The Development of Assays for Atractyloside and its Localisation in Rat Tissue

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Submitted in partial fulfilment
of the requirements for the degree
of Doctor of Philosophy
in the
Department of Biochemistry
University of Natal

Pietermaritzburg *1991*

This thesis is dedicated to Ron Berry

as a token of appreciation for all his

support, advice and assistance

during the course of this study

PREFACE

The experimental work described in this thesis was carried out in the Department of Biochemistry, University of Natal, Pietermaritzburg from January 1987 to December 1991, under the supervision of Prof. M.F. Dutton, the co-supervision of Dr T.R. Anderson, and at the Roben's Institute for Industrial and Environmental Health and Safety, University of Surrey, Guildford, England under the direction of Dr Peter Bach.

The studies represent original work by the author and have not been submitted in any other form to another University. Where use was made of the work of others, it has been duly acknowledged in the text.

Sandra Noël Bye

Twelth day of December 1991.

ABSTRACT

An extract of the tuber of *Callilepis laureola* is regarded as the source of a powerful therapeutic agent, known as *Impila*. Its use is associated with fatal hepatic and renal necrosis, the renal toxin being atractyloside (ATR). The aims of this study were three-fold. Firstly, to generate a model set of biological specimens (urine, serum, liver and kidney) from rats dosed with 5-25 mg ATR/kg bwt. Secondly, to develop a competitive ELISA and HPLC method for the diagnosis of ATR poisoning employing the model specimens as test samples. Thirdly, to localise the target organs, cells and organelles of ATR, *in vivo*.

The HPLC method necessitated the systematic development of the derivatisation of ATR with 9-anthryldiazomethane, sample clean up employing hexane, methanolic hydrochloric acid and a silica minicolumn, as well as the chromatographic conditions. Optimal resolution was obtained with a 3.9 × 150 mm NovaPak reverse phase column, fluorescence detection (excitation = 365 nm, emission = 425 nm) and a solvent system of MeOH:1M ammonium acetate:1M glacial acetic acid:water (38:2:2:58). This method has a detection limit of 0.001 ng ATR, shows a mean recovery of 89% and detected approximately 6.7 µg ATR/g wet weight of tuber tissue. The toxin was also detected in some of the urine samples at levels of about 200 pg/ml, but not in the serum.

The production of antibodies to ATR for use in the ELISA and immunocytochemical investigations required the investigation of the conjugation procedure, carrier type, host species and immunization protocol. Optimal antibody yield, specificity and affinity was obtained with an acid-treated *Salmonella minnesota* bacterial carrier conjugated to ATR by carbodiimide, although there were indications of class switch inhibition and T-lymphocyte suppression by ATR. The development of the ELISA yielded a protocol involving the coating with a bovine serum albumin-ATR conjugate, blocking with bovine serum albumin, incubating the primary antibody at 4°C and detection with a secondary antibody-alkaline phosphate conjugate. This method detected ATR in both urine and serum from ATR-dosed rats and shows a detection limit of 10 ng. Since the less sensitive ELISA detected ATR in samples where the HPLC did not, this suggested that ATR is biotransformed *in vivo*, such that its retention time on a reverse phase column is affected, but not its epitope determinants.

The results of the organ function assays demonstrated that, when administered intraperitoneally, ATR is not hepatotoxic, but is a powerful nephrotoxin, targeting for the microvilli of the brush border of the proximal tubules, and compromising glomerular

permselectivity and distal tubular function. In addition, this toxin inhibits proline transport in the proximal tubule, and therefore probably affects protein biosynthesis. Renal regeneration is noted 3 days post-dosing, as demonstrated by calcium excretion.

Immunocytochemistry was optimised on tuber tissue and necessitated the intracellular fixation of the toxin, using carbodiimide, to prevent leaching out of the ATR. The toxin was encapsulated in vesicles in the tuber tissue. Atractyloside was also located in the kidney of ATR-treated rats, up to 72 hours after exposure, targeting the microvilli of the proximal tubule brush border, the mitochondrial cristae and specific sites on the Golgi apparatus membrane. Microvilli disruption and mitochondrial swelling was noted within 24 hours after exposure to the toxin while after 72 hours, loss of mitochondrial integrity was observed.

The development of these diagnostic assays for ATR have provided the means to monitor the levels of this toxin in plant extracts and mammalian body fluids. Future work should include the identification of the hepatotoxin associated with *Impila*, the effects of the route of administration on the toxicity of this remedy and furthermore, the identification of a suitable antidote, which could include the use of duramycin and stevioside. The association between compounds blocking the ADP/ATP antiporter in the c-state and Reye's syndrome should also provide an interesting area of research.

ACKNOWLEDGEMENTS

An undertaking of this magnitude is not completed singlehandedly, and I wish to express my sincere gratitude to the following people for their contribution:-

My supervisor Prof. Mike Dutton, for his enthusiasm, support and open-mindedness in allowing me the freedom to pursue my own field of interest, and also for encouraging and enabling me to interact with overseas scientists, both at conferences and at the Robens Institute for Industrial and Environmental Health and Safety, not to mention the Haverigg Working Mens' Club;

My co-supervisor Dr Trevor Anderson, for his critical appraisal of the draft manuscript and his encouragement;

Dr Clive Dennison, for all his advice and encouragement;

Theresa Coetzer, for introducing me to the field of immunology, her invaluable advice and generous gifts of immunoreagents;

Edith Elliott, for her friendship, advice and assistance, particularly with the immunocytochemistry, not to mention the generous gift of her precious gold probe;

Jenny Schwartz, Lesley Brown, Cheryl Brown, Mel Webber, Elias Mchunu and George Mdladla, a very big thank you for all their patience and help during my five years in the Department;

John Geyser and Sherwin Chetty, for unflinchingly pandering to my whims, fixing the equipment and, in particular, redecorating my laboratory red;

The librarians, who patiently accepted my overdue books and in particular the inter-library loan staff;

Tony Bruton, Vijay Bandu, Belinda White, Priscilla Donnelly and Geraldine Bruce of the Electron Microscope Unit, Pietermaritzburg for their patience, concern and unstinting support;

AECI for their more than generous support for the major part of this study which enabled me to attend the International Association of Forensic Scientists' Toxicology Congress in Glasgow (1989), the First Ethnopharmacology Congress in Strasbourg (1990) and visit the Robens Institute. Thanks also to my mentors, Les Pallent, Colin Kenyon and Rocky Skeef;

The CSIR Foundation for Research and Development for the financial assistance at the start and towards the end of this study;

Dr Peter Bach for generously inviting me to visit the Robens Instituite and for sharing his knowledge with me. A special thanks to Dr Ligia Delacruz, Merce Moret, Sally Nicholls, Ian MacManus, Gail Sutherland, Lisa Wilkes, Joanne, Monique, Margaret, Michelle MacClean and Prof. Patricia Berjak (who first suggested the visit);

Dr Graham Thurman, Fred Kruger and the staff at the Biomedical Resource Centre, University of Durban-Westville, for their professionalism, advice and assistance, far beyond the call of duty;

Prof. J.P. Nel, who first suggested this project and who was a great source of inspiration;

Dr Linda-Gail Becker and the laboratory staff at the Eshowe Hospital for their co-operation;

Marion Hundley, for her encouragement and loan of the Beckman System Gold;

The staff at the King Edward VIII, hospital for willingly carrying out the AST and ALT liver function tests;

In no specific order, a very big thank you to, the late Richard Butler (Premier Technologies) for his interest, cheerfulness and support both to myself and the Department; Nicci Difford (Weil Organisation) for her amazing information retrieval system and the "trial packs"; Elaine Hampton (Protea) for her cheerfulness and allowing us the use of the Kontron Integration Pack; Lizette Linder (Millipore Waters) for her friendship, use of the photodiode array and capillary electrophoresis, as well as her relentless interest in this project; Steve Walford (Millipore Waters) and Daniel Garside (Beckman) for their relentless pursuit of an HPLC method for atractyloside; Lorraine Smith and Mike Adams (Beckman) for their invaluable assistance, particularly in mediating the AST and ALT analyses. Arthur Peterson (Perkin Elmer) for all his support and encouragement;

My dear friends Jeanette Mackay, Janet Lee, Janet and Neville Spurr, Dr Andre van der Hoven for all the support and encouragement; Sally Schwarbe, for her help with the diagrams and both Jeanette Mackay and Jackie Woolfe-Piggott for their help with the proof-reading;

My office mates Frieda Dehrmann and Ron Berry, for their friendship, patience and help throughout the course of this study; it certainly made life much easier;

Finally to my grandparents, brothers Ian and Alan and my parents who never stopped believing in me, my sincerest gratitude.

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LIST OF ABBREVIATIONS

®	registered trade mark
α	separation factor
Ab	antibody
ABTS	2,2'-Azino-di(3-ethylbenzthiazoline sulphonic acid)
ACN	acetonitrile
ADAM	anthryldiazomethane
ADP	adenosine -5'-diphosphate
Ag	antigen
ALT	alanine transaminase
AP	alkaline phosphatase
AST	aspartate transaminase
ATP	adenine-5'- triphosphate
ATR	atractyloside
ATR-ADAM	atractyloside derivatised with anthryldiazomethane
ATR-ovalbumin	atractyloside conjugated to ovalbumin
B-B	BSA reacted with carbodiimide
BMRC	Biomedical Resource Centre
BSA	bovine serum albumin
BSA-ATR	bovine serum albumin-atractyloside conjugate
CAMOR	carrier agent-modified residues
CATR	carboxy-atractyloside
conc.	concentrated
CV	Coefficient of variation
Da	Daltons
Dept.	Department
DMF	N,N-dimethyl formamide
DNA	deoxyribonucleic acid
$\Delta\psi$	membrane potential; the difference in electric potential between two phases separated by a membrane
Earles' BSS	Earles' Buffered Salt Solution
EDTA	ethylene diamine tetra-acetic acid
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
env	envelope protein from HIV
FP	false positive

GA	Golgi Apparatus
GGT	γ-glutamyl transferase
GMT	Greenwich Mean Time
GN	glomerular nephritis
HEPES	N-2-Hydroxyethylpiperazine-N'-ethanesulphonic acid
HIV	human immunodeficiency virus
HPLC	high performance liquid chromatography
hr	hour
i.p.	intraperitoneal
Ig	immunoglobulin
IgG	immunoglobulin G
IgM	immunoglobulin M
IgY	immunoglobulin isolated from egg yolks
k'	capacity factor
KDO	2-keto-deoxyoctonate
kg bwt	kilograms body weight
λ	wavelength
LDH	lactate dehydrogenase
MeOH	methanol
NAD^+	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide (reduced)
$NADP^+$	nicotinamide adenine nuclectide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
O-O	ovalbumin reacted with carbodiimide
PAPS	adenosine-3'-phosphate-5'-phosphosulphate
PBS	phosphate-buffered saline
PEG	polyethylene glycol
pers. comm.	personal communication
P_i	inorganic orthophopshate
R-R	RSA reacted with carbodiimide
R.H.	relative humidity
RAST	radioallergosorbent
RER	Rough Endoplasmic Reticulum
RIA	radioimmunoassay
RIIEHS	Robens Institute for Industrial and Environmental Health and Safety
RNA	ribonucleic acid

RSA	rabbit serum albumin
RSA-ATR	rabbit serum albumin-atractyloside conjugate
s.c.	subcutaneous
saline-Tween	TBS containing 0.01% Tween® 20
SD	standard deviation
TBS	0.02 M tris buffered saline, pH 7.4
THF	tetrahydrofuran
TLC	thin layer chromatography
TNBS	2,4,6-Trinitrobenzenesulphonic acid
Tris	Tris (hydroxymethyl) aminomethane
Tween® 20	polyoxyethylene sorbitan monolaurate
UV	ultraviolet
v/v	volume by volume
VBS	veronal buffered saline
V_M	column void volume
V_R	retention volume
w/v	weight by volume
w/w	weight by weight
ψ	electrical potential
z	relative incorporation of radiolabelled amino acid
™	trademark
μ	ionic strength
Ω	electric resistance (ohm)

GLOSSARY

Insangoma	Zulu diviner who consults with the ancestors to identify the cause of an illness
Inyanga	Zulu herbalist
Umuthi omhlophe	(Zulu) white medicine, used for the amelioration of physical symptoms
Umuthi omnyama	black medicine, used to exorcise evil spirits
Impila	Zulu medicine prepared from the tuber of *Callilepis laureola*, which is believed to have magical properties
ikhubala	Zulu name for a medicine which is prepared from the hypogean organs of a plant
muthi	Zulu = remedy
Gogo	Zulu = grandmother
Atractyloside	a toxic diterpene glycoside
Atractylosides	analogues of atractyloside containing the basic diterpene ring and glycoside moeity

The Third Defence

"... what is there in all that God has created that is not blessed with goodness for the benefit of mankind. Why then should a poison be cast aside and condemned, since we are exploring not the poison itself, but the nature from whence it came. ... Omnis purgatio venenum si non sereutur dosis (every cathartic is a poison if it is not administered in the proper dose): let us start with your cathartics. In all your books, do you find a purgative that is not a poison or a substance that could not cause death when administered if not needed, and when the dose is not given proper consideration. Now consider the primary point, namely, that it (the dose) must not be too much nor too little. The one who chooses the middle dose, does not contribute to an intoxication. ... If you want to explain each poison correctly; what is there that is not poison, all things are poison and nothing (is) without poison. Solely the dose determines that a thing is not a poison. Nil sine veneno presertim dosi non seruari (nothing is without poison if the quantity is not considered): for example, every food substance and every drink is poisonous when ingested above its dose; this has been proven by the effects. ... You should understand one thing, namely, whether your prescriptions, which you say are harmless, can or cannot heal ... or whether you can cure with your sugar of roses (rosat) St. Vitus dance, the "lunaticos" or similar diseases. Truly you did not cure these diseases with sugar and you will not. ... You do not admit that it (Vitriol - sulphuric acid) is a poison; you say that it is a "corrosive". Tell me just how you render it corrosive, else, it is not a corrosive. If it can be changed in to a corrosive then it can also be converted into a "dulcidinem" (diethyl ether - sweet spirit of vitriol), this depends on the preparation. It is this way with vitriol as well as with any other substance which can undergo chemical changes. For instance, the food placed on the table: if eaten by man, it becomes the flesh of man; if ingested by a dog, it is converted to dog flesh and in the cat, to cat flesh. With the medicine it is the same, its fate depends on the species or what you do with it. It is possible that something good will cause harm, just as it is possible that something harmful may become beneficial. No one should condemn something whose "transmutation" he does not understand. And while a thing may be a poison, it may not cause poisoning. ... A person who wants to reprimand others should first acquire knowledge so that he might not be rebuked. ... Recognise that that which is beneficial for man is not a "poison", only that is a poison which does not benefit him and which is harmful ... ".

Paracelsus (1492-1541) taken from the translation by Deichmann et al. (1987)

CHAPTER 1

INTRODUCTION

Reliance on the therapeutic properties of indigenous plants is common to peoples of developing countries, and the Zulu population of Southern Africa is no exception. These people present the classic scenario of culture and economics influencing choice of health care. The Zulus maintain disease to be a manifestation of disharmony between a person and the ancestors (Bryant, 1909). Sorcery and medicine are inextricably linked and it is the function of the traditional healers, regarded as the "protectors of society", to be diagnosticians, apothecaries and diviners (Krige, 1981).

The *Insangoma* or diviner is the diagnostician and augur of society, identifying an affliction by consulting with the spirits. The herbalist or *Inyanga* has no spiritual association, merely dispensing various remedies prepared from roots, berries, animals and insects (Kiernan, 1978). The tangible link between magic and medicine has resulted in two distinct categories of traditional medicine. White medicine (*Umuthi omhlophe*) is used to ameliorate physical symptoms, while black medicine (*Umuthi omnyama*) exorcises evil spirits. Invariably *umuthi omnyama* is an emetic, prepared from leaves or roots which are either bruised or powdered, prior to extraction in a suitable solvent (Krige, 1981).

Despite routine use of some 400 herbs for medicinal purposes, the pharmacological properties are unknown and many contain toxic principles (Gundidza, 1985). While some remedies are beneficial, or at worst innocuous, others are highly toxic. The precise incidence of herbal-mediated deaths is unknown however, as no specific diagnostic assays are available. Therefore, officially, such intoxications do not occur and the indiscriminate fatalities continue unchecked.

One of the more popular Zulu *umuthi omnyama*, called *Impila*, is prepared from the tuber of *Callilepis laureola* and there is evidence that each year this herb is responsible for numerous deaths from liver and kidney necrosis. For instance, over the period 1971 to 1977, this remedy was reputed to be the major aetiological factor in at least 74 deaths at the King Edward VIII Hospital, in Durban. *Impila* contains the nephrotoxin atractyloside (ATR) which is believed to be the major causative agent for the high mortality rate among patients using *Impila* (Bhoola, 1983). It was Prof. J.P. Nel's (Head of the Dept. Forensic Medicine, University of Natal, Durban) observations concerning the repeated incidences of *C. laureola*-mediated deaths in Natal, that stimulated him to suggest the subject of this study.

Atractyloside was first isolated from the thistle *Atractylis gummifera* (Santi and Luciani, 1978), the tuber of which is also used for medicinal purposes. Atractyloside is a diterpene glycoside (Fig 2.3, page 11), with the carbohydrate portion consisting of a single glucose molecule, to which are bound two bisulphate esters and isovaleric acid. The aglycone portion, atractyligenin, contains a penhydrophenanthrenic structure (Bruni *et al.*, 1962). A structural analogue to adenosine diphosphate (ADP), this toxin competitively associates with the ADP binding site on the ADP/ATP antiporter, terminating nucleotide exchange and therefore, oxidative phosphorylation (Chappell and Crofts, 1965).

At present, no reliable and specific diagnostic assay exists for the detection of *Impila* or more specifically, ATR poisoning. The toxin is detected by thin layer chromatography (TLC) (Brookes, 1979), but the detection limits of this method restrict its diagnostic potential. Thus, *Impila* poisoning may only be identified by histological means and the results are not conclusive. The suspected high incidence of ATR intoxication, not only in Southern Africa, but possibly throughout Africa and other parts of the world, warrants development of a specific assay for ATR, which could be used both for aiding early diagnosis so that prompt therapeutic action may be taken, and also for post-mortem investigations. Armed with statistical information relating to the incidence of *Impila* poisoning or fatalities, authorities could be pressured to take cognisance of the situation and act appropriately to limit the use of *Impila*.

The objectives of this study were three-fold. It aimed, first, at generating a model set of biological specimens (urine, serum, liver and kidney) from rats dosed with different levels of ATR. The second objective was to develop high performance liquid chromatography (HPLC) and enzyme-linked immunosorbent assays (ELISA) for the estimation of ATR in these specimens, with the ultimate intention of providing a method that could be applied to the screening of human body fluids, tissues and medicinal plants. Since no routine analytical HPLC method for ATR existed, it was necessary to develop the entire procedure, which included optimisation of sample preparation, derivatisation, and the stationary and mobile phases of the chromatographic system. Similarly, the development of an ELISA for ATR necessitated production of antibodies, a procedure which also had to be optimised, as there are no previous reports of antibodies being raised to this toxin.

The third aim of this study was to sequentially localise the target organs, cells and organelles, of this toxin *in vivo* by screening for liver and kidney function markers in the serum and urine respectively, and employing histochemical and immunocytochemical

techniques. Since immunocytochemical detection of ATR had not been carried out prior to this study, a substantial amount of procedure optimisation was necessary, both on *C. laureola* tuber and rat tissue.

This thesis deals with each aim in a separate chapter, the results of which are ultimately correlated in the final discussion. As the study involved a considerable amount of method development, only the final optimised procedures are detailed in the Materials and Methods (Chapter 3), whereas the developmental aspects of the methodology are covered separately in their respective chapters (4 to 8).

CHAPTER 2

LITERATURE REVIEW

The high incidence of liver and renal necrosis in the Negroid population of Natal prompted investigations aimed at identifying the causative agent. It was serendipitously discovered that a herbal remedy called *Impila* was responsible for this pathology. The purpose of this literature review is to give a description of the botanical characteristics and geographical distribution of *C. laureola*, the herb from which *Impila* is prepared, and the alleged therapeutic benefits of this remedy. The description of the preparation and use of this herb is in part the result of personal interviews with *Insangomas* and their clientele. Thereafter, the clinicopathology of *Impila* poisoning is presented as a synopsis of the currently available information pertaining to the physical symptoms, as well as the biochemical and morphological alterations in humans and animals following exposure to *Impila*, ATR or plants biosynthesising this toxin. The chemistry of ATR is outlined, together with the incidence of ATR in plant species other than *C. laureola*. As a conclusion, the chronological elucidation of the action of ATR is given, together with its effects on intra-mitochondrial calcium stores and post-translational sulphation in the Golgi apparatus (GA).

2.1 Classification and distribution of *Callilepis laureola* and its use in herbal remedies

Reclassified as a member of the family Asteraceae (previously Compositae), *C. laureola* is commonly called the ox-eye daisy, wildemargriet or jakkelsgras (Brookes *et al.*, 1983). This perennial bears a tuberous root, likened to a sweet potato (Fig 2.1). The leaves are ovoid, occasionally bearing pilose hairs. Stems average 60 cm in length and when mature, both stem and leaves dry a characteristic black. The plant flowers from August to November, bearing a solitary off-white flower with a deep purple disc (Fig 2.2) (Dyer, 1975; Hilliard, 1977; Palmer, 1985).

Callilepis laureola is widely distributed in Natal, Southern and Eastern Transvaal, Swaziland, Transkei, Lebombo, Moçambique (Dyer, 1975; Hilliard, 1977) and the Eastern Cape (Steyn, 1949) but does not grow at altitudes above 1 800 m (Hilliard, 1977). The plant thrives in full sun and is pyrophytic (T. Edwards, pers. comm., 1988). Fresh tubers have little odour until sliced, but desiccated rootstock releases a pungent smell, comparable to dried peaches (Wainwright *et al.*, 1977) which permeates confined areas.

Fig 2.1 The tuber of *Callilepis laureola*, collected in 1989 from World's View Pietermaritzburg, Natal

Fig 2.2 The flowers of *Callilepis laureola*, picked in November, 1989 at World's View, Pietermaritzburg, Natal

The Nguni people (includes Zulu and Sotho) regard *C. laureola* as a valuable *muthi*. Random surveys in the Umlazi Township, Durban (urban) indicate that 30% of those questioned valued *C. laureola* as a medicinal herb and furthermore, had used or, were using it on a routine basis (Wainwright *et al.*, 1977). The decoction, which is known to the Zulu as *Impila* and the Xhosa as *"intsika yo-mhlaba"*, is prepared from the tuber (Debetto, 1978) although leaf infusions are reputed to have limited curative properties (Bryant, 1909). Classified as an *iKhubala* or "medicine which is dug up", the tuber is harvested in winter (June/July), dried, and crushed (Bryant, 1909; Ellis, 1986). Alternatively, a piece of fresh tuber the size of a forefinger is bruised and chopped (Wainwright *et al.*, 1977), whereupon the pulp is boiled for 30 minutes in a suitable volume of water and the decoction given orally or rectally (*Insangoma*, pers. comm., 1987). A warm effusion may be prepared in milk, or the raw tuber simply chewed and swallowed (Ellis, 1986), but these methods are less popular.

Essentially a broad spectrum *muthi*, *Impila* is taken to ameliorate gastrointestinal disorders and tapeworm infestations (Bryant, 1909), to promote fertility (Debetto, 1978), as an expectorant and whooping cough remedy (Maberley, 1905) as well as an appetite promoter. Of the twenty patients questioned in a Drakensburg hospital (rural), ten admitted using *Impila*; eight for stomach disorders and two for coughs (Ellis, 1986). The protective powers of the plant are, however, considered its greatest attribute with a tuber buried in the vicinity of a home believed to intercept evil directed towards the household (C. Dlamini, pers. comm., 1988), while consumption of the liquor extract prior to celebrations is thought to protect one from evil (Wainwright *et al.*, 1977). A daily spoonful of the infusion is considered to promote well-being and may also be added to a child's bathwater (*Gogo* (grandmother), pers. comm., 1987). *Impila* may also be prepared in conjunction with other herbs (Bryant, 1909), or worn about the neck for protection.

Unfortunately, *Impila* is not an innocuous *muthi*, and there is evidence to implicate the use of this decoction with a multiplicity of life-threatening conditions. These are discussed in the next section.

2.2 The clinicopathology of *Impila* poisoning

There has been a significant amount of research into the use of *C. laureola* in remedies although one may question such general interest, since it is only one of 400 herbs used for medicinal purposes. In the mid-1970s the high incidence of centrilobular zonal liver necrosis, regarded as the commonest cause of fatal hepatic necrosis in Negroid Natalian residents, was cause for concern. Of unknown aetiology, over the period 1958 to 1977, this

necrosis, which was invariably accompanied by renal damage, accounted for 2.06% of deaths at the King Edward VIII Hospital, Durban (Bhoola, 1983). Extensive hepatic zonal necrosis can result from exposure to toxins (Netter, 1979). Since 85% of Negroid patients have consulted a traditional healer prior to hospital admission (Bhoola, 1983), Wainwright *et al.* (1977) were able to investigate the potential hepatotoxicity of five common herbal remedies. All these remedies, however, failed to elicit the characteristic hepatic and renal damage in laboratory animals.

Unfortunately, *Impila* was not included in the above studies. The nephrotoxicity of *Impila* was first noted in 1971, when a man demonstrated renal failure after being treated with this herb for impotence, by an *Inyanga*. As he survived and had no evidence of hepatic damage, no correlation was made between the use of this herb and centrilobular liver necrosis (Seedat and Hitchcock, 1971). The association was only made in 1977, when a six-month old child died from liver and renal failure after receiving a herbal enema. The tuber used by the mother to prepare the enema was identified as *C. laureola* and subsequent tests confirmed the toxicity of the herb (Wainwright *et al.*, 1977; Bhoola, 1983).

A methanol extract from the tuber, and subsequent purification of the major extraction product, indicated that the predominant toxin was the diterpene glycoside ATR, which is produced by several species of Compositae (Wainwright *et al.*, 1977). While it is appreciated that plants containing atractylosides may biosynthesise other toxins, for the sake of clarity, the clinicopathology presented by animals or humans poisoned by ATR-containing plants will be considered as if ATR is the primary source of toxification.

Tables 2.1 to 2.3 outline the clinicopathological changes in rats, man, calves and pigs, following administration of ATR, carboxy-atractyloside (CATR), or plants known to biosynthesise these toxins. The three tables, which constitute an extensive review by the present author, cover physical manifestations of poisoning, biochemical changes in serum or urine and morphological alterations, respectively. As seen in Table 2.1, coma is common to all species, regardless of dose, form, or route of administration, while vomiting is also prevalent. Hypoglycaemia is noted, as is liver and renal necrosis. There is a dispute as to whether ATR *per se* is hepatotoxic and whether this diterpene specifically targets the proximal renal tubule, as distal tubule alteration has also been observed in rodents (Hatch *et al.*, 1982). The main conclusions drawn are that regardless of the species of mammal under investigation, atractylosides induce both hypoglycaemia and renal necrosis. The nature of the hepatotoxic agent has not been clearly identified and it was suggested that this could be a metabolite of ATR, as rats only demonstrated liver necrosis 24 hours after being given an *Impila* extract (Wainwright *et al.*, 1977).

Table 2.1 Physical symptoms manifested in man, rats, pigs and cattle following intoxication by either atractylosides or plants biosynthesising these diterpene glycosides.

Plant or Compound	Species	Route and Dose	Symptoms*	References
Callilepis laureola	Man	Oral/rectal	A, B, C, D	Bhoola (1983)
Atractylis gummifera	Man	Oral/rectal?	A, B, C, D, E, F	Georgiou *et al.*, (1988)
CATR	Male CD rats	1, 2, 4, 8, 16, and 32 mg/kg bwt , i.p.	A, B, H, I, J	Hatch *et al.*, (1982)
CATR	Pigs	Oral 25-100mg/kg bwt	A, C, L (3-24 hrs), N	Stuart *et al.* (1981)
	Pigs	5-100 mg/kg bwt, i.v.	do.	
Xanthium strumarium	Feeder pigs	Oral 0.75-3% m/m single dose	N	Stuart *et al.* (1981)
(seedlings)		3% daily oral dose	A, B, K, N	
		10/20/30% w/w in feed	10% L	
Xanthium strumarium	Pigs	Oral (aqueous extract)	A, C, M, N	Stuart *et al.* (1981)
Xanthium strumarium	Hereford calves	Oral	A, B, G, N	Martin *et al.* (1986)

* A = coma, B = vomiting, C = epigastric pain, D = jaundice, E = respiratory failure, F = brain edema, G = gastritis and enteritis, H = increased depth of respiration, I = seizures, J = cerebral inoxia, K = 100% fatality, L = no symptoms, M = increased vascular permeability, N = Depression, recumbency, muscular weakness

Table 2.2 Biochemical alterations in urine and serum of rats, man, calves and pigs following intoxication with atractylosides or plants known to biosynthesise these diterpene glycosides

Plant or Compound	Species	Route and Dose	Biochemistry*	Other Symptoms	Ref.†
ATR	Male albino rats	I.P. 10-100 mg/kg bwt	A, D, E	Uremia, excretion of protein, glucose and ketone bodies, K^+ and Na^+. Serum urea ↑ 3 × with ↓ in urinary urea	1
Callilepis laureola	Man	Oral/rectal	A, B, C, D, E	Urine contained albumin, blood, glucose, ketone bodies and bilirubin	2
Atractylis gummifera	Man	Oral/rectal?	A, B, D, E	–	3
Xanthium strumarium	Hereford calves	Oral	F	Neutropenia	4
Xanthium strumarium (seedlings)	Feeder pigs	Oral 0.75-3% w/w	A, B, D, E, F,	–	5
		(Single dose)	G, H, I	–	
		Oral 3% daily dose	do.	–	
		10/20/30% in feed	do.	–	
Xanthium strumarium	Pigs	Oral	B, D, E, F, G, H, I	–	5

* A = Increased serum Bilirubin, B = Hypoglycæmia, C = Elevated serum lactate dehydrogenase, D = Elevated serum aspartmine transaminase, E = Elevated serum alanine transaminase, F = Elevated γ-glutamyl transferase, G = Elevated isocitrate dehydrogenase, H = Elevated sorbitol dehydrogenase, I = Elevated alkaline phosphatase, ↑= increased, ↓ = decreased.

† 1 = Carpenedo *et al.* (1974), 2 = Bhoola (1983), 3 = Georgiou *et al.* (1988), 4 = Martin *et al.* (1986), 5 = Stuart *et al.* (1981).

Table 2.3 The histological and morphological alterations following exposure to atractyloside or atractyloside containing plants

Plant or Compound	Species	Histology*	Other Symptoms	Ref.†
ATR from tuber	Rats	B, E	No alteration in heart, liver or lung morphology 1 hr: mild nephrotubular damage 3 hr: widespread tubular necrosis, casts and debris in tubules 5 hr: frank, widespread necrosis 24 hr: established lesion 48 hr: evidence of regeneration	1
ATR (pure)	Rats	B, E,	As above	1
ATR	Rats	B, E	No liver changes. After 150-180 mins, inner zone of kidney cortex had deep lesions confined to distal portion of the proximal convoluted tubules. Distal tubules were filled with granular casts. Glomerulus intact. Regeneration 2 days post-dosing	2
CATR	CD Rats	A	Vacuolated hepatocytes with single large cytoplasmic vacuoles and necrosis. Distal not proximal renal tubule damage noted. If animals survived 24 hr after dosing, recovered completely	3
Callilepis laureola	Rats	A, B	–	1
Callilepis laureola	Man	A, B, C, D, E	Liver pale yellow and speckled. Hepatocytes peripheral to necrosis contain lipid vacuoles. Some reticular frame-work collapse. Defective clotting mechanism. Kidney mass increased by ± 30%. Renal cortex pale and swollen. Medulla congested. No difference in gender susceptability. Most victims< 14 years. 91% fatal	1
Atractylis gummifera	Man	A, B, C	Diffuse hepatocyte necrosis, collapse of interstitial conective tissue. Tubule renal necrosis, brain œdema. Pleomorphism of nuclei and fatty degeneration of hepatic cells. Intra-hepatic cholestasis	4
Xanthium strumarium	Calves	A, B, C, D	Blood covered faeces. Liver tan with mottled capsular surface. Rectal mucosa vessels showed vascular engorgement and surface epithelial necrosis. Mucosal surface of œsophagus and abomasum had multifocal ulceration	5
Xanthium strumarium	Pigs	A, B, C, D	Liver lesions noted. Serofibrinous ascites and œdema of gall-bladder wall. Fatty gastro-hepatic ligament. Large fibrous strands or gelatinous clots on liver surface and other viscera. Liver congested and had centrilobular necrosis. Some pericardial, pleural, subcutaneous or intramuscular serous effusion. Gall-bladder wall distended by proteinaceous fluid. Acute diffuse centrilobular liver necrosis. Often necrosis of whole lobule. Extensive karyolysis and karyohexis of hepatic nuclei. Mild to moderate acute tubule renal degeneration. Mild to moderate gastric congestion and hæmorrhage. Brain œdema present in some pigs	6

* A = Centrilobular liver necrosis, B = Proximal tubular renal necrosis, C = Hæmorrhage, D = Blood-filled liver sinusoids, E = Glomerulus unchanged

† 1 = Bhoola (1983), 2 = Carpenedo *et al.* (1974), 3 = Hatch *et al.* (1982), 4 = Georgiou *et al.* (1988), 5 = Martin *et al.* (1986), 6 = Stuart *et al.* (1981)

Since ATR is the predominant toxin isolated from plants such as *C. laureola*, consideration will be given to the chemistry and occurrence of this toxin and its associated analogues in the next section.

