cyanide levels compared to those patients that received solely amygdalin. Further to this, clinical signs of mild cyanide poisoning were also observed in the patients treated with both amygdalin and sweet almonds. Patients treated exclusively with amygdalin displayed no typical signs of cyanide poisoning (Moertel *et al*, 1981).

An additional observation from the amygdalin cancer treatment trial was the "dramatic increase in blood cyanide level from 0.5 to 3.5 μg/ml" for a patient who doubled one 0.5 g oral amygdalin dose to make up for a previously missed dose. Intravenously administered amygdalin was found to be free of clinical toxicity and no cyanide could be detected in the blood. This result affirms the trial by Eason *et al.* (personal communication) for the release of cyanide from amygdalin by natural gut *β*-glucosidases. Knowing that stomach acid is concentrated and that concentrated acids hydrolyse cyanogenic glycosides to their corresponding 2-hydroxy acids, sugar and ammonium ions, it would be highly unlikely that chemical hydrolysis of the cyanogenic glycoside to form cyanide would have occurred (**Figure 1.4.1.1**).

From the findings of their trial, Moertel and co-workers (1981) suggested that patients treated with amygdalin should be "warned of the possible hazard of cyanide toxic reaction" by amygdalin overdose, and the probable hazard of simultaneous ingestion of foods containing *β*-glucosidases. Examples of these foods include raw nuts, particularly almonds, lettuce, mushrooms, and celery. Other cyanide containing food sources for humans include cassava, sweet potatoes, yams, maize, millet, bamboo, sugarcane, peas, beans, almond kernels, lemons, limes, apples, pears, cherries, apricots, prunes and plums (DOC, 1987).

The aim of analysing sweet almonds was to determine whether almonds have the potential to liberate lethal doses of cyanide to possums. Experimental analysis included incubating almond macerates with the substrates amygdalin and mandelonitrile over a pH range of 3.0 to 8.0 at 37°C (possum body temperature). Analysis of HCN liberation from amygdalin treated with either emulsin or MNL enzymes gave data for comparing HCN release from amygdalin using almond macerates.

5.2 Results and Discussion

5.2.1 Amygdalin Hydrolysis using Commercial Emulsin

An initial trial investigating the hydrolysis of amygdalin by emulsin enzymes was performed to determine the pH optima for amygdalin hydrolysis by *β*-glucosidase enzymes. These data would indicate both the maximum HCN release possible from the amygdalin-using *β*-glucosidase, and indirectly where in the possum's digestive tract the most HCN would be released. A series of analyses were completed in which 5 mg of amygdalin was incubated at 37°C with 1.5 mg of commercial emulsin. These tests were performed over the same range of pH's as used in the clover analysis.

The emulsin enzymes were active over a wide range of different pH values (pH 4.0 to pH 8.0), **(Figure 5.2.1.1)**. The level of cyanide liberation in acidic conditions (pH 3.0) was extremely low with just 0.015 mg (15 μg) being released over a 24 hour period. With a pH increase from just 3.0 to 4.0 there was a marked increase for the cyanide liberated in two hours from 2 percent for pH 3.0 to 59 percent for pH 4.0 incubations **(Table 5.2.1.1)**. As the pH of the incubations were increased up to pH 6.8, the liberation of cyanide likewise increased. The pH optima for emulsin to hydrolyse amygdalin was between pH 5.0 and 6.8, with as much as 0.25 mg of cyanide being produced in only two hours.

	% liberation of HCN	% liberation of HCN
Time (minutes)	**pH 3.0**	**pH 4.0**
10	0.5	7.5
60	1	32
120	2	59
240	3	78
360	3.5	92
600	5	93
1440	5.5	97
1920	5.5	95

Table 5.2.1.1 Percentage of HCN liberation from amygdalin using emulsin
5 mg samples of amygdalin (Sigma) were incubated in the outer well of modified erlenmeyer flasks with 1.5 mg emulsin (Worthington) dissolved in 3 ml of either pH 3.0 or 4.0, 0.1 M sodium acetate buffer. 1 ml of 1 M NaOH was added to the inner well of each flask. All samples were incubated in a 37°C shaking water bath for up to 32 hours before undergoing the quantitative cyanide analysis procedure of Lambert *et al.* (1975).

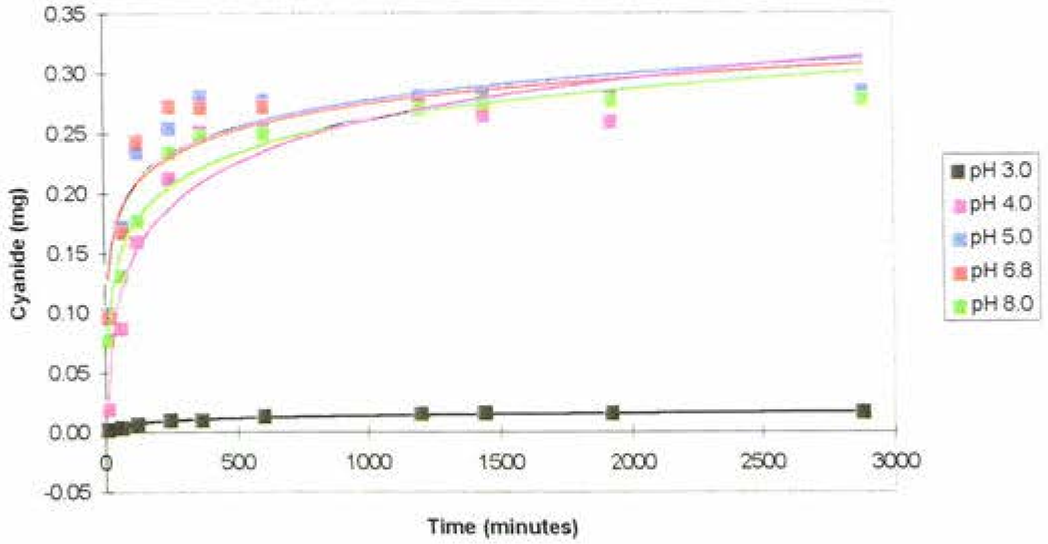


Figure 5.2.1.1 Time course analysis of amygdalin hydrolysis by emulsin
5 mg samples of amygdalin were incubated in the outer well of modified erlenmeyer flasks with 1.5 mg emulsin in 3 ml of either 0.1 M sodium acetate buffer (pH 3.0, 4.0 or 5.0), or 0.1 M sodium phosphate buffer (pH 6.8 or 8.0). The inner well of each flask contained 1 ml of 1 M NaOH. All samples were incubated in a 37°C shaking water bath for up to 48 hours before undergoing the quantitative cyanide analysis procedure of Lambert *et al.* (1975).

There was 100 percent cyanide liberation with the microdiffusion technique within four hours for the pH 6.8 incubations and six hours for the pH 5.0 incubations (**Table 5.2.1.2**).

	% liberation of HCN - emulsin & amygdalin	% liberation of HCN - emulsin & amygdalin	% liberation of HCN - almonds & amygdalin	% liberation of HCN - almonds & amygdalin
Time mins	**pH 5.0**	**pH 6.8**	**pH 5.0**	**pH 6.8**
10	36	36	7.5	8
30	ND	ND	22	23
60	63	61	44	45
90	ND	ND	50	55
120	86	89	55	60
150	ND	ND	65	71
180	ND	ND	81	77
240	93	100	79	85
360	100	99	ND	ND
600	100	100	ND	ND
1200	100	100	ND	ND
1440	100	100	96	99
1920	100	100	100	100
2880	100	100	95	99

Table 5.2.1.2 Percent liberation of HCN from amygdalin using emulsin and almond enzymes (37°C)

5 mg samples of amygdalin were incubated in the outer well of modified Erlenmeyer flasks with 1.5 mg emulsin dissolved in 3 ml of either pH 5.0, 0.1 M sodium acetate buffer or pH 6.8, 0.1 M sodium phosphate buffer. Samples containing 5 mg of amygdalin with 1 g of almond macerate in either 3 ml of the pH 5.0 or pH 6.8 buffer were run in parallel. 1 ml of 1 M NaOH was added to the inner well of each flask. All samples were incubated in a 37°C shaking water bath for up to 48 hours before undergoing the quantitative cyanide analysis procedure of Lambert *et al.* (1975). ND = Not Determined.

5.2.2 Amygdalin Hydrolysis using Macerated Almonds

The literature (Moertel *et al*, 1981) indicates that natural almonds contain the *β*-glucosidase enzymes needed for cyanide liberation from cyanogenic substrate. The initial analyses for cyanide liberation from macerated almonds therefore involved adding the substrate amygdalin. These tests were performed at the optimal pH's 5.0 and 6.8 for *β*-glucosidase catalysed amygdalin hydrolysis.

The results showed almond enzymes hydrolysed cyanogenic glycosides to a lesser degree than the emulsin enzymes with 100 % cyanide liberation occurring after 24 hours rather than only four to six hours in the case of the emulsin incubations (**Table 5.2.1.2**). This data therefore indicates that a larger quantity of almond macerate is required to release the same amount of cyanide from a standard dose of amygdalin.

5.2.3 Amygdalin Hydrolysis using Hydroxynitrile Lyase

The next step was to find the effect of hydroxynitrile lyases on amygdalin hydrolysis. Amygdalin was again incubated at 37°C at pH 5.0 and 6.8 but this time with 5 µl of MNL enzymes added. With both acidities there was minimal cyanide release with a peak of only 0.025 mg (**Figures 5.2.3.1 and 5.2.3.2**). This result was not surprising as MNL only acts on the unstable hydroxynitrile intermediates of cyanogenic glycoside hydrolysis. *β*-glucosidases are required for the formation of the hydroxynitriles on which hydroxynitrile lyases (e.g. MNL) then work. Without the addition of *β*-glucosidase enzymes (from either almond macerate or commercial emulsin) to amygdalin there are likely to be no hydroxynitriles to be hydrolysed to HCN and benzaldehyde (**Figure 5.1.1**).

A comparison for cyanide liberation from amygdalin incubated with pH 6.8 buffer, and amygdalin incubated with 5 µl MNL (in pH 6.8 buffer), indicated that a small amount of amygdalin may have been hydrolysed to the intermediate mandelonitrile (**Figure 5.2.3.2**) due to a slightly higher HCN release in the MNL incubations. Alternatively there may have been some *β*-glucosidase contaminants present in the MNL used.

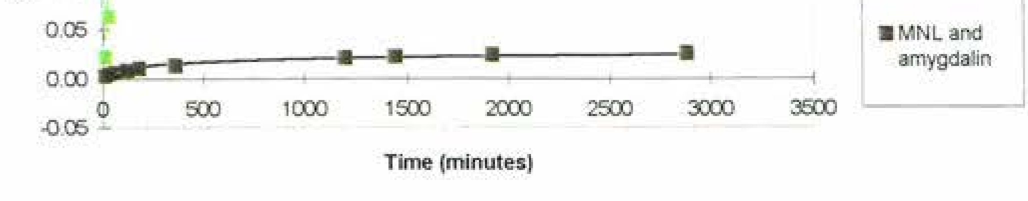


Figure 5.2.3.1 Amygdalin hydrolysis by emulsin, almond and MNL enzymes (pH 5.0)

5 mg samples of amygdalin were incubated in the outer well of modified Erlenmeyer flasks with 3 ml of 0.1 M sodium acetate buffer (pH 5.0). To each flask either 1 g of macerated almonds, 1.5 mg of emulsin (Worthington) or 5 µl of MNL (Sigma) were also added to the outer well. 1 ml of 1 M NaOH was placed in the inner well of each flask. All samples were incubated in a 37°C shaking water bath for up to 48 hours before undergoing the quantitative cyanide analysis procedure of Lambert *et al.* (1975).

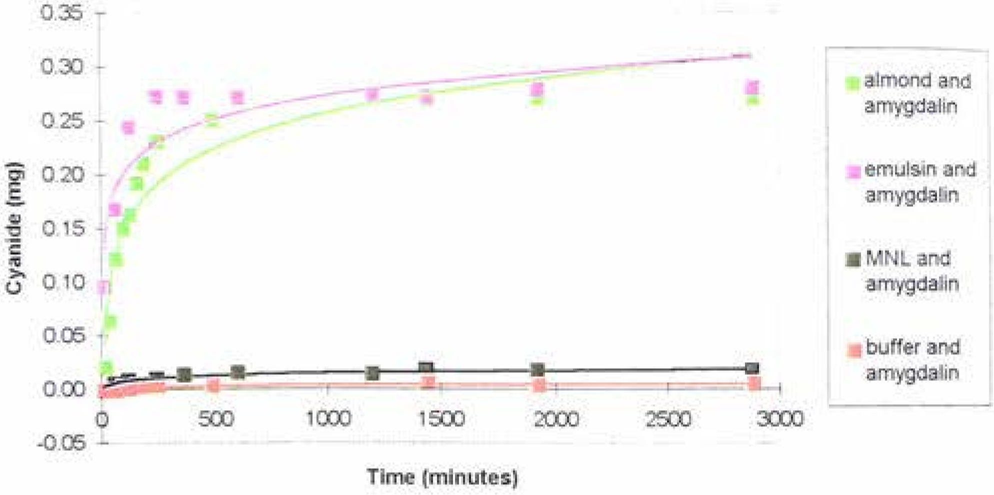


Figure 5.2.3.2 Amygdalin hydrolysis by emulsin, almond and MNL enzymes (pH 6.8)

5 mg samples of amygdalin were incubated in the outer well of modified Erlenmeyer flasks with 3 ml of 0.1 M sodium phosphate buffer (pH 6.8). Control samples with no enzymes added were run in parallel with each of the remaining flasks in which either 1 g of macerated almonds, 1.5 mg of emulsin (Worthington) or 5 µl of MNL (Sigma) were added. 1 ml of 1 M NaOH was placed in the inner well of each flask.. All samples were incubated in a 37°C shaking water bath for up to 48 hours before undergoing the quantitative cyanide analysis procedure of Lambert *et al.* (1975).

5.2.4 Testing for the Presence of HNL's in Almonds

Testing for the presence of hydroxynitrile lyase enzymes in almonds involved incubation of the hydroxynitrile, mandelonitrile (MN), with almond macerates at pH 4.0, 5.0 and 6.8. These experiments were run in parallel with a control containing 20 µl of MN incubated with 5 µl of MNL in 3 ml of buffer. A further control with only MN and buffer was run in parallel as MN is a relatively unstable hydroxynitrile. Incubations of almond macerates with MNL were also analysed to check for the presence of any natural stable hydroxynitriles in the almonds.

All the almond and MN incubations resulted in a high percentage of HCN liberated (**Figures 5.2.4.1, 5.2.4.2 and 5.2.4.3**). Comparison with the data for the buffer/MN controls however showed that MN undergoes spontaneous hydrolysis although not as much as when macerated almond was also present. The MN/MNL control incubations released an intermediate amount of HCN. This data indicated that either there is more active MNL present in the almond macerates than in the commercial MNL or there may be some substrate in the almond macerate that only the commercial MNL will act on. This latter theory was disproved by the almond/MNL analysis as no HCN was detected.