2.3 The chemistry and occurrence of the atractylosides

For the purposes of clarity, ATR refers to the diterpene glycoside, atractyloside, while atractylosides refers to analogues containing the penhydrophenanthrenic structure and glycoside moiety. The first chemical investigation into the structure of ATR (Fig 2.3) was made in 1867, by the French apothecary, Lefranc. Having isolated this toxin from the tuber of *Atractylis gummifera*, Lefranc proposed the structure of ATR, but his work was harshly criticised and disregarded. Angelico renewed investigations in 1906 and confirmed Lefranc's findings (Piozzi, 1978).

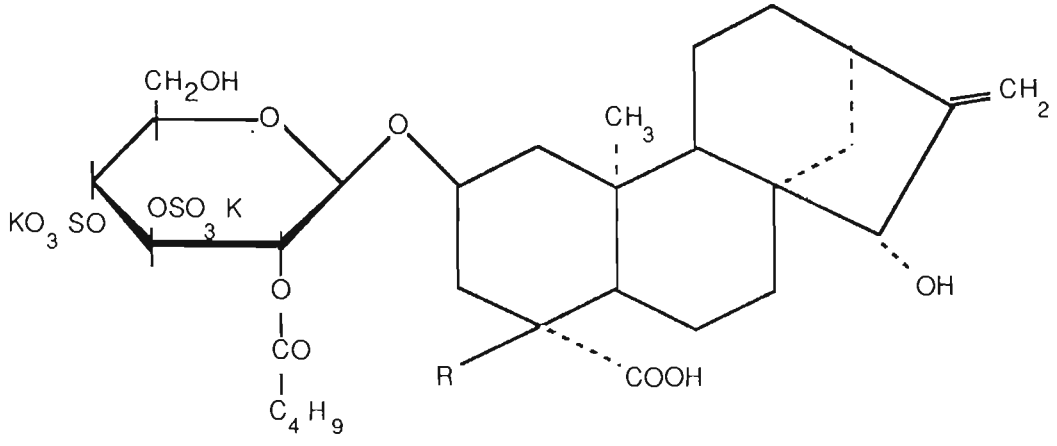

Fig 2.3 The structure of the atractylosides (potassium salt)
R=H, atractyloside (ATR); R=COOH, carboxy-atractyloside (CATR)

Potassium atractylate has the formula $C_{30}H_{44}O_{14}S_2K_2$ and a molecular mass of 803.0 Da (Dawson *et al.*, 1986). The hydrolysis products of ATR consist of one mole D (+) glucose, two moles sulphuric acid and one mole each of isovaleric acid and atractyligenin ($C_{19}H_{28}O_4$). The first studies into the elucidation of the structure of ATR were carried out on the diterpene ring, atractyligenin (Piozzi, 1978). This acidic hydrophenanthrenic structure contains a carboxyl group on C_4 of the diterpene ring, which was confirmed by chemical and infra-red spectral analysis and is apparently vital to the toxicity of the molecule (Piozzi, 1978).

Diterpenes are generally non-volatile and found in the resins of higher plants (Vickery and Vickery, 1981). Most tetracyclic diterpenes contain a basic 18-nor(-)kaurene

skeleton; kaurenes are a precursor to the gibberellins (Takahashi *et al.*, 1986) and atractyligenin is no exception, comprising a nor-diterpenoid acid of (-)kaurene (Ghisalberti *et al.*, 1968). Elucidation of the structure of atractyligenin was followed by work on ATR, specifically the glucose unit, and it was determined that the isovaleric acid and bisulphate esters are situated on positions 2', 3' and 4' respectively of the glucose moiety. Atractyligenin is joined to the glucose moiety via a β–glycosidic linkage (Brookes, 1979; Brookes *et al.*, 1983).

A precursor, carboxy-atractyloside (CATR) (Fig 2.3) has been extracted from thistle tubers (Bombardelli *et al.*, 1972). It has a second carboxyl group, on C_4 of the diterpene ring, and is 40 times more toxic than ATR. Carboxy-atractyloside is predominant in fresh tubers and undergoes decarboxylation during ageing or desiccation, to form ATR (Bombardelli *et al.*, 1972).

The toxicity of ATR is dependent upon the maintenance of its structural integrity (Vignais *et al.*, 1978). The aglycone is 150 times less toxic than ATR, indicating the importance of the glycoside moiety in potentiating toxicity. Removal of the isovaleric acid (apo-ATR) or the bisulphate esters reduces the toxicity some ten-fold. The carboxyl group on C_4 of the diterpene ring is, however, most crucial to ATR's inhibitory action, and reduction of this group to, for example, an alcohol (atractylitriol) renders the molecule non-toxic (Vignais *et al.*, 1978).

For centuries, the toxicity of *A. gummifera* has been documented and it has been used for suicidal and criminal poisonings throughout the ages (Piozzi, 1978). In the first century Discordes wrote of the toxicity and therapeutic properties of *A. gummifera*, in his "*De Materia Medica*". Previously named *Chamæleon* (reflecting the changing colours of its flowers), this plant was recommended as a treatment for drowsiness, depression, toothache and bleeding gums, intestinal parasites, snake-bite, ulcers and oedema (dropsy). In the sixteenth century an angel reputedly revealed *Chamæleon* to be a cure for bubonic plague. Despite the therapeutic potential of this herb, Theophrastas noted as far back as 300 BC that *Chamæleon* was fatal to animals, and its toxicity has been alluded to throughout the ages (Santi and Luciani, 1978). Today, the plant is used for the treatment of syphilis, as an abortifacient, to bleach teeth, for its emetic properties (Lemaigre *et al.*, 1975; Capdevielle and Darracq, 1980) to stop haemorrhage, and to facilitate childbirth (Melek *et al.*, 1989). A gum secreted from the leaves is used by children as chewing gum and to capture birds. *Atractylis gummifera* is often mistaken for the innocuous *Atractylis aucaulis*, the latter having anti-pyretic and diuretic properties (Georgiou *et al.*, 1988).

Atractyloside has also been isolated from *Wedelia glauca*, a plant indigenous to Argentina, Uruguay and Southern Brazil, where it is known for its lethality to livestock (Schteingart and Pomilio, 1984). The Australian plant *Wedelia asperima* is also highly toxic and synthesises wedelioside, an acylaminoglycoside diterpene related to ATR (Eichholzer *et al.*, 1981). The rhizomes of atractyloides (*A. lancea*, *A. japonica* or *A. ovata*) are used in Japan as diuretics and to stimulate gastric activity. *Atractylis lancea* tubers contain several atractylosides whose structures have been elucidated (Yahara *et al.*, 1989). This Chinese medicinal plant is reputed to heal gastric ulceration (Kubo *et al.*, 1983), increase bile secretion (Yamahara *et al.*, 1983), and is also used in China and Japan for rheumatism, abdominal distension, poor appetite and nausea, fluid retention and phlegm, oedema and mild diarrhoea. An acetone extract has anti-anoxic effects (Yamahara *et al.*, 1990), and interestingly, *A. gummifera* has been used to treat oxyurosis (Georgiou *et al*, 1988), indicating that this species of plant has some effect on the oxygen state in humans.

Coffea arabica L. beans contain atractylosides whose metabolites have been isolated from the urine of coffee drinkers (Piozzi, 1978). These ATR analogues have been implicated as mediators of pancreatic cancer in habitual coffee consumers (Pegel, 1981), but this has yet to be fully substantiated. The atractylosides derived from coffee beans differ from those isolated from *C. laureola* and *A. gummifera*, in that the glucose moiety (Fig 2.3) is replaced by glucuronic acid, which is devoid of the bisulphate esters and isovaleric group (Piozzi, 1978). Atractylosides are also present in the cotyledonary seedlings of the burr weed or cocklebur *Xanthium strumarium* (Craig *et al.*, 1976), and there have been repeated reports of livestock poisoning by this plant (Stuart *et al.*, 1981; Hatch *et al.*, 1982; Martin *et al.*, 1986).

It is clear from the literature that atractylosides occur in a wide variety of plant species which are frequently used for medicinal purposes, in many parts of the world, thus emphasising the urgency for a diagnostic assay for ATR. The toxicity of these plants is well documented, yet there are still reports of fatal intoxications (Capdeville and Darracq, 1980; Bhoola, 1983; Georgiou *et al.*, 1988). It is a personal observation that *Impila* poisonings are not uncommon in South Africa; the primary victims being infants. As increasing numbers of native women are opting to give birth in hospitals, there are now more opportunities to educate them on the dangers of administering herbal remedies. Blame would seem to lie, however, with the grandparents, who continue to value traditional medical practices and often administer the remedies against the wishes of, or unbeknown to, the parents. The situation is further exacerbated by the economic situation, as, soon after giving birth, the mother returns to her place of employment and the child is left in the

care of the *Gogo*. It is a personal opinion, therefore, that only continued education will limit the use of dangerous traditional remedies such as *Impila* and prevent these unnecessary deaths, as there is little chance of antidotes to these "remedies" being developed in the foreseeable future. The development of an antidote for ATR will be particularly difficult, as this toxin apparently has a number of inhibitory effects on the mitochondria, GA and mitochondrial calcium sequestration, and treatment would therefore have to be approached multidirectionally. Details of the action of ATR on subcellular function are presented in section 2.4.

2.4 The mechanisms of toxicity of atractylosides

The toxicity of ATR has been well documented over the centuries, but it was only in 1958 that the precise pharmacological action of this toxin was first investigated (Santi, 1958). Further elucidation of the precise inhibitory action of ATR resulted from its use as a biochemical probe in the study of its chronic effects on mitochondrial and Golgi function, as well as on calcium regulated systems. These effects are outlined in this section.

2.4.1 The effect of atractyloside on mitochondrial function

In 1958, Santi showed that when administered to rodents, rabbits and dogs, ATR induces transient hyperglycaemia, followed by hypoglycaemia, acidosis and decreased oxygen consumption (Santi, 1958; Luciani *et al.*, 1978). Santi proposed that ATR acts at a subcellular level, inhibiting the tricarboxylic acid cycle as well as systems regulating oxidative phosphorylation, and adenine trinucleotide synthesis (Santi, 1958). Subsequently, mitochondria were identified as the target organelles of ATR (Vignais *et al.*, 1962) and at the same time it was noted that the inhibitory action of this toxin is potentiated by magnesium ions. It was speculated that the magnesium ions neutralise the anionic sulphate esters on ATR, facilitating the binding of the toxin to the mitochondria (Bruni *et al.*, 1962).

Initially, the inhibitory action of ATR was considered to be similar to that of oligomycin (Bruni *et al.*, 1962), which blocks the catalytic activity of ATPase by binding to the hydrophobic region of the F_0 particle (Gale *et al.*, 1981), but subsequent experiments on liver mitochondria demonstrated that a distinction could be made between the inhibitory action of ATR and oligomycin (Chappell and Crofts, 1965). By 1965, it was established that ADP and ATR have a common binding site on the inner mitochondrial membrane (Kemp and Slater, 1964; Bruni *et al.*, 1965). In their classic experiment, Kemp and Slater incubated rat

liver mitochondria with [^{32}P]-P_i and monitored the effect of ATR on the incorporation of radioactivity into ATP and the results are shown in Fig 2.4.

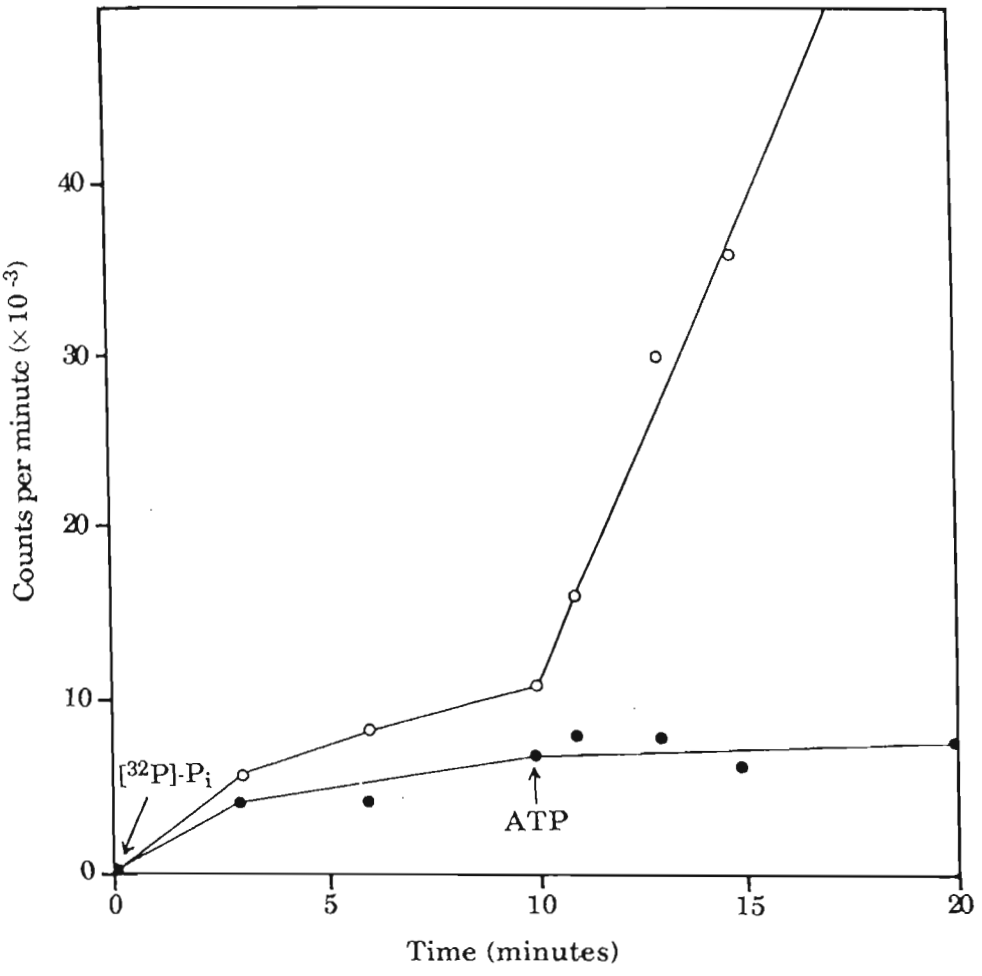

Fig 2.4 The effect of ATR on [^{32}P]-P_i incorporation into rat liver mitochondria (after Kemp and Slater, 1964) (• = in the presence of ATR; o = in the absence of ATR). Aliquots were removed at pre-determined time intervals and after 10 minutes incubation, ATP was added and further representative aliquots were removed. The samples were deproteinised with trichloroacetic acid (TCA) and the free [^{32}P]-P_i was extracted into benzene-isobutanol as a phosphomolybdate conjugate

From their results shown in Fig 2.4, Kemp and Slater concluded that the initial rate of labelling of endogenous ATP was not significantly less in the presence of ATR and from this they deduced that ATR does not inhibit oxidative phosphorylation *per se*, as residual ADP within the mitochondria was still being phosphorylated. They proposed that the site of action of ATR is on the mitochondrial membrane where it inhibits the reaction between

intra-mitochondrial ATP and extra-mitochondrial ADP (Fig 2.5). This proposal was later proven (Chappell and Crofts, 1965). From these results, it is personally believed that there is little basis on which to identify the site of action of ATR, and all that can be concluded is that ATR does not directly inhibit the synthesis of ATP, but rather prevents the access of ADP to the site of phosphorylation.

The experiment of Kemp and Slater (1964) could have furnished more information had it been further refined. Strictly speaking, after removal of the sample aliquot, the protein should have been precipitated in a solution of TCA containing "cold"-P_i to prevent non-specific adsorption of the radiolabel after sampling. In addition, a control devoid of mitochondria, and another containing mitochondria pre-treated with TCA, should have been included, to ensure that the measured radioactivity was truly representative of the amount of labelled ATP, and was not residual [^{32}P]-P_i which had not been removed by the extraction procedure. Nevertheless, the work of Kemp and Slater (1964) together with that of Bruni *et al.* (1964) and Santi (1964) provided the first critical clue to the precise inhibitory action of ATR. Further in depth studies by Chappell and Crofts (1965) demonstrated that ATR did not affect metabolism of internalized adenine nucleotides, but prevented use of cytoplasmic adenine nucleotides by the mitochondria. From these results it was concluded that ATR either inhibited transfer of adenine nucleotides across the mitochondrial membrane, or prevented inorganic phosphate from entering the mitochondrial matrix. The data collected supported both hypotheses, but the final conclusion was that ATR "...perhaps revealed the existence of a 'permease' that allows adenine nucleotides to be transferred across the mitochondrial membrane..." (Chappell and Crofts, 1965) and this hypothesis was to become a very significant finding in the elucidation of the mechanism of oxidative phosphorylation and cellular energy production.

It was proposed from the work of Chappell and Crofts (1965) and that of others, that ATR and ADP have a common binding site on the inner mitochondrial membrane (Bruni *et al.*, 1965) but it was Allman *et al.* (1967) who finally demonstrated that ATR binds to the cytoplasmic side of the inner mitochondrial membrane. In their following experiment, beef heart mitochondria were isolated; part of which were sonicated to produce submitochondrial particles. The intact mitochondria and submitochondrial particles were incubated separately in the presence of ATR and the exchange of ATP-P_i was calculated as a percentage of nucleotide exchange relative to a sample devoid of toxin. The results showed a 77% inhibition of ATP-P_i exchange in mitochondria incubated with ATR, whereas in the sonicated subparticles this inhibition was only 24%. Furthermore, in mitochondria that were preincubated with ATR prior to sonication and reincubated with ATR, the inhibition was 76%. Finally in submitochondrial particles where the inner

membrane was damaged, ATR inhibited ATP-P_i exchange by only 15%. From these results it was conclusively shown that the inhibitory action of ATR is dependent upon the maintenance of the inner mitochondrial membrane integrity and that ATR binds to a site on the cytoplasmic side of this inner mitochondrial membrane. Subsequently, it was also proven that ATR binds to a inner mitochondrial membrane antiporter, which specifically controls the permeability of the inner membrane (Poochiari and Silano, 1968). In doing so, the toxin inhibits nucleotide exchange, as the inner mitochondrial membrane is selectively impermeable to the passive transport of charged hydrophiles (Zalman *et al.*, 1980; Harold,1986).

As shown in Fig 2.5, ATP production and oxidative phosphorylation occur in the matrix of the mitochondria. The proton-motive force, necessary for energy transduction and oxidative phosphorylation, is dependent upon the establishment of both a pH and an electropotential gradient (ΔpH and $\Delta\psi$) across the inner mitochondrial membrane (Klingenberg, 1978; Nicholls, 1982). Atractyloside competitively inhibits the transport of endogenous (cytosolic) ADP across the inner mitochondrial membrane, by binding to the ADP-binding site on the inner membrane antiporter. Consequently, in terminating the passage of ADP across the inner membrane, ATP synthesis is prevented (Nicholls, 1982). The ADP-binding site on the antiporter is blocked by virtue of the analogous size and structure of ATR and ADP, as the phosphate esters, ribose moiety and purine ring of ADP are similar to the sulphate esters, glycoside moeity and diterpene ring of ATR respectively (Allman *et al.*, 1967). The aglycone, atractyligenin, is significantly less toxic than ATR, demonstrating the importance of the bisulphate ester-glycoside moiety and isovaleric acid residue in potentiating toxicity. The hydrophobic diterpene ring penetrates the inner mitochondrial membrane, but the strongly hydrophilic glucose moeity maintains ATR at the surface of the membrane, locking the antiporter (Vignais *et al.*, 1978).

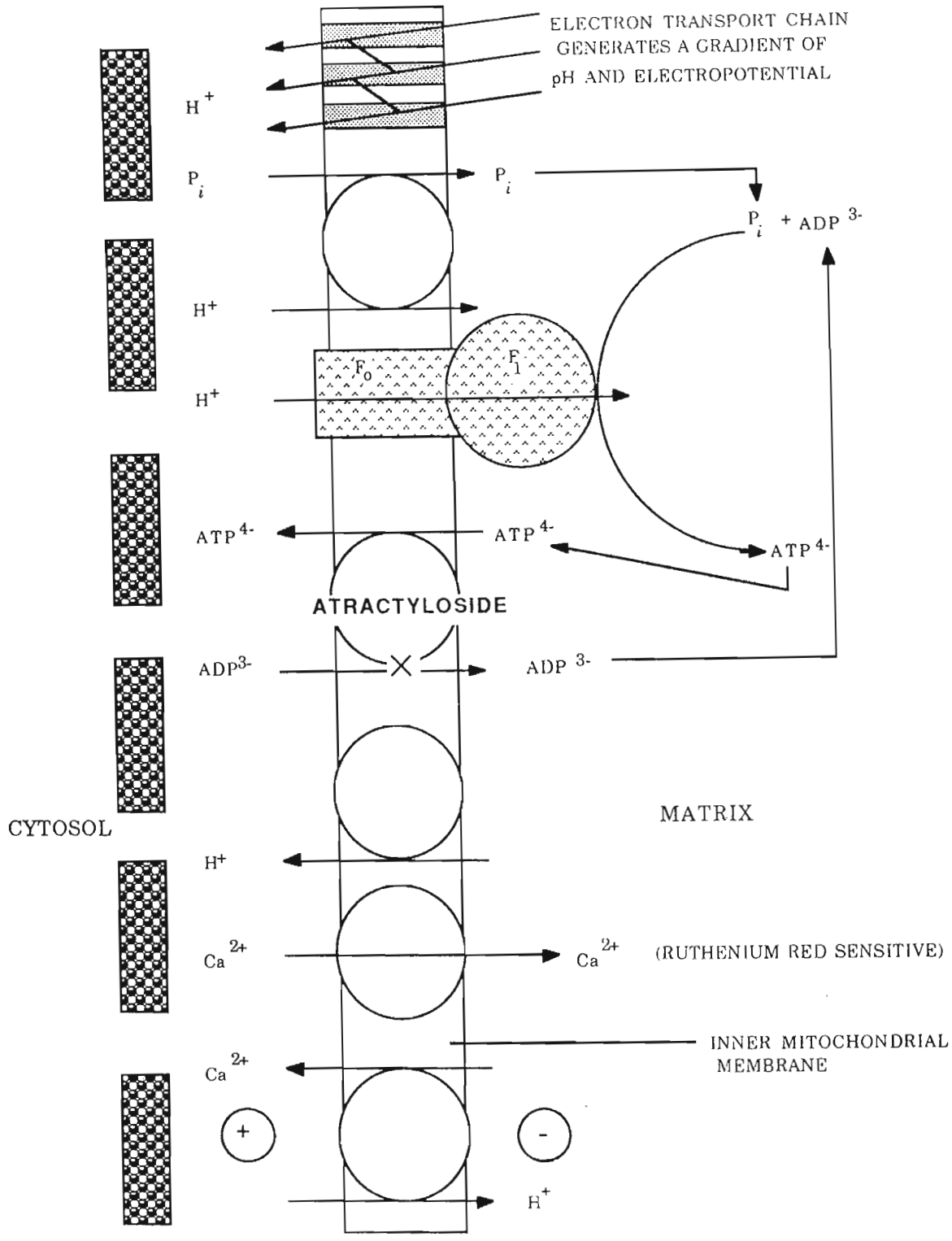

Fig 2.5 The role of the ADP/ATP antiporter in mitochondrial oxidative phosphorylation and the site of action of atractyloside, which binds to the ADP/ATP antiporter, terminating nucleotide exchange. Details of calcium exchange are also included, as described in section 2.4.2, showing the ruthenium red sensitive calcium uniporter and the calcium antiporter, which are involved in mediating cellular calcium homeostasis

The inhibitory effect of this toxin is not confined to the blocking of the ADP/ATP antiporter and thus, brief consideration is given to the effect of ATR on intra-mitochondrial calcium stores and sulphation in the GA in sections 2.4.2 and 2.4.3 respectively.

2.4.2 The effect of atractyloside on calcium accumulation

Cellular injury, mediated by various toxins, is invariably associated with an alteration in the metabolism and storage of trace elements, in particular calcium ions. An accumulation of cytoplasmic calcium is accompanied by cessation of cell growth and ultimately cell lysis (Lynn *et al.*, 1989). Calcium ions are important regulatory agents of intracellular metabolic processes, and depots of this cation are accumulated in the mitochondria. As shown in Fig 2.5, calcium ion uptake is mediated by a specific uniporter which is sensitive to the inhibitory action of ruthenium red dye (Harold, 1986). The divalent nature of calcium generates an electropotential across the membrane and it is the electrophoretic import of calcium which results in the high accumulation of this ion in the mitochondria. The concentration of the intra-mitochondrial calcium cannot, however, exceed 1 mM, as this would result in the irreversible precipitation of calcium with the inorganic phosphate present in the matrix. Homeostasis is dependent upon the presence of a second calcium carrier, a calcium ion-H^+ antiporter (Fig 2.5) (Harold, 1986). Being electroneutral, this carrier tends towards an equilibrium distribution across the membrane, differing to that favoured by the uniporter. As the calcium ion concentration increases, the pH also rises with the extrusion of H^+. Release of calcium is therefore favoured by the antiporter, and as a consequence, precise regulation of extra-mitochondrial calcium levels is facilitated by steady-state cycling of the calcium across the inner mitochondrial membrane. Consequently, mitochondria play a vital role in maintaining intracellular homeostasis (Bygrave, 1978; Nicholls, 1978; Nicholls and Scott, 1980; Bernardi and Azzone, 1983; Harold, 1986; Lê Quôc and Lê Quôc, 1988).

Atractyloside induces a collapse of the trans-membrane potential ($\Delta\psi$) and this loss of the electropotential gradient is noted as an early event in the mechanism of calcium discharge (Nicholls and Crompton, 1980). Atractyloside is known to induce the efflux of intra-mitochondrial reserves of calcium into the cytoplasm (Nicholls and Crompton, 1980; Vercesi, 1984; Moreno-Sanchez, 1985; Chavez and Osornio, 1988; Lê Quôc and Lê Quôc, 1988). This was first shown by the experiment of Asimakis and Sordahl (1977), who incubated rabbit heart mitochondria with calcium and examined the effect of varying concentrations of ATR on the calcium discharge, as shown in Fig 2.6.

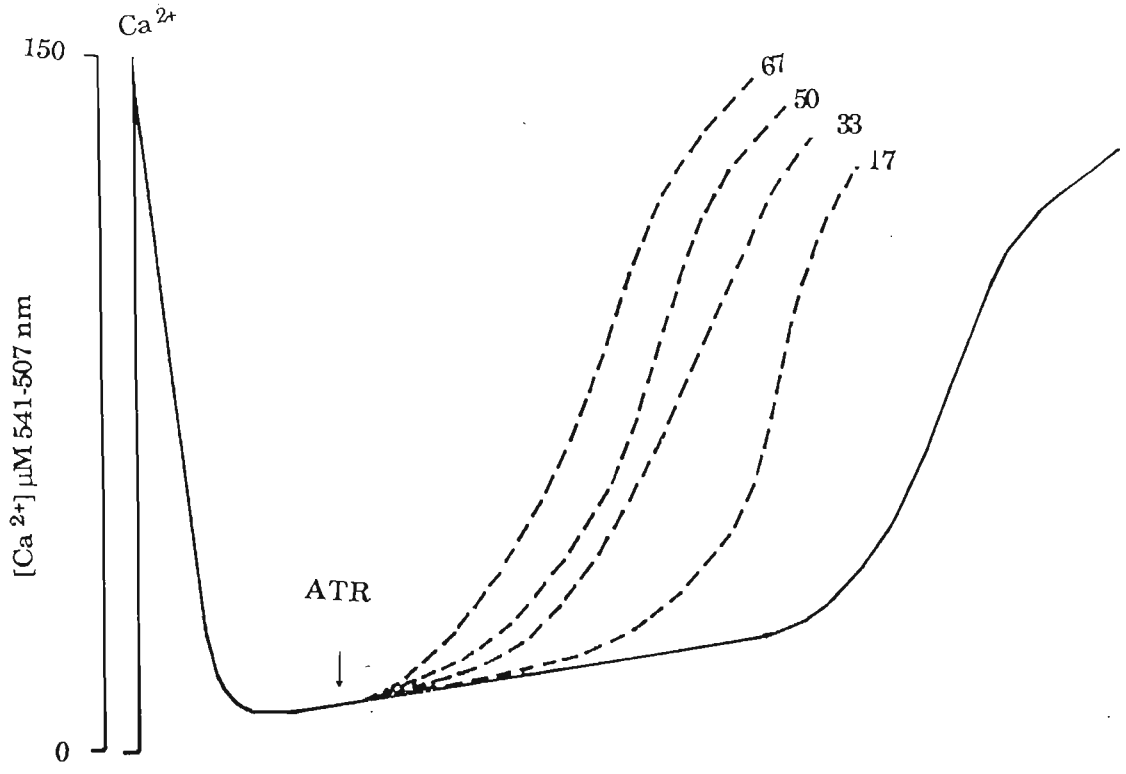

Fig 2.6 Effects of increasing concentrations of atractyloside on calcium discharge from rabbit heart mitochondria, (Asimakis and Sordahl, 1977). Mitochondria were incubated with calcium and incubated with ATR (17-67 µM/ml) and the discharge of calcium was monitored spectrophotometrically using the calcium sensitive dye, murexide

The results (Fig 2.6) given by a spectrophotometric trace show the addition of the calcium to the mitochondria (upward deflection) and subsequent sequestration of the calcium by the mitochondria (downward deflection). On the addition of ATR, the calcium is released into the surrounding milieu, in a time indirectly proportional to the concentration of toxin.

The influence of calcium on membrane permeability has been well documented and discharge of accumulated calcium induces practically irreversible modification of the inner mitochondrial membrane properties, causing so-called "leaky" membranes; an alteration noted following incubation with ATR (Nicholls and Ackerman, 1982). This membrane destabilisation is believed to be the result of activation of a calcium-dependant phospholipase A, with formation of lysophosphatides. The mechanism by which certain reagents or conditions induce increased membrane permeability and calcium release is unknown, but the generation of peroxides and free radicals which accompanies this membrane permeabilisation is believed to be a significant reaction. Addition of free radical scavengers (such as butylhydroxytoluene) to mitochondria, in the presence of membrane permeability promoters, results in complete restabilisation of the membrane. Carbonera and Azzone (1988) proposed that processes involving the release of

mitochondrial calcium and the increased permeability of the inner mitochondrial membrane merge into a common step involving generation of free radicals. It is a personal suggestion, therefore, that free radical scavengers could be administered to patients with *Impila* poisoning, as this may promote recovery by restoring membrane stability and preventing further calcium leaching.

The effects of ATR on membrane $\Delta\psi$ and calcium release point to a possible participation of the antiporter in the regulation of mitochondrial permeability to cations (Chavez and Orsonio, 1988). The dimeric ADP/ATP carrier protein spans the inner mitochondrial membrane and, depending on the pre-treatment of mitochondria, the carrier can be isolated in one of two distinct conformations (Fig 2.7). The antiporter exists in the c-state conformation (i.e., the nucleotide binding site facing the cytosol) in the presence of ATR and in the m-state (i.e., nucleotide binding site facing the matrix) after pre-treatment with bongkrekic acid (Klingenberg *et al.*, 1978).

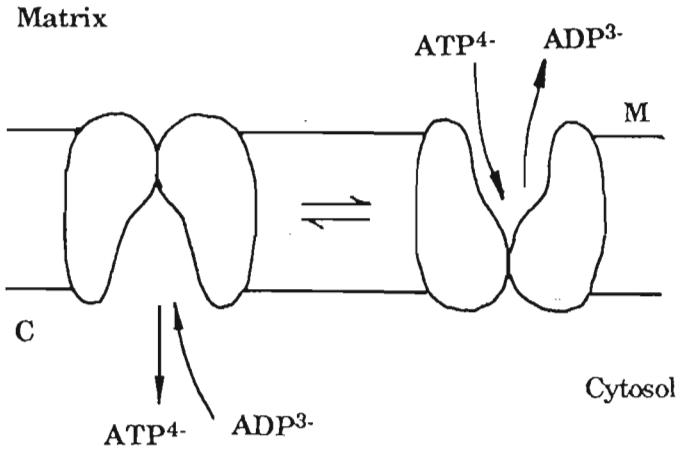

Fig 2.7 The ADP/ATP antiporter showing the proposed c and m-state conformations (Harold, 1986).

Palmitoyl CoA, like ATR, promotes c-state stabilization of the antiporter (Lê Quôc and Lê Quôc, 1988). These two inhibitors induce alteration in the ability of the mitochondria to control both cation storage and exchange, resulting in the leakage of accumulated mitochondrial calcium and mitochondrial swelling. Only when the antiporter exists in the c-state is calcium seen to exert an effect on membrane permeability, and from this it has been concluded that the conformational state of the carrier could be regarded as the primary factor allowing, or impeding, the damaging effects of calcium on membrane permeability. All agents which induce and block the antiporter in the c-state, such as ATR,

will have a deleterious effect on the membrane permeability of calcium-loaded mitochondria (Lê Quôc and Lê Quôc, 1988).

Atractyloside also promotes mitochondrial swelling (Davidson and Halestrap, 1987) which suggests that the antiporter may mediate alterations in the size of the mitochondria. The amplitude of this effect is temperature dependent and is not evident when mitochondria are incubated in the presence of ATR at 4°C. It is suggested that the size of the mitochondria is influenced by the lipid fluidity of the membranes, accounting for the temperature dependence of this phenomenon (Chavez and Orsonio, 1988).

The effect of ATR on mitochondrial amplitude and calcium stores is a very interesting phenomenon and this toxin may be associated with Reye's syndrome. Reye's syndrome has many clinical similarities to *Impila* poisoning, including vomiting, hypoglycaemia and coma (Bhoola,1983). Bhoola compared the centrilobular liver necrosis of *Impila* poisoning with the acute liver necrosis of Reye's syndrome and concluded that there are differences, particularly with the morphological alteration in the liver. While in the 1970s it was proposed that Reye's syndrome was caused by a viral infection, in recent years, it has been suggested that a toxin or toxins may be involved in the aetiology of this disease. Salicylate has been implicated as a potential candidate for this pathology, as the symptoms of salicylate poisoning and Reye's syndrome show some resemblance. In addition, a correlation between the consumption of aspirin and the manifestation of this syndrome has been noted (Starko *et al.*, 1980), even though the levels of salicylate in the serum are not significantly high (Rosenfeld and Liebhaber, 1976). It was proposed that the mitochondrial damage associated with Reye's syndrome, including increased permeability of the inner mitochondrial membrane and loss of NAD^+, is the result of the toxic agent potentiating calcium efflux from the mitochondria (Martens *et al.*, 1986).