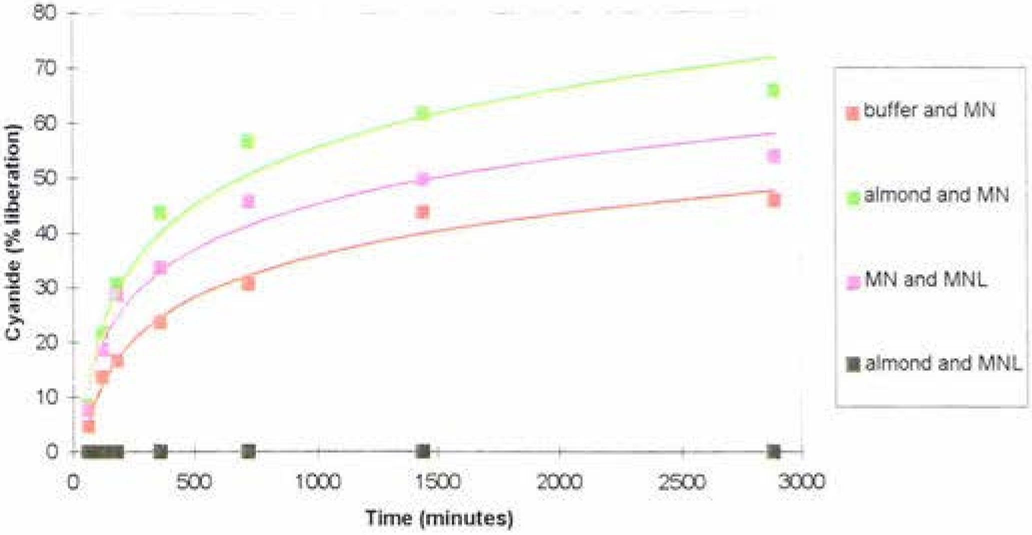


Figure 5.2.4.1 Percent liberation of HCN from amygdalin and MN (pH 4.0)
20 μl samples of MN were incubated in the outer well of modified Erlenmeyer flasks with 3 ml of 0.1 M sodium acetate buffer (pH 4.0). The outer wells of each of the test samples also contained either 1 g of macerated almonds or 5 μl of MNL. Control samples containing 20 μl MN in 3 ml buffer, or 1 g of almond macerate with 5 μl MNL in 3 ml buffer were run in parallel with the test samples. 1 ml of 1 M NaOH was added to the inner well of each flask. All samples were incubated in a 37°C shaking water bath for up to 48 hours before undergoing the quantitative cyanide analysis procedure of Lambert *et al.* (1975).

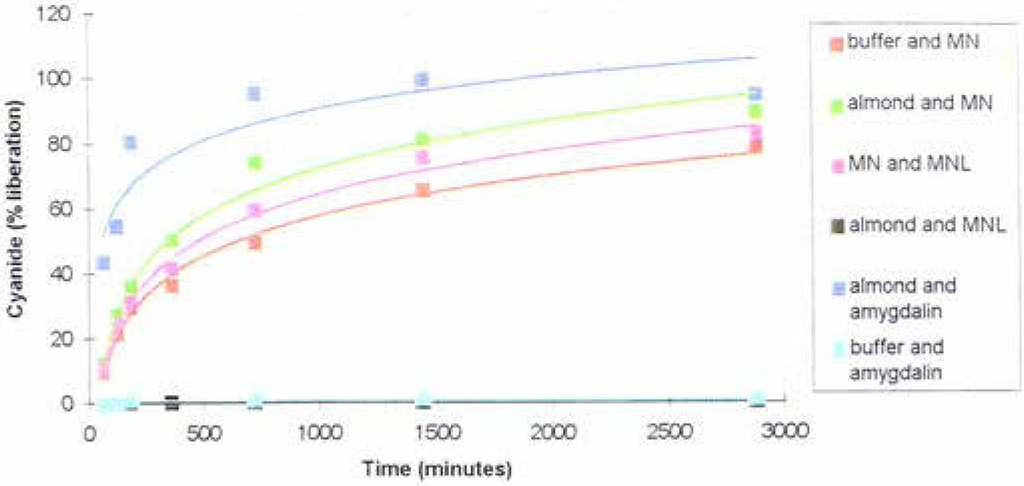


Figure 5.2.4.2 Percent liberation of HCN from amygdalin and MN (pH 5.0)
20 µl samples of MN were incubated in the outer well of modified Erlenmeyer flasks with 3 ml of 0.1 M sodium acetate buffer (pH 5.0). The outer wells of each of the test samples also contained either 1 g of macerated almonds or 5 µl of MNL. Control samples containing 20 µl MN in 3 ml buffer, or 1 g almond macerate with 5 µl MNL in 3 ml buffer were run in parallel with the test samples. Flasks containing 3 ml buffer, 5 g amygdalin and 1 g almond macerate, and 3 ml buffer with 5 mg amygdalin were run in parallel for comparison of cyanide release. 1 ml of 1 M NaOH was added to the inner well of all the flasks. All flasks were incubated in a 37°C shaking water bath for up to 48 hours before undergoing the quantitative cyanide analysis procedure of Lambert *et al.* (1975).

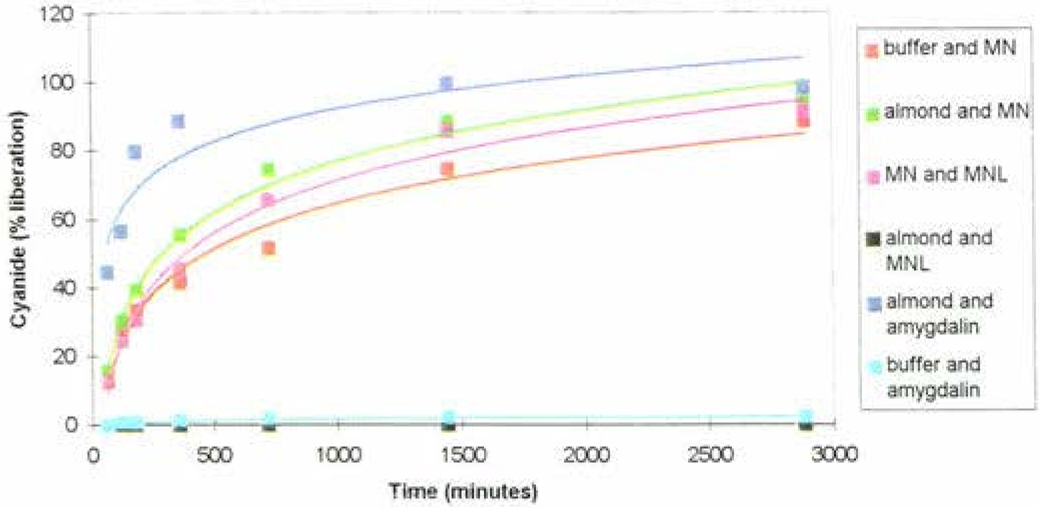


Figure 5.2.4.3 Percent liberation of HCN from amygdalin and MN (pH 6.8)
20 µl samples of MN were incubated in the outer well of modified Erlenmeyer flasks with 3 ml of 0.1 M sodium phosphate buffer (pH 6.8). The outer wells of each of the test samples also contained either 1 g of macerated almonds or 5 µl of MNL. Control samples containing 20µl MN in 3 ml buffer, or 1 g almond macerate with 5 µl MNL in 3 ml buffer were run in parallel with the test samples. Flasks containing 3 ml buffer, 5 g amygdalin and 1 g almond macerate, and 3 ml buffer with 5 mg amygdalin were run in parallel for comparison of cyanide release. 1 ml of 1 M NaOH was added to the inner well of all the flasks. All flasks were incubated in a 37°C shaking water bath for up to 48 hours before undergoing the quantitative cyanide analysis procedure of Lambert *et al.* (1975).

5.2.5 Analysis of Wild Almonds for Cyanogenicity

A small supply of locally found almonds were tested for their cyanide liberating ability. The almonds were known to be an inedible variety although the species could not be identified. *In vitro* analysis, in which 1 g samples of the almond nuts were macerated and incubated for 24 hours in 3 ml of pH 6.8 buffer, showed that these almonds were highly cyanogenic. 1 g of the almond nuts produced on average 1.93 mg of HCN (**Table 5.2.5.1**). When compared with the results of HCN release from 1 g of sweet almonds incubated with 5 mg of amygdalin the data shows that these bitter almonds must contain at least 35 mg of amygdalin per gram of nuts that is hydrolysed. A lethal dose of HCN for 90 percent of test possums weighing 2.5 kg is estimated at about 45 mg therefore only 24 grams of these almonds in theory could kill the average possum.

Incubation Period (hours)	Wild Almonds (mg HCN)	Sweet Almonds (mg HCN)
24	1.93	0.271

Table 5.2.5.1 HCN release from Wild Almonds

1 g samples of wild and sweet almonds were incubated in the outer well of modified Erlenmeyer flasks with 3 ml of 0.1 M sodium phosphate buffer (pH 6.8). 5 mg of amygdalin was also added to the outer well of each of the flasks containing sweet almonds. The inner well of each flask contained 1 ml of 1 M NaOH. All flasks were incubated in a 37°C shaking water bath for 24 hours before undergoing the quantitative cyanide analysis procedure of Lambert *et al.* (1975).

From this set of experiments further analysis was performed on the HCN liberating abilities of sweet almonds. Due to lack of the amygdalin substrate only one set of experiments was performed in which 30 mg of amygdalin was incubated with 1 g of macerated sweet almond. The experiments were performed in pH 5.0 buffer at a temperature of 37°C. As shown in **Figure 5.2.5.1** by the release of 1.6 mg of HCN (per gram of almond macerate) from 30 mg of amygdalin in 24 hours, the HCN liberation from the previous experiments were limited by the amount of substrate added and that sweet almonds contain an excess amount of β-glucosidase enzymes.

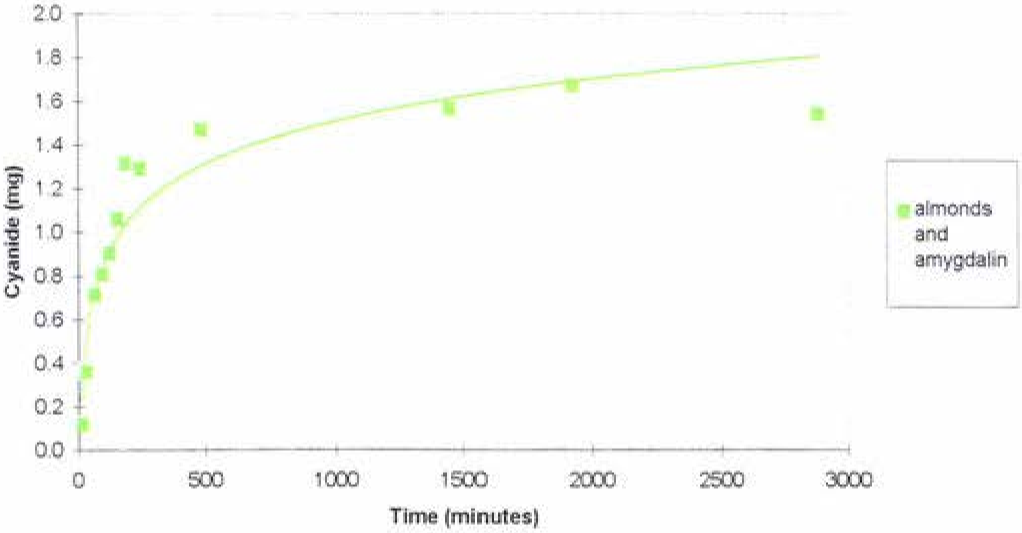


Figure 5.2.5.1 Sweet almonds incubated with 30 mg of amygdalin (pH 5.0)
1 g samples of macerated sweet almonds were incubated in the outer well of modified Erlenmeyer flasks with 30 mg of amygdalin and 3 ml of 0.1 M sodium acetate buffer (pH 5.0). The inner well of each flask contained 1 ml of 1 M NaOH. All flasks were incubated in a 37°C shaking water bath for 24 hours before undergoing the quantitative cyanide analysis procedure of Lambert *et al.* (1975).

5.2.6 Analysis of the Flavour Enhancer for the *In vivo* Trials

The amount of cyanide liberated in studies using almond β-glucosidases *in vitro* were sufficient to warrant *in vivo* testing in possums. A suitable carrier to mask the bitter flavour of amygdalin was required. Peanut butter, which has a flavour attractive to possums was used in conjunction with sugar to help reduce the bitter taste of the amygdalin substrate. The quantities of substrate and flavour enhancers tested were 30 mg of amygdalin, 2 g of peanut butter and 0.3 g of white sugar. The samples were incubated for up to three hours.

As detailed in **Table 5.2.6.1** the peanut butter/sugar incubations liberated a very small amount of HCN. These quantities were not significant to affect the *in vivo* study as this amount of HCN is easily detoxified by the body. The peanut butter/sugar incubations with amygdalin added released a slightly larger amount of HCN, although still only measurable in microgram quantities. Thus we can conclude from the data that the peanut butter/sugar paste is a suitable vector for administering amygdalin to the trial possums assuming the possums find it palatable.

Time (minutes)	Peanut butter and sugar (µg cyanide)	Peanut butter, sugar and amygdalin (µg cyanide)
10	0	0
60	0.101	0.122
90	0.133	0.199
120	0.156	0.247
150	0.23	0.263

Table 5.2.6.1 Effect of peanut butter and sugar on amygdalin hydrolysis (pH 4.0)

Samples comprising 30 mg of amygdalin mixed in 2 g of peanut butter and 0.3 g of white sugar were incubated in the outer well of modified Erlenmeyer flasks with 3 ml of pH 4.0 buffer. Control samples containing the same quantity of peanut butter and white sugar were run in parallel. The inner well of each flask contained 1 ml of 1 M NaOH. All flasks were incubated in a shaking water bath for up to 3 hours at 37°C before undergoing the quantitative cyanide analysis procedure of Lambert *et al.* (1975).

5.3 Summary

Amygdalin and emulsin were used to test the effectiveness of the microdiffusion technique in liberating HCN. The technique was very effective for HCN liberation and capture, with 100% HCN liberation recorded in just four hours for the pH 6.8 (37°C) samples. Incubations at lower acidities (pH 4.0 and 5.0) released HCN at a slightly slower rate and in the case of pH 3.0 samples, negligible cyanide release was recorded.

Analysis of the cyanide liberating abilities of raw almonds showed that 1 g of almond macerate took longer to liberate HCN from amygdalin than could be liberated by 1.5 mg of emulsin . The almonds did however completely hydrolyse amygdalin within 24 hours, with 60% of the hydrolysis occurring in the first two hours. This rate of cyanide liberation was deemed enough to proceed with *in vivo* analyses of amygdalin hydrolysis by almond macerates in possums.

Addition of hydroxynitrile lyase to amygdalin did not cause any significant HCN liberation therefore indicating that the amygdalin samples did not contain any hydroxynitriles. The rapid hydrolysis of amygdalin (60% in two hours) when incubated with almond macerate indicates that amygdalin requires the presence of β-glucosidases for HCN liberation.