Martens *et al.* (1986) investigated the effects of certain toxins on mitochondrial structure and calcium transport in the following manner. Rat liver mitochondria were isolated and incubated with calcium, and calcium transport was measured using a similar method to that of Asimakis and Sordahl (1977) (Fig 2.6), but in this case the calcium binding dye was antipyrlazo II. On addition of the calcium, it was taken up by the mitochondria. When salicylate, allantoin or the plasma from Reye's syndrome patients (Reye's plasma) was added to the incubation mixture, the mitochondria demonstrated large amplitude swelling and spontaneously released calcium. The results of the salicylate experiment are shown in Fig 2.8 below. Similar results were noted for allantoin and Reye's plasma.

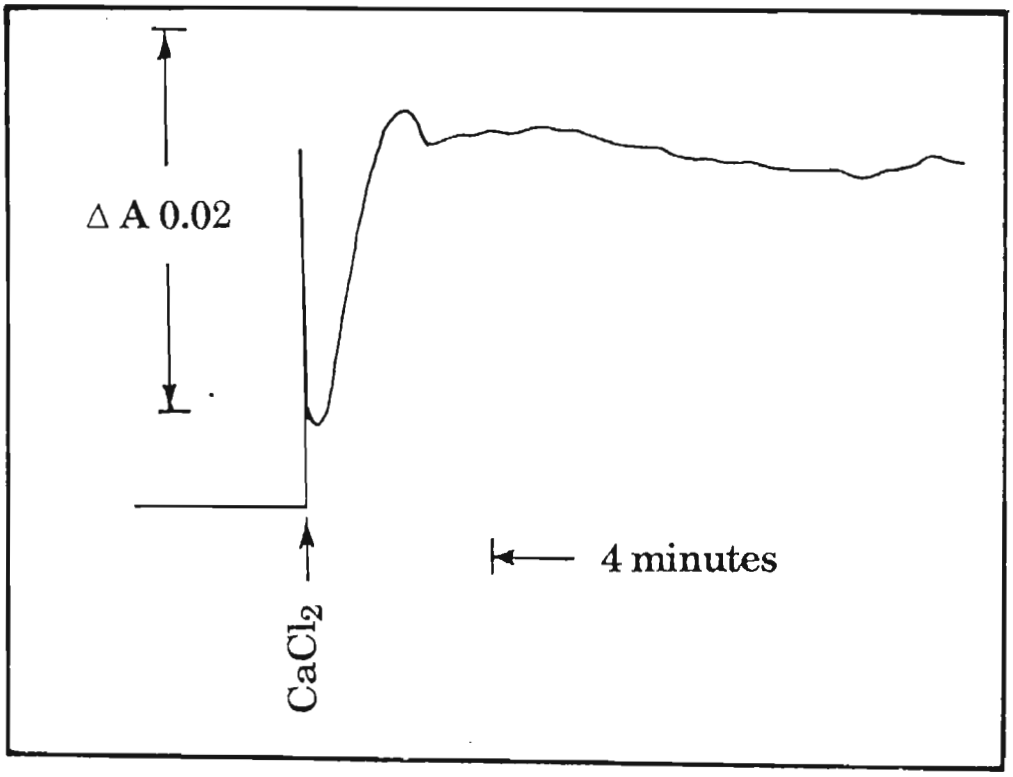

Fig 2.8 Effects of salicylate on calcium discharge from rat liver mitochondria (after Martens *et al.*, 1986)

It will be seen from the results in Figs 2.6 and 2.8, that both salicylate and ATR have similar effects on calcium sequestration in the mitochondria, as do allantoin and Reye's plasma (data not shown) and furthermore, salicylate, allantoin, Reye's plasma and ATR all induce mitochondrial swelling (Martens *et al.*, 1986; Davidson and Halestrap, 1987). Martens *et al.* (1986) noted that the effect of these toxins (excluding ATR), could be prevented by including either ATP or ADP in the incubation medium, but that the protective effect of these nucleotides was lost if CATR was also added. They observed that CATR potentiated the effects of the salicylate, but made no correlation between the effects of the atractylosides on intra-mitochondrial stores, mitochondrial swelling and their consequences. Martens *et al.* (1986) concluded that salicylates, allantoin or Reye's plasma induce calcium release from the mitochondria, causing inner mitochondrial membrane destabilisation, in a manner as yet not elucidated.

It is present author's view that these compounds may somehow induce the c-state conformation of the antiporter, causing calcium release and membrane permeabilisation, and that ATR may also induce Reye's syndrome. The correlation between *Impila* consumption and the incidence of this pathology should be thoroughly investigated, since what is diagnosed as Reye's syndrome may be sub-chronic intoxication by *Impila*. Furthermore, if toxins are involved in the aetiology of Reye's syndrome, it is a personal opinion that it is probably a non-specific pathology caused by agents which lock the ADP/ATP antiporter in the c-state conformation.

The present author also suggests that duramycin may provide a suitable therapeutic agent for both *Impila* poisoning and Reye's syndrome. This polypeptide is produced by *Streptoverticillium cinnamomeus* (Sokolove *et al.*, 1989), formerly *Streptomyces cinnamomeus*, and is active against Gram-positive bacteria, some yeasts, and fungi (Shotwell *et al.*, 1958). Work on mitochondria isolated from rat cardiac tissue showed that at low concentrations, duramycin increases the inner mitochondrial permeability to monovalent cations, but when incubated with ATR, this increase in permeability is blocked, suggesting that the ATR reverses the effects of the antibiotic (Sokolove *et al.*, 1989). These results are confusing as, from the information detailed above, ATR ultimately has a similar effect to duramycin, *viz.*, increased inner mitochondrial membrane permeability. Yet apparently the duramycin-altered membrane is stabilised in the presence of ATR (Sokolove *et al.*, 1989). There has been no experimental work carried out on the reverse reaction, i.e., incubation of mitochondria with ATR, followed by addition of duramycin, but the results of such an analysis might reveal that duramycin is able to stabilise the ATR-damaged mitochondrial membrane. It is the author's opinion therefore, that this antibiotic may be useful as a therapeutic agent for the treatment of both *Impila* or ATR poisoning, as well as Reye's syndrome, a possibility that certainly warrants future investigation.

Although the effects of ATR on the mitochondria are quite well documented, the action of this toxin is not confined to nucleotide exchange or calcium sequestration, but also affects the GA, an aspect which is briefly reviewed in the next section.

2.4.3 The effect of the atractylosides on the functioning of the Golgi Apparatus

The GA and the Rough Endoplasmic Reticulum (RER) are sites of post-translational processing, i.e., glycosylation and sulphation, primarily of secretory proteins. During this processing, a protein enters the RER and is labelled with a precursor oligosaccharide. The sugar residues are transported as sugar-nucleotides across the the semi-permeable

RER membrane, in mechanisms only poorly elucidated. The glycoprotein complex is then exported to the GA where further processing occurs, such as glycosylation and sulphation. The sugar-nucleotides involved in these processes are transported across the GA membrane by specific carriers (Lee *et al.*, 1984; Coste *et al.*, 1986; Hirschberg and Snider, 1987). Adenosine-3'-phosphate-5'-phosphosulphate (PAPS) is the protein sulphate donor in the GA. Both ATR and CATR inhibit the passage of PAPS across the GA membrane, possibly by interacting with the PAPS translocase, which has structural features in common with the ADP/ATP antiporter. Though of similar molecular mass to the ADP/ATP antiporter, the PAPS carrier is not identical, as demonstrated by the fact that PAPS *per se* cannot traverse across the inner mitochondrial membrane, despite the presence of the ADP/ATP antiporter (Capasso and Hirschberg, 1984; Lee *et al.*, 1984).

Temperature-dependent inhibition of casein kinase by ATR has also been noted in GA isolated from mammary tissue of lactating rats. Casein kinase, present in the GA lumen, requires both ATP and calcium for its catalytic activity (West and Clegg, 1984) and thus this observed inhibition is hardly surprising considering the effects of ATR on both ATP synthesis and cytoplasmic calcium.

In conclusion, it is clear that ATR inhibits processes involving the translocation of sugar-nucleotides across membranes, those requiring energy in the form of ATP, and reactions which are either inhibited or enhanced by the presence of cytoplasmic calcium. Atractyloside has been and continues to be used as a powerful biochemical tool in the elucidation of various metabolic processes. It is a matter of concern that the rationale used to explain the effects of ATR is superficial and classic cases are presented by the work of Martens *et al.* (1986) and Sokolove *et al.* (1989). Despite knowledge of the primary inhibitory action of this toxin, the cascade of secondary effects are quietly ignored. This is certainly an area for future research and results obtained in the past need careful re-assessment.

Despite there being considerable information pertaining to the action of ATR *in vivo*, from the South African perspective, and in view of the potentially catastrophic effects of ATR, there is clearly a need for an accurate and reliable assay for this toxin. In this manner, potentially harmful *muthi* may be screened and diagnosis made where *Impila* poisoning is suspected. At the time this study was initiated, the only means of detecting ATR was by TLC (Brookes, 1979) but the detection limits of this method restricted its applicability. The next chapter (Chapter 3) outlines the final optimised methods used in this study for the detection and cellular localisation of ATR, and this is followed by details of the sequential development of a chromatographic and immunological detection method for ATR

(Chapters 4 to 6). The localisation of the target organ(s), cells and subcellular organelles of ATR is described in Chapter 7 and the results of the entire study are correlated in the final discussion (Chapter 8).

CHAPTER 3

MATERIALS AND METHODS

This chapter details all materials employed in these studies, including the preparation of tissue and urine samples from ATR-treated rats, which were subsequently used to validate the various assays developed in this project. Since this thesis constitutes a substantial amount of method development, only the final optimised procedures are detailed in this chapter, with the developmental aspects of the methodology being covered in individual chapters. In the interests of clarity, a flow diagram and key of the major experimental methods performed during the course of this study is given in Fig 3.1 below.

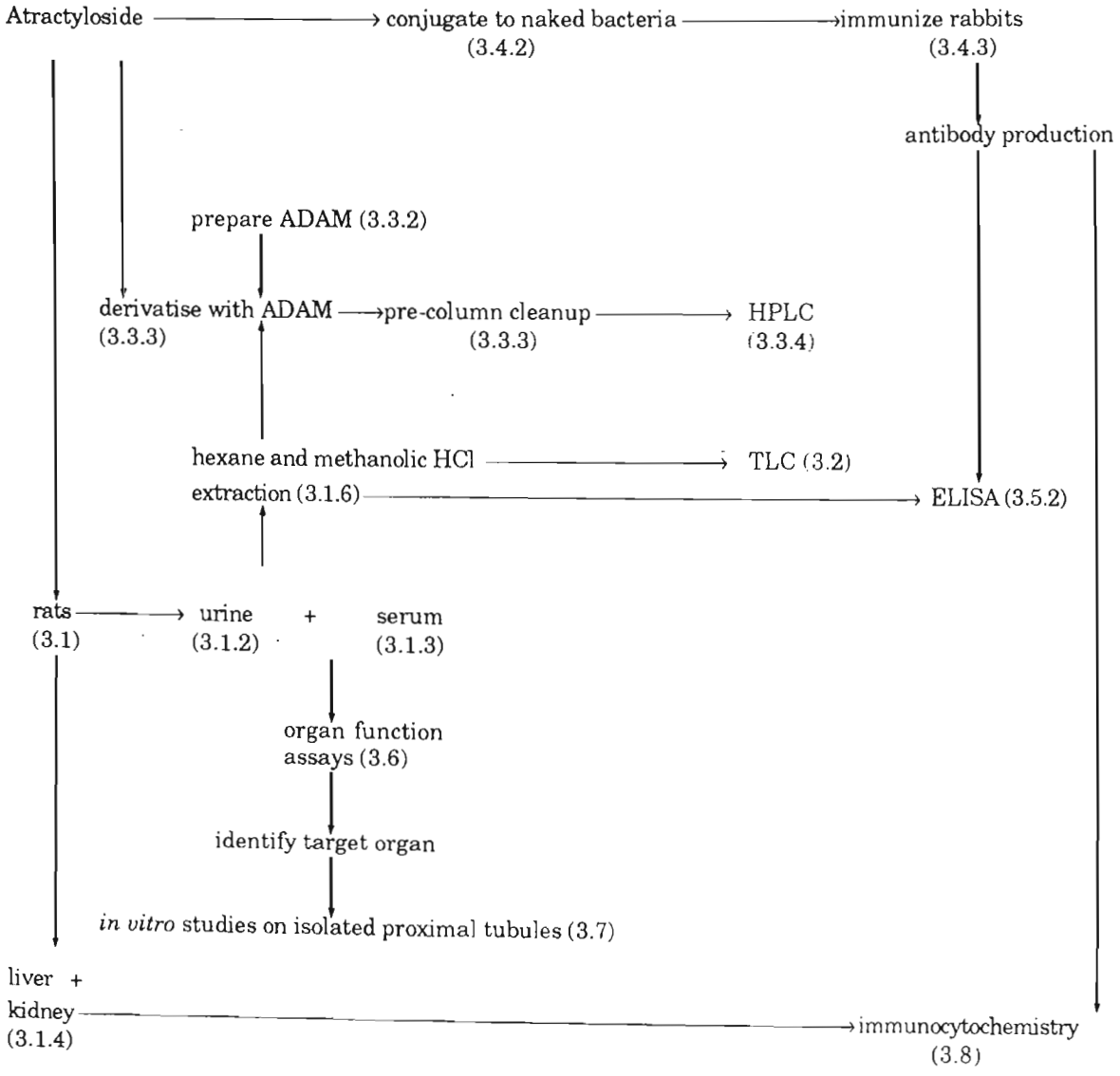

Fig 3.1 Overview of the experiments carried out in the course of this study, with the relevant sections, detailing the experimental procedures given in brackets

All dosing, tissue collection and processing for the urine and organ collection was carried out at the Robens Institute for Industrial and Environmental Health and Safety (RIIEHS), University of Surrey, Guildford, UK, while the serum collection was carried out at the Biomedical Resource Centre (BMRC), University of Durban-Westville. Tissue sectioning, immunolabelling, HPLC and the ELISA were performed at the University of Natal, Pietermaritzburg. The housing of the rabbits and their immunization for anti-ATR antibody production, was carried out at the University of Durban-Westville, BMRC.

3.1 Generation of a model set of tissue and urine samples from atractyloside-treated rats

3.1.1 Specialised chemicals and materials

Atractyloside (potassium salt) (Sigma Chemical Company (LTD), Poole, Dorset, UK). All other chemicals used in the study were of analytical grade and of the highest purity commercially available.

3.1.2 Urine samples

Male Wistar rats (Porton Strain, random bred closed colony, maintained by Experimental Biology Unit, University of Surrey, Guildford) were used for the urine and histological analysis. Rats were handled regularly prior to dosing, to minimize stress responses during experiments. Ordinarily, they were housed in translucent "shoebox" type cages (North Kent Plastic Cages LTD, UK); dimensions 60 × 35 × 18 cm (up to nine rats per cage) on sterile soft wood shavings (Lee and Son, Chertsey, Surrey, UK) and acclimatized to a controlled environment (12 hours light:dark schedule with the light cycle starting at 07.30 hr GMT). The temperature of the room was maintained at 23 ± 1°C, humidity 40 ± 10% and had 15 air changes per hour. To facilitate collection of urine, animals were housed in metal metabolic cages (Metabowl™) designed for separate collection of urine and faeces. Animals were marked on the tail by a colour coding system for each dose regime and with a number for inter-group identification, which was renewed each day if the markings were seen to be fading.

Dosing with ATR was performed as described in section 3.1.5 below. The 18 hour urine samples were collected twice prior to dosing, i.e., on day −1 and day 0. Following dosing (on day 0), the animals were immediately placed in the Metabowls for 18 hours. The urine samples were collected over ice to retain enzyme activity for the entire 18 hour period,

during which time the rats were fasted, but had free access to water at all times. When the urine was harvested, the rats were given access to food for 6 hour periods each day (09.00 hr-15.00 hr GMT) (Spratts Laboratory Animal Diet Number 1 obtained from Spratts, Barking, UK) whereupon they were returned to the cleaned Metabowls. This procedure was repeated until day 4, when the trial was terminated.

The urine was immediately centrifuged at 5 000 $\times g$ for 15 minutes, to separate debris and particulate matter. The supernatant was collected and enzyme activity measured (section 3.6.2) on the day of collection. Samples were stored at –20°C for the calcium and glucose analysis (section 3.6.2) which were performed within one week of collection. Prior to analysis of the samples by TLC (section 3.2), HPLC (section 3.3) or ELISA (section 3.5), sample clean up was performed as described in section 3.1.6 below.

3.1.3 Serum samples

Twenty male Wistar rats, both genetically and microbiologically defined, of mass 150-200 g, were randomly selected from the stock at the BMRC, University of Durban-Westville. The procedure was approved by the Ethics Committee at the University of Durban-Westville (Ethical clearance number 91023 B). Animals were housed in individual cages, conforming to nationally and internationally recognised standards, as outlined in the *National Code for Animal Use in Research, Education, Diagnosis and Testing of Drugs and Related Substances in South Africa*. The temperature remained at a constant level, between 19-21°C and the relative humidity 50 ± 10% R.H. The filtered air flow had 15-20 air changes per hour. Artificial lighting was adequate and remained a constant 12-14 daylight hours per day. Diet consisted of irradiated standard commercial pelleted rat feed (Epol, Pietermaritzburg, South Africa) and both water and feed were available *ad libitum*. At initiation of the trial, animals were examined for their general state of health. Following administration of toxin, the rats were monitored daily, to observe any untoward effects of the toxin.

Animals were randomly allocated into four groups of five, designated control, low, medium and high corresponding to the relative level of dosing with ATR, the details of which are described in section 3.1.5 below. At the onset of the experiment (on Day 0) animals were weighed, labelled individually with a colour code and a number, indicating the dose level and inter-group identification respectively. Blood (1 ml per rat) was aseptically collected from the tail-vein prior to dosing (representing the control) and thereafter at 24, 48, 72 and 96 hours post-dosing. At termination of the trial, rats were euthanased using CO_2 and the carcasses were incinerated.

The collected blood was allowed to clot and centrifuged at 5 000 × *g* for 10 minutes. Sera aliquots (200 µl) were taken to monitor serum aspartate transaminase (AST) and alanine transaminase (ALT) levels (see liver function tests, section 3.6.3). Care was taken to prevent haemolysis as this interferes with the enzymatic studies. The remainder of the sample was stored at –20°C until analysed by HPLC or immunoassay (Sections 3.3 and 3.5 respectively).

3.1.4 Liver and kidney samples

Thirty six male Wistar rats (Porton Strain, random bred closed colony, maintained by Experimental Biology Unit, University of Surrey, Guildford) were used for the analysis. The rats were given *ad libitum* access to Spratts Laboratory Animal Diet Number 1 (Spratts, Barking, UK) and tap water. They were housed as described in section 3.1.2 in translucent "shoebox" type cages. Rats were randomly divided into four groups of nine, designated control, low, medium and high groups corresponding to the relative dosing with ATR, the details of which are described in section 3.1.5 below. Three rats per dose level were euthanased at 24 hour intervals for a total period of 72 hours.

Before euthanasia, rats were anaesthetised as follows. A measured volume of diethyl ether (50 ml) was poured onto a cotton-wool pad, secured in a wire basket in an air-tight bucket, which formed the anaesthetising chamber. Animals were placed in the chamber for a suitable period until completely anaesthetised, removed and euthanasia was performed by cervical dislocation.

Following euthanasia, the animal's abdominal cavity was opened via mid-line incision from pubic symphis to xiphoid process and the peritoneum carefully incised with scissors. This was carefully retracted, together with the skin flaps, to expose the intestines and the intestinal and alimentary canal, which were removed to the left hand side of the animal thus exposing the liver and kidneys. Extreme care was taken at all times to minimize mechanical damage to the tissue during excision. The kidneys were dissected intact, after surrounding fat and fascia had been removed by blunt dissection. Kidneys were depoled using a single edged razor blade and the left kidney notched for identification. The liver was also excised intact and a section 5 mm from the edge of the fourth lobe removed for fixation.

Tissue was fixed according to the method of Bancroft and Stevens (1982) and Gregg *et al.* (1990) for histological examination. Simultaneously, kidney tissue was collected for fixation and embedding in preparation for immunocytochemistry (section 3.8).

3.1.5 Dosing of animals with atractyloside

The dosing regime was the same for all the above rat experiments. Animals were given a single dose administered intraperitoneally (i.p.) of ATR (potassium salt) at dosages of 0, 5, 10 and 25 mg/kg bwt respectively (control, low, medium and high) dissolved in sterile saline. A final volume of 1 ml was administered in the instance of the high dose regimen, which is acknowledged as being a very large amount, but the poor solubility of the ATR limited lower volumes being prepared. The medium and low dose regimen received approximately 500 µl of ATR.

3.1.6 Sample preparation for thin layer and high performance liquid chromatography and the immunoassay

Serum (section 3.1.3) and urine (section 3.1.2) samples were defatted and deproteinized, as follows, prior to being analysed by TLC, HPLC and ELISA. An aliquot (200 µl) was vortexed with an equal volume of hexane, allowed to settle and submerged in an acetone dry ice-bath and the hexane layer containing extracted lipids was decanted from the frozen aqueous sample (Lim, 1986). The sample was thawed and mixed with 400 µl of a solution of methanolic hydrochloric acid (2 ml conc. HCl/litre methanol) which resulted in the spontaneous formation of a thick white precipitate. The sample was vortexed for a further 30 seconds, centrifuged at 5 000 $\times g$ for 10 minutes and the supernatant removed for further analysis. The extraction procedure did not interfere with any of the analyses performed. All samples were stored at 4°C until analysed by TLC, HPLC or ELISA (sections 3.2, 3.3 and 3.5 respectively).

3.2 Thin layer chromatography

Thin layer chromatography was used extensively in the present studies to assist in the optimisation of the derivatisation of ATR (section 4.4) sample clean up (section 4.5) and the HPLC method (section 4.6). Analytical TLC was carried out as described by Brookes (1979) on 10 cm × 10 cm aluminium backed, non-fluorescent silica gel TLC plates (Merck, Germany). Atractyloside (1 mg/ml), urine, serum or derivatised toxin (ATR-ADAM, section 4.4) was developed in a solvent system of freshly prepared butanol:glacial acetic acid:chloroform:water (6:2:2:1). Saturation of the development tank was hastened by

insertion of filter paper discs into the solvent for 10 minutes prior to development of the plate. Following development, the plates were sprayed with anisaldehyde spray reagent, prepared by mixing anisaldehyde (500 µl), glacial acetic acid (10 ml), conc. sulphuric acid (5 ml) and methanol (85 ml). Plates were heated to 100°C for a few minutes after spraying and ATR or ATR-ADAM was visualised as a pink spot. Prior to anisaldehyde treatment, the ATR-ADAM was also visualised as a blue fluorescent spot under UV light at a wavelength of 365 nm.

3.3 High performance liquid chromatography of atractyloside

The first step in the chromatographic detection of ATR was preparation of the derivatisation agent, 9-anthryldiazomethane (ADAM). Those aspects of the synthesis involving method development are described in section 4.3, while the final optimised method is detailed in section 3.3.2 below. Likewise for the derivatisation of ATR, sample clean up and chromatographic conditions, the developmental aspects are described in sections 4.4, 4.5 and 4.6, while optimised procedures are detailed in sections 3.3.2, 3.3.3, and 3.3.3 again, respectively. High performance liquid chromatography was carried out on all the urine and serum samples from the ATR-treated rats, the preparation of which is described in section 3.1.2 and 3.1.3 respectively.

3.3.1 Specialised chemicals

All the chemicals used in the study were of analytical grade and of the highest purity commercially available, unless otherwise stated. All solvents were prepared in ultra-pure water, obtained from a Milli-Q plus system, which produces organic free 18 Ωcm^{-1} resistivity water, filtered to 0.22 µm. Solvents for HPLC were degassed prior to use by ultrasonication and stored in glass bottles. Plastic bottles were not used as organic solvents can leach "plasticisers" from the containers, causing spurious peaks on the chromatogram (Johnson and Stevenson, 1978). Anisaldehyde (BDH, Bucks., UK); Anthracen-9-carbaldehyde, hydrazinium hydroxide (80%), reverse phase-18 F 254S TLC plates, thickness 0.25 mm and aluminium backed silica gel plates (Merck, Germany); Mercuric oxides (Hopkins and Williams, Essex, UK); Silica gel 60 (for chromatography); (Fluka, Switzerland).

3.3.2 Preparation of derivatising agent

The method of Nakaya *et al.* (1967) for the preparation of 9-anthrylhydrazone was employed without modification. Unless otherwise stated, all reactions were carried out at

room temperature. A solution of 8.8 g 9-anthryldehyde in 15 ml absolute ethanol was stirred with 8.5 g hydrazine hydrate, for 3 hours. The solid product (9-anthrylhydrazone) was removed by filtration, dried under vacuum and recrystallised from ethanol and stored desiccated at 4°C until used. The synthesis of ADAM from the hydrazone is a modified procedure of Miller (1959) and Nakaya *et al.* (1967) (section 4.3). Fresh catalyst was prepared as follows. Mercuric chloride (10 g) was mixed with 50 ml of ethanol and 39 ml of a saturated solution of potassium hydroxide in distilled water. The resulting yellow precipitate of mercuric oxide was filtered and dried under vacuum for immediate use. To 2.9 g of 9-anthrylhydrazone was added 3 g anhydrous sodium sulphate, 40 ml of diethyl ether, 1 ml of ethanol saturated with potassium hydroxide and the mercuric oxide (approximately 7 g) prepared as described above. The solution was stirred for 5 hours, in a pressure bottle wrapped in wet towels to contain a possible explosion. The reaction mixture was filtered and the solvent was removed from the filtrate under reduced pressure to leave a dark red oil which was dissolved in petroleum ether (boiling point 30-60°C) and filtered through Whatman N°1 filter paper. The solvent was removed from the filtrate under reduced pressure, yielding red 9-anthryldiazomethane (ADAM) crystals, which were not recrystallised. The crystals were stored, desiccated at 4°C until they were used to derivatise the ATR, details of which are given below in section 3.3.3.

3.3.3 Derivatisation of atractyloside and pre-column clean up

The derivatisation of ATR was a modification of the method of Matthees and Purdy (1979). A 15% (v/v) solution of ADAM was prepared in diethyl ether and approximately 50 µl was added to 100 µl of the serum, urine or standard ATR (prepared as described in section 3.1.6). If there was sufficient derivatisation agent, the sample solution turned bright red, but on depletion of the derivatisation agent the solution turned yellow. More ADAM solution was added until the solution remained red for at least one hour. The unreacted reagent and decomposition products were removed from the sample in a pre-column clean up step, using a pasteur pipette plugged with glass fibre and filled with silica gel 60. The columns were activated with ethyl acetate:MeOH (3:1) and the crude derivatised material was applied to the column surface. The column was rinsed with approximately 7 ml of ethyl acetate:MeOH (3:1) which resulted in the removal of yellow contaminant. Application of 5 ml of a chloroform:MeOH (2:3) solvent resulted in the elution of the derivatised ATR from the column. The sample was evaporated to dryness in a stream of nitrogen with gentle heating and redissolved in 2 ml of mobile phase (section 3.3.4). The derivatised sample was analysed by HPLC as described in section 3.3.4 below.

3.3.4 Spectrophotometry and chromatographic conditions

All the UV spectrophotometric determinations as well as the absorption spectrum for ATR and ATR-ADAM were carried out on a Hitachi 220 spectrophotometer. The excitation and emission wavelengths for the ATR-ADAM derivative were determined on a Hitachi F2 000 fluorescent spectrophotometer and found to be 360 and 425 nm respectively. The HPLC was carried out on Millipore Waters equipment (Millipore Corporation, Milford, USA) and included the following: Mod. 710B WISP autosampler, Fluorescence detector Mod. 420-AC (fitted with a 365 nm excitation filter and a 420 nm band-pass emission filter), Mod. 501 pump and a Mod. 994 programmable photodiode array detector. An in-line pre-column guard filter was used at all times and all samples were filtered through Waters HV filters (0.45 µm). Integration was carried out either using the Waters 840 chromatography data station, Beckman System Gold or a Kontron PC Integration Pack.

Separation was carried out at room temperature on 4 µm Nova-Pak® reverse phase C_{18} columns of dimension 3.9 × 150 mm from Millipore Waters. An aliquot (20 µl) of the semi-purified sample was injected directly onto the column and eluted at a flow rate of 1.5 ml/minute in a solvent system of methanol:1 M ammonium acetate:glacial acetic acid: water (38:2:2:58). The eluted solutes were monitored sequentially at a UV wavelength of 254 nm and for fluorescence at an excitation wavelength of 360 nm and emission wavelength of 425 nm.

3.4 Antibody production

Antibodies to ATR were produced for use in the ELISA (Chapter 6) and for immunocytochemistry of tuber and kidney tissue (Chapter 7). Since the entire antibody production protocol had to be optimised, the developmental aspects of the methodology are given in the various materials and methods sections, in Chapter 5, whereas the final optimised method is detailed in this section.

3.4.1 Specialised chemicals, materials and equipment

All the chemicals used in the study were of analytical grade and of the highest purity commercially available, unless otherwise stated. All buffers were prepared in ultra-pure water, filtered through a Milli-Q plus system, which produces organic free 18 Ωcm^{-1} resistivity water, filtered to 0.22 µm. Acid treated *Salmonella minnesota* R595 (Bioclones, South Africa); Agarose (for electrophoresis) (Unilab, South Africa);1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, dialysis tubing (D9652), Freund's adjuvants

(complete and incomplete), goat anti-rabbit IgG-alkaline phosphatase conjugate, p-nitrophenyl phosphate tablets, rabbit serum albumin (RSA) Fraction V, 2,4,6-trinitrobenzenesulphonic acid (TNBS) and Tris base (Sigma, St. Louis, MO); polyethylene glycol (6000) (PEG) (Merck, West Germany); bovine serum albumin (BSA)(Fraction V) (Boehringer Manneheim, West Germany); Gelatin (Difco Bacto., UK); Nitrocellulose membranes (Schleider and Schnell, Dassel, West Germany); N,N'-dicyclohexylcarbodiimide, diethanolamine, N,N-dimethyl formamide (DMF) (British Drug Houses (LTD) (BDH), Eastleigh, Hampshire, UK); NUNC microwell module U16 immunoquality (Weil, South Africa); Ovalbumin (Grade 111) (Koch Light Labs., Bucks, UK). All ELISA plate readings were carried out on a Biotek microtitre spectrophotometer.

3.4.2 Conjugation of atractyloside to *Salmonella minnesota* bacteria and bovine serum albumin

The procedure outlined below was a modification of the method of Bellstedt *et al.* (1987) and P. Hoffman (pers. comm.,1988). Lyophilised naked bacteria (2.5 mg) were rehydrated for 1 hour at 37°C, in 0.1 M borate buffer, pH 8.5. Atractyloside (19 mg) was dissolved in a 66% (v/v) solution of DMF and stirred for 30 minutes, at room temperature with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (12 mg). This solution was added to the hydrated "naked bacteria" and stirred overnight at 4°C. The naked bacteria were pelleted by centrifugation at 5 000 × *g* for 10 minutes. The supernatant was removed and the pellet washed twice in 0.1 M phosphate buffer, pH 7.0 and finally resuspended in this buffer (1 ml). The conjugate was used to immunize rabbits and the dosing and bleeding regimen are described in section 3.4.3.

A BSA-ATR conjugate was prepared for use as the coating antigen in the immunoassay, using a modification of the method supplied by P. Hoffman (pers. comm.,1989). Atractyloside (120 mg) was dissolved in a solution of 66% (v/v) DMF. To this was added 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (72 mg) and the solution was stirred for 30 minutes, at room temperature. Bovine serum albumin (100 mg) was dissolved in 0.5 M borate buffer, pH 8.5 and the ATR solution was added dropwise, with stirring, the pH being monitored and maintained above pH 8.0 throughout the procedure. The conjugate was stirred overnight at 4°C and dialysed exhaustively against 0.1 M borate buffer, pH 8.0.

The degree of binding of ATR to BSA, or RSA, was monitored by the method of Habeeb (1966) as follows: Protein solution (BSA or RSA, 1 mg/ml, 100 µl) was diluted with 0.1 M borate buffer, pH 8.5 (900 µl) and mixed with 1 ml of 0.1% (v/v) 2,4,6-trinitrobenzenesulfonic acid (TNBS) in distilled water. The solution was incubated for

2 hours at 40°C and mixed with 1 ml of 10% (w/v) SDS, prepared in the same borate buffer, followed by 0.5 ml of 0.1 M HCl. The absorbance of the solution was determined at 335 nm, against a distilled water blank, treated as described above. The absorbance readings of the conjugates were compared to that of the BSA and RSA controls, prepared simultaneously, and from these results the degree of binding of ATR to the BSA and RSA carriers was determined.

3.4.3 Immunization procedure

New Zealand white rabbits randomly selected from the BMRC breeding stock, University of Durban-Westville were used in the immunization procedure (Ethical Clearance Number 90009 B). The animals were housed in individual cages, conforming to internationally recognized standards. The temperature was kept between 16-20°C and the relative humidity 50 ± 10% R.H. The filtered air flow was changed 10-20 times per hour. Artificial light was adequate and was a constant 12-14 daylight hours, per day. The animals were allowed *ad libitum* access to food and water, which consisted of irradiated standard commercial pelleted feed and autoclaved Tef hay.