Almonds were later tested for the presence of HNL's by incubation with the hydroxynitrile, mandelonitrile. Controls run in parallel, containing only MN and buffer, showed that MN spontaneously dissociates to form HCN. However, the HCN liberation from MN was not as high in the controls therefore indicating that there is probably some HNL activity in almond macerates.

The wild bitter almonds that were found locally contained both cyanogenic substrate and enzymes and therefore were highly cyanogenic. Only 24 g of the raw almonds were calculated as being capable of liberating 45 mg of HCN. This, in a single dose, would be easily enough to kill the average 2.5 kg possum. Encouraging the possums to consume this dose of bitter almonds however would be extremely difficult due to the taste associated with the high cyanogenic glycoside content.

The amygdalin/peanut butter/sugar paste developed as a carrier for the amygdalin in the *in vivo* trials was found to be very stable with only 0.263 μg of HCN released in 2.5 hours. This HCN liberation was similar to the HCN liberation from a control peanut butter/sugar paste (0.230 μg). It was therefore found to be a suitable amygdalin vector so long as the possums found it palatable.

CHAPTER SIX

IN VIVO POSSUM STUDY

6.1 Introduction

The effects of orally administered amygdalin in *Trichosurus vulpecula* has been researched by Charles Eason of Landcare Research, Christchurch. Eason and co-workers (personal communication) gavaged possums with amygdalin (100 mg/kg) with the prospect that midgut β-glucosidase activity would liberate sufficient cyanide to cause death. However, at the concentrations administered, cyanogenic glycoside tolerance resulted, presumably due to aglycone detoxification.

6.1.1 Lethal Doses of HCN for Possums

The LD_{50} for HCN poisoning in possums is 11 mg/kg. **Table 6.1.1.1** below displays the LD_{50} and an estimated LD_{90} for each of the experimental possums involved in this study based on their body weights.

POSSUM	**A**	**B**	**C**	**D**
Possum weight (kg)	2.85	2.50	2.20	1.60
LD_{50} (11mg/kg) mg cyanide	31.35	27.50	24.20	17.60
LD_{90} (estimated @ 18 mg/kg) mg cyanide	51.30	45.0	39.60	28.80

Table 6.1.1.1 The LD_{50} and estimated LD_{90} of HCN for each possum

Knowing that amygdalin contains 5.7% cyanide, complete hydrolysis of 1 g amygdalin by β-glucosidase should yield 57 mg of cyanide, this is assuming the reaction goes 100% to completion.

6.1.2 *In vitro* HCN Recovery Rate at pH 4.0

An *in vitro* study used to determine the effectiveness of cyanide liberation and capture using the microdiffusion technique was performed with commercial emulsin and amygdalin. The recovery rates at pH 4.0, a pH similar to that of the possum stomach, were as follows (**Table 6.1.2.1**).

Time (hours)	% Recovery
1	32
2	59
4	78
6	92

Table 6.1.2.1 HCN recovery rate using the microdiffusion technique (pH 4.0)
5 mg samples of amygdalin (Sigma) were incubated in the outer well of modified Erlenmeyer flasks with 1.5 mg emulsin (Worthington) dissolved in 3 ml of pH 4.0, 0.1 M sodium acetate buffer. 1 ml of 1 M NaOH was added to the inner well of each flask. All samples were incubated in a 37°C shaking water bath for up to 6 hours before undergoing the quantitative cyanide analysis procedure of Lambert *et al.* (1975).

The data shows that 100% cyanide recovery does not occur from this method in a reasonable time. Discrepancies in the percent recovery may result from incomplete hydrolysis of substrate where maceration is not completed properly, through loss of HCN gas via the flask lid seals, and/or the cyanide may be in a form that is not easily released.

The recovery figures were used to calculate the approximate quantity of cyanide released within the possums' stomachs over several hours, given a particular dose of amygdalin and emulsin (**Table 6.1.2.2**).

POSSUM	A	B	D
mg amygdalin	1500	1000	1250
mg cyanide 1 hr (32 %)	20.1	13.4	16.7
mg cyanide 2 hrs (59 %)	45.3	30.2	37.7
mg cyanide 4 hrs (78 %)	64.1	42.7	53.4
mg cyanide 6 hrs (92 %)	75.2	50.2	62.7

Table 6.1.2.2 Estimated cyanide release within each possum

6.2 Results and Discussion

6.2.1 *In vivo* Trial One

During the first six days of captivity all of the possums were pre-fed between 5 and 20 grams of almonds each day along with their normal diet of fruit and bread. This was to determine the palatability of the natural almonds to the possums. In this pre-trial period possum A and D always consumed all of the allocated almonds while possums B and C would eat only a few of the almonds. On the seventh day of captivity the almonds were omitted from the diet.

The palatability of the amygdalin/peanut butter/sugar paste was assessed on day eight. This paste was made up with 0.5 grams of amygdalin, approximately half of that used in the paste for the actual trials. The paste was fed to the possums along with a small portion of the standard fruit and bread diet. All four possums ate all of the regular diet along with a small portion of the amygdalin paste (up to 30%). It was concluded from this result that the amygdalin paste was reasonably palatable.

For trial one the concentration of the amygdalin in the paste was stronger so that the portion of paste required to be consumed would be less. Additional to this the possums were also starved for the 12 hours immediately prior to the trial to ensure they would be hungry and more likely to consume the required amount of paste.

On the ninth day the possums were fed only fruit and bread. The following day the possums were all fed 80 grams of almonds. This feeding was to determine whether the possums would consume the required amount of almonds for adequate β-glucosidase levels to be achieved, and also to load as much almonds into the possums before trial one began the next day.

Possums A and D ate all the almonds made available (**Table 6.2.1.1**), the difference between the almonds fed (80 g) and those actually eaten (72.1 g and 78.02 g respectively) was due to the loss of some almonds through the bottom of the possums' cages. Possum B ate 40.98 grams - only half the available almonds. Possum C ate the least almonds (8.76 grams). As this possum was unlikely to eat enough almonds for the actual trial, it became the control.

The eleventh day of captivity was the day of the first trial. Due to the nocturnal nature of possums they are more active and feed the most during the early hours of the morning hence this first trial started at 4 am. Changes in behaviour indicative of the early stages of cyanide poisoning (i.e. impaired balance and co-ordination) would also be accentuated during these hours when the possums are generally awake and moving about.

All of the possums were fed the amydalin paste. Possums A and C were fed 17.3 grams of paste containing a total of 1.3 grams of amygdalin while possums B and D were fed 17 grams of paste containing 1 gram of amygdalin. Possums A, B and D were also fed 60 grams of natural almonds. Possum C, the control in this experiment had no almonds made available for consumption. All of the possums were then observed for the first four hours post feeding.

Within the first half hour of the trial possum A had eaten 37.73 grams of almonds. Over the next nine minutes 4.57 grams (26.4%) of the amygdalin paste was eaten. This equates to 343 mg of amygdalin being consumed. During this period possum A also drank water. For the next hour and a quarter this possum appeared moderately restless with periods of cleaning himself and wandering around the cage. This was followed by a light dozing period of 30 minutes before cleaning himself again and observing his surroundings. Three hours into the trial possum A was fed a quarter orange which had

had its pips removed. Although the orange was eaten immediately no further almonds or amygdalin paste were consumed. The possum then sat with his head down for the remaining hour that he was observed. Other than slight restlessness in the first hour and a quarter no other signs indicative of mild cyanide poisoning were observed.

POSSUM	**A**	**B**	**C**	**D**
DAY 10 almonds ingested (g)	72.10	40.98	8.76	78.02
DAY 11 almonds ingested (g)	37.73	4.80	X (control)	6.21
DAY 11 amygdalin ingested (g)	0.343	X	0.663	X

Table 6.2.1.1 Almonds and amygdalin consumed in trial one
Each possum was pre-fed up to 80 g of almonds on day 10 of captivity. All but the control possum C were fed a further 60 g of almonds on day 11. Each possum was given the amygdalin paste on day 11 as follows: possum A and C were fed 17.3 g of paste containing 1.3 g of amygdalin and possums B and D were both fed 17 g of paste containing 1 g of amygdalin. Each possum was observed closely for the first four hours and then rechecked 20 hours later.

Possum B ate only 4.8 grams of almonds in this first trial. From the *in vitro* study this was not nearly enough almonds to provide adequate levels of active β-glucosidase in the possums digestive tract should all the amygdalin paste have been eaten. No amygdalin paste was eaten by possum B, hence there were no signs of cyanide poisoning. Possum B slept for the first hour of the trial. She then ate a couple of almonds, and sat down or squatted for the next half hour. A few more nuts were then eaten and some water drunk before she cleaned herself and sat observing me. Two and a half hours into the trial she ate a few more almonds, searched for some more palatable food (of which she decided there was none as the amygdalin paste remained untouched). She was fed quarter of an orange which was eaten immediately. For the remainder of the observation period possum B spent sitting down with her head down.

Possum C, the control in this trial, had eaten only 8.76 grams of almonds on day ten. On the day of the trial she was fed only amygdalin paste. In the first half hour she ate 8.83 grams of paste (663 mg of amygdalin). For the remaining three and a half hours she slept or squatted and dozed. No further paste was eaten during the four hour observation period. The orange was left untouched during the observation period.

As with possum B, possum D did not eat many almonds. In all only 6.21 grams of almonds were eaten and no amygdalin paste was consumed. He sat observing me for over an hour before foraging for food. The almonds and paste did not appeal and therefore were not eaten. After realising no other more palatable food was on offer, possum D went to sleep. Upon feeding the orange he awoke and ate it immediately. Possum D then resumed a restful slumber for the remainder of the observation period.

At no time during the four hour observation period did any of the possums show signs of salivation, retching, vomiting or any other forms of suffering. When the possums were checked again at 4 am the following morning possum C, the control, was dead and rigor mortis was apparent. There were no physical signs indicating the possum had been distressed (i.e. no fur in cage or wounds on animal). The orange had also been eaten sometime during the time the possum was left unattended. A post-mortem was performed on possum C at 11.30 am the same morning. Samples of liver and muscle tissue along with the possum's stomach contents were sent to the Ministry of Agriculture and Fisheries (MAF) for cyanide analysis.

The MAF report found only trace cyanide levels in the stomach contents, with none recorded from liver or muscle tissue. The cyanide found was not believed to be of a significant amount to cause death. The pathologist noted a distinct almond odour in the lung and liver material along with some nutty smelling semi-solids in the small intestines and mouth. The heart was in good condition and there was no indication that death was a result of heart failure. The pathologist diagnosed death by cyanide poisoning.

Of note is the rapid degradation of cyanide and the time lapsed between death, post-mortem and MAF tissue analysis. Time of death was estimated at about 4 pm on day eleven - 19.5 hours before the autopsy was performed. The MAF samples were processed several days after the post-mortem along with other samples to be cyanide tested. This may have affected the level of cyanide detected in the tissue samples by MAF.

Whether there was HCN release from the amygdalin because of residual almond enzymes in the gut from the day 10 diet is unknown. This seems unlikely as only about 9 g of almonds were eaten and possum A, the only other possum to eat any amygdalin on day 11, did not show any signs of cyanide poisoning. The *in vitro* analysis of amygdalin with buffer only (**Figures 4.2.4.2 and 4.2.4.3**), showed negligible HCN release therefore it is unlikely that the amygdalin spontaneously hydrolysed. The research by Moertel *et al.* (1981), however indicated that there are natural β-glucosidases in the human digestive tract. It may therefore be possible that possums also have β-glucosidase enzymes present and that possum C contained enough of the appropriate enzymes in her gut to liberate a lethal dose of HCN from the 663 milligrams of amygdalin she ate.

The standard diet of fruit, bread and a few almonds was resumed for possums A, B and D on the twelfth day of capture and continued until the day prior to trial number two, eight days later.

6.2.2 *In vivo* Trial Two

As the possums would not voluntarily eat the required volume of almonds and amygdalin paste, gavaging was required. Possums A and B were involved in this trial of which B was the control. Initially intra muscular (i/m) medetomidine and its reversal agent atipamezole were used to induce and reverse anaesthesia, however this anaesthetic did not give sufficient relaxation of the jaw muscles for effective gavaging. Food grade carbon dioxide was subsequently used as the anaesthetic. The possums were individually anaesthetised by first placing them in a large plastic bin filled with carbon dioxide. This lowered each possums level of consciousness rapidly to a level at which they were safe to handle and start the gavage procedure. Anaesthesia was maintained with a light flow of carbon dioxide via a face mask. On completion of the gavage procedure the mask was removed and the possums replaced in their individual cages. Recovery from anaesthesia was in the order of a few seconds.

Shortly before the possums were gavaged an almond paste was made by pulverising raw almonds with water. 50 mls (or 18.88 g dry weight of almonds) of this paste was the maximum volume that could be comfortably used to gavage possum A without over expanding his stomach. A further solution containing 1000 mg amygdalin dissolved in 15 ml of water was also administered to possum A. Possum B was gavaged with the amygdalin solution only (**Table 6.2.2.1**).

POSSUM	**A**	**B**
DAY 20 **almonds ingested (g)**	39.2	X
DAY 21 **almonds ingested (g)** **(50 ml paste)**	18.88	X
amygdalin ingested (g)	1.0	1.0

Table 6.2.2.1 Substrate and enzyme consumed in trial two
Possums A and B were gavaged with 1 g of amygdalin in 15 ml of water whilst anaesthetised with food grade carbon dioxide. Possum A also received 50 ml of an almond/water paste made up of 18.88 g almond in milliQ water. The anaesthetic was then removed and both possums were observed closely for the following four hours.

Neither possum presented with any conclusive signs of cyanide poisoning. Possum A was slightly restless for the first 15 minutes after the gavage procedure which was entirely appropriate behaviour for the stressful situation he had just encountered. For the remainder of the observation period possum A cleaned himself and slept. Control possum B displayed no period of restlessness and merely slept and cleaned herself the for the entire four hour observation period. After four hours each possum was fed two slices of bread and a decored apple Possum B ate the food immediately but possum A did not eat anything until nine hours later. The possums were checked regularly over the 24 hour period following this trial. Neither possum died or showed any signs of suffering.

The results from this trial were interesting as both possums received more amygdalin (1 g) than the control (possum C) in trial one that died (0.663 g). The fact that neither possum in trial two showed any signs of HCN poisoning tends to dispel the theory that possum C died from HCN poisoning due to the amygdalin being hydrolysed from her own gut β-glucosidases. The rate at which 1 g of amygdalin is hydrolysed to 57 mg of HCN by 18.88 g of almond enzymes must be similar to the rate at which the possums can detoxify HCN. Therefore in the next *in vivo* trial, all three possums were gavaged with different and larger amounts of amygdalin and emulsin.

6.2.3 *In vivo* Trial Three

In the third trial in which the three remaining possums were gavaged whilst anaesthetised with CO_2, possum A appeared to suffer a moderate degree of cyanide poisoning before recovering, possum B showed no signs of any poisoning while possum D died from HCN poisoning. Substrate and enzyme purchased from the Sigma and Worthington Biomedical chemical companies were diluted in milliQ water and used for this gavage procedure.