Pre-immune serum was collected prior to the rabbits being immunized with 0.2 ml of the naked bacteria-conjugate (prepared as described in section 3.4.2). The antigen was administered without emulsification in Freund's adjuvant, as the bacteria possess adjuvant-like properties (Bellstedt *et al.*, 1987). Where a protein-hapten conjugate was administered, rabbits were immunized with the conjugate (1 mg protein) which had previously been triturated with Freund's complete adjuvant. Care was taken to ensure that a stable water-in-oil emulsion was formed, by periodically testing the emulsion in cold water. The maintenance of a discrete drop of emulsion on the surface of the cold water, without evidence of dispersion was considered "stable" and only this was used to immunize the rabbits (Catty and Raykundalia, 1988). Rabbits were injected subcutaneously using a 25 gauge needle at a single site, there being no advantage to multiple site immunizations (Tijssen, 1985).

The bleeding procedure was optimised as follows. Rabbits were handled as regularly as possible, to minimise the stress response and this negated their restraint for bleeding purposes. Instead, they were placed on a table, preferably in the sun and if unconfined, remained calm. Any apprehension by the bleeder resulted in spontaneous venous constriction and a failure to bleed. Animals' ears were clipped free of hair with scissors, swabbed with alcohol and a thin layer of petroleum jelly was applied to the prepared area. This inhibits clotting and facilitates bleeding (Tijssen, 1985). If the central vein did not

dilate sufficiently, a cotton-wool swab, soaked in xylene was applied to the dorsal and lateral sides of the tip of the ear, causing dilation of the ear veins. Exposure of the rabbit to the toxic xylene was only used in extreme cases. Often, allowing the animal to relax in a warm environment was sufficient to dilate the veins. The syringe needle (25 gauge) was bent slightly and inserted bevel up into the central ear vein, care being taken to ensure that the needle remained as parallel as possible to the surface of the ear, and once the needle was inserted under the skin it was lifted fractionally to ensure that it penetrated the vein. Blood was allowed to flow freely into a sterile container and approximately 10-15 ml of blood was collected.

Animals were bled from the central ear vein at fortnightly intervals and the blood allowed to clot for 1 hour, at 37°C. Following release of the clot from the walls of the container, the samples were left overnight at 4°C to permit further clot retraction. The red blood cells were removed from the serum by centrifugation at 12 000 $\times g$ for 10 minutes. The antisera was never pooled, but divided into 100 µl aliquots and stored at –70°C until used in the ELISA (section 3.5) and immunocytochemistry (section 3.8.3). Titre was monitored using the immunoassay of Bye *et al.* (1990) (see details section 5.3.2), which was a preliminary version of the immunoassay described in section 3.5. Only when the primary response receded were animals boosted with the same concentration of antigen. When the secondary response began to recede, animals were anaesthetised with Alphaxalone and Alphadalone (Saffan, Galaxo), at a dosage of 9 mg/kg bwt, administered in the marginal ear vein and antisera was collected via cardiac puncture. The animals were not bled when the secondary response peaked, but as this response began to recede, since it has been observed that the affinity for the antigen is higher when the secondary response is beginning to recede, as compared to when response is maximal (S. Fawaz, pers. comm., 1990).

Thereafter, animals were rested for 6 months, immunized with fresh conjugate and the procedure repeated. At termination of the experiment, animals were anaesthetised with 0.3 ml anaesthetic, given intravenously in the central ear vein and exsanguinated by cardiac puncture. If necessary, euthanasia proceeded by barbiturate overdose, given intra-cardiac whilst animals remained under anaesthesia, following which the carcasses were incinerated. If the animals died during exsanguination, the carcasses were fed to the resident crocodiles at the BMRC.

The antibodies were characterised and the antisera with the best titre was used in the ELISA described in section 3.5 as well as for immunocytochemistry, as described in section 3.8 below.

3.5 Enzyme-linked immunosorbent assay (ELISA)

The ELISA detailed in this section was applied to both the urine and serum samples, from ATR-treated rats, as described in section 6.7. This method, however, required substantial development, the details of which are given in the relevant methods sections in Chapter 6.

3.5.1 Buffers

0.05 M carbonate buffer, pH 9.6: Sodium carbonate (0.424 g) and sodium bicarbonate (0.504 g) were dissolved in 1 litre of distilled water.

0.02 M Tris buffered saline, pH 7.4 (TBS): Tris base (2.42 g) and NaCl (9 g) were dissolved in about 800 ml of distilled water, titrated to pH 7.4 with 1 M HCl and made up to 1 litre with distilled water.

0.02 M Tris buffered saline, pH 7.4 containing 0.01% Tween®20 (saline-Tween): Tris base (2.42 g) and sodium chloride (9 g) were dissolved in about 800 ml of distilled water with 100 µl Tween 20, titrated to pH 7.4 with 1 M HCl and made up to 1 litre with distilled water.

0.1 M diethanolamine buffer, pH 9.6: Diethanolamine (97 ml) was added to 800 ml of distilled water containing $MgCl_2.5H_2O$ (101 mg), the pH adjusted to pH 9.6 with 1 M HCl and the buffer made up to 1 litre with distilled water.

3.5.2 Immunoassay

Where appropriate, buffers were pre-equilibrated to the incubation temperature. Microtitre plates were coated with a BSA-ATR conjugate (prepared as described in section 3.4.2) at a concentration of 0.001 mg/ml in 0.05 M carbonate buffer, pH 9.6 (200 µl/well). Plates were covered with cling film and incubated overnight at 4°C. Plates were blocked for 1 hour at room temperature with 200 µl/well of 0.1% (w/v) BSA in TBS. The antisera (section 3.4.3) were prediluted 1:150 in TBS. To each well was added 100 µl sample (diluted 1:10 and 1:100, in TBS) or standard ATR (over the range 1 mg to 10 ng ATR/ml) and an equal volume of the appropriate antiserum to give a final primary antibody solution of 1:300. All samples were tested in quadruplicate. Wells were incubated for 16 hours at 4°C, washed thrice in saline-Tween and incubated for a further 2 hours, at 37°C, with 200 µl/well of a 1:5 500 dilution of goat anti-rabbit IgG-alkaline phosphatase conjugate, in TBS. Plates were

then washed three times in saline-Tween and incubated for a further two hours at 37°C with p-nitrophenyl phosphate (1 mg/ml) in 0.1 M diethanolamine buffer, pH 9.6 (200 µl/well). The absorbance was monitored using a Biotek spectrophotometer.

Results were determined by calculating the mean of the replicates and comparing the mean absorbance of the 1:10 dilution with that of the 1:100 dilution. Theoretically, an increase in absorbance is expected with an increase in dilution, as less atractylosides would be present in the 1:100 dilution than in the 1:10 dilution and in the former, more antibody would be available to bind to the coating antigen. The increase in absorbance between the 1:10 and 1:100 dilution was calculated and a 10% or greater increase in absorbance was classified as positive (+).

3.6 Organ function assays

Organ function assays were employed as described in sections 7.2 and 7.3, on urine and serum, in order to gain information as to the target organ (kidney or liver) for ATR toxicity. The assays described in this section were used without any significant modification.

3.6.1 Specialised chemicals

Test kits for COBAS-BIO centrifugal analyzer: alkaline phosphatase (AP), opt, Calibrator I, γ-glutamyl transferase (GGT) Uni-Kit II, Glucose HK Uni-Kit II, lactate dehydrogenase (LDH) opt. DGKC MA-Kit 10, Calcium, Protein, Albumin (Roche Products Ltd. Welwyn, Herts., UK); Universal containers (30 ml) (Sterilin Ltd., Middlesex, UK); *Decision* control serum, aspartate aminotransferase reagent (AST) and alanine aminotransferase reagent (ALT) (Beckman Instruments, Durban, South Africa).

3.6.2 Kidney function assays

Metabolite and enzyme analyses were performed on a COBAS-BIO centrifugal autoanalyzer. This enables sequential analysis of several parameters in up to 24 samples per rotor. Representative urine sample (150-200 µl), prepared as described in section 3.1.2, were pipetted into sample vials, held in a plastic rotor. The reagent(s) required for the analysis, a starter solution and, if necessary, standards, were placed in a suitable reagent rack. Sample, reagent and starter were automatically transferred to plastic cuvettes and centrifugally mixed. Glucose, albumin, total protein and calcium levels as well as GGT, AP and LDH activity was measured using Roche diagnostic kits as detailed in section

3.6.1. The levels of AP, GGT and LDH were expressed as units of activity/litre of urine (U/litre) while glucose, calcium, albumin and total protein are given as mg/ml urine/18 hour urine volume.

3.6.3 Liver function tests

Aliquots (200 µl) of sera (prepared as described in section 3.1.3) were taken to monitor AST and ALT levels. Enzyme levels were monitored by staff at the King Edward VIII hospital, Durban using an automated Beckman Synchron CX clinical system and Beckman analytical test kits for AST and ALT as detailed in section 3.6.1. The results of these analyses are given in Chapter 7.

3.7 *In vitro* amino acid incorporation

The following *in vitro* analytical method was carried out, whereby the effect of ATR on the incorporation of tritium labelled proline into isolated viable proximal tubules was monitored. It was envisaged that this experiment would yield further evidence as to the specific target tissue of ATR toxicity and details of the *in vitro* method are given below.

3.7.1 Specialised chemicals and equipment

Atractyloside (potassium salt) (Sigma Chemical Company (LTD), Poole, Dorset, UK); Optiphase "safe" scintillant liquid (LKB, Wallac, Finland); Plastic scintillation vials, plastic cuvettes (FSA Laboratory Suppliers, Loughborough, Leics., UK); [^{3}H]-L-proline; specific activity 555 GBq/mM (Amersham Int. plc, Aylesbury, Buckinghamshire, UK); stainless steel sieves, woven wire mesh series of diameter 100 mm and pore size 75, 150 and 250 µm (Endecotts, London, UK).

Earles' buffered salt solution (Earles' BSS): NaCl (6.8 g), KCl (0.4 g), $CaCl_2.2H_20$ (0.247 g), $MgSO_4$ (0.1 g), NaH_2PO_4 (0.125 g), Glucose (1.0 g), Phenol red (0.01 g) and HEPES (6.7 g) were dissolved in 1 litre of distilled water.

3.7.2 Preparation of proximal tubules

Groups of ten male Wistar rats (150-200 g) from the University of Surrey's breeding stock were anaesthetised and killed by cervical dislocation (as described in section 3.1.4). Kidneys were immediately excised and placed in ice-cold Earles' BSS. Thereafter, the kidneys were decapsulated, cut longitudinally and both the papilla and inner medullary

tissue discarded. The remaining tissue (approximately 20 g) was finely minced with a single edge stainless steel blade and suspended in a minimum volume of fresh Earles' BSS. The glomerular and proximal tubule fragments were isolated by forcing the tissue through a series of three graduated sieves, of decreasing mesh size, *viz.*, 75, 150 and 250 µm. Proximal tubules were retained by the 150 µm sieve, while the glomeruli were harvested from the 75 µm sieve. Fractions were resuspended in a approximately 20 ml of Earles' BSS, washed twice with the same buffer followed by centrifugation at 5 000 × *g* for 3 minutes, and finally resuspended in approximately 20 ml of ice cold buffer (Earles' BSS). An overview of the procedure is given in Fig 3.2. Aliquots of the suspension were used immediately for the amino acid incorporation experiment (section 3.7.3) and for protein determination as described in section 3.7.4.

3.7.3 Amino acid incorporation and experimental method

As outlined in Fig 3.2, aliquots (500 µl) of glomerular or tubular preparations were incubated in a shaking water bath (120 cycles/min) at 37°C, in the absence or presence of 500 µl of ATR solution of concentration ranging from 1-6 µM. To each vial of tubule fractions was added 50 µl of [^{3}H]-L-proline (92.5 kBq, in Earles' BSS). Representative control samples devoid of toxin were collected at times 0, 1, 2 and 3 hours respectively, to ensure that the proximal tubules were viable for the entire incubation period, whereas the tests samples containing the toxin were collected only at time 3 hours. Incubation was terminated by addition of 3 ml of ice-cold TCA (6%, v/v) containing 1 mM of non-radio-labelled L-proline to prevent non-specific adsorption of the labelled compound. Samples were stored at 4°C until processed. The samples were transferred to glass centrifuge tubes, washed and centrifuged thrice for 5 minutes at 5 000 × *g* . A representative 250 µl sample was collected from each vial and transferred to plastic scintillation vials, to which was added 200 µl of 0.5 M NaOH. The sample was left to hydrolyse for 24 hours, mixed with 4 ml Optiphase "safe" scintillation fluid and counted on a LKB 1219 Scintillation counter.

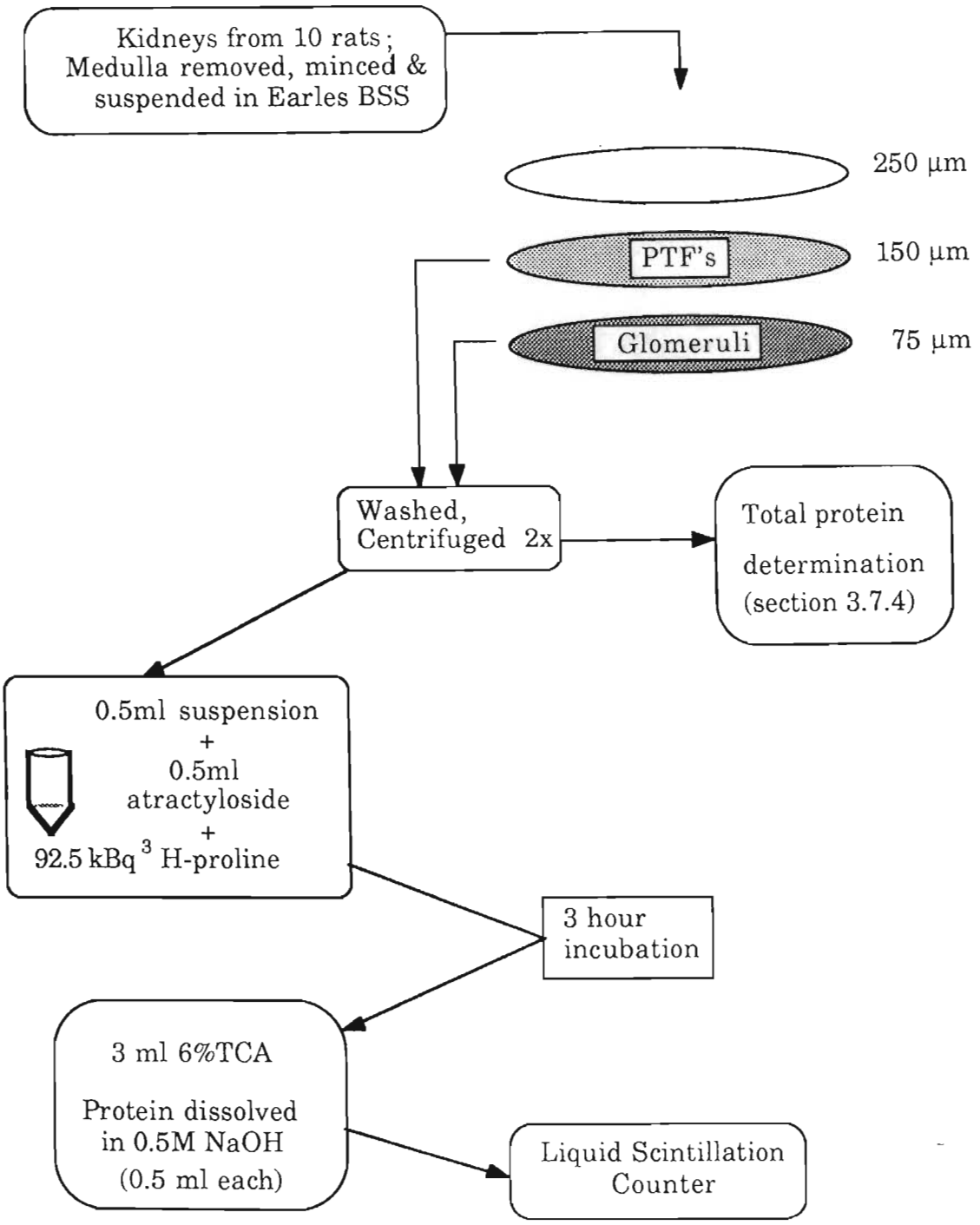

Fig 3.2 Diagrammatic representation of the isolation of viable proximal tubules (PTF) and glomeruli from rat kidneys (adapted from the diagram of M.Wilkes, pers. comm., 1990). Detailed methodology is described in the text, section 3.7.2

The protein content of the tubules and glomeruli was determined as detailed in section 3.7.4 and the results calculated as described in 3.7.5 below.

3.7.4 Total protein determination

Aliquots (500 µl) of the tubule or glomerular mixture were dissolved in 500 µl 0.5 M NaOH for 48 hours and protein content was measured in triplicate by the Coomassie blue method of Bradford (1976).

Reagents

Coomassie Brilliant Blue G (100 mg), absolute alcohol (46.7 ml) and conc. orthophosphoric acid (89 ml) were made up to 1 litre with distilled water. The stock solution was filtered through Whatman N°1 filter paper after preparation and again immediately prior to use.

Procedure

Distilled water blanks, standards of BSA over the concentration range 25-200 mg/ml or sample (200 µl) were pipetted into plastic cuvettes. Glomerular samples were used undiluted, while the tubule fractions were diluted 1:4 because of their higher protein concentration. Filtered Coomassie blue stock solution (3.8 ml) was added to the cuvettes and after 10 minutes equilibration the absorbance was read at a wavelength of 595 nm. The final protein concentration was calculated from the standard curve for BSA and expressed as mg total protein/ml. These values were used in the final calculation for the determination of amino acid incorporation as detailed in section 3.7.5 below.

3.7.5 Calculation of results

Amino acid incorporation (z) was calculated from the following formula and expressed in units of picomoles of L-proline incorporated/mg protein (P.Bach, per. comm.,1990):

$$z = \frac{[\text{dpm sample-dpm background}] \times [4.5 \times 10^{-4}]}{\text{specific activity of substance} \times [\text{protein content}]}$$

The effects of ATR are expressed as the relative incorporation of [^{3}H]-L-proline, incorporated into the protein, at time three hours, as a percentage of the control, i.e., the z value obtained for the ATR samples was calculated as a percentage of the z value for the control after 3 hours incubation*. Relative toxicity is shown as the concentration of toxin required to inhibit amino acid incorporation in the isolated fragments by 50%, when compared to the controls (IC_{50}). Results are expressed as the mean of triplicate determinations.

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* relative incorporation of [^{3}H]-L-proline in the presence of ATR = $\frac{z \text{ sample}}{z \text{ control at time 3 hours}} \times 100$

3.8 Immunocytochemistry of tuber and kidney tissue

Immunocytochemistry was carried out on tuber tissue as well as on the kidney tissue from the rats dosed with 25 mg/kg bwt ATR. This section details the optimised procedure for immunocytochemistry, whereas the developmental aspects of the method are described in the materials and methods sections in section 7.5.

3.8.1 Specialised chemicals and materials

All buffers and reagents were prepared in ultra-pure water, filtered through a Milli-Q plus system, which produces organic free 18 Ωcm^{-1} resistivity water, filtered to 0.22 µm. Standard electron microscopy reagents for resin embedding were provided by the Electron Microscope Unit of the University of Natal, Pietermaritzburg or the RIIEHS, University of Surrey, Guildford, UK. Protein A-gold probe was prepared and supplied by E. Elliott (Dept. Biochemistry, University of Natal, Pietermaritzburg). Tubers of *C. laureola* were collected from World's View, Pietermaritzburg, Natal during the first quarter of 1989. Tissue processing was initiated within an hour of collection. Rat kidney was collected as described in section 3.1.4.

The anti-ATR antibodies were produced as described in section 3.4. The antisera was stored at –70 °C as small, undiluted aliquots. Once the antisera was thawed, it was not refrozen and all antibody solutions were centrifuged for 1 minute at 5 000 × *g* , prior to use, to remove all aggregates.

3.8.2 Fixation of tissue

The method of Sossountzov *et al.* (1988) was followed, with modifications (as detailed in section 7.5). Tissue was sectioned into 3 mm^3 pieces and fixed in 2% (w/v) 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide in 0.05 M sodium cacodylate buffer, pH 6.87 (for plant material) or in 0.02 M cacodylate buffer, pH 7.2 (for mammalian tissue) under a vacuum of 750 mm Hg for 30 minutes. Thereafter the carbodiimide and cacodylate solution was replaced and tissue was left for a further 4 hours at room temperature. Samples were fixed in 3% glutaraldehyde, 0.05% caffeine and 0.05 M sodium cacodylate buffer, pH 6.87, (plant tissue) or in Karnovsky's fixative (for animal tissue) (Karnovsky, 1965) at 4°C, overnight. Samples were rinsed in two changes of the appropriate buffer (total period 1 hour) and dehydrated for 10 minutes each in a graduated ethanol series of 10, 30, 40, 50, 60 and 70% (v/v) ethanol. Tissue was stored in the 70% ethanol solution overnight at 4°C, and thereafter dehydration continued in 80, 90 and 3 × 100% ethanol (10 minutes each).

Specimens were incubated in two changes of propylene oxide (30 minutes each) and infiltrated with 25 and 50% (v/v) Spurrs resin in propylene oxide for 1 hour each, overnight in 75% Spurrs resin, 8 hours in 100% at room temperature and finally embedded overnight in 100% resin, at 45°C.

Following embedding, the tissue was sectioned, etched and immunolabelled as described in section 3.8.3.

3.8.3 Immunocytochemical labelling

The immunolabelling procedure is an adaptation of the method of E. Elliott (pers. comm., 1989). All labelling and washing steps were carried out at room temperature, unless otherwise stated, on a piece of parafilm™ which was attached to the benchtop by a film of water. Aliquots of the reagents (100 µl, unless otherwise stated) were spotted onto the parafilm, forming discrete droplets. The grids were floated, section-side down, on the surface of the droplets. During the incubation procedure, the grids and reagents were covered with a perspex lid to afford protection from dust and other particulate matter. A 2 mm loop was prepared from 0.2 mm diameter copper wire, and this was used to transfer the grids to the reagents. The transfer loop was rinsed in distilled water and dried on fibre-free paper between all steps, to minimise the chances of cross-contamination. Similarly, the grids were briefly blotted onto fibre-free paper between all steps, to remove residual reagent. Care was taken to ensure that the grids did not dry out.

The embedded samples were sectioned and the thickness of the section was judged by the interference patterns given under an oblique light beam. By preference, gold sections of approximately 150 nm thick (Hayat, 1986) were mounted on nickel grids and etched for 1 minute in bromine vapours. After etching, grids were rinsed in eight changes of distilled water (1 minute each), blocked with a solution of TBS containing both 5% (w/v) foetal calf serum and 0.02 M glycine (designated buffer A) for 30 minutes. Primary antiserum was diluted 1:100 in buffer A and aliquots of 100 µl were spotted onto the parafilm, on which the sections were incubated overnight at 4°C. The grids were rinsed in buffer A (2 × 2 minutes and 1 × 5 minutes). The Protein A-gold was diluted 1:200 in buffer A and samples were incubated for an hour on 7 µl aliquots of the probe. The grids were washed in buffer A (6 × 3 minutes) and distilled water (4 × 2 minutes) and counterstained in uranyl acetate and lead citrate before being viewed in a Jeol TEM-100 CX transmission electron microscope.

CHAPTER 4

DEVELOPMENT OF A HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) METHOD FOR THE ANALYSIS OF ATRACTYLOSIDE

4.1 Introduction

From personal observations and reports in the literature, it is apparent that the incidence of ATR poisoning is not uncommon. The diagnosis of such intoxication is based on the clinical symptoms demonstrated by the patient or histological investigations on post-mortem tissue, but the results are inconclusive. Until such time as a specific diagnostic assay is produced and statistics on the incidence of such poisoning are available, the indiscriminate deaths seem likely to persist. From a cultural perspective, it is unlikely that the collection and retail of toxic herbal remedies such as *Impila* will ever be abolished, and therefore a compromise must be made to prevent these needless fatalities.

The first priority is to determine the precise extent of ATR poisoning by positive diagnosis. This information would also indicate if there is a correlation between the incidence of *Impila* poisoning and the season. *Callilepis laureola* is usually gathered in winter (Bryant, 1909) and deaths may be prevented simply by restricting collection periods to summer months, as hypogean organs tend to accumulate secondary metabolites in winter. Certainly, levels of ATR rise in the tuber of *A. gummifera* during winter periods (Contessa and Fassina, 1978) but whether the same pertains to *C. laureola* is not known. A specific, quantitative analysis for ATR would provide such useful information and by monitoring the seasonal variability of plant ATR levels, periods of low toxin accumulation in the plant could be identified. Penalties could be imposed for collection of the tuber during "peak" periods and in this manner, a compromise could be reached without inhibiting cultural practices.

It is therefore important to develop some form of sensitive diagnostic assay for the quantification of ATR in biological fluids or plant extracts. When this project was initiated, TLC was the standard detection method for this toxin (Brookes, 1979), but this is only semi-quantitative and furthermore, detection limits restrict the routine application of this technique. Atractyloside may be resolved on silica gel plates in a solvent system of butanol:glacial acetic acid:chloroform:water (6:2:2:1) and visualised by spraying with an

anisaldehyde spray reagent, which stains atractylosides pink (Brookes, 1979). Anisaldehyde-sulphuric acid is a non-specific spray reagent which is used for the detection of sugars, steroids and terpenes (Stahl, 1969).

Since HPLC is ideal for the analysis of compounds of molecular mass less than 1 000 Da (Lim, 1986), it was proposed that this method could provide a sensitive detection system for ATR, which has a molecular mass of 803.0 Da (Dawson *et al.*, 1986). There is a single reference in the literature to the HPLC detection of ATR, in which this toxin was derivatised with dansyl chloride, for use as a fluorescent probe to investigate the mitochondrial ADP/ATP antiporter (Boulay *et al.*, 1983). The 6'-O-Dansyl ATR derivative was purified on a μBondapak C_{18} column with a solvent system of glacial acetic acid:1 M ammonium acetate:water (1:1:98, v/v) and a linear gradient of acetic acid:1 M ammonium acetate :MeOH (1:1:98, v/v, 20-80% in 30 minutes) at a flow rate of 2 ml/minute. The 6'-O-Dansyl ATR derivative had a retention time of approximately 35 minutes and was monitored at an absorbance of 260 nm (Boulay *et al.*, 1983). This HPLC method, however, is only suitable for preparative purposes, and thus there are no known reports of the routine analytical detection of ATR by HPLC.

In view of the properties of ATR, namely its small size, as well as the requirement for a high resolution method to separate trace amounts of the toxin expected to be present in the tissues or body fluids, HPLC could prove a most useful analytical method. The aims therefore, were to develop an analytical HPLC method for the quantification of ATR. This necessitated the systematic optimisation of every stage of the process, including sample clean up, preparation of a derivatisation agent, derivatisation of the sample, further clean up of the derivatised sample, as well as selection of the solvent, detector and stationary phase. These aspects are reported in detail in separate subsections in this chapter, with the final optimised procedure also included in Chapter 3, section 3.3.

4.2 Preliminary investigations into chromatography conditions and detection methods for atractyloside

In view of the fact that there was no known analytical HPLC procedure available for the estimation of ATR, it was necessary to thoroughly investigate and test all possible stationary and mobile phases and methods of toxin detection.

As a first priority it was necessary to investigate the spectroscopic properties of ATR to establish whether the toxin could be detected after chromatography. Since ATR does not fluoresce, the possibilities of fluorescence detection were ruled out. By contrast the

absorbance spectrum for ATR (Fig 4.1) shows an absorbance maximum at 213 nm, which offered the possibility of employing UV detection after HPLC. Three problems however presented themselves, firstly, the absorbance at 213 nm is not very strong (Fig 4.1) and therefore could limit the sensitivity of detection and, secondly this wavelength would exclude the use of the important reverse phase solvent, tetrahydrofuran (THF), which has a UV cutoff point of 212 nm (Johnson and Stevenson, 1978), below which the solvent would interfere with the detection. Thirdly, monitoring ATR at this wavelength would limit the selectivity of detection, since many compounds absorb strongly below 250 nm (for example, aromatics, nitrates and conjugated double bond systems) (Lim, 1986). The chromatogram of a sample of biological origin would therefore contain many peaks, making identification and quantification very difficult.

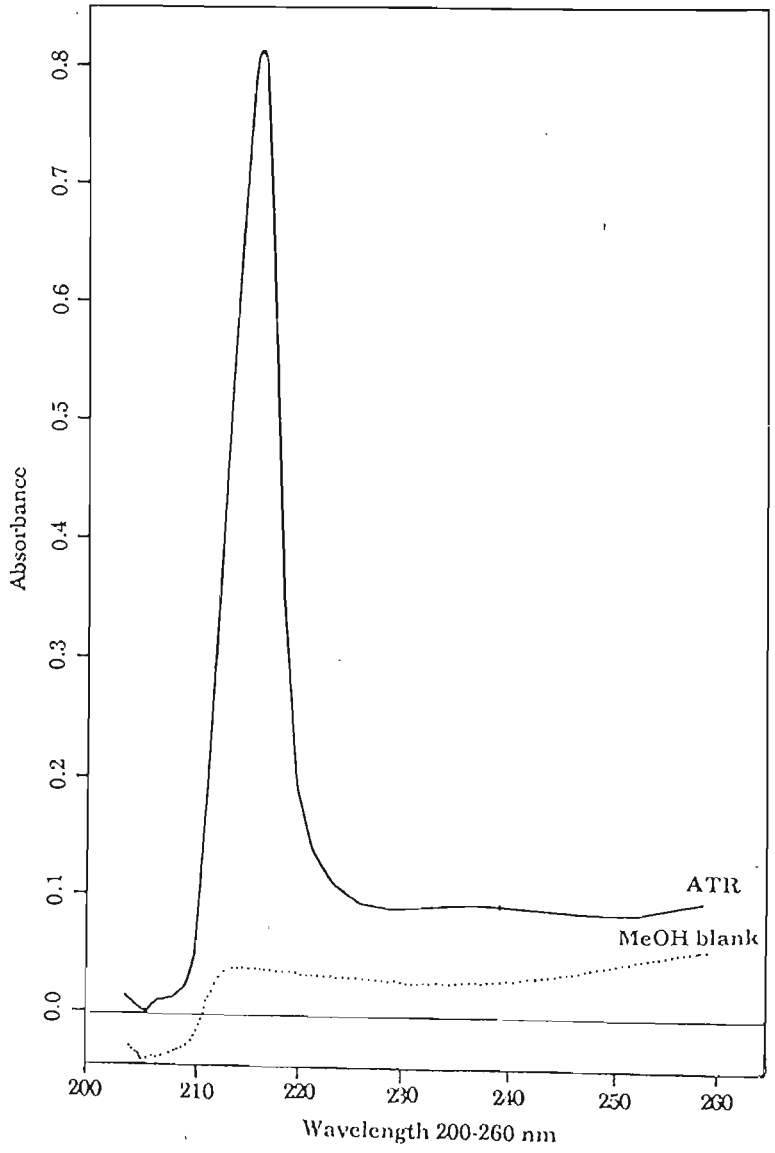

Fig 4.1 The absorbance spectrum over the wavelength 200-250 nm for atractyloside (1 mg/ml) dissolved in methanol. The spectrum was run on a Hitachi 200 spectrophotometer as described in section 3.3.4

Nevertheless, bearing the above problems in mind, it was decided to investigate possible stationary and mobile phases for ATR separation, employing this detection method. Based on the selection table of Johnson and Stevenson (1978) (since ATR is primarily water soluble) and the fact that 90% of all HPLC is carried out on reverse phase columns (Engelhardt *et al.*, 1985; Lim, 1986), a reverse phase C_{18} HPLC column was initially employed. Tests were carried out to select the best organic solvent for the elution of ATR, attempting to optimise k' (capacity factor) and α values (separation factor)§. With reverse phase columns, a polar solvent is selected and methanol (MeOH), acetonitrile (ACN), THF and water are commonly used. Selection of a suitable solvent system may be carried out in one of three ways, *viz.*, trial and error, statistical design and computer based models or mathematical computation.

Initially, selection of the solvent type for the resolution of ATR was carried out by equilibrating the column with a solution of 100% organic phase (MeOH, ACN or THF in this case) bearing in mind that with ACN the k' is usually less than with MeOH (Johnson and Stevenson, 1978; Engelhardt *et al.*, 1985; Lim, 1986). The pure ATR, dissolved in MeOH, was injected onto the column and an attempt was made to elute this compound, using ACN. Since it was noted that ACN induced spontaneous and irreversible precipitation of pure ATR, causing column blockage, it was discounted as a suitable solvent and was replaced by MeOH. The procedure was repeated using 100 % MeOH and a small peak was detected within 10-15 minutes. Since ATR was apparently being eluted from the column, the ratio of organic phase was decreased to 80% (v/v) and the column re-equilibrated. The ATR was re-injected and the retention time noted, bearing in mind that the higher the proportion of water in the eluant, the greater the k' value. Working on the premise that if the k' increases significantly, then the optimal ratio of organic phase lies between 80-100% (v/v) or, alternatively, if k' is very small, further adjustments are

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The k' value can lie between zero and infinity, depending on whether the solute is excluded from the column and passes out with V_M (k'= 0) or is irreversibly bound to the column (k'= ∞). The V_M value is characteristic for a given solute under standardised conditions. When two solutes (a and b) are to be separated, a solvent system is chosen so that k'_a and k'_b are different. This is represented by the separation factor α, where

$$\alpha = \frac{k'_b}{k'_a} \qquad \text{and } k'_a < k'_b$$

The greater α, the better resolution of the two peaks. As a corollary, if $\alpha = 1$, the two solutes will co-elute. By altering the composition of the mobile phase, it is possible to increase α and afford successful resolution of solutes (Hupe *et al.*, 1985; Lim, 1986).

required by decreasing the solvent ratio to less than 80 % organic phase until a suitable k' and α value are obtained, where $0.5 \leq k' \geq 20$, different solvent ratios were sequentially tested. Generally, a 10% (v/v) change in the proportion of organic phase in the eluant results in an effective two-fold change in k' (Johnson and Stevenson, 1978; Engelhardt *et al.*, 1985; Lim, 1986). It was noted that by changing the MeOH ratio to 80 %, the k' for ATR was still very small and thus further changes were implemented, but the subsequent chromatography runs still produced peaks with random retention times. Since the addition of 1% (v/v) glacial acetic acid to the eluant can improve resolution, particularly of solutes containing carboxyl groups (Engelhardt *et al.*, 1985), this acid was added to the MeOH eluant, but results were not reproducible. The MeOH solvent system was replaced by THF, but little improvement was afforded, also bearing in mind that the cutoff wavelength of this solvent limited its applicability.