Possum A with 526 mg amygdalin per kg body weight (**Table 6.2.3.1**) showed definite signs of near lethal cyanide poisoning. He later recovered with no apparent ongoing problems. Within 20 minutes from ingestion of the substrate and enzymes the possum was semi-conscious, with body twitching. He remained this way for the next 40 minutes. A period of unbalanced standing followed for 10 minutes before the possum once again lay down semi-conscious. Two hours and 15 minutes after gavaging the possum assumed a 'sitting dog' position and remained in a rather "dopey" state for the next 2 hours. Eight hours post gavage the possum's level of consciousness had improved although he was still not eating. After 18 hours the possum appeared to be back to normal and eating well again.

Possum B was given the lowest dose of amygdalin at only 400 mg per kg body weight (**Table 6.2.3.1**). This possum regurgitated some of the amygdalin during the gavage procedure hence the exact amount that remained in the stomach could not be determined. At no stage during trial three did possum B show any indication of cyanide poisoning. It spent most of its day either sitting and dozing, or sleeping.

POSSUM	A	B	D
Emulsin (mg)	450	300	375
amygdalin (mg)	1500	1000	1250
mg amygdalin per kg body weight	526	400	780

Table 6.2.3.1 Substrate and enzyme consumed in trial three
Possums A, B and D were gavaged with between 1 and 1.5 g of amygdalin in water (1 g/15 ml) whilst anaesthetised with food grade carbon dioxide. Each possum also received between 300 and 450 mg of commercial emulsin dissolved in milliQ water (100 mg/ml). The anaesthetic was then removed and each of the possums were observed closely for the following four hours.

At 780 mg amygdalin per kg body weight (**Table 6.2.3.1**) possum D received the largest dose of amygdalin. For the first 25 minutes his behaviour was normal, followed by a period of loss of balance (13 minutes) then a period of unconsciousness and minor body twitching (27 minutes). One hour and five minutes after gavaging possum D died. At no stage were there any convulsions, salivation, retching or vomiting. Because there were obvious signs of severe cyanide poisoning immediately prior to death a post mortem was not performed.

Following the third trial possums A and B were given their standard diet again for four days. No other adverse effects were observed in the possums over this period. At the end of the four days they were killed by lethal injection of sodium pentobartitone.

6.3 Summary

In the first trial three of the four experimental possums were fed almond nuts and an amygdalin/peanut butter/sugar paste, the quantities of which were determined from prior *in vitro* analysis. The fourth possum, the control, was fed the amygdalin paste only. None of the possums ate all of the trial food made available and only possums A and C (control) ate any of the amygdalin paste. During the first four hours of observation there were no signs of HCN poisoning in any of the possums however 20 hours later the control possum was found dead. The post-mortem indicated death by HCN poisoning although the MAF tissue analysis report indicated only trace cyanide levels that were not indicative of death by cyanide poisoning.

Due to the difficulty in getting the possums to voluntarily consume the amygdalin and almonds, in trial two the test possums were gavaged with a macerated almond and water paste followed by amygdalin dissolved in water. Neither of the two possums gavaged showed any signs of HCN poisoning at any stage over the following 24 hours.

From the trial two results, it appeared that the possums were able to detoxify the cyanide at a rate close to the production rate of the cyanide hence there were no signs of any HCN poisoning. Therefore in the final gavage trial, each of the three remaining possums was gavaged with a greater amount of amydalin (1 g to 1.5 g) and β-glucosidase enzymes commercially produced.

The possum with the lowest dose of amygdalin (400 mg/kg body weight) showed no signs of HCN poisoning at any stage. The possum dosed at 526 mg of amygdalin per kg of body weight displayed signs of severe cyanide poisoning for a two hour period before complete recovery. The third possum, which received 780 mg of amygdalin per kg body weight, died from cyanide poisoning one hour after the gavage procedure.

There were obvious limitations in this *in vivo* study due to the experimentation involving only four possums. The results showed that possums used in this study appeared to detoxify HCN at an extremely fast rate, and that the use of a cyanogenic glycoside/catabolic enzyme pest control would require a large amount of HCN to be liberated in the digestive tract in a very short space of time.

CHAPTER SEVEN

CONCLUSION AND FUTURE WORK

7.1 Conclusion

The release of HCN from cyanogenic plants is thought to be a pest deterrent (Vicari and Bazely, 1993). The fact that some plant species have evolved to contain only the substrate or catabolic enzymes has made them non-cyanogenic and therefore more attractive to the diet of many animals. Linking the single cyanogenic moiety (either substrate or enzyme) within one of these plants with a bait containing the complementary cyanogenic moiety became the basis for developing a species-specific toxin. Although the overall research aim was to locate a plant species within the possum diet that contains a single cyanogenic moiety and to develop from this a bait that is complementary for cyanide liberation, initial analyses performed in this research were aimed at determining whether such a project would be viable.

Initial tests involved analysing plants identified as being highly cyanogenic ('aran' clover) or from species of high cyanogenicity (cherries and almonds). Several combinations of substrates or enzymes were incubated with the test plants to maximise cyanide release. Once the plant tissues liberated sufficient cyanide to be lethal in possums they were tested *in vivo*.

7.1.1 Clover

Although 'aran' clover is one of the most highly cyanogenic species of clover in New Zealand, the analyses from this study showed that the HCN released from unadulterated leaves was insufficient to be toxic to possums. Addition of a combination of hydrolysing enzymes did however increase the rate and extent of cyanide release (about 30%) but not to the degree required to be lethal to possums. These findings could still be promising however, if a plant species within the possum diet contained the substrate and either no or low levels of catabolic enzymes. A complementary bait containing the appropriate enzymes could then be produced which when consumed in conjunction with the plant species, would release a lethal dose of HCN to possums.

There was a concurrent increase in HCN liberation with the introduction of surplus substrate to the clover macerates. Substrate controls run in parallel identified this increase to be as a result of spontaneous hydrolysis rather than a result of excess catabolic enzymes within the plant tissue.

7.1.2 Cherries

Both the sweet and the sour cherries contained β-glucosidase enzymes but no substrate. Although it seems unlikely that cherries could produce enough cyanide in conjunction with an appropriate cyanogenic glycoside bait, there may be a possibility for orchard pest control of other animals. Birds are the greatest hazard to cherry orchards and whether the catabolic enzyme content of cherry pericarps can be used to target birds could provide an interesting orchard pest control solution.

7.1.3 Almonds

Of the three plant varieties analysed in this study, the almond macerates liberated the most HCN after addition of substrate. Comparison of the HCN liberation from amygdalin using almond macerates (96% liberation at pH 5.0 over 24 hours) or cherry macerates (29% liberation at pH 5.0 over 24 hours) indicated that almonds contain more enzymes per gram of tissue, or more active β-glucosidases than cherries.

Sweet almonds were found to contain an excess of β-glucosidase enzymes when in a trial incubating 30 mg of amygdalin with just 1 g of almond macerate, over 1.6 mg of HCN was liberated. This level of HCN liberation was sufficient to proceed with an *in vivo* possum study

7.1.4 *In vivo* Possum Study

This *in vivo* study was limited by the use of only four experimental possums. While two of the possums did die during the trials, only one death was an obvious result of cyanide overdose. A third possum did display signs of near lethal HCN poisoning before making a full recovery. At no stage during any of the trials did the fourth possum show any signs of cyanide poisoning.