Since the determination of the optimal solvent ratios is also possible using linear gradients by increasing the proportion of the organic phase from 10-100% over 20 minutes (Johnson and Stevenson, 1978), gradients of MeOH, THF and mixtures of both solvents in combination with acetic acid, were tested as potential eluants for ATR, but all failed to afford good resolution of the toxin. However, since the use of gradients is not recommended, unless absolutely necessary, due to complications with baseline drift and the presence of spurious peaks (Johnson and Stevenson, 1978), attention was rather turned to the mathematical determination of eluant ratios, based on the method of Snyder *et al.* (1981). However, while in theory this equation is useful for method development, in practice its application was found to be too complicated, especially if multicomponent solvent systems are used. Alternative methods for the selection of suitable solvent system were therefore sought. Since optimisation of α may also be afforded by changing the stationary phase, the column temperature (limited applicability), the mobile phase flow rate or the chemical nature of the eluant (Snyder *et al.*, 1981), attention was turned to the use of ion pair chromatography.

Ion-pair chromatography has had particular application in the separation of hydrophilic organic acids; an ion bound to a matrix may be extracted into the mobile phase as a neutral ion pair, in the presence of a counter ion such as tetra-alylammonium salts (Wahlund 1975; Lim, 1986). The principle of ion pair chromatography is theoretically suited to the resolution of ATR (Hoffman and Liao, 1977; Wahlund, 1975) but despite altering various parameters such as the buffer type and pH, solvent ratios and ion pair reagent, a reproducible k' could not be obtained. Alternatively, ammonium acetate added to the eluant can act as a counter ion, forming an ion pair with the solute, or the ammonium ions may bind to the matrix, effectively resulting in a stationary phase with ion exchange

properties (Lim, 1986). When, however, ammonium acetate was added to the MeOH eluant, it proved totally unsuccessful in the resolution of this toxin. It was suggested that ATR may be randomly binding to residual silanol groups on the reverse phase matrix, thereby rendering the results non-reproducible. The danger of chemically modifying silica bases is that residual, unreacted silanol moieties on the packing can cause peak tailing and variable k' values. This may be overcome by reacting the bonded phase with trimethylchlorosilane, which effectively "end-caps" the column, blocking potential non-specific binding sites (Engelhardt *et al.*, 1985). An end-capped ODS column (PhaseSep Spherisorb ODS2) was tested for the resolution of ATR, but no improvement was observed.

Carbohydrates have been resolved on alkylamine or alkylnitrile-substituted silica columns (Hounsell, 1986). In view of the glycosidic nature of ATR it was suggested that the separation of ATR might be possible on such a column. However, although many different solvents were tested with an alkylamine bonded phase column (Varian MicroPak-NH_2), satisfactory resolution was not obtained. Since ion exchange columns have found successful application in the resolution of amino acids and other charged molecular species (Lim, 1986), and the anionic nature of ATR (Fig 2.3) tends towards the use of such a column, a Waters IC-PAK anion exchange column was tested, but separation could not be satisfactorily optimised. More recently, mixed-mode columns have been manufactured which exhibit both reverse phase and ion exchange properties by virtue of bound octadecyl (C_{18}) and amino-propyl residues. Such a mixed mode column (PhaseSep S5 MM) was also tested using a variety of buffers, and while some success was afforded, retention times were random and therefore, the method was abandoned. The first stationary phase for HPLC was made of silica (Engelhardt *et al.*, 1985); separation being based on the differences in affinities of the solutes for the acidic silanol groups on the silica (Engelhardt *et al.*, 1985). Since ATR may be resolved on silica gel TLC plates, normal phase HPLC was attempted using a silica column (Waters μPorasil) with hexane, iso-octane, heptane, methylene chloride and chloroform as solvents. Once again though the resolution of peaks was unacceptable.

While selection of both the stationary and mobile phase is important for the resolution of a specific solute, the efficiency of the separation process is negated if the solutes cannot be detected, or as mentioned above, if the solvent systems inhibit detection. In the present studies the detectability of ATR at 213 nm was a major limiting factor, such that it was decided to subject the toxin to either pre- or post-column derivatisation. A number of diverse derivatisation agents are available, all of which when bound to the solute, impart UV or fluorescent properties to the molecule of interest, thereby facilitating detection. However, the derivatisation reaction must be rapid, must occur under mild conditions, be

reproducible and, above all, the derivatised compound must be stable (Johnson and Stevenson, 1978).

Derivatisation occurs through functional groups inherent to the molecule of interest and these include amines, alcohols, phenols, thiols, carboxylic acids, aldehydes and ketones (Imai and Toyo'oka, 1988). Since fluorescent detection is more selective and sensitive than UV detection, a fluorescent derivative was sought for the derivatisation of ATR which would enhance its detection and, in doing so, slightly alter the chemistry of the molecule, thereby facilitating chromatographic separation. Previously, ATR has been derivatised through C_6' on the glycoside moiety (Fig 2.3), using dansyl chloride (Boulay *et al.*, 1983) but when attempted by the present author, the method was not very successful. This failure was attributed to an inability to maintain the strict anhydrous conditions required by the reaction mechanism, when derivatising samples of biological origin. Therefore, this method is not practicable for serum or urine samples, for which the assay is required. Since the carboxyl group on the ring could also provide a suitable route for the derivatisation of the ATR, a suitable derivatisation agent, for this group, was sought.

In 1979, Matthees and Purdy (1979) reported that the detection of fatty acids by HPLC is facilitated by derivatising these molecules with aryldiazoalkanes. An example of such a derivatisation agent is 9-anthryl diazomethane (ADAM) which reacts without a catalyst, at room temperature with free carboxyl group to form esters (Nimura and Kinoshita, 1980). Advantages of this method are that it may be adapted for either UV or fluorescence detection, ADAM is compatible with many organic solvents and anhydrous reaction conditions are not necessary. Solutions of diazoalkanes are coloured red, but on reaction with carboxylic acids, they turn yellow. Once the derivatising reagent is added to the sample, the red colouration must be maintained for at least an hour, and should the solution turn yellow within this period, more reagent must be added. In this manner it is possible to determine the correct concentration of reagent to be added and ensure complete derivatisation of the relevant solutes (Matthees and Purdy, 1979).

In view of the above considerations, it was decided to derivatise ATR with ADAM and attempt HPLC on this derivative. Some of the studies below constitute the first description of the anthryldiazomethane derivatisation of ATR and its resolution by HPLC. Since it was necessary to develop and test each stage of the procedure, the following was investigated: synthesis of ADAM, derivatisation of ATR. determination of detection properties, sample clean up and finally, resolution by HPLC, both of standards and authentic biological samples.

4.3 Synthesis of derivatisation agent

4.3.1 Introduction

The aims of the study presented in this section were to synthesise ADAM for use as a derivatisation reagent. The synthesis of ADAM is a two step procedure as outlined in Fig 4.2, involving the production of a hydrazone species, which under the catalytic activity of mercuric oxide is converted to ADAM (Miller, 1959; Nakaya *et al.*, 1967).

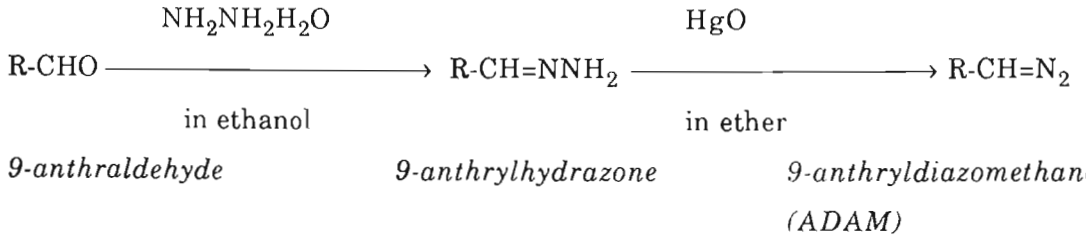


Fig 4.2 The overall reaction sequence for the synthesis of 9-anthryldiazomethane from 9-anthraldehyde (where R = 9-anthryl)

These methods necessitated a fair amount of manipulation in order to optimise the yield, the details of which are discussed in section 4.3.3 below.

4.3.2 Methods

The method of Nakaya *et al.* (1967) for the synthesis of the hydrazone did not require further modification and was applied as detailed in section 3.3.2. For the synthesis of ADAM from 9-anthrylhydrazone, substantial developmental work was necessary to optimise the procedure. This involved testing both the method of Miller (1959) and that of Nakaya *et al.* (1967), with the final optimised procedure being a combination of both (detailed in section 3.3.2).

4.3.3 Results and discussion

The synthesis of the hydrazone gave a 64% yield of crystals with a melting point of 123°C, as compared to a yield of 90% and a melting point between 124-126°C, reported by Nakaya *et al.* (1967). Since repeated efforts did not improve the yield, it was decided to move onto the next stage of the process. As described by both Miller (1959) and Nakaya *et al.* (1967) the conversion of the hydrazone to ADAM involves the catalyst mercuric oxide and is carried out under basic conditions (ethanol saturated with KOH), in the presence of anhydrous sodium sulphate (which absorbs water) in a solvent of diethyl ether. In the present studies,

the hydrazone was observed to be sparingly soluble in the diethyl ether and it was presumed that on conversion to the diazomethane, the compound went into solution. At termination of the reaction period, there were low yields of ADAM and, it was noted that considerable amounts of unreacted hydrazone were still present in the reaction mixture, even if the incubation time was increased to 5 hours. The low yields were presumed to be the result of the poor solubility of the hydrazone in the diethyl ether and thus the solvent was sequentially replaced by ethanol, MeOH, petroleum ether, hexane and chloroform, but with no further success. Attention was, therefore, turned to the catalyst as a means of improving the yield.

Initially only orange mercuric oxide was available which resulted in low yields of diazomethane. Miller (1959) however investigated the use of mercuric oxides as catalysts in the synthesis of diazomethane and concluded that yellow mercuric oxide was a more efficient catalyst than the red oxide, for the synthesis of diphenyldiazomethane. This, in part, was proposed to be due to the smaller particle size of the yellow oxide, but it was also suggested that its method of synthesis could be of significance. Red mercuric oxide is synthesised by igniting mercuric nitrate whereas yellow mercuric oxide is prepared by adding base to a mercuric salt. Contaminating base in the yellow mercuric oxide is thought to be important in the preparation of the diazomethane (Miller, 1959). As a trial, in the present studies, both yellow and red mercuric oxide were used as catalysts in the preparation of ADAM and, while the yield was improved in the presence of the yellow mercuric oxide, particularly if freshly prepared, the final ADAM yield of 18% was significantly lower than that quoted in the literature (65%, Nakaya *et al.*, 1967) This result, however, represented an improvement on previous efforts and provided sufficient reagent to carry out the derivatisation steps. The ADAM crystals had a melting point of 63°C which is in agreement with that quoted in the literature (63-64°C, Nakaya *et al.*, 1967).

Once the synthesis of the ADAM crystals had been optimised (see details in section 3.3.2), the reagent was used to derivatise ATR and this procedure, together with the monitoring of the derivatisation, is given in section 4.4 below.

4.4 Optimisation of the derivatisation of atractyloside

4.4.1 Introduction

The derivatisation agent ADAM was initially developed for the detection of fatty acids (Matthees and Purdy, 1979), its advantage being in its stability and high reactivity with carboxylic acids. Previously, non-derivatised fatty acids were detected at 210 nm or below,

but limited solvent selection and poor selectivity at this wavelength warranted derivatisation of these acids (Nimura and Kinoshita, 1980). As a similar detectability problem was experienced with ATR, an attempt was made to derivatise this toxin with ADAM, in the hope that the diazomethane would react with the carboxylic acid group on C_4 of the diterpene ring (Fig 2.3), thus improving both the detectability and the hydrophobicity of ATR. The developmental work that was systematically undertaken to optimise this derivatisation of ATR, is listed in section 4.4.2 and the results are discussed in section 4.4.3 below.

4.4.2 Methods

The following approaches were sequentially carried out :-

1. Selection of compatible solvents for ATR and ADAM;
2. Determination of the extent of derivatisation, as monitored by TLC, employing the method as detailed in section 3.2;
3. Preparative TLC (section 3.2) for the isolation of a semi-pure ATR-ADAM extract;
4. Determination of the absorbance spectrum and the excitation and emission wavelengths of this semi-pure extract, by the methods described in section 3.3.4.

4.4.3 Results and discussion

Selection of the reaction solvent

Initially the derivatisation procedure of Matthees and Purdy (1979) was used, whereby the ADAM was dissolved in hexane, chloroform or ether and added to the ATR solution (prepared in buffer, water or methanol). However, phase separation frequently occurred, particularly if the ATR was prepared in an aqueous solution. Some success was afforded if the ATR was prepared in MeOH and thus attention was turned to the derivatisation procedure of Nimura and Kinoshita (1980) who dissolved fatty acids in methanol, and treated this with 0.1% (w/v) ADAM solution prepared in methanol. It was noted by the present author that when preparing the 0.1% solution, that the ADAM did not readily dissolve in the MeOH and, consequently, a considerable proportion of the reagent was wasted. This problem was overcome by adding sufficient ADAM crystals directly to the ATR test solution to maintain a dark red colour, for at least an hour and adding more reagent if the solution turned yellow. It was subsequently found, however, that reaction conditions were most successful if the ADAM was dissolved in diethyl ether and added to a sample prepared in methanol.

This approach was incorporated into the final optimised method detailed in section 3.3.3.

The extent of derivatisation

The extent of derivatisation was monitored by the one-dimensional TLC method, detailed in section 3.2. As can be seen from Fig 4.3, this method efficiently resolved ADAM, ATR-ADAM and pure ATR from each other and thus enabled efficient monitoring of the extent of the derivatisation. When the plates were viewed under UV light (365 nm), prior to staining with anisaldehyde, a blue fluorescent spot at an R_f of 0.574 was noted in the reaction mixture, lane 3. When the plates were subsequently sprayed with the anisaldehyde spray reagent, the same blue fluorescent spot stained pink and furthermore, its R_f value was higher than that of the non-derivatised ATR (R_f 0.314) in lane 2. From these results it was concluded that ATR had been derivatised by ADAM and the reaction had gone to completion as there was no evidence of free ATR in lane 3. This result was vital to ensure that the HPLC method for ATR was fully quantitative. Since however, the crude ATR-ADAM solution still contained free ADAM and a bright yellow contaminant (see solvent front, Fig 4.3) it was necessary to employ preparative TLC to purify the derivative. This is described in the next section.

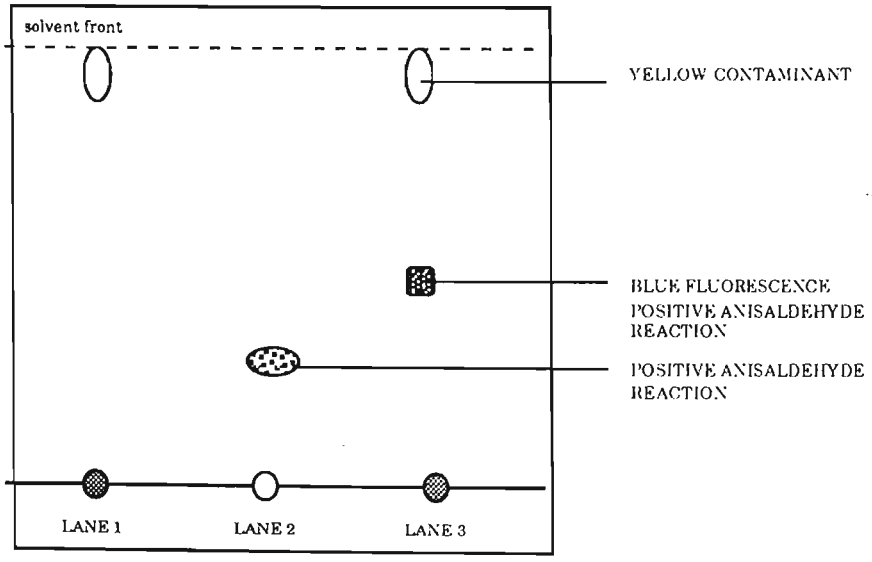


Fig 4.3 Comparative resolution of ADAM (LANE 1), ATR (LANE 2) and ATR derivatised with ADAM (LANE 3) using TLC on silica gel plates in a solvent system of butanol:acetic acid:water:chloroform (6:2:2:1). Atractyloside and ATR-ADAM were detected with anisaldehyde, while ADAM by-products and the ADAM conjugated to the ATR were detected by fluorescence at 365 nm, prior to spraying with anisaldehyde spray reagent

Isolation and spectroscopic properties of ATR-ADAM

Preparative TLC was carried out to isolate the ATR-ADAM conjugate from the free ADAM, reaction by-products and other contaminants, and the semi-pure ATR-ADAM was used to determine the UV spectrum of the molecule. As shown in Fig 4.4, ATR-ADAM has an absorbance maximum at 254 nm probably due to the presence of its anthracyl moiety. An attempt was made to elucidate the structure of the ATR-ADAM isolate using gas chromatography-mass spectroscopy, but the salt-like properties and the non-volatile nature of ATR-ADAM prevented conclusive results being obtained.

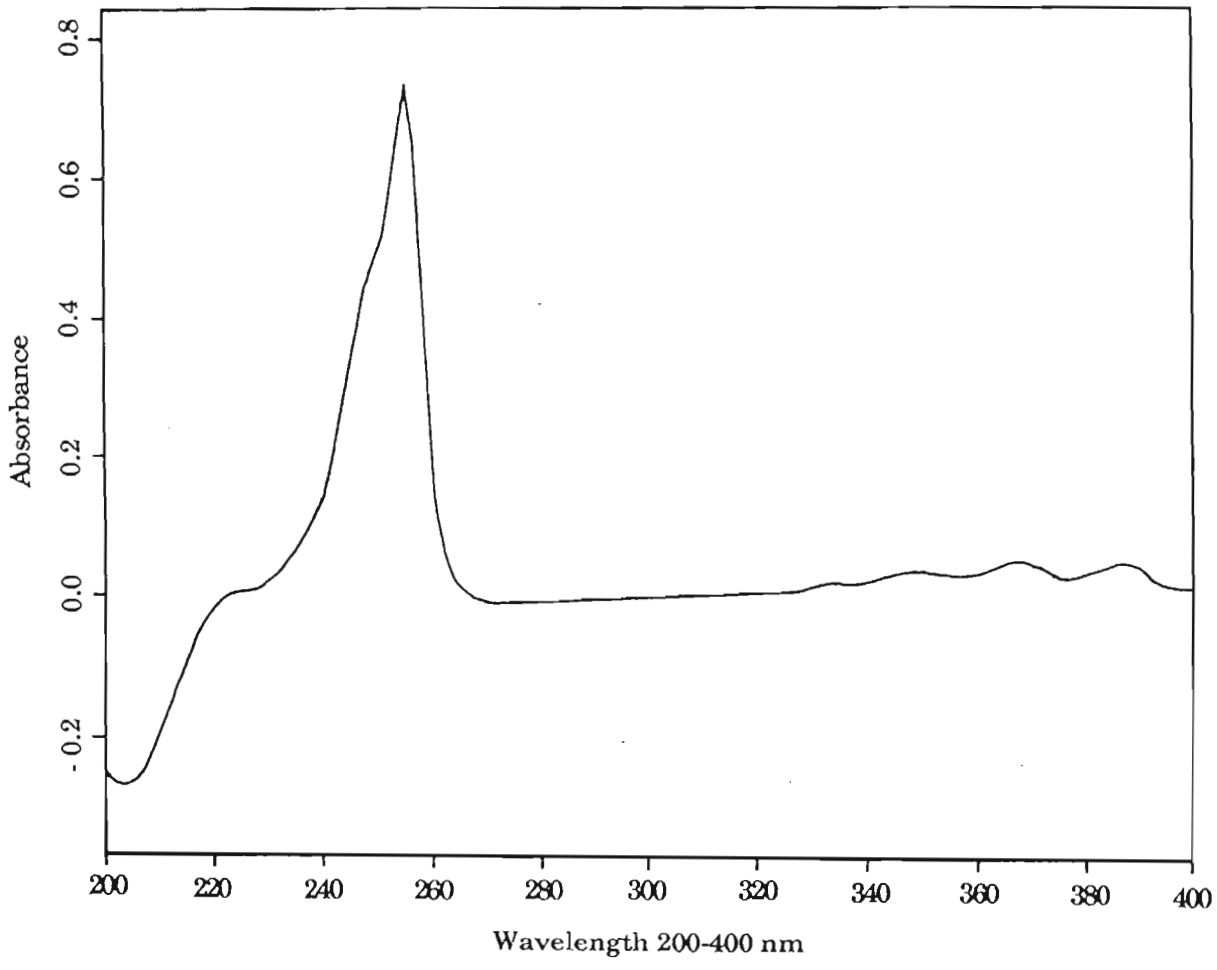


Fig 4.4 Absorbance spectrum over the range 200-400 nm for the semi-purified ATR-ADAM derivative, separated by TLC and extracted into methanol

The advantage obtained by derivatising compounds with ADAM is that they may be detected both by UV absorbance (at 254 nm) or with a fluorescence detector. An important consideration when employing fluorescence detectors though is the solvent choice and in this regard non-halogenic solvents are recommended (Johnson and Stevenson, 1978). The solvent can also affect fluorescent intensity and for example, quinoline does not fluoresce in hexane, but does so in ethanol (Johnson and Stevenson, 1978). In the present studies the fluorescence of the semi-purified ATR-ADAM was investigated and found to have an

excitation wavelength of 360 nm and an emission wavelength of 425 nm, in either ACN or MeOH. It was also noted with interest that, while ATR precipitates in the presence of ACN (see section 4.2), the ATR-ADAM derivative does not, thus permitting the use of ACN as a solvent for the HPLC of this derivative.

To conclude, the above developmental studies resulted in a highly acceptable derivatisation method with optimal modification of ATR and the resulting ATR-ADAM derivative had ideal UV adsorption, fluorescence and solvent properties for HPLC. Since it was clear from the TLC investigation that the sample derivatised with ADAM contained a number of reaction by-products and other contaminants and, furthermore, that the biological samples would contain fatty acids and other molecules which would react with the ADAM, it was decided to eliminate some of these extraneous compounds. For this purpose, a sample clean up protocol was developed which involved the defatting and deproteinisation of the sample and removal of most of the reaction contaminants. The results of these studies are presented in the next section.

4.5 Optimisation of sample clean up

4.5.1 Introduction

It was anticipated that the presence of contaminants in the biological fluids would interfere with both the derivatisation and resolution of ATR. Thus, it was important to implement a sample clean up procedure to remove as much of the contaminating protein as possible. Two methods were considered. The first was the precipitation of proteins with a 10% (v/v) TCA solution and, while this is a useful reagent for the denaturation and precipitation of proteins, TCA is very corrosive of stainless steel, a common alloy used in HPLC equipment (Johnson and Stevenson, 1978). An alternative protein denaturant is methanolic hydrochloric acid which has been used by Flanagan and Ruprah (1989) to extract herbicides from urine and serum. While hydrochloric acid is also corrosive of stainless steel, the concentration used in the extraction procedure is only 0.2% and thus its deleterious effect may be considered negligible.

Pilot studies were initiated to monitor the efficiency of methanolic hydrochloric acid as a solvent for the extraction of ATR from biological fluids, together with the use of hexane as a solvent for the extraction of fatty acids and removal of the derivatisation reaction by-products using a minicolumn system. The methods of this clean up system are described in section 4.5.2 while the results are discussed in section 4.5.3.

4.5.2 Methods

Rat urine or serum (section 3.12 and 3.1.3), spiked with 1 mg ATR/ml, was first mixed with hexane and plunged into an acetone ice-bath. This caused the serum sample to freeze, but the hexane containing the extracted lipids remained in solution and thus could be decanted off (Lim, 1986). Thereafter, methanolic hydrochloric acid solution was added (Flanagan and Ruprah, 1989), briefly centrifuged in a bench top centrifuge and the supernatant was collected while the pellet from the precipitated protein was discarded. The defatted, deproteinised sample was derivatised with ADAM, and further sample clean up was carried out on a silica minicolumn system, as described in section 3.3.3. One-dimensional TLC (section 3.2) was used to monitor the efficiency of the clean up steps. Details of the final optimised sample clean up method are presented in section 3.3.3.

4.5.3 Results and discussion

When the extraction method of Flanagan and Ruprah (1989) was applied to spiked rat serum or urine, the anisaldehyde test demonstrated that ATR had been extracted, although steroids were also probably present, since they too are stained by anisaldehyde (Stahl, 1969). The process though had clearly removed significant quantities of protein from the ATR preparation. The methanolic hydrochloric acid extraction, therefore, appears to be an efficient and acceptable method for the extraction of ATR from biological material and fortuitously, methanol is miscible with the derivatising solvent diethyl ether, thus facilitating derivatisation of ATR.

When the extracted ATR in methanolic hydrochloric acid was derivatised with ADAM and the sample monitored using TLC, it was found that the extraction solvent did not affect the derivatisation procedure. However, when the spiked derivatised samples were resolved on a C_{18} column, it was apparent that the sample preparation method was inadequate in removing certain contaminating molecules which had reacted with the ADAM; presumed to be either lipids or fatty acids. This complicated interpretation of the chromatogram and thus, as a preliminary clean up step before treatment with the methanolic hydrochloric acid, the sample was pre-treated with hexane (see details in section 3.1.6) which extracted the lipids (Lim, 1986).

Resolution of the sample, prepared as described above, using HPLC, demonstrated that the hexane extraction had removed a significant proportion of contaminating molecules. However, the low k' values obtained for the elution of ATR-ADAM from a reverse phase column presented difficulties when the same method was attempted on spiked biological

samples. Despite altering the polarity of the eluant, the k' value could not be changed significantly and, when the derivatised samples were injected directly onto the column without any form of pre-column clean up, the decomposed product of the ADAM and excess ADAM was eluted with a k' very close to 0, in a manner comparable to that reported by Matthees and Purdy (1979). This meant that α was less than one as these reaction by-products were co-eluting with the ATR-ADAM, thus indicating that the sample still required further clean up.

In an attempt to improve the purification of the crude ATR-ADAM derivative, a minicolumn system filled with silica gel was employed (section 3.3.3). When the column, loaded with sample, was rinsed with ethyl acetate:MeOH (3:1) a yellow contaminant was removed. Thin layer chromatography of the same sample on silica gel plates demonstrated that this solvent system resulted in the deposition of ADAM by-products at the solvent front, while the ATR-ADAM conjugate remained fixed to the origin. Since it was assumed that the ATR-ADAM had been attached to the silica column, it was decided to investigate a second solvent for the elution of the ATR-ADAM. Thus, when further TLC studies showed that the ATR-ADAM conjugate could be moved from the origin to the solvent front in a solution of chloroform:MeOH (2:3), this solvent system was tested on the silica column, after first washing with the ethyl acetate:MeOH solvent (3:1). The results showed that the chloroform:MeOH (2:3) did indeed remove the ATR-ADAM from the column.

To conclude, treatment with hexane, then methanolic hydrochloric acid and finally the above two-solvent method on a silica column, thus provided an efficient procedure for the clean up of ATR-ADAM from a crude serum or urine sample. At this stage therefore, the semi-purified samples were ready for final separation and analysis by HPLC. Attention was thus turned to the development of an HPLC method for the detection of the ATR-ADAM derivative which is described in the next section.

4.6 Optimisation of the HPLC procedure employing ATR-ADAM and spiked biological samples

4.6.1 Introduction

From the results of the TLC (Fig 4.3) it was concluded that ADAM successfully derivatises ATR, in a stable and reproducible manner and not only imparts fluorescent properties to the toxin, but in addition, as noted from the R_f values obtained by TLC, the diazomethane alters the polarity of the ATR. Attention was therefore turned to the optimisation of the solvent system for the resolution of ATR-ADAM by HPLC. Since the separation of fatty

acid-ADAM derivatives was carried out on a reverse phase C_{18} column using a solvent system of ACN or MeOH (Matthees and Purdy, 1979; Nimura and Kinoshita, 1980), it was decided to attempt the HPLC separation of ATR-ADAM on a reverse phase column, using a similar system. Preliminary developmental work to identify the most suitable solvent was carried out on the semi-purified ATR-ADAM extract using reverse phase C_{18} TLC plates. Although separation was afforded by both ACN or MeOH, it was noted that the results of the TLC experiments could not be directly extrapolated to HPLC. Further developmental work was therefore done directly on the HPLC system and details of this methodology are given in section 4.6.2.

4.6.2 Methods

Several alternative solvent systems were tested on a 3.9 × 150 mm NovaPak C_{18} column, at a flow rate of 1.5 ml/minute, using both a UV detector set at 254 nm and a fluorescence detector set at an excitation wavelength of 360 nm and an emission wavelength of 425 nm. These included, in sequence of testing:-

1. 70-30% (v/v) ACN;
2. 45% (v/v) ACN;
3. 40% (v/v) MeOH;
4. 40% (v/v) MeOH with 1% (v/v) glacial acetic acid;
5. Ion pair chromatography
6. glacial acetic acid:1 M ammonium acetate:water (1:1:98, v/v) with a linear gradient of 20-80% glacial acetic acid: 1 M ammonium acetate: MeOH (1:1:98, v/v) (Boulay *et al.*, 1983);
7. 30-70% (v/v) ACN with 2% (v/v) 1 M ammonium acetate and 2% (v/v) glacial acetic acid;
8. 30-70% (v/v) MeOH with 2% (v/v) 1 M ammonium acetate and 2% (v/v) glacial acetic acid;
9. MeOH:1 M ammonium acetate:glacial acetic acid: water solvent mix (38:2:2:58, v/v).

Method 1 was carried out on the semi-purified ATR-ADAM extract (section 4.5), while methods 2-9 also involved work on rat serum and urine samples, from non-ATR treated Wistar rats. Samples were first spiked with ATR and then derivatised. In the case of the semi-purified ATR-ADAM extract, TLC (section 3.2) was used to identify the nature of the resolved peaks. The results of the resolution of ATR-ADAM, and ATR-ADAM in biological samples, using the above solvent systems, are discussed in section 4.6.3 below, with the final optimised solvent system being detailed in section 3.3.4.

4.6.3 Results and discussion

When a series of solvent systems ranging from 70-30% (v/v) ACN (method 1 above) were tested on the semi-purified ATR-ADAM, a solution of 40% (v/v) ACN was found to yield the best resolution but, since the k' and value for this compound was very low, it remained to be seen how efficient the method would be for the detection of ATR in complex biological samples. Indeed, when spiked biological samples were subjected to HPLC with the same solvent system, there was considerable co-resolution of peaks, despite the pre-chromatographic clean up described in section 4.5. This necessitated further developmental work on solvent optimisation to improve the α value of the system.

When the ratio of ACN:water was adjusted marginally (from 40% to 45%, method 2), in an attempt to optimise both k' and α, the resolution remained poor. Subsequently, when the ACN was replaced by MeOH (method 3), a small improvement in α was noted. The addition of glacial acetic acid (1%, v/v, method 4) sharpened the peaks (Johnson and Stevenson, 1978), but the resolution was still unsatisfactory. Thus attention was again turned to the use of ion pair chromatography (method 5) and, while marginal improvement was noted with the urine standard, the resolution of the spiked serum samples was still inadequate. Close scrutiny of all the chromatograms showed that a combination of MeOH, glacial acetic acid, water and an ion pair reagent might be more ideal, and thus attention was again given to the HPLC method of Boulay *et al.* (1983) for the separation of the 6'-O-Dansyl derivative of ATR. Scrutiny of their solvent system, as detailed in method 5, section 4.6.2, suggests that the MeOH decreases the polarity of the solvent, while the glacial acetic acid improves peak sharpness and resolution (Engelhardt *et al.*, 1985). The ammonium acetate may act as either an ion pair reagent or impart ion exchange properties on the column (Lim, 1986). Based on the above studies and work carried out by the present author, it was decided to use a similar solvent system for the elution of the ATR-ADAM derivative (method 6), except avoiding the use of gradients. This would keep the elution system as simple as possible, preventing baseline drift and long column run times; which result from having to re-equilibrate the column between runs.

Both ACN and MeOH showed potential as eluants, but initially various ratios of ACN were tested with a set proportion of 2% ammonium acetate and 2% (v/v) glacial acetic acid (method 7). The results were encouraging, but when the ACN was replaced by MeOH (method 8), improved resolution was afforded. Subsequent work on the optimisation of this solvent combination resulted in the use of a methanol:1 M ammonium acetate:glacial acetic acid: water solvent mix (38:2:2:58, v/v) with an optimal flow rate of 1.5 ml/minute.

The chromatogram of such a separation, carried out on the semi-purified ATR-ADAM standard is shown in Fig 4.5.

The identity of the ATR-ADAM peak was carried out by monitoring the peak spectra, using the photodiode array detector. The second peak at time 2.96 minutes gave the characteristic spectrum as shown in Fig 4.4, while that of the first peak was significantly different, indicating that the second peak was the ATR-ADAM. The eluant containing the resolved peaks was collected separately, concentrated, spotted onto TLC plates and sprayed with anisaldehyde spray reagent. The collected peak with a retention time of 2.96 minutes stained pink, which indicated a predominance of ATR whereas the peak eluting before this, at approximately 1.5 minutes, gave a negative result with anisaldehyde. These observations thus confirmed the assumption made on the basis of the photodiode array spectra.

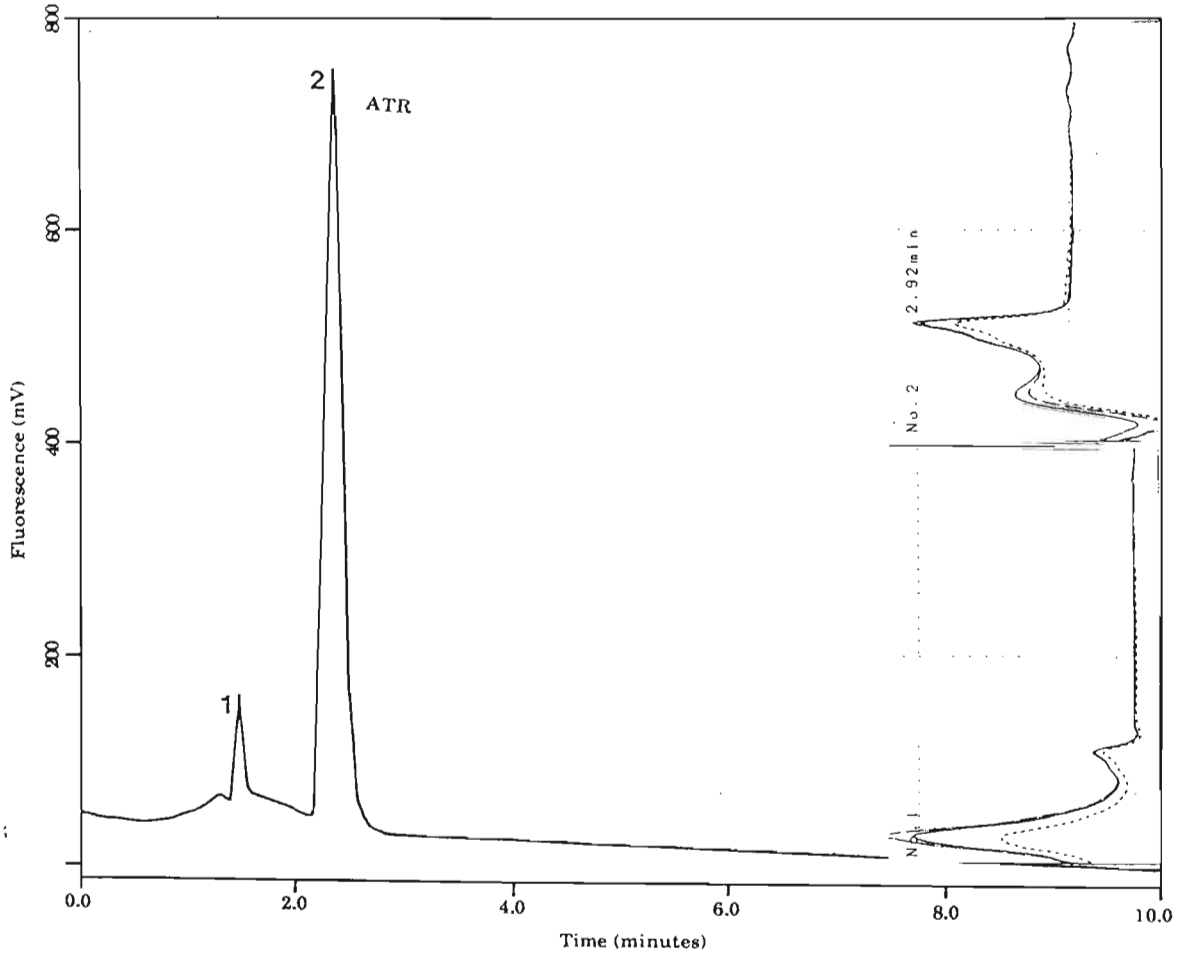


Fig 4.5 Chromatogram of a semi-purified ATR-ADAM derivative
Column: 3.9 × 150 mm NovaPak C_{18} column, solvent:MeOH:1 M ammonium acetate:glacial acetic acid:water (38:2:2:58), flow rate: 1.5 ml/minute, detection: fluorescence; λ_{ex} = 360 nm, λ_{em} = 425 nm. The diode array spectra for the two peaks is included, showing three spectral lines for each plot, taken at three time points

Having checked the performance of the solvent system with a standard sample, it was important to fully evaluate its ability to resolve ATR-ADAM from other ADAM modified compounds in authentic samples of urine and serum that had been spiked with ATR. The results of the chromatographic separation of a sample of semi-purified ATR-ADAM, a serum sample spiked with 1.5 mg ATR/ml and the same spiked serum sample mixed 1:1 (v/v) with the semi-purified ATR-ADAM standard, are presented as an overlay plot in Fig 4.6. It was noted from the chromatogram in Fig 4.6, that the semi-purified ATR-ADAM gave rise to four peaks, the major of which was eluted at time 3.02 minutes. By comparison the ATR-spiked serum sample gave rise to eight predominant peaks, once again including a peak at time 3.06 minutes, which correlated directly with that of the ATR-ADAM standard. When the standard and the spiked serum were mixed, in a ratio of 1:1 (v/v), it was anticipated that a peak at time ± 3.0 minutes would be eluted and by virtue of dilution factors, should have a peak height intermediate to that of the spiked serum and the ATR-ADAM standard. This is clearly evident in Fig 4.6 and thus from these results it was concluded that this solvent system afforded good resolution of both the semi-purified ATR-ADAM standard and complex biological fluids containing this toxin and furthermore, that the results were reproducible. Similar results were obtained for a spiked urine sample (data not shown). From a consideration of the amount of ATR used to spike the serum and urine samples, taken through the entire clean up process, the recovery of ATR was calculated to be 86 and 92% respectively. These differences in the recoveries suggest that the toxin does indeed bind to serum proteins and that possibly some ATR remained bound during the extraction process, thus assecting its recovery. Based on these recovery determinations, it was thus decided to correct all results by factors of 1.16 or 1.08 for the serum or urine analyses respectively.

Having optimised the derivatisation, sample preparation and chromatographic elution of both semi-purified ATR-ADAM and ATR-ADAM spiked urine and serum, and found it to be efficient, reproducible and give high recoveries of ATR, attention was turned to the efficacy of the system in detecting endogenous ATR in authentic biological specimens.

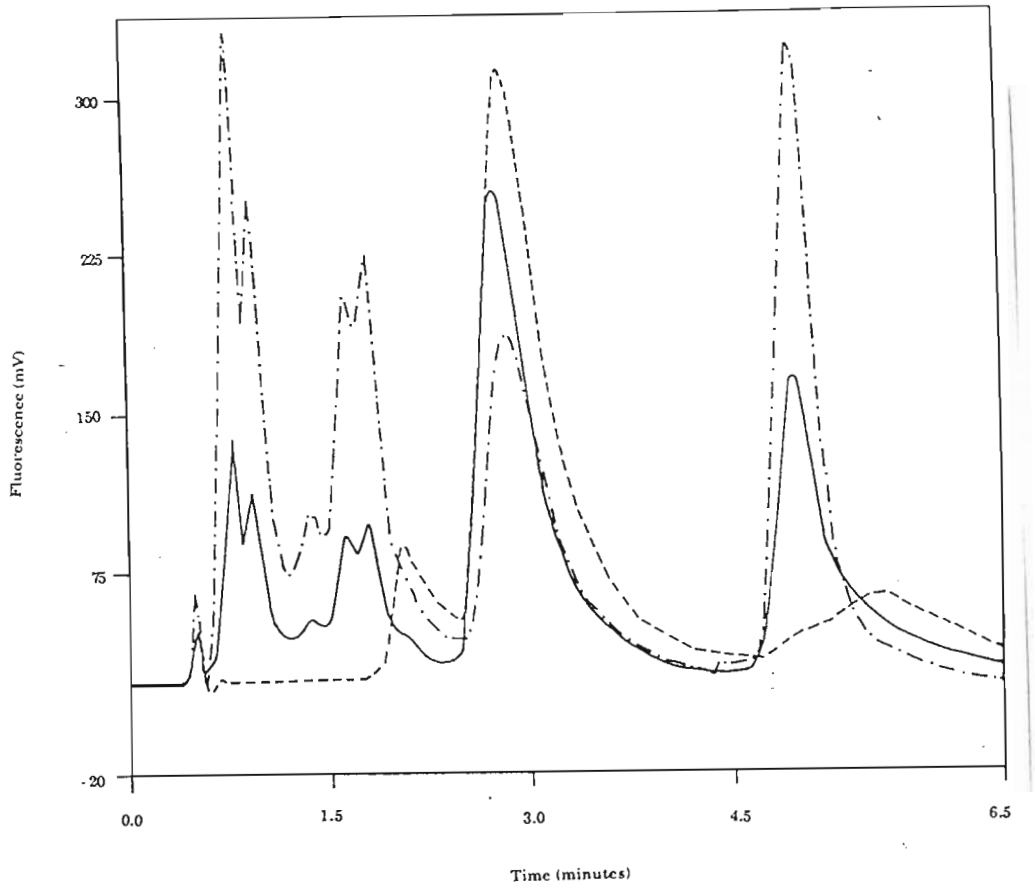


Fig 4.6 Overlay chromatogram of a semi-purified ATR-ADAM conjugate, a serum sample spiked with ATR and derivatised (serum spike) and the same derivatised spiked serum sample mixed 1:1 (v/v) with the ATR-ADAM isolate (serum spike + ATR-ADAM). Chromatographic conditions were as for Fig 4.5 (ATR-ADAM = --, serum spike = ---, serum spike + ATR-ADAM (1:1, v/v) = –). In all cases, 20 µl of sample were analysed, as detailed in section 4.6.2 and 3.3.4

4.7 Analysis of ATR in the tuber of *Callilepis laureola*

4.7.1 Introduction

To date, most work relating to the levels of ATR in plants has been carried out on the tuber of *A. gummifera* and it has been determined that the concentrations of this toxin are dependent upon the season and also the region from where the plant was collected (Contessa and Fassina, 1978). These workers found that the concentration of ATR in this thistle is higher in winter than in summer, correlating with the observation that hypogean organs accumulate secondary metabolites during quiescent periods (Contessa and Fassina, 1978). Furthermore it was also determined that thistles collected from Sicily are significantly more toxic than those from Sardinia, indicating that environmental conditions may also

affect toxin production. This apparent increased biosynthesis and accumulation of secondary metabolites is frequently associated with conditions of stress, a prime example being the production of Δ^9-Tetrahydrocannabinol from the *Cannabis* species (Prof. J.P. Nel, pers. comm, 1989). This plant is ubiquitous in the Transkei, where the soils are very poor, and it is a well known fact that marihuana originating from this area is of exceptional quality (Anon. foreign tourist, pers. comm., 1988). Unfortunately, there are no reports on the seasonal levels of ATR in *C. laureola*, information which may be of extreme significance, since the herb is frequently gathered in winter (Bryant, 1909), when essentially it could be most toxic. Thus, the aim of this investigation was to establish if ATR could be detected in an extract of the tuber of *C. laureola*. Details of the methodology are presented in section 4.7.2, with the results and discussion in section 4.7.3.

4.7.2 Methods

The HPLC determination of ATR was carried out on a methanolic extract of the tuber of *C. laureola*, as prepared by F. Dehrmann (1991). Briefly, fresh tuber was milled, extracted with methanol and the extract clarified by elution from an NH_2 solid phase column. The semi-purified plant extract was derivatised and cleaned up as described in section 3.3.3. Thereafter the sample was injected onto the HPLC column and subjected to HPLC as described in section 3.3.4. Standard ATR-ADAM over the range 0.5 to 16 ng ATR was also analysed by HPLC and a standard curve of the amount of ATR versus peak area was generated.

4.7.3 Results and discussion

From the chromatogram shown in Fig 4.7 (a), for the derivatised tuber extract, it is clear that this extract contains ATR, a fact which was confirmed by TLC. As further confirmation that the peak eluting with a k' of ± 3.0 minutes was in fact ATR, the derivatised plant sample was spiked with the semi-purified ATR-ADAM standard and rechromatographed. The results presented in Fig 4.7 (b) show that the peak with an elution time of approximately 3 minutes was observed to increase in height on addition of the standard spike, thus confirming the presence of the ATR in the plant tuber.

In order to quantify the amount of ATR in the tuber, and correct for any losses of ATR due to the clean up and chromatography process, it was necessary to prepare a standard curve for graded amounts of toxin which also could be used to check the sensitivity of the detector and the linearity of its response. Fig 4.8 shows a plot of peak area versus the amount of ATR, derivatised with ADAM. From this figure it is apparent that a linear relationship

exists between the peak area and ATR concentration. The detection limit of this method is 0.001 ng ATR*. The standard curve was used to calculate the quantity of ATR in the tuber and it was found that there was 6.7 µg ATR/g "wet weight" of tuber tissue. Assuming that *Impila* is prepared from approximately 10 g of material, this would account for a dosage of 67 µg ATR. The LD_{50} for ATR in rats, dosed i.p., has been determined as 210 mg/kg bwt by Luciani *et al.* (1978), who state that "the toxicity increasing from mouse and rat to dog suggests a very high ATR toxicity for man". Clearly though from the results of the HPLC investigation, the levels of ATR administered in *Impila*, are significantly lower than the levels discussed in the LD_{50} investigation. Furthermore, the levels of toxin administered to rats in this study, were significantly lower than the LD_{50} quoted by Luciani *et al.* (1978) and a level of 25 mg ATR/kg bwt was sufficient to induce severe kidney damage. This emphasises the high toxicity of both ATR and *Impila*.

* If a sample of 200 µl is injected onto the column and the detector is set to × 128

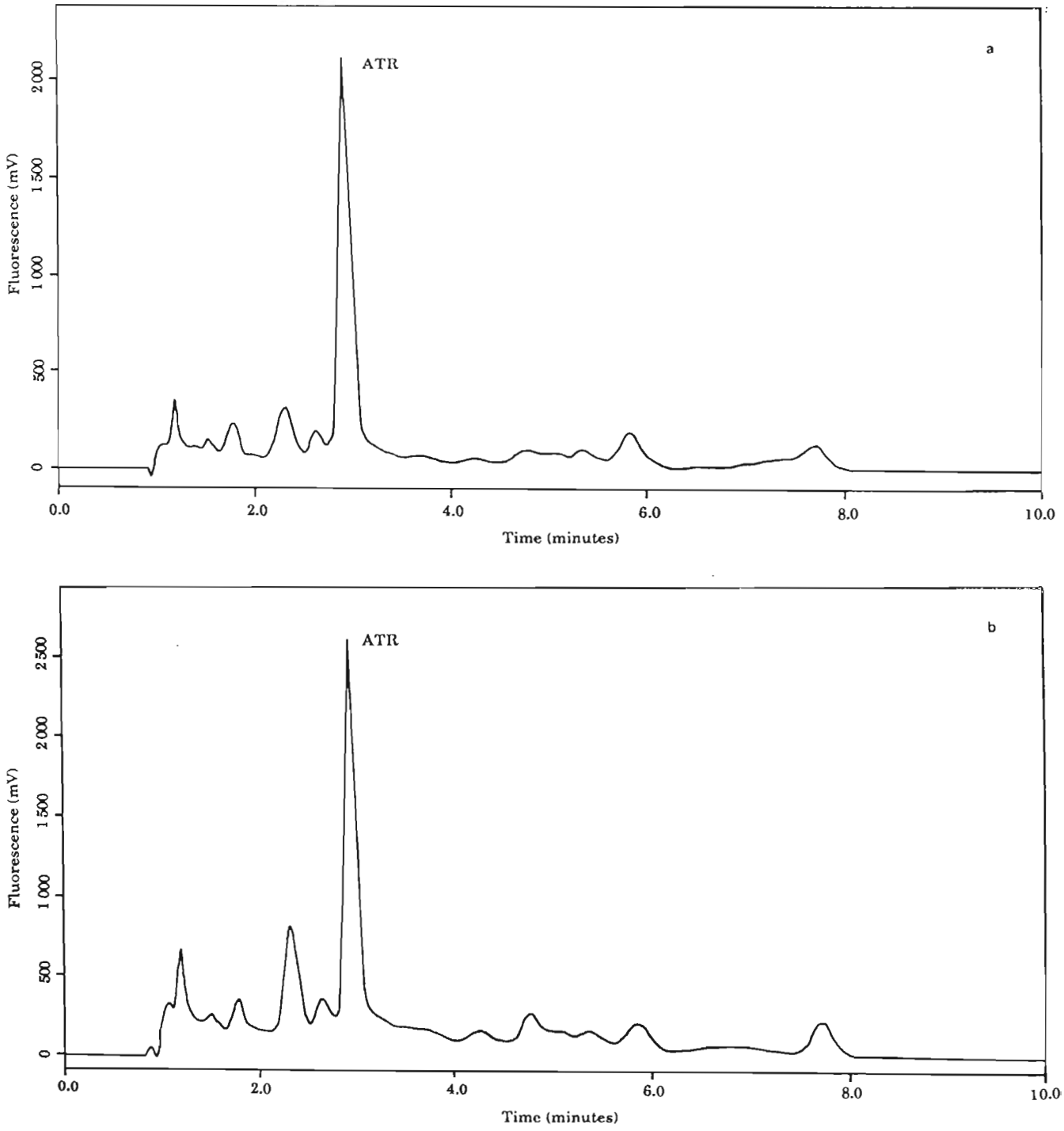


Fig 4.7 (a) Chromatogram of a methanolic plant extract of *C. laureola* derivatised with ADAM

(b) Chromatogram of a methanolic plant extract from *C. laureola*, derivatised with ADAM and spiked 1:1 with a semi-purified ATR-ADAM standard.

Chromatographic conditions were as for Fig 4.5

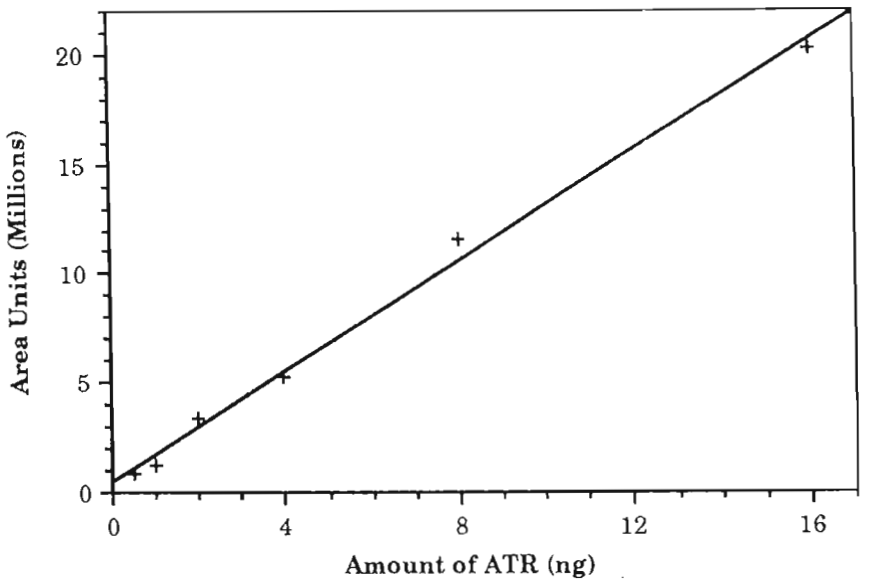


Fig 4.8 Standard curves of ATR derivatised with 9-anthryl diazomethane, showing the peak area of the derivative versus injected amount of ATR. The standard solutions were subjected to the same clean up steps as described in section 3.1.6. Serial dilutions of standard ATR-ADAM were injected (20 µl) onto the column with the detector set at a sensitivity of × 4

This quantitative method for the monitoring of ATR in *C. laureola* shows great promise for the monitoring of the seasonal variation in ATR levels in the tuber, as well as the relative concentrations of the toxin in plants from different regions. In this way, recommendations could be made regarding the best time to harvest the herb and furthermore, less-toxic strains could be selected for commercial cultivation. Since the method could be applied to the analysis of endogenous ATR in plant extracts, attention was turned to the suitability of this method for the detection of the toxin in urine and serum samples collected from rats dosed with this toxin and this investigation is described in the next section.

4.8. Analysis of atractyloside in urine and serum from ATR-treated rats

4.8.1 Introduction

The ability of the HPLC method to detect ATR in plant extracts led to further investigation as to its potential in identifying this toxin in biological fluids collected from animals exposed to ATR. A number of important issues had to be addressed, namely whether urine or serum should be collected for use in the analysis and furthermore, whether this toxin is

biotransformed *in vivo*. There has been no work of this nature carried out on ATR, but a brief investigation was performed by Richter and Spiteller (1978) who isolated analogues of ATR, originating from coffee beans, from the urine of coffee drinkers. The analogue, as detected by gas chromatography, was excreted into the urine having not been radically altered *in vivo*,. Based on this work, it was decided that in the case of ATR, the toxin was most likely to be found in the urine. The methodology for the analysis of the rat urine and serum is given in section 4.8.2 with the final results and discussion in section, 4.8.3.

4.8.2 Method

The urine and serum samples collected from rats dosed with 0, 5, 10 and 25 mg ATR/kg bwt (section 3.1) were cleaned up and derivatised with ADAM as described in section 3.3.3. The samples were analysed by TLC, as described in section 3.2 and by HPLC, as described in section 3.3.4. The results are given in section 4.8.3 below.

4.8.3 Results and discussion

Although TLC was carried out on the derivatised urine and serum samples, the ATR-ADAM derivative could not be detected probably due to the poor detection limits of this method. High performance liquid chromatography was performed on the serum and urine samples but ATR could not be detected in the serum samples, either because it was present in too low a concentration, i.e., less than 1 pg per ml, or the toxin was not present in the serum after 24 hours. Atractyloside, was however present in some but not all of the urine samples. Typical elution profiles of a control urine sample, collected from a rat prior to dosing, and a sample collected from the same rat after dosing with ATR, are shown in Fig 4.9 a and b respectively. The results show clearly that ATR (peak with a retention time of ± 3.0 minutes) was present in high concentrations in the urine of ATR-treated rats but not in the controls. Furthermore, it will be noted from the two chromatograms, that two other peaks eluting at times of 6.56 and 7.04 minutes, respectively, were detected in the urine from rats dosed with toxin but were absent from the control. Some of the urine samples from rats dosed with ATR gave negative results for ATR, but still showed these additional peaks eluting at about 7 minutes. It is the opinion of the author that the presence of these peaks is in some way related to the dosing with ATR and are possible metabolites of this toxin. Radiotracer studies will be necessary to confirm the identity of these peaks.

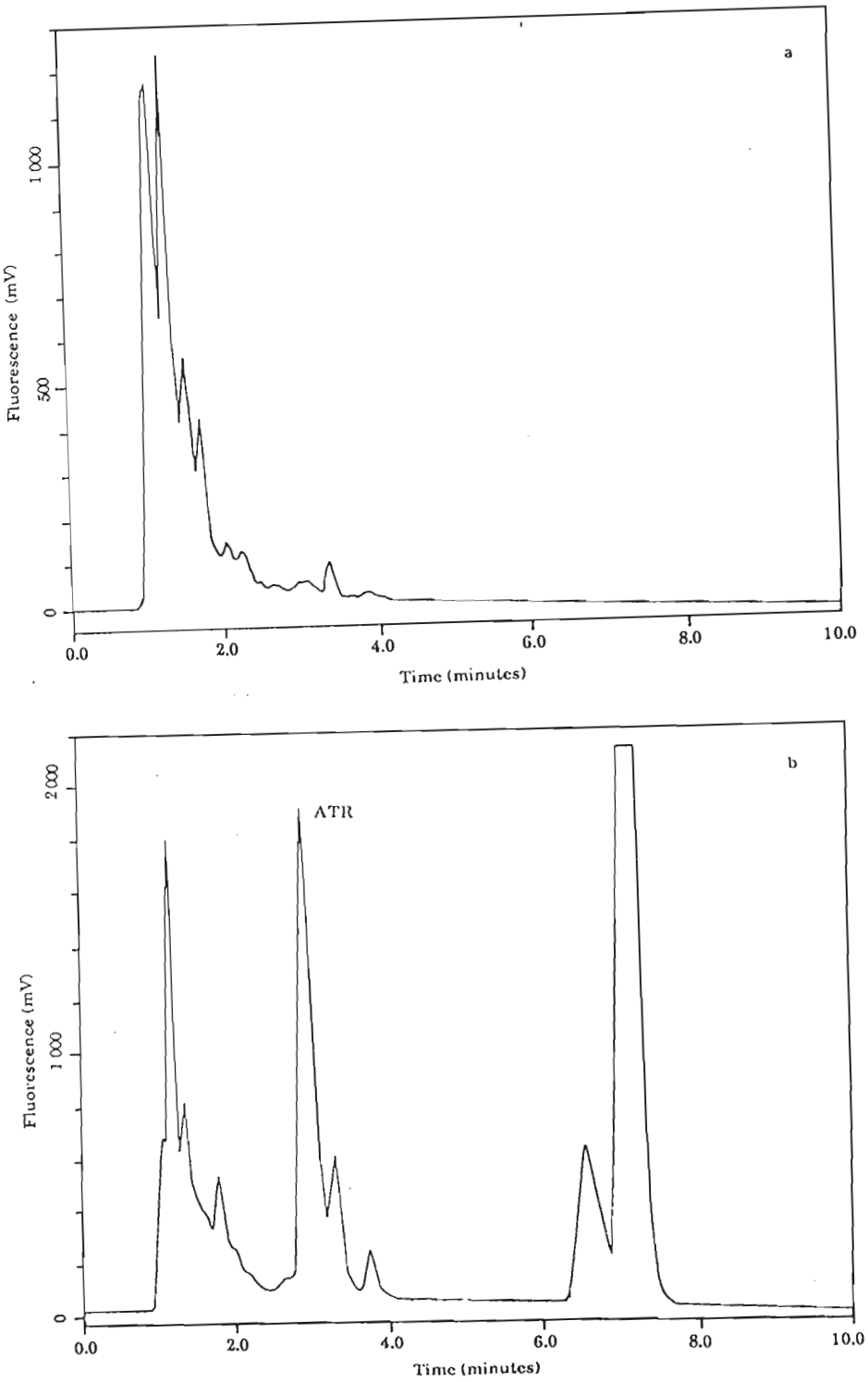


Fig 4.9 Representative chromatograms of rat urine derivatised with ADAM
(a) Control rat urine collected prior to dosing
(b) Urine collected from the same rat dosed with 25 mg ATR/kg bwt, on day 1; chromatographic conditions were as for Fig 4.5

The overall results of the HPLC on the rat urine samples are tabulated in Table 4.1, and were calculated by converting the area under the ATR peak into quantity of ATR, employing the standard curve given in Fig 4.8. From these results it will be seen that, as expected, no ATR is detected in the urine of the control rats. Furthermore, ATR was not detected in the urine of rats dosed with 5 mg ATR/kg bwt, suggesting that the toxin has a

long residence time in the rat and would be excreted at some point after the trial was terminated. Alternatively, it may have been present in the urine at a level too low to be detected or was excreted as a metabolite which could not be identified by HPLC.

Table 4.1 Daily excretion of ATR (detected as ATR-ADAM) in the urine of rats dosed with 0, 5, 10 and 25 mg ATR/kg bwt. Atractyloside was derivatised with ADAM as described in section 3.3.3 and monitored by reverse phase HPLC as described in section 3.3.4. Results were corrected by a factor of 1.08 to allow for the loss of ATR during sample preparation

dose mg /kg bwt	rat	[ATR] (pg/ml urine)				
		day 0	day 1	day 2	day 3	day 4
25	1	-	-	247.3	-	236.5
	2	-	-	217.1	-	48.6
	3	-	262.4	-	-	-
	4	-	212.8	-	-	-
	5	-	1965.6	-	-	-
10	1	-	-	-	41.04	-
	2	-	-	-	-	-
	3	-	-	-	-	-
	4	-	-	-	3.43	-
	5	-	-	-	2.12	284.1
5	1	-	-	-	-	-
	2	-	-	-	-	-
	3	-	-	-	-	-
	4	-	-	-	-	-
	5	-	-	-	-	-
0 (control)	1	-	-	-	-	
	2	-	-	-	-	-
	3	-	-	-	-	-
	4	-	-	-	-	-
	5	-	-	-	-	-

The rats receiving the high dose of toxin (25 mg/kg bwt) could be divided into two groups. The first group consisting of rat 1 and 2 were found to excrete ATR into the urine on day 2 post-dosing, and the concentrations of ATR were very similar (approximately 200 pg ATR/ml). There was no evidence of ATR being excreted by these rats on day 3, but on day 4 when the trial was terminated, there was evidence of ATR being excreted again. The second group comprising rats 3, 4 and 5, dosed with 25 mg ATR/kg bwt, excreted ATR into the urine on day 1 post-dosing only. The levels excreted by rats 3 and 4 were similar to that excreted by rats 1 and 2, but rat 5 apparently excreted a 10-fold higher amount of ATR than the other rats in this dose regimen.

Toxin was only detected in the urine of the medium dosed (10 mg ATR/kg bwt) rats on day 3 (rats 1,4 and 5) and the peak areas were not very large, demonstrating that the levels of excreted ATR were not very high. Rats 2 and 3 from the same dose group did not excrete ATR after dosing. On day 4 however, when the trial ended, rat 5 excreted a considerable proportion of toxin into the urine equivalent to that of the higher dose regimen, but this pattern was not observed in any of the other medium dosed animals.

Although the results do not demonstrate any noticeable trend, the following four explanations are possible. The first is that toxin was excreted each day, but not always at a level high enough to be detected by this method. The second proposal was that the toxin is excreted daily but was metabolised to the extent that the biotransformation altered the molecule's k' under the HPLC conditions described, thus preventing positive identification of the ATR metabolite. Work by Richter and Spiteller (1978) demonstrated that ATR analogues in the urine of coffee drinkers were found to be slightly modified from the parent molecule, and although this investigation was carried out on an ATR analogue (Richter and Spiteller, 1978), it is likely that ATR may undergo a similar form of biotransformation *in vivo*. The third proposal is that the toxin remains "fixed" or bound within the animal, either in the kidney or in the serum, although the latter is unlikely as the toxin was not detectable in the serum samples. The "fixed" ATR is then gradually released from its site of binding, thus accounting for the random appearance of the toxin in the urine samples. The fourth and perhaps the most likely possibility is that all three situations may occur simultaneously, such that some ATR is released untransformed, while some remains "fixed" in the tissue, only to be randomly excreted, while the remaining toxin is biotransformed and thus not recognised as ATR when excreted in the urine. It is interesting to note from Table 4.1, that those rats dosed with the highest level of ATR excreted the toxin earlier than those rats receiving the medium dose. If this is indeed a real trend, then it is possible that the rats dosed with 5 mg ATR/kg bwt released their ATR sometime later than day 4.

This is the first report of the derivatisation of ATR with ADAM, the analytical determination of ATR by HPLC and furthermore, the detection of ATR in mammalian biological fluids. The results indicate that ATR is excreted into the urine and that for diagnostic purposes this is the biological fluid of choice. This is the first report that ATR is excreted into the urine and will be of benefit in both future studies and for diagnostic purposes. An overall discussion of the optimisation procedure and the results of the HPLC determination of ATR in biological fluids is given in section 4.9 below

4.9 Overview of results and conclusions

This chapter dealt with the sequential development of an HPLC method for the detection of ATR in biological fluids. Up until this time, the standard detection method was by TLC (Brookes, 1979) although there was reference to the preparative clean up of a dansyl chloride derivative of the toxin (Boulay *et al.*, 1983). The development of an HPLC detection method for this toxin proved more complex than initially envisaged and it soon became apparent that the poor detectability and amphipathic nature of the molecule hindered both resolution and detection. Derivatisation seemed the approach of choice and although the method of Boulay *et al.* (1983) was attempted, the pre-requisite for anhydrous conditions presented practical problems when using biological samples. Attention was drawn to the anthryldiazomethane derivatisation of fatty acids, mediated through the carboxyl group on the acid. It has been previously shown that the toxicity of ATR is dependent upon the retention of structural integrity and removal or alteration of the carboxyl group effectively renders the molecule non-toxic (Vignais *et al.*, 1978). Since the toxicity of ATR is undisputed, it was concluded that the carboxyl group on C_4 of the diterpene ring would provide a suitable site for derivatisation as it was unlikely to be a site of biotransformation *in vivo*.

The purpose of using ADAM was three fold: the site of derivatisation was convenient, the derivatisation agent could be detected by both UV or fluorescence, improving the detectability of the ATR and furthermore, the hydrophobic nature of the ADAM would effectively enhance the overall hydrophobicity of the ATR, possibly facilitating chromatographic resolution.

The initial step in this derivatisation procedure was the synthesis of ADAM and although the methodology was well documented, this still necessitated developmental work. Miller (1959) noted the importance of an alkaline mercuric chloride catalyst, hence the use of yellow mercuric oxide (instead of the red oxide) and this was verified by the present author.

However, the reaction only went to completion if the mercuric oxide was freshly prepared. Despite optimisation of the synthesis, the yields obtained were much lower than those quoted in the literature and no explanation can be given for this. Melting points were however, essentially equivalent.

The derivatisation of ATR was very successful and, when monitored by TLC, it was apparent that the reaction had gone to completion. This method is not dependent upon the maintenance of anhydrous conditions, being ideally suited for use with biological samples. The amount of reagent added to each sample was arbitrary and could be strictly monitored by observing a colour change from red to yellow. The reaction takes place under mild conditions and the derivatised ATR conjugate remains stable for several weeks. Unfortunately, the derivative reacts with carboxyl groups and bound to a number of other metabolites in the sample, giving rise to complicated chromatographs, which hindered interpretation. Verification of the ATR-ADAM peak was made possible by carrying out semi-preparative TLC on the ATR-ADAM, isolating the fluorescent blue band, which also stained pink on reaction with an anisaldehyde-sulphuric acid spray reagent, and this isolate was used in the optimisation of the HPLC conditions. An attempt was made to further characterise this isolate, using gas chromatography-mass spectroscopy, but this proved unsuccessful.

Although the fluorescent properties of the derivatisation agent were the final means of detection, use was made of the photodiode array detector to aid peak identification, based on a UV spectral profile for the semi-purified TLC fraction (as presented in Fig 4.4). The eluted solutes were monitored sequentially at a UV wavelength of 254 nm and for fluorescence, at an excitation wavelength of 360 nm and emission wavelength of 425 nm. The fluorescence detection limits were much lower than that of absorbance and fluorescence is therefore recommended as the detection method.

After perfecting the solvent system for the semi-pure ATR-ADAM derivative, attention was turned to the detection of the toxin in biological fluids. Both urine and serum were spiked with the toxin and derivatised. Interpretation of the chromatograms was complicated and this necessitated extensive sample clean up, both prior to and post derivatisation in order to improve resolution. Nevertheless, there was apparent co-resolution of peaks and α had to be optimised by altering the solvent system to MeOH:1 M ammonium acetate:glacial acetic acid: water (38:2:2:58, v/v).

After optimisation of the HPLC method, employing spiked biological samples, attention was drawn to its application to authentic samples. As a starting point, a methanolic extract

of the tuber of *C. laureola* was prepared and the presence of ATR was demonstrated by TLC. Following derivatisation of the extract with ADAM and sample clean up the results of the chromatogram (Fig 4.7) confirmed the presence of ATR in the tuber and furthermore demonstrated that this HPLC method could be applied to the detection of ATR in other biological samples. Urine and serum samples collected from rats dosed i.p. with ATR (Fig 4.9 and Table 4.1) demonstrated that ATR could not be detected in the serum samples, excluding the use of this biological fluid for diagnostic purposes. However, ATR was detected in some of the urine samples, providing the first positive evidence that this toxin is excreted into the urine and furthermore, that the period of excretion was apparently inversely proportional to the level of toxin administered. It was difficult to identify a specific trend in the excretory pattern of ATR as different rats within a specific dose regimen displayed variable degrees of toxin elimination. Toxin was apparently not excreted all at once, or over a period of several successive days but it appeared as if the toxin was "fixed" within the animal and at different time points, was released. This phenomenon is apparently not uncommon, as drugs may bind to macromolecules establishing an equilibrium between bound and free compound; the latter being available for biotransformation and excretion (Bradford, 1990). The bound fraction acts as a depot and is released once the free form of the xenobiotic is excreted, thus re-establishing equilibrium (Bradford, 1990). A similar situation could occur with ATR and this could explain the differential rates of excretion of toxin into the rat urine. Without further studies on the pharmokinetics of ATR this proposal will remain highly speculative.

Attention was also drawn to the appearance of spurious peaks eluting at approximately 7 minutes, present in the chromatograms of the urine of rats dosed with ATR. The fact that these peaks were absent from the control samples suggests that this compound was related to exposure to ATR. It was proposed that this may be a metabolite of ATR but no further attempt was made to identify this compound.

The purpose of the study presented in this chapter was to develop an analytical method for the detection of ATR in biological fluids, and from the evidence presented it is clear that this has been accomplished. From the results of this investigation the presence of ATR in the tuber of *C. laureola* was confirmed and it is proposed that this method will prove ideal for the monitoring of seasonal changes of ATR in the plant. As mentioned in the introduction to this chapter, this information may prevent further deaths due to accidental ATR poisoning, simply by restricting herb gathering times, but obviously this is an area for further investigation. The HPLC carried out on the rat urine and serum samples demonstrated that this toxin is released into the urine and furthermore that the rate of excretion is apparently dose dependent. It was also noted that the appearance of the toxin in

the urine is not sequential over a period of several days but rather sporadic, suggesting that the toxin is bound within the animal and released at different periods. The presence of spurious peaks on the chromatograms of the urine from animals exposed to ATR intimated that metabolites of this toxin were present and therefore that ATR undergoes some form of biotransformation *in vivo*. In conclusion therefore, a sensitive method for the HPLC detection of ATR has been developed and this method has proven suited to the detection of ATR in plant extracts and mammalian biological fluids. Attention was then turned to the development of an alternative assay for the detection of this toxin and the following two chapters (5 and 6) describe the optimisation of antibody production and an immunoassay.

CHAPTER 5

THE PRODUCTION AND CHARACTERISATION OF ANTIBODIES TO ATRACTYLOSIDE

5.1 Introduction

As outlined in Chapter 1, the primary aims of this investigation were to develop a diagnostic assay for ATR and identify the target organs and organelles of this toxin. Accidental poisoning by ATR in both humans and livestock is widespread (Bhoola, 1983; Hatch *et al.*, 1982; Martin *et al.*, 1986; Schteingart and Pomilio, 1984; Georgiou *et al.*, 1988) but the unavailability of an assay for the specific detection of this toxin makes diagnosis nebulous. The development of a diagnostic assay would be extremely beneficial for a number of reasons. Firstly, specific diagnosis would facilitate the immediate application of appropriate therapy should a patient be intoxicated by this poison. Secondly, from a long term perspective, data relating to the precise incidence of ATR poisoning would alert authorities to the dangers of using toxic medicinal herbs such as *C. laureola* or *A. gummifera* and relevant action could be taken. Thirdly, although no antidote to this toxin is presently available, prompt identification of *X. strumarium* poisoning in livestock could prevent further loss, provided the farmer is alerted to the danger and can move his herd to alternative grazing areas.

The development of an assay for ATR was approached from both a chromatographic and immunological perspective, and Chapter 4 describes the sequential development of an HPLC method for ATR. The use of HPLC is restrictive however, in that the technique requires both skilled labour and expensive equipment. Thus, an assay was required for use in the field which had to be specific for ATR poisoning, but did not necessarily have to be quantitative. For this purpose an immunoassay was deemed appropriate, but since antibodies have not previously been raised to ATR, their production necessitated considerable optimisation of every stage of the process, including choice of carrier and conjugation of ATR to the carrier, selection of host species, dosing regimen, as well as collection of antibodies and their characterisation. These aspects are reported in detail in separate subsections in this chapter, with the final optimised procedure also included in Chapter 3, section 3.4. Not only did the availability of antibodies to ATR enable subsequent development of a diagnostic assay (Chapter 6) but, in addition, provided the means to locate the toxin *in vivo* by immunocytochemical methods, which was the second primary aim of this investigation (see Chapter 7).

5.2 Preliminary investigations into antibody production for atractyloside

The immunogenicity of a molecule is reliant on it being foreign to the host species and possessing sufficient structural complexity to stimulate an immune response. The ability to initiate antibody production is not inherent to all non-self molecules. Compounds which are antigenic (i.e., capable of reacting with antibodies) but are not immunogenic (i.e., incapable of inducing an immune response unless coupled to a carrier) are classified as haptens (Bach, 1982; Tijssen, 1985). Generally haptens are molecules with a mass of less than 5 000 Da, but sometimes even compounds with a mass exceeding this cut-off point are not immunogenic (Bach, 1982; Tijssen, 1985; Catty and Raykundalia, 1988). By this definition, ATR is classified as a hapten, as this molecule has a molecular mass of 803.0 Da (Dawson *et al.*, 1986). To be rendered immunogenic, a hapten such as ATR must be covalently coupled to a carrier, the choice of carrier being essentially limitless.

In view of the fact that there was no known procedure available for the production of antibodies to ATR, it was necessary to thoroughly investigate and test different carriers, conjugation procedures, host species and the dosing regimen. Since this toxin contains an ent-kaurene backbone (a precursor to the gibberellins) (Fig 2.3) it was decided to conjugate ATR to the carrier using a modified procedure to that employed for the conjugation of gibberellins to carriers (Fuchs and Fuchs, 1969). When, however, the efficacy of both BSA and ovalbumin as immune carriers for ATR were tested, employing Ross brown hens, antibodies (IgY) isolated from the egg yolks by the method of Polson *et al.* (1985) were of poor quality. This prompted a change in host species from chicken to rabbit but once again it became apparent that the quality of antibodies raised to both ATR-BSA and ATR-ovalbumin conjugates were very poor with the resulting 1:40 working dilution of anti-ATR antibody being inadequate for routine use (Bye *et al.*, 1990). These results led to serious consideration of the entire immunization procedure and the factors influencing antibody titre and the immune response in general.

Poor antibody titre may in part be attributed to ATR having an as yet unidentified inherent immune toxicity; a phenomenon apparently not uncommon in toxic haptens (Hunter *et al.*, 1985; Ragelis, 1985). Hunter *et al.* (1985) demonstrated that spontaneous dissociation of the mycotoxin T_2-toxin from its protein carrier inhibited protein biosynthesis, and effectively induced immunological paralysis. Similarly, it was noted that antibodies raised against a conjugate of highly poisonous tetrodotoxin, coupled to BSA, were of low titre (Ragelis, 1985; Watabe *et al.*, 1989). It was also demonstrated that an amanitin-BSA conjugate had a high specific toxicity for sinusoidal cells of the liver, macrophages and the proximal tubules of the

kidney (Cessi and Fiume, 1969; Barbanti-Brodano and Fiume, 1973; Bonetti *et al.*, 1974) and that this conjugate induced very poor antibody response. It was only by altering the conjugation procedure and foregoing boosting of the rabbits until seven weeks after priming, that a final anti-amanitin antibody working dilution of only 1:200 was achieved. However, of the eight rabbits immunised by Faulstich *et al.* (1975), four died within a week of priming, a further two died after the fourth booster and the condition of the remaining two deteriorated sufficiently to warrant euthanasia.

Like in the case of T_2-toxin, tetrodotoxin or amanitin, low working dilutions of anti-ATR antibodies may also be the result of this toxin being an immunosuppressant, a property which is compounded by successive immunizations. Frequent boosting may render the secondary response partially inoperative as a result of a high serum concentration of antigen still in the process of being eliminated by primary antibodies. However, while an inherent toxicity of the hapten and frequent immunization may result in a poor antibody response, the metabolic fate of the antigen may also affect antibody production. In this regard, it is known that antigens which are poorly metabolised in the body are poor immunogens (Bach, 1982), and though little is known about the metabolic fate of ATR *in vivo*, analogues of ATR isolated from the urine of coffee drinkers appear to be only slightly modified from the parent molecule (Richter and Spiteller, 1978). Hence, the effects of ATR toxicity, poor ATR metabolism and too frequent immunization of the host species, may account for the low quality of antibody produced.

In view of the above considerations, the procedure for the production of anti-ATR antibodies was revised, with specific attention being given to the type of carrier, the conjugation procedure, the host species and, above all, the immunization regimen.

5.3 Optimisation of the conjugation of atractyloside to a carrier and production of antibodies

5.3.1 Introduction

The first priority for the production of antibodies to ATR was the optimisation of the conjugation procedure. The site of conjugation was an important consideration, particularly for the immunocytochemical investigations which followed (Chapter 7). Landsteiner observed that an antibody is generally directed to the (hydrophobic) region of the hapten, furthest removed from the linkage (Tijssen, 1985), and it was speculated that anti-ATR antibodies would therefore be directed towards the penhydro-region of the phenanthrenic ring of ATR, should the carboxyl moeity be used as a site of conjugation. The carboxyl group

on the diterpene ring is crucial to the toxicity of the molecule, apparently associating directly with the ADP binding site on the antiporter, while the stereology of the remaining portion of the molecule maintains ATR "locked" in this position (Vignais *et al.*, 1978). The success of an immunocytochemical investigation would, therefore, be dependent upon the antibody having access to an exposed portion of the ATR molecule, namely that furthest removed from the carboxyl portion.

Landsteiner is credited with establishing the rules for the coupling of haptens to carriers, which is extensively reviewed by Tijssen (1985). Briefly, stable, covalent conjugation is mediated through reactive groups on the protein/carrier (including ε and α amino moieties, phenolic or carboxyl groups) and the hapten (Bach, 1982; Tijssen, 1985). Thus, molecules with inherent carboxyl groups, such as ATR, may be directly conjugated to the carrier without further modification. The mixed anhydride (Fig 5.1a) or carbodiimide methods (Fig 5.1b) are most frequently used, whereby the carboxyl group on the hapten is converted to an acid anhydride, which in turn reacts with free amino groups on the carrier (Erlanger, 1980; Bach, 1982; Tijssen, 1985). Both these coupling methods were investigated by the present author as a means of coupling ATR to a carrier (see section 5.3.3 below).

a) $R'COOH + RO.CO.Cl \longrightarrow R'CO.O.CO.OR + R''NH_2 \longrightarrow R'CO\text{-}NHR''$

Hapten — mixed anhydride — carrier — conjugate

b) $R'COOH + R''NH_2 + RN{=}C{=}NR \longrightarrow R'CO\text{-}NHR'' + RNH\text{-}CO\text{-}NHR$

Hapten — carrier — carbodiimide — conjugate

Fig 5.1 Proposed overall reaction for the conjugation of a hapten to a protein carrier, mediated by a) mixed anhydride and b) the carbodiimide method (after Erlanger, 1980; Bach, 1982)

The choice of carrier is limitless and as previously mentioned, serum albumins are commonly used (Bach, 1982). More recently novel carriers such as naked bacteria have been utilized as immune conjugates (Bellstedt *et al.*, 1987), the advantages being that they are highly immunogenic and in addition, antibodies raised against the carrier do not interfere with the immunoassay, a problem which frequently occurs if albumin carriers are used.

The Enterobacteriaceae, including the genus *Salmonella*, produce lipopolysaccharide endotoxins, which form constituents of the outer cell wall. The lipopolysaccharides may be divided into two distinct regions; a hydrophobic lipid portion (Lipid A) which is in close

contact with the cell wall and a hydrophilic polysaccharide portion. The hydrophilic region comprises a basal core of branched polysaccharides containing N-acetylglucosamine, glucose, galactose, heptose, 2-keto-deoxyoctonate (KDO) and O-specific chains. It is the O-specific polysaccharides which impart the serological specificity of both the lipopolysaccharide and the bacterial parent strain and provide the basis for the Kauffmann-White classification scheme (Galanos *et al.*, 1971). R-mutant strains are devoid of the O-specific chains and contain defective basal cores. *Salmonella minnesota* R595 contains acid labile ketocyclic links between the KDO in the core region and Lipid A. Mild acid hydrolysis cleaves off the hydrophilic portion, exposing the hydrophobic region of the bacterial cell wall, yielding so-called naked bacteria. Exposed functional groups on the bacteria may be used to chemically couple haptens such as ATR to the bacteria and this complex is used to immunize animals. A further benefit to the use of this carrier is that the lipoprotic nature of the bacteria functions as an immune stimulant, obviating the need for adjuvants (Luderitz *et al.*, 1966; Galanos *et al.*, 1971; Bellstedt *et al.*, 1987).

Rabbits and chickens are most routinely used in polyclonal antibody production; the antibodies being isolated from serum or egg yolks respectively. Both host species were used by the present author. Site of immunization and dose regimen vary considerably depending on the host, the antigen and the desired response. In the case of chickens, immunogen is injected directly into the pectoral muscles, while rabbits are generally immunized subcutaneously; the immunogens being emulsified in adjuvants which potentiate and prolong the immune response (Tijssen, 1985; Catty and Raykundalia, 1988). Most adjuvants, such as the routinely used Freund's adjuvants, are water-soluble surfactants or have the capacity to form a surface interface (oil-emulsions). This adjuvant can however, result in severe granularmatomous inflammation and other undesirable effects, distressing the animal (Woodard, 1989). Hence the added advantage of using naked bacterial carriers.

The objectives of this investigation were, therefore, to compare the mixed anhydride and carbodiimide methods of conjugation, employing BSA and RSA as protein carriers and secondly, to employ the selected conjugation method for the preparation of ATR conjugates with BSA, RSA and naked bacteria, which would then be compared for their ability to generate a strong immune response specific for ATR and not the carrier.

5.3.2 Materials and methods

Mixed anhydride procedure (Method I)

Atractyloside was conjugated to either BSA or RSA through the carboxyl group on C_4 of the diterpene ring, using a modified procedure of Fuchs and Fuchs (1969). Atractyloside (60 mg)

was dissolved in 1,4 dioxan (0.5 ml). To this was added N,N'-dicyclohexyl carbodiimide (25 mg) and the solution stirred for 30 minutes, at room temperature. The resulting white precipitate was removed by centrifugation for 1 minute, at 5 000 $\times g$ and the solution (containing the acid anhydride) was added dropwise to a solution containing the protein carrier (50 mg) dissolved in 0.1 M borate buffer, pH 8.5 (5 ml). The solutions were stirred overnight at 4°C and dialysed exhaustively against 0.1 M borate buffer, pH 8.0.

Carbodiimide procedure (Method II)

Atractyloside was coupled to BSA and RSA, using a modification of the method supplied by P. Hoffman (pers. comm., 1988) for conjugating gibberellins to BSA. Atractyloside (120 mg) was dissolved in a solution of 66% (v/v) DMF (the toxin appeared more soluble in this solvent than in the 1,4 dioxan used in Method I). To this was added 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (72 mg) and the solution was stirred for 30 minutes, at room temperature. Protein carrier (RSA or BSA, 100 mg) was dissolved in 0.5 M borate buffer, pH 8.5 and the ATR solution was added dropwise, with stirring; pH being monitored and maintained above pH 8.0 throughout the procedure. The conjugate was stirred overnight at 4°C and dialysed as for Method I. A set of control conjugates, devoid of hapten, were prepared concomitantly.

Estimation of percentage of amino groups on the protein carriers occupied by atractyloside

The two conjugation methods (I and II above), were compared by estimating the percentage of amino groups on the proteins occupied by ATR, prior to dialysis, using the method of Habeeb (1966), details of which are given in section 3.4.2. This procedure involves the treatment of a protein solution with TNBS, which reacts specifically with free amino groups on the surface of the protein, to give trinitophenyl derivatives which can in turn, be measured colorimetrically. The control consisted of BSA or RSA, treated as described under the methodology for Methods I and II, but in the absence of ATR.

Conjugation of atractyloside to *Salmonella minnesota* bacteria by Method II

A modification of the methods of Bellstedt *et al.* (1987) and P. Hoffman (pers. comm., 1988) was employed as described in section 3.4.2.

Immunization procedure

The conjugates prepared as described above and detailed in section 3.4.2, were used to immunise six New Zealand white rabbits by the procedure described in section 3.4.3. Two rabbits were allocated for each carrier-hapten conjugate, i.e., rabbits 1 and 2 were immunized with the ATR-BSA conjugate, rabbits 3 and 4 were immunized with the ATR-naked bacterial conjugate and rabbits 5 and 6 were given the ATR-RSA conjugate. Briefly, animals were

dosed with the antigen and antisera were collected at fortnightly intervals and the titre was monitored. When the primary response was seen to recede, the animals were boosted and the titre was again monitored every fortnight. Once the secondary response had peaked and then receded, antisera were collected by cardiac puncture whilst the animals remained under anaesthesia (section 3.4.3). The collected antisera from these six rabbits was used in both the titre determination and characterisation studies described in this chapter.

Titre determination

The titres for the six rabbit antisera were monitored fortnightly, as follows, using an adaptation of the method of Bye *et al.* (1990) (see Appendix A).

An ATR-ovalbumin conjugate prepared using carbodiimide Method II, was diluted to a concentration of 0.01 mg/ml in 0.05 M carbonate buffer, pH 9.6 (section 3.5.1). Microtitre plates were coated overnight at 4°C with this conjugate (200 µl/well). Antisera samples collected from the six rabbits were serially diluted 10-fold in TBS containing 0.1% (w/v) ovalbumin respectively. Plates were incubated overnight at 4°C, washed thrice with tap water and incubated for a further 2 hours at 37°C with a 1:3 000 dilution of Goat anti-rabbit IgG-alkaline phosphatase. Ovalbumin controls were included in each assay. Plates were washed and incubated for 1 hour at 37°C with a solution of p-nitrophenyl phosphate (1 mg/ml) in 0.1 M diethanolamine buffer, pH 9.6 (section 3.5.1). The absorbance was measured at 405 nm and results are given in section 5.3.3 below.

5.3.3 Results and discussion

The results for the percentage of amino groups on the BSA and RSA carrier proteins, bound by ATR as compared to that of the controls, are shown in Table 5.1.

From these results it was concluded that Method II (the carbodiimide method) yielded the most efficient binding of ATR to the protein carrier and would be the procedure of choice. This was attributed in part to better solubility of the hapten in DMF, compared to 1,4 dioxan, thus enhancing the reaction between hapten and carrier. A mean 6.61% binding for ATR to BSA and RSA protein carriers was calculated, as monitored by the method of Habeeb (1966). Assuming that there are 60 potential binding sites on a BSA molecule (Habeeb, 1966), a 6.61% binding rate would represent the attachment of four molecules of ATR/molecule of BSA. By the standards of Dintzis *et al.* (1989) this ratio is too low but Fuchs and Fuchs (1969) reported a similar, low binding ratio of 6-14% for gibberellins bound to a protein carrier and still succeeded in producing antibodies to the gibberellins.

Table 5.1 Percentage binding of atractyloside to BSA and RSA protein carriers. BSA = bovine serum albumin, RSA = rabbit serum albumin. The results were calculated as a percentage of the control and are expressed in units of % occupied amino groups as compared to the controls. Methods I and II use the mixed anhydride and carbodiimide procedures respectively, as detailed in section 3.4.2

Conjugate	Method I	Method II
RSA-atractyloside	5.70%	8.83%
BSA-atractyloside	5.22%	6.68%

The results of the titre determination of the antisera collected from the rabbits primed and boosted with an ATR-BSA (rabbits 1 and 2), ATR-naked bacteria (rabbits 3 and 4) or ATR-RSA conjugate (rabbits 5 and 6) are given in Fig 5.2.

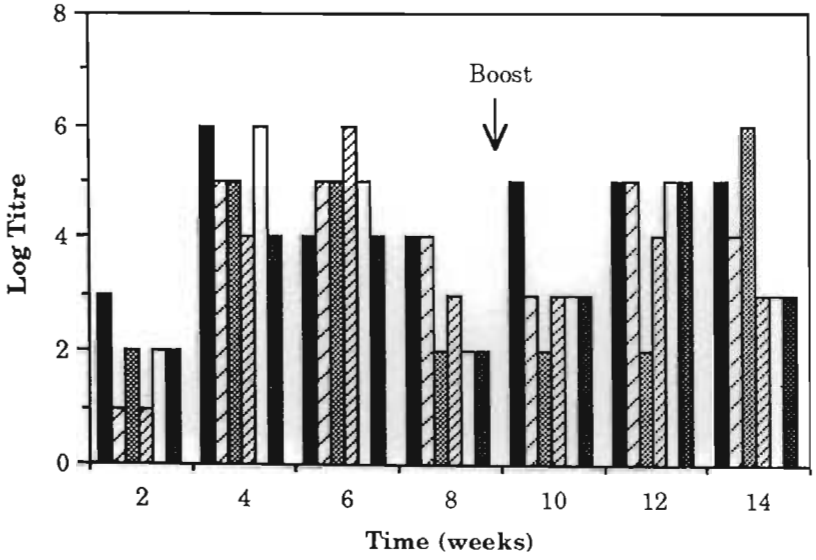


Fig 5.2 Titre responses in rabbits immunized with ATR-carrier conjugates. Rabbits were primed and boosted with an ATR-BSA (rabbits 1 ■ and 2 ▨), ATR-naked bacteria (rabbits 3 ▩ and 4 ▨) or ATR-RSA (rabbits 5 □ and 6 ▩) conjugate, respectively. The details of the method are given in section 5.3.2

Theoretically, humoral response to an antigen may be divided into two stages, namely the primary and secondary response. Primary response results from first exposure to an antigen,

and a predominant population of IgM molecules are produced. This antibody concentration declines and, on subsequent exposure to the same antigen (boosting), a secondary response occurs with an associated class switch and emergence of IgG antibodies (Tijssen, 1985). It will be seen from Fig 5.2, that the primary response by the rabbits to their respective antigens was not uniform, but generally, the titre peaked between weeks 4 and 6, post-priming. Specifically, the response by rabbit 1 (primed with an ATR-BSA conjugate) was almost immediately discernible, as was the response by rabbits 3, 5 and 6 (primed with ATR-naked bacteria, RSA-ATR and RSA-ATR respectively). The response by rabbit 2 (primed with BSA-ATR) and rabbit 4 (primed with an ATR-naked bacterial conjugate) was very low, but rose sharply by week 4 and 6 respectively. Rabbit 1 had a maximal response at week 4, but thereafter, although the response to the antigen decreased, it was still maintained at an average above that of the remaining rabbits. Rabbits 5 and 6 had a maximal response by week 4 and although this response was seen to decline in rabbit 5, the titre of the antiserum from rabbit 6 was the same for weeks 4 and 6, after which time the response declined.

By week 8 the primary response had receded and thus the animals were boosted at week 9. Subsequently rabbit 1 maintained a high titre for the remaining 5 weeks, while the titre of the antiserum from rabbit 2 (dosed with ATR-BSA) peaked at week 12, only to decline by week 14. Rabbit 3, dosed with an ATR-naked bacterial conjugate had a delayed response with the titre only increasing at week 14. Rabbit 4, dosed with the same conjugate had a maximal response at week 12. Rabbits 5 and 6, which were both dosed with ATR-RSA, showed very similar titre responses which peaked at week 12 and then receded.

The major conclusion that can be drawn from these results is that, when rabbits were dosed with an ATR-carrier, the secondary response was not significantly greater than the primary response. Although this was seen as a general trend, the absolute titre from individuals within a group, immunized with a given antigen, varied greatly. In addition it was also established that the carbodiimide method was more efficient in conjugating ATR to a carrier. Although three carriers were tested in their relative potential to produce antibodies to ATR, at this point it was not possible to select the most suitable carrier based on the titre response and this necessitated further characterisation of the antisera, as described in section 5.5.

During the course of this study, at different periods during the immunization regimen, the two rabbits immunized with the RSA-conjugate died of unknown causes. Despite no evidence to the contrary about the use of autologous protein carriers, the demise of these rabbits led to serious consideration about the effects of injecting RSA into rabbits and the autoimmune response. This aspect is discussed in the next section.

5.4 Examination for possible autoimmunity in rabbits

5.4.1 Introduction

Generally, the immune system does not react against its own constituents, so the phenomenon whereby an individual recognises self as non-self, is termed autoimmunity (Bach, 1982). Autoantibodies are thought to be involved in the disposal of cellular breakdown products but when produced by humans, against soluble antigens in the cytoplasm, induce systemic lupus erythmatosis, manifested by widespread pathological changes with non-specific lesions (Bach, 1982). Autoimmunity may be induced if new epitopes appear on the autologous antigen due to incomplete or incorrect synthesis, breakdown or cross-reactivity with exogenous antigens. The incorporation of Freund's adjuvant also frequently causes the stimulation of an immune response by autologous proteins since the essentially hydrophobic environment induced by the oil-water emulsion may result in conformational changes in the protein (Roitt, 1988). This alters the exposed epitopes on the protein, which are now recognised as non-self.

Autoimmunity in rabbits may be identified in two ways: firstly, by an increase in the presence of immune complexes in the serum and, secondly, by glomerular nephritis (GN) (Bach, 1982). Autoimmunity manifested in the form of GN may be detected as early as day 10, post-priming and on day 5 after boosting. While GN is histologically detectable, provided re-exposure to the antigen does not occur, this damage subsides within 2-3 weeks. Practically, it is difficult to detect GN, and therefore measurement of any alteration in serum immune complex levels may provide an easier means of monitoring autoimmunity (Bach, 1982).

Accurate measurement of serum immune complexes is, nevertheless, exceedingly difficult, as it is hard to discriminate between complexed and normal immunoglobulin. The assays for the detection of immune complexes, in the presence of native IgG, rely on the differences in relative solubility and molecular mass between free and complexed immunoglobulins. These procedures are more reliable than methods based on the radioallergosorbent (RAST) cell assays or complement fixation assays but are less sensitive. For the assay of immune complexes, use is made of polyethylene glycol which precipitates proteins in proportion to their molecular size and concentration. Thus 2% (w/v) PEG solubilises free IgG, but will precipitate an immune complex. If the concentration of PEG is increased to 15% (w/v), both free IgG and immune complexes will be precipitated. Thus, by manipulating the PEG concentration it is possible to selectively precipitate out the immune complexes, to the exclusion of the free IgG (Hudson and Hay, 1989).

As it is difficult to detect GN in the rabbit, the aim of this aspect of the work was to monitor the levels of immune complexes in the blood, following exposure to RSA. Firstly four rabbits were immunized with RSA and twelve days later, sera was collected. The immune complexes were precipitated from the sera and the concentration of the immune complexes (effectively autoantibodies) were determined by a single immunodiffusion test. These results were compared with the concentration of immune complexes five days after boosting with the same antigen.

5.4.2 Materials and methods

Immunization protocol

Four adult New Zealand white male rabbits (designated a, b, c and d), from the BMRC, University of Durban-Westville, were immunized subcutaneously with a stable emulsion of RSA (1 mg/ml) in Freund's Complete adjuvant. The albumin was previously reacted with carbodiimide (Method II, section 5.3.2), in the absence of ATR, to mimic any modifications that may have been introduced by the coupling reaction. Twelve days post-priming, approximately 5 ml of blood was collected from the marginal ear vein and allowed to clot. Nine weeks after priming, animals were boosted with an emulsion of the albumin in Freund's incomplete adjuvant. Five days post-boosting, blood was collected from the marginal ear vein. After collection of sera and centrifugation at 5 000 $\times g$ for 10 minutes, the presence of immune complexes was monitored as outlined below. Pre-immune rabbit serum was collected from the four rabbits and used to isolate IgG as described below.

Isolation and quantification of rabbit IgG

Serum was mixed with two volumes of 0.05 M borate buffer, pH 8.35 and to this was added 15 % (w/v) PEG, the solution mixed well and centrifuged at 12 000 $\times g$ for 10 minutes. The pellet was re-dissolved in one-fifth of the original serum volume, in the same borate buffer. An aliquot was diluted 1:100 in borate buffer and the absorbance measured at 278 nm. The extinction coefficient for rabbit IgG at 278 nm is given as 1.35 mg ml^{-1} cm^{-1} (Tijssen, 1985) and from an absorbance of 0.124, as given by the antibody solution in question, the IgG concentration was calculated as follows:-

absorbance = concentration $\times$ extinction coefficient $\times$ pathlength

0.124 = concentration $\times$ 1.35 mg ml^{-1} cm^{-1} $\times$ 1 cm

$$\text{concentration} = \frac{0.124}{1.35 \text{ mg ml}^{-1} \text{ cm}^{-1} \times 1 \text{ cm}} \text{ mg IgG/ml}$$

$$= \underline{0.092 \text{ mg IgG/ml}},$$

but this is a 1: 100 dilution

therefore final concentration = 9. 2 mg IgG /ml

From this a series of IgG solutions were prepared ranging from 23 to 184 ng IgG/ml respectively for use in the radial immunodiffusion assay.

Immune complex determination

The following solutions were prepared for the isolation of the immune complex in rabbit serum according to the method of Hudson and Hay (1989):

Veronal buffered saline (VBS): Sodium chloride (85.0 g), sodium barbitone (3.75 g) and barbitone (5.75 g) were dissolved in 2 litres of distilled water. This buffer is five times the working concentration used in the assay, but is more stable when stored as a concentrated stock and diluted appropriately before use.

Polyethylene glycol solution (PEG solution): A 20% (w/v) PEG (6 000) stock solution was prepared in VBS.

Ethylene diamine tetra-acetic acid (EDTA) disodium salt solution: A 0.2 M EDTA solution was prepared and titrated to pH 7.6 with 0.1 M NaOH.

All reactions and centrifugation steps were carried out at 4°C unless otherwise stated. The PEG solution was adjusted to a working concentration of 12% (w/v) by mixing 6 ml of the PEG stock solution with 3 ml EDTA solution and 1 ml VBS. Duplicate aliquots of serum (150 µl) were mixed with the PEG working solution (30 µl) and incubated overnight at 4°C. Solutions were centrifuged at 2 000 $\times g$ for 20 minutes, the tubes placed on ice and the supernatant carefully removed and discarded. The precipitate was resuspended in ice-cold PEG working solution (2 ml) and centrifuged at 200 $\times g$ for 20 minutes following which, the supernatant was again removed and the precipitate redissolved over 48 hours in VBS (150 µl), without stirring. To ensure complete dissolution of the precipitated immune complexes, the solutions were incubated for a further hour at 37°C and precipitated immune complex levels were quantified by single radial immunodiffusion as outlined below.

Single radial immunodiffusion

The following procedure for the radial immunodiffusion assay represents a modification of the procedures of Buttle *et al.* (1988) and Hudson and Hay (1989). Microscope slides were washed in ethanol, dried and coated with a thin layer of 0.5% (w/v) agar in distilled water, to ensure good adhesion of the next layer, i.e., the analytical agar gel. After the coating gel had set, the analytical agar was prepared by dissolving 2 g of agar in 100 ml of 90 mM Tris/ 80 mM boric acid buffer, pH 8.3. The agar was dissolved by heating and allowed to cool to about

56°C. To 2 ml of the agar was added 30 µl of a previously prepared sheep anti-rabbit IgG serum (prepared by T.H.T Coetzer, Dept. of Biochemistry, University of Natal, 1988) in 3.8 ml of the Tris-borate buffer. This analytical agar was carefully layered onto the pre-coated microscope slides and allowed to cool, whereupon a gel punch was used to cut out sufficient wells for analysis.

Duplicate samples (7 µl) of each IgG standard solution (see above) were added to each well on the precoated slides, to form the standard concentration series, from which an estimate of immune complex concentration in the unknown solutions could be determined. Remaining wells were incubated with the unknown precipitin samples. Solutions were allowed to infiltrate the gels at room temperature for 1 hour, before being incubated at 4°C. Since after 24 hours incubation, there was little evidence of precipitin band formation, the slides were left to incubate for a further 24 hours. Free protein was removed by rinsing the gels in repeated washes of PBS for 24 hours followed by a final wash for a further 24 hours in 0.9% (w/v) saline. The gels were then stained in Coomassie brilliant blue for 10 minutes (Hudson and Hay, 1989) and differentiated in a solution of 20% glacial acetic acid. Thereafter, the slides were covered with a layer of lint-free, dampened filter paper and incubated for 24 hours at 37°C to dehydrate and preserve the slides. By measuring the mean diameter (horizontal and vertical directions) of the precipitin rings, a standard curve was prepared of ring area versus IgG concentration (Fig 5.3). The concentration of serum immune complexes in the unknown samples was read from this graph, as was that of a control serum. The procedure was repeated on samples collected after both priming and boosting and a comparison of the immune complex concentrations was made.

5.4.3 Results and conclusions

The standard curve in which ring area was plotted against IgG concentration is depicted in Fig 5.3. This curve was found to be considerably more linear than when ring diameter is plotted instead of ring area. Two sets of data points are given for each IgG concentration and these represent the areas obtained for two successive radial ring assays, i.e., the first after priming and the second after boosting. This indicates that although the diffusion assays were carried out at different times, the results did not differ significantly and hence in any alterations in the levels of immune complexes in the rabbit sera between priming and boosting, would not be artifact.

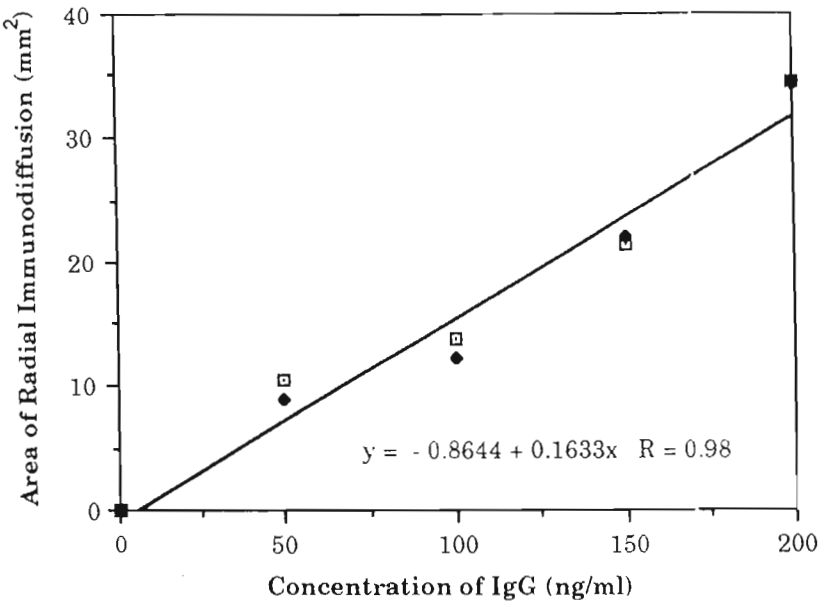


Fig 5.3 Graphical representation of the single radial immunodiffusion area versus concentration of a standard concentration series rabbit IgG. The two sets of coordinates were the results following priming (◆) and boosting (□). Method details are given in section 5.4.2

The results of the autoimmune study are given in Table 5.2, where the area of the radial immunodiffusion ring of the unknown samples was measured and used to determine the approximate concentration of the immune complex from the standard curve in Fig 5.3.

Table 5.2 The concentration of sera immune complexes in rabbits immunized with rabbit serum albumin after priming and boosting, as described in section 5.4.2

Rabbit	Concentration immune complex (ng/ml)	
	Prime	Boost
a	124.9	342.6
b	214.8	278.0
c	82.9	212.5
d	22.7	469.9
control	3.5	9.3

From these results it is clear that for all four rabbits there was a significant increase in the concentration of immune complexes in the sera, after boosting. Thus, this confirmed that the immunization of the rabbits with RSA, emulsified in Freund's adjuvant, induced autoimmune reactions. Further investigations would have been necessary in order to fully

substantiate whether these autoimmune reactions were severe enough to cause the death of the two rabbits. The fact remains though that the use of autologous proteins as immune carriers has the potential to induce autoimmune reactions and should therefore be avoided.

In the light of this experiment, doubt was cast on the usefulness of RSA-ATR conjugates for the production of antibodies in rabbits. Following this work, further investigations were carried out to select the carrier most suited for production of anti-ATR antibodies, by characterising the antisera collected from the rabbits immunized with the ATR-carrier conjugates. The details of this characterisation and selection are given in section 5.5 below.

5.5 Characterization and selection of antisera

5.5.1 Introduction

The characterisation of the antisera produced against the various ATR conjugates (see section 5.3.2) involved the determination of the antibody affinity for ATR (as detailed in section 5.5.2 below), precipitin reactions of the antisera with various ATR-protein conjugates (section 5.5.3) and determination of the cross specificity of the antisera (section 5.5.4). These characterisation steps were essential prerequisites for the selection of antisera with antibodies of high affinity and specificity for ATR.

5.5.2 Determination of antibody affinity

5.5.2.1 Introduction

The affinity of an antibody for an antigen is a measure of the binding strength and stability between an antigen and an antibody, at equilibrium (Bach, 1982). Hudson and Hay (1989) state that the qualitative precipitin test developed by Heidelberger and Kendall is the basis of all qualitative studies of antibody-antigen interaction. Increasing amounts of antigen are added to a constant concentration of antibody and the amount of precipitate formed in each tube, is determined. Maximum precipitation occurs just after the point of equivalence and thus, is an indication of the affinity for the antibody to the antigen. The affinity of the antibodies raised in the rabbits in response to the various ATR conjugates (section 5.3.2) was determined by the method of Hudson and Hay (1989).

5.5.2.2 Methods and materials

Atractyloside (1 mg/ml in 0.1 M phosphate buffered saline, PBS, pH 7.23) and undiluted antisera (sections 3.4.2 and 5.3.2) were mixed in the proportions outlined in Table 5.3, in duplicate (Hudson and Hay , 1989).

Table 5.3 Proportions of antigen (Ag) (ATR, 1 mg/ml in PBS) and antibody (Ab) (antisera prepared as described in section 3.4.3) used in the determination of antibody affinity

Tube	1	2	3	4	5	6	7	8	9	10
Ag (µl)	0	10	20	50	100	150	200	250	350	450
Ab (µl)	50	50	50	50	50	50	50	50	50	50
PBS (µl)	450	440	430	400	350	300	250	200	100	0

Reagents were mixed and incubated for 1 hour at 37°C followed by 10 days at 4°C. Tubes were spun at 5 000 × *g* for 5 minutes, the supernatant removed, and the precipitate washed twice with ice-cold PBS (1 ml/tube). Between centrifugation steps, tubes were kept on ice. After removal of the final wash buffer, the resulting precipitate was incubated overnight at 4°C in 1 ml of 0.1 M NaOH. The absorbance was read at 280 nm, against a blank of 0.1 M NaOH. From the results, the point of maximal precipitation for each set of antibodies was calculated and the antiserum with the greatest affinity for ATR was identified.

5.5.2.3 Results and discussion

The results of the affinity assay are presented in Table 5.4 below. From these results it is clear that the point of maximal precipitation in the antisera/ATR solutions was spread over a concentration range of 0.04 to 0.5 mg ATR/ml. The antiserum produced against the naked bacteria-ATR conjugate by rabbit 4, had the highest affinity for the toxin (i.e., 0.04 mg ATR/ml 0.1 M NaOH) and would therefore be the antiserum of choice, given this criterion of selection.

Table 5.4 Affinity of various antibodies for atractyloside. Antisera from the six rabbits was prepared as described in section 5.3.2.

Carrier	Rabbit Number	Atractyloside concentration at maximal precipitation (mg/ml)
BSA	1	0.5
	2	0.3
Naked Bacteria	3	0.4
	4	0.04
RSA	5	0.3
	6	0.3

Further characterisation of the antisera was attempted using Ouchterlony precipitin gel reactions, and this is reported in section 5.4.3 below.

5.5.3 Precipitation in gels

5.5.3.1 Introduction

As a further step in the characterisation of the antisera to ATR, Ouchterlony diffusion assays were attempted to determine the cross-specificity of the antisera raised to the ATR conjugates (prepared as described in section 5.3.2). A modification of the methods of Buttle *et al.* (1988) and Hudson and Hay (1989) were employed.

5.5.3.2 Method

Agarose (2%, w/v) was added to a 90 mM Tris/80 mM boric acid buffer, pH 8.3 and dissolved by heating. The molten gel was poured into petri-dishes, allowed to solidify, and with the aid of a template, several ring sets were cut consisting of a central well, surrounded by six outer wells. In the first set of assays, a serial dilution series of ATR solutions (initial concentration 1 mg/ml, in the Tris-borate buffer) was incubated in the outer wells, while the central well was left vacant for the addition of the appropriate test antiserum. All six sets of antisera were tested in the same manner. In a second set of gels, the outer wells were occupied by the six antisera in question while the central wells contained either BSA-, RSA- or naked bacterial-ATR conjugates, ovalbumin, RSA or BSA, respectively. Gels were incubated at room temperature until all the solutions had diffused into the gel and thereafter plates were inverted and incubated overnight at room temperature.

Since after incubation, there was no evidence of precipitin bands, gels were washed in several changes of 0.15 M PBS, pH 7.2 for 24 hours, followed by 0.9 % (m/v) saline for a further 24 hours, to remove residual protein (Hudson and Hay, 1989). The gels were then stained in Coomassie brilliant blue for 10 minutes (Hudson and Hay, 1989) and differentiated in a solution of 20% glacial acetic acid. Since this approach still showed no evidence of precipitin bands, despite repeated efforts, an alternative approach was tested. This involved the addition of 4% (w/v) PEG (6 000) to the agarose, prior to casting the gel slabs. This can improve the sensitivity of the assay, by enhancing precipitation (Hudson and Hay, 1989). The results obtained are discussed below in section 5.5.3.3 below.

5.5.3.3 Results and discussion

Weak precipitin bands were evident between the wells containing anti-BSA-ATR antiserum (raised in rabbit 1) and both BSA and BSA-ATR respectively. No other bands could be visualised. This is, however, not altogether unexpected as Bach (1982) reported that monovalent haptens do not bring about precipitation because each molecule binds to only one of two possible antibody binding sites, and such complexes remain soluble. This explanation would account for the lack of precipitin bands in the Ouchterlony test, but does not account for the presence of the precipitation in the affinity test described in Section 5.5.2. Bach (1982) further reputed that over a lengthy, but unspecified, period of time, aggregation of several identical monovalent haptens may induce aggregation and precipitation. Since the affinity assay proceeded over a period of 11 days, while the Ouchterlony was only carried out for 24 hours, this time difference could account for the precipitation in the affinity assay, but not in the Ouchterlony assay.

The physicochemical aspects of the precipitation of antibody-antigen complexes have been defined by Bach (1982) and are thought to occur in two phases. The first rapid phase involves the formation of a soluble lattice. This in turn enters a slow phase, during which the lattice complexes form aggregates, with tight ionic bonds and visible precipitates. These precipitates may not be present when antibody-antigen complexes form cyclic complexes or when determinants are closely repeated on an antigen surface, as is the case with a hapten-carrier conjugate (Bach, 1982). Thus prolonged incubation of the Ouchterlony gel over a period of several weeks may indeed result in the formation of precipitin bands, but logistically, the risk of microbial contamination would hamper such a method. This aspect was not investigated any further.

The failure of the precipitin reaction to demonstrate any cross-reactivity of the antisera meant that an alternative method was required to test this cross-specificity. For this purpose both an immunodot blot and an ELISA were tested, the results of which are presented and compared in section 5.5.4 below.

5.5.4 Determination of cross-specificity of antibodies protein coats

5.5.4.1 Introduction

Theoretically, when ATR was coupled to the various carriers for immunization purposes, antibodies would have been raised to both the hapten and the carrier. The purpose of this study was, therefore, to determine the cross reactivity of the antisera to BSA, RSA and ovalbumin.

Even coupling agents used to conjugate a hapten to a carrier have the potential to elicit antibody production (Goodfriend *et al.*, 1964; Briand *et al.*, 1985), as these reagents can modify carrier residues. When injected into animals, these "coupling agent modified residues" (CAMOR) may induce anti-CAMOR antibody production, contributing to false readings in immunoassays, especially if a protein-hapten conjugate is used to coat ELISA plates. Such anti-CAMOR antibodies may also be elicited by carrier molecules that have reacted with carbodiimides. These antibodies react with both the same carrier treated with the conjugation reagent in the absence of bound hapten, and with unrelated carriers treated with the same coupling compound. Since this phenomenon is often over-looked when characterising antisera (Briand *et al.*, 1985) it was thoroughly investigated by the present author.

The experiment described below, therefore, was designed to investigate the presence of anti-CAMOR antibodies, and to monitor the cross-reactivity of the antisera to different proteins. Anti-CAMOR antibodies were tested by an ELISA, while cross-reactivity was determined by both ELISA and an immunodot blot (section 5.5.4.2). The immunodot blot was developed by F. Dehrmann (pers. comm., 1991).

5.5.4.2 Methods

Enzyme immunoassay

This immunoassay is a modification of the assay of Bye *et al.* (1990) (see Appendix A). Ovalbumin, BSA and RSA were reacted with carbodiimide, as described in section 5.3.2, but in the absence of ATR, hereafter referred to as O-O, B-B and R-R respectively. Microtitre wells were coated with 200 µl/well of a 0.1 mg/ml solution of either unadulterated or carbodiimide-modified protein, prepared in 0.05 M carbonate buffer, pH 9.6 (section 3.5.1) and the plates were incubated overnight at 4°C. Antisera, containing the primary antibody, from each of the six rabbits (prepared as described in section 3.4.2) was diluted 1:500 or 1:1 000 in TBS. A 200 µl volume of each primary antibody was added to each well, for each representative protein coat. Plates were incubated for 2 hours at 37°C, washed thrice with tap water and incubated for a further 2 hours, under the same conditions, with a 1:3 000 dilution of goat anti-rabbit IgG-alkaline phosphatase conjugate. Plates were washed thrice with tap water and incubated for 1 hour, at 37°C with p-nitrophenyl phosphate (1 mg/ml in 0.1 M diethanolamine buffer, pH 9.6; section 3.5.1) and the absorbance was monitored using a Biotek spectrophotometer.

Dot immunobinding assay

This method was developed by F. Dehrmann (pers. comm., 1990). Unless otherwise stated, all incubations were carried out at room temperature, in the dark. Nitrocellulose membranes were washed in distilled water for 5 minutes and air dried. A 1 µl volume of protein (BSA, RSA or ovalbumin,1 mg/ml) was spotted onto the membrane and dried. The sheets were rinsed twice in TBS, once in TBS containing 0.05% (v/v) Tween 20 and again in TBS. The free sites on the membrane were blocked with 3% (w/v) "Elite" milk powder in TBS, for 3 hours. Membranes were washed as described above and incubated with appropriate antisera (prepared as described in section 3.4.2), diluted 1:20, 1:100 or 1:200 in the blocking solution. Membranes were incubated overnight. Washing was repeated and membranes were incubated for 3 hours with the secondary antibody-enzyme conjugate, diluted 1:4 000, in the blocking solution. The sheets were washed and incubated with 4-Bromo-3-chloro-indolyl phosphate (1 mg/ml in 0.1 M diethanolamine buffer, pH 9.6; section 3.5.1) for 1 hour. A positive reaction was noted by development of a blue precipitate. Appropriate controls were included throughout. The results of the dot blot and the ELISA are summarized in section 5.5.4.3 below.

5.5.4.3 Results and discussion

The results of both the ELISA and the immunodot blot are presented in Table 5.5 below. Clearly, the ELISA appears to be more sensitive than the immunodot blot since the ELISA was often positive while no reaction was noted for the immunodot blot. It was also noted in the immunodot blot, that antisera from rabbit 3 targeted for RSA, but this result was not confirmed by the ELISA. Since rabbit 3 was inoculated with a naked bacteria conjugate, the cross reaction with RSA was unexpected. No explanation can be offered for this observation, other than experimental error, but this is unlikely, since the results were reproducible.

Table 5.5 Results of the immunoassay and immunodot blot carried out on antisera of rabbits immunized with an ATR-carrier conjugate, to monitor cross reactivity to bovine serum albumin, rabbit serum albumin, ovalbumin and carbodiimide modified proteins. The immunodotblot was carried out by F. Dehrmann (pers. comm., 1990). Antisera were from the six rabbits immunized with the ATR-carrier conjugates as detailed in section 3.4.2

Rabbit	ATR carrier	BSA E/D*	RSA E/D	ovalbumin E/D	B-B	R-R	O-O
1	BSA	+ / +	+ / +	+ / +	+	+	+
2	BSA	+ / -	+ / -	- / -	+	-	-
3	bacteria	- / -	- / +	- / -	-	-	-
4	bacteria	- / -	- / -	- / -	-	-	-
5	RSA	- / -	+ / +	- / -	-	-	-
6	RSA	+ / -	- / -	+ / -	-	-	-

* E=ELISA; D=immunodot blot; +=positive; -=no reaction, BSA = bovine serum albumin, RSA = rabbit serum albumin, B-B = BSA reacted with carbodiimide, R-R = RSA reacted with carbodiimide, O-O = ovalbumin reacted with carbodiimide

The antiserum from rabbit 1, reacted very strongly with all the proteins under test; in particular with ovalbumin. This is due to so called "reciprocal cross reactions" which are not uncommon since rabbits immunized with BSA often produce antibodies which react with ovalbumin (Bach, 1982). The antiserum from rabbit 2, also immunised with a BSA conjugate, however, did not cross react with ovalbumin, but only with RSA, suggesting epitope homology on the albumin molecules. Rabbit 1 antiserum notably reacted with the

CAMOR proteins, but the reaction was as intense as with the unadulterated protein, and therefore, it was concluded that even if anti-CAMOR antibodies existed, their effect was negligible. Based on these results and those of the affinity assay (section 5.5.2.3), showing the degree of affinity of the antibodies to the ATR, the antiserum from rabbit 1 was not selected for future use in immunoassays.

Antiserum collected from rabbit 2 reacted with both BSA and RSA, as shown by the ELISA results, but not confirmed by the dot blot. Likewise, this antiserum reacted with the B-B conjugate, but as there was no reaction with the other CAMOR proteins, this reaction was thought to be the result of the antibodies targeting the BSA epitopes and not the result of anti-CAMOR antibodies. Thus this antiserum was not deemed to be suitable for the immunoassay.

Rabbit 3 was immunized with a naked bacterial conjugate and as mentioned previously, the antiserum appeared to target for RSA which is unexpected and may be an artifact. Despite this, the specificity of this antiserum made it a potentially good source of antibodies for the ATR ELISA, although the quality of the antiserum produced by rabbit 4 was even better. Rabbit 4 was also immunised with a naked bacteria conjugate but as shown in Table 5.5, the antiserum did not react with any of the protein test samples. Furthermore, there was no evidence of anti-CAMOR antibodies. This antiserum contained antibodies with a high affinity for ATR and, as shown in section 5.5.2, effectively achieved maximal precipitation of ATR at a concentration one-tenth of that of any of the other antisera produced. Based on these results, the antiserum from rabbit 4 became the antiserum of choice for all subsequent studies reported in this thesis.

The antiserum from rabbit 5, immunized with RSA-ATR, cross-reacted with RSA, which was unexpected, as this is an autologous protein. However, it has been previously stated that Freund's adjuvant can stimulate an immune response by autologous proteins, due to conformational changes in the protein (Roitt, 1988), which could explain the presence of anti-RSA antibodies. Anti-CAMOR antibodies were excluded as there was no reaction with any of the CAMOR proteins. Antiserum from rabbit 6, immunized with the same carrier, cross-reacted with the BSA and ovalbumin. There is no obvious explanation for the existence of antibodies to BSA and ovalbumin but the theory of reciprocal cross- reactivity (Bach, 1982) may apply in this instance.

The results of both the affinity assay (section 5.5.2.3) and the experiments reported in this section enabled selection of the antiserum most suited for use in future immunoassays and clearly, based on these criteria of selection, rabbit 4, immunized with the naked bacterial-

ATR conjugate, produced the antiserum of choice. However, a number of important issues came to light from these results. Firstly, it was evident that considerable heterogeneity exists between the ability of different animals of the same species to produce antibodies to the same antigen, a fact which reiterates the importance of not pooling antiserum samples. Secondly, while anti-CAMOR antibodies appear to be commonly produced by animals immunized with peptide-carrier conjugates, this does not apply to all hapten-protein complexes. Finally, the use of autologous proteins can stimulate an immune response or, more specifically, an autoimmune response which can have a deleterious effect on both the welfare of the animal and ultimately on antibody production.

5.6 General discussion

The aim of the work presented in this chapter was to produce high affinity antibodies, specific for ATR, but this preliminary, seemingly routine procedure was dogged by anomalies and inconsistencies which in some cases necessitated extensive developmental studies particularly with regard to the most suitable carrier, conjugation procedure, dosing regimen and site of antigen administration. In addition, the chemical nature of ATR was also taken into consideration as properties inherent to the antigen may either enhance or inhibit antibody production.

Foreigness and size of the immunogen are the first criteria for immunoreactivity and while ATR satisfied the first requirement, size limited the immunogenicity of this molecule. This was overcome by conjugating the hapten to a carrier; a routine method. Nevertheless, the working dilution of the resulting antibody was very low (1:40) and in stark contrast to antibodies raised, for example, against the hapten fenpropimorph, where a primary antibody working dilution of 1:200 000 was used for their immunoassay (Jung *et al.*, 1989).

Although the shape of the immunogen apparently has no effect on the immune response, a high charge density may decrease antigenicity as noted with protamines, histones and sialic acids (Absolom and van Oss, 1986). Thus, the anionic nature of ATR may decrease the antigenicity of the toxin, an observation previously noted by Weiler and Weiczorek (1981) who concluded that protonated gibberellins competed more efficiently for antibody binding sites than anionic gibberellate. If charge can affect antibody-binding capabilities it may therefore also influence immunogenicity. Unless chemically modified, the net negative charge on ATR could not be neutralised and therefore, attention was focused on alternative properties affecting antigenicity, but which could also be advantageously manipulated.

The binding of ATR to the carrier was the first parameter to be investigated. Titre has been improved by changing the coupling procedure when the antigen is a hapten, as noted by Weiler (1981) and Ohtani *et al.* (1988) and, as shown in section 5.3.3, the carbodiimide procedure produced a superior conjugate to the mixed anhydride method. The success of a conjugation procedure is reliant on the degree of binding by the hapten to a carrier and in this regard it is proposed that a minimum number of haptens must be spatially grouped in close proximity on the surface of the carrier, forming an "immunon", before an immune response can be stimulated (Dintzis *et al.*, 1989). In the case of a dinitrophenyl phosphate-polyacrylamide complex, a minimum of 20 hapten molecules were required per molecule of polyacrylamide (molecular mass 100 000 Da), before antibodies could be produced (Dintzis *et al.*, 1989). Since, however, too many molecules bound to a carrier may induce immune tolerance (Tijssen, 1985) it is important to reach a compromise.

The percentage binding of ATR to protein carrier, was monitored by the method of Habeeb (1966), and a mean of 6.61% binding was calculated. Assuming that there are 60 potential binding sites on a BSA molecule (Habeeb, 1966), a 6.61% binding rate would represent the attachment of four molecules of ATR/molecule of BSA. By the standards of Dintzis *et al.* (1989) this ratio is too low but Fuchs and Fuchs (1969) reported a similar, low binding ratio of 6-14% for gibberellins bound to a protein carrier. As they succeeded in raising antibodies to this plant growth hormone and since ATR is structurally related to these compounds, the degree of binding obtained by the present author was considered acceptable.

An infinite variety of macromolecules have been used as carriers, and albumins are no exception. Since ovalbumin is susceptible to the denaturing effects of the conjugation reagents it is therefore not recommended as a carrier (Ohtani *et al.*, 1988) whereas BSA is commonly used, but "reciprocal cross reactions" with such proteins as ovalbumin can result in false positive results. Autologous carrier proteins may prevent such cross reactions, but can induce an autoimmune response, the implications of which are not fully understood. Such a response may have been responsible for the deaths of the two rabbits (5 and 6) in the present studies.

Naked bacteria were shown in the present study to be suitable alternative carriers to albumins and advantageously, the antisera produced had minimal cross-reactivity with components used in the immunoassay, such as blocking and coat proteins. In addition, the use of adjuvants is negated by the lipoprotic nature of the bacterial carrier (Bellstedt *et al.*, 1987). The detrimental effects of adjuvants to both the animal and the researcher (if accidentally injected with the emulsion) is a further recommendation for the use of naked bacteria carriers. A comparison of the antisera produced against ATR, coupled to BSA, RSA

or naked bacteria conclusively showed that the naked bacteria conjugates produced antibodies to ATR of the highest quality. This novel carrier is therefore recommended for future antibody production against this toxin.

The antibody response may also be suppressed if the antigen is poorly metabolised *in vivo* (Bach, 1982) and although the metabolic fate of ATR *in vivo* is unknown, analogues originating from coffee beans isolated from the urine of coffee drinkers appeared only slightly modified, if at all (Richter and Spiteller, 1978). Similarly for the present studies (Chapter 4) HPLC analysis demonstrated that ATR is excreted into the urine of rats and that not all the injected ATR is sufficiently biotransformed *in vivo* so as to alter its k'. In addition, the toxicity of ATR is dependent upon the maintenance of its structural integrity and removal of the bisulphate esters, glycoside moeity or the isovaleric acid significantly decreases its toxicity, while loss of the carboxyl group on C_4 of the diterpene ring renders ATR non-toxic (Vignais *et al.*, 1978). Hence if the toxin is poorly metabolised, this could contribute to the immunogen showing a lower titre than expected.

The dose and route of antigen administration, is subject to personal preference. From a humane perspective it was suggested that animals be dosed either subcutaneously or intramuscularly (G. Thurman, pers. comm., 1989). Animals were boosted when the primary response receded but on monitoring the secondary response it was noted that, unlike reports in textbooks (Roitt, 1988), this secondary response was not significantly higher than that of the primary. Initially little attention was paid to this observation, but this together with the potential of ATR to toxify the immune system, could not be dismissed.

Immunotoxicological studies have demonstrated that the immune system is very sensitive to chemical insult (Luster *et al.*, 1987). Researchers however, have had difficulty defining the immunosuppressive effect of the myriads of immunotoxicants, and the problem has been further complicated by their multidirectional effect on immune function. Immunotoxins can act in one of three ways: by targeting specifically for the lymphocytes, by altering biochemical or cellular events common to cells associated with the immune system or by a general antiproliferative or cytolytic activity (Luster *et al.*, 1987). The immunosuppressive effects of ATR are not fully understood, but could account for the poor antibody response to this hapten.

If the fate of an immunogen following its administration to an animal is considered, the following scenario is likely to occur. An antigen (such as BSA-ATR) emulsified in Freund's adjuvant, when administered subcutaneously, remains localised for some time at this site or in adjacent lymph nodes. Macrophages then congregate at this site of inflammation and

engulf the soluble antigen by pinocytosis (Weir, 1984). A close association between these macrophages and lymphocytes (B- and T-cells), suggests that macrophages are involved in stimulation of antibody production. This association has been confirmed experimentally, since the removal of macrophages from a lymphocyte suspension is correlated with a suppression in antibody production. This suppression of antibody production in the absence of macrophages, is most notable with soluble, weakly immunogenic or thymus independent antigens (Bach, 1982). It is possible that when ATR is phagocytosed by macrophages the toxin may inhibit energy production in these mitochondrial-rich cells, thus either destroying or depleting the macrophage population. Macrophages, in turn, are implicated in the induction of antibody production by lymphocytes and, if macrophages are destroyed by ATR, this would ultimately depress lymphocyte stimulation, and account for the poor antibody response. Theoretically though, destruction of the macrophages would limit all antibody production whereas the fact that antibody production to ATR was only depressed rather than prevented, suggests that only inhibition of B- and T-cell function occurred.

B- and T-lymphocytes are involved in humoral and cell mediated immunity (Tijssen, 1985) whereby B-cells are thought to produce antibodies to thymus dependent antigens, with the help of T-cells. However, if the antigen is a hapten-carrier, the response is shared by the B- and T-cells; B-cells producing anti-hapten antibodies and the T-cells anti-carrier antibodies. Although a division of labour exists between the T- and B-cells with respect to the production of antibodies to a carrier and its hapten moieties, the two lymphocyte populations are nevertheless closely associated (Bach, 1982).

It is feasible that ATR could prevent ATP synthesis in the T-cells, causing them to die but this toxin also induces the release of intra-mitochondrial calcium into the cytoplasm, and this may have a far more detrimental effect on the lymphocytes. Cytoplasmic calcium is thought to be an intracellular signal for cell growth inhibition, and chronic levels of this cation in the cell are an early indicator of cell injury, particularly in immune cells. It has been noted that infection of T_4^+ lymphoid cells by the human immunodeficiency virus (HIV) (an immunosuppressant) is associated with enhanced permeability of the cell membrane, causing an influx of divalent ions, particularly calcium (Lynn *et al.*, 1988). The envelope protein (*env*) of the HIV or an *env*-gene product is thought to alter the membrane permselectivity, resulting in this influx of calcium ions. Calcium impairs macromolecule anabolism and depresses the uptake of other divalent ions, such as zinc. The activity of zinc-dependent enzymes, involved in DNA and RNA replication and ultimately protein biosynthesis is, therefore, suppressed. This increased concentration of cytoplasmic calcium causes cessation of growth and ultimately, cell lysis (Lynn *et al.*, 1988). Immune suppression by HIV is well

documented and the elevation of cytoplasmic calcium levels is thought to be involved in this inhibition (Lynn *et al.*, 1989).

Atractyloside also alters membrane structure, inducing efflux of calcium from intra-mitochondrial stores into the cytoplasm, and thus effectively both HIV and ATR cause cytoplasmic calcium concentrations to increase. It is feasible, therefore, that ATR may also affect T-cells in the same manner as HIV and by suppressing T-cells, B-cells cannot produce antibodies which decreases the immune response.

Although B-cells are mainly involved in the production of antibodies to haptens, the T-cells are apparently also required although their precise function is not known. The T-cells are imperative in the stimulation of a secondary response, by the B-cells, to a hapten. It has been demonstrated that when an animal is primed with a hapten conjugated to a carrier (such as keyhole limpit haemocyanin), boosting with the same antigen induces a secondary immune response, the result of immunological memory. This secondary response is associated with antibody class switch from IgM to IgG antibodies and T-cells are thought to be imperative for this class switch to occur although T-cell memory is directed towards the carrier, while that of the B-cells is directed towards the hapten. If however, the animal is primed with a hapten-keyhole limpit haemocyanin conjugate and boosted with an immunogen where the carrier is now ovalbumin, there is no secondary response since the T-cells do not recognise the carrier (Bach, 1982; Tijssen, 1985). Thus, T-cells are involved in both class switch and promoting B-cell antibody production and consequently, if these T-cells were destroyed by the ATR, this could suppress the antibody response to the hapten by the B-cells and furthermore, T-cell memory could be lost. On subsequent boosting with the ATR-carrier, there should be a secondary response but if immunological memory was lost, the apparent secondary response could be a primary response. This could account for the similar titre levels in both the primary and secondary response to an ATR-carrier conjugate (section 5.3).

If the secondary response did not occur, then theoretically class switch would also not have taken place. Therefore, the antibodies produced by the B-cells to ATR post-boosting would be IgM not IgG antibodies. It was accidently found that the primary antibodies raised against ATR only bind to the antigen at 4°C and not 37°C and these results are shown in Chapter 6. So called "cold-antibodies" are usually IgM and not IgG antibodies and this would support the proposal that ATR inhibits T-cell function and class switch.

Unfortunately, the phenomena of antibody production, class switch and immune suppression are not fully elucidated and this makes interpretation of results very difficult. The proposals

presented are, therefore, highly speculative but it was nevertheless concluded from this study that ATR is not a good immunogen and has the potential to act as an immunosuppressive agent. Use was however made of the antiserum raised by the rabbit immunized with the naked bacterial conjugate (Rabbit 4) for the development of an immunoassay, and the details of the optimisation of this immunoassay are given in Chapter 6 and ultimately in the immunocytochemical investigation detailed in Chapter 7.

CHAPTER 6

THE DEVELOPMENT OF AN ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR ATRACTYLOSIDE

6.1 Introduction

The availability of antibodies to ATR (the production of which is described in Chapter 5), provided the means to develop an immunological detection system for this toxin. Although, historically, haptens have been detected by radioimmunoassays (RIA), the quantitative determination of antigens by non-isotopic, enzyme immunoassays (EIA) has evolved as a natural progression from RIA and provided an alternative, but not necessarily superior, detection system (Engvall and Perlmann, 1971; Maggio, 1980)

Enzyme-bound immunoreactants were initially developed in the mid-sixties for identification and localization of antigens in histological specimens, and for the identification of immunodiffusion and immuno-electrophoresis precipitin bands (Nakane and Pierce, 1967). It was only in the 1970s, however, that this technique was routinely used as an analytical procedure, when it was noted that antigens and antibodies could be immobilized onto solid phases (Engvall and Perlmann, 1971; Van Weemen and Schuurs, 1971; Maggio, 1980; Tijssen, 1985; Kemeny and Chantler, 1988). One such solid phase technique that was developed is the enzyme-linked immunosorbent assay (ELISA