The control possum in trial one was the first to die. In this study the control was fed amygdalin but no almonds therefore it should not have died from HCN poisoning. A pathology report however suggested death by cyanide poisoning (a contradictory analysis from the MAF report) therefore indicating that the possum somehow hydrolysed the amygdalin itself. Previous research on orally administered amygdalin in humans (Moertel *et al*, 1981) did indicate that the stomach does contain β-glucosidases capable of hydrolysing amygdalin. The presence of β-glucosidases within the control possum's stomach may therefore have been the cause of cyanide liberation. The only other possum to voluntarily eat any amydalin ate half the quantity that the control did and showed no signs of cyanide poisoning.

The second trial tends to dispel the theory that the control possum died from amygdalin hydrolysed by its own gut enzymes as in this trial the possums were subjected to higher quantities of amygdalin without showing any signs of HCN poisoning. The cause of death of the control possum is therefore inconclusive. This trial revealed that the possums were capable of detoxifying cyanide at an extremely quick rate.

The results of the third trial gave a vague indication of how much amygdalin is required to be lethal to possums. At 526 mg amygdalin/kg body weight the dose was slightly too low as the possum recovered from very severe HCN poisoning. A dosage of 780 mg/kg killed the possum in a relatively short time with no obvious signs of suffering.

7.2 Future Work

During this research there was no literature available examining amygdalin toxicity in possums and in particular whether these animals contain sufficient β-glucosidase activity in their gut to hydrolyse amygdalin. An interesting extension of this thesis would therefore be to analyse possum digestive juices for its β-glucosidase activity. Should this research show that the possums do contain adequate levels of cyanogenic enzymes then their would be no purpose in analysing plants within the possum diet for cyanogenic substrates as obviously the possums do not eat them. The production of a palatable cyanogenic glycoside bait however may be a good idea, although other animal species likely to consume the bait would need to be eliminated from being 'at risk' by checking their β-glucosidase activities.

If the possums are found to be unable to hydrolyse amygdalin then further investigation into plants species commonly browsed by possums that are cyanogenic would be the next logical step. As indicated by this research project the plants investigated would need to contain extremely high levels of cyanogenic glycosides or their catabolic enzymes.

In order to create a more realistic *in vitro* model of the possums digestive tract, the model could mimic the changes in pH as the plant tissue travels through the digestive system (i.e. from near neutral pH in the mouth to about pH 4.0 in the stomach and then about pH 8.0 in the intestine). This would involve altering the pH of the incubations at different times during the incubation and recording any associated changes in HCN liberation.

REFERENCES

Agricultural Pest Destruction Council (1971) Use of 1080 poison for agricultural pest control. 17-21.

Allen, S. (1989) The possum trap. *Listener.* **124**, 33-34.

Atkinson, K. (1991) Opossum potions fail to fire. *The Dominion.* **14-3-1991**, 6.

Conn, E.E. (1978) Cyanogenic glycosides. *Encycl. Plant Physiol.* **8**, 461-492.

Conn, E.E. (1979) Cyanide and cyanogenic glycosides. *Herbivores: Their interaction with secondary plant metabolites* **Chapt. 10**, 387-412.

Conn, E.E. (1980) Cyanogenic compounds. *Ann. Rev. Plant Physiol.* **31**, 433-451.

Corkill, L. (1940) Cyanogenesis in white clover (*Trifolium repens* L.). I. Cyanogenesis in single plants. *N.Z. J. Sci Tech.*, **22**, 65B-67B.

Cuddihy, M. (1993) Possum control programme. *New Zealand Forestry* **38**, 17-18.

Department of Conservation (1997) Toxins and poisons. *Vertebrate pest control manual.*

Edwards, F. (1990) Shifting the balance. *For. and Bird* **21(3)**, 30-33.

Eyjolfsson , R. (1970) Recent advances in the chemistry of cyanogenic glycosides. *Fortschr. Chem. Org. Naturst.* **28**, 74-108.

Feratox pellets for possum control. (1997) *Feratox Brochure.* Feral Control, 36B Sir William Ave, Auckland.

Gee, D. (1995) Contraceptive vaccine could halt possum population boom. *The Christchurh Press* 11 August, 14.

Glasgow, L. (1990) NZ declares war on the possum. *New Scientist* **126**, 30.

Greer, M.A., Stott, A.K. and Milne, K.A. (1966) Effect of thiocyanate, perchlorate and other anions on thyroidal iodine metabolism. *Endocrinology* **79**, 237-247.

Gregory, N.G., Milne, L.M., Rhodes, A.T., Littin, K.E., Wickstrom, M. and Eason, C.T. (1997) Effect of potassium cyanide on behaviour and time to death in possums. *NZ Veterinary Journal* **46**, 60-64.

Kelly, M. (1990) Possum stalls fight against bovine TB. *The New Zealand Farmer* **112**, 5-7.

Lambert, J.L., Ramasamy, J. and Paukstelis, J.V. (1975) Stable reagents for the colorimetric determination of cyanide by modified Konig reactions. *Anal Chem* **47**, 916-918.

Millar, J.M. and Conn, E.E. (1980) Metabolism of hydrogen cyanide by higher plants. *Plant Physiol.* **65**,1199-1202.

Moertel, C.G., Ames, M.M., Kovach, J.S., Moyer, T.P., Rubin, J.R. and Tinker, J.H. (1981) A Pharmacologic and Toxicological Study of Amygdalin. *J. Amer. Med. Assoc.* **245,** 591-594.

Montgomery, R. D. (1969) Cyanogens. *Toxic constituents of plant foodstuffs* **Chapt. 5,** 143-157.

Paim, N.R. and Dean, C.E. (1976) Characteristics of cyanogenic and acyanogenic white clover plants. *Proceedings.* **35**, 18-21.

Poulton, J.E. (1990) Cyanogenesis in plants. *Plant Physiol.* **94**, 401-405.

Salkowski, A.A. and Penney, DG. (1994) Cyanide poisoning in animals and humans: A review. *Vet. Human Toxicol.* **36**, 455-466.

Seitzer, S. (1992) Possum: an ecological nightmare. *New Zealand Geographic* **13**, 42-70.

Selmar, D., Lieberei, R., Biehl, B. and Conn, E.E. (1989) α-Hydroxynitrile lyase in *Hevea brasiliensis* and its significance for rapid cyanogenesis. *Physiologia plantarum* **75**, 97-101.

Swain, E., Li, C.P. and Poulton, J.E. (1992a) Development of the potential for cyanogenesis in maturing black cherry (*Prunus serotina* Ehrh.) fruits. *Plant Physiol.* **98**, 1423-1428.

Swain, E., Li, C.P. and Poulton, J.E. (1992b) Tissue and Subcellular Localization of Enzymes Catablizing (*R*)-Amygdalin in Mature *Prunus serotina* Seeds. *Plant Physiol.* **100**, 291-300.

Till, I. (1987) Variability of expression of cyanogenesis in white clover (*Trifolium repens* L.). *Heredity.* **59**, 265-271.

Till-Bottraud, I. and Gouyon, P.H. (1992) Intra-versus interplant batesian mimicry? A model on cyanogenesis and herbivory in clonal plants. *Am. Nat.* **139,** 509-520.

Torres, E., Pereira, J.P., Brinker, A.M. and Seigler, D.S. (1988) Determination of Total Nitrogen and Cyanide Nitrogen in Cassava. *J. Sci. Food Agri.* **42,** 149-156.

Vicari, M. and Bazely, D.R. (1993) Do grasses fight back? The case for antiherbivore defences. *Tree* **8**, 137-141.

Woolhouse, A.D. and Morgan, D.R. (1995) An evaluation of repellents to suppress browsing by possums. *Journal of clinical ecology.* **21**, 1571-1583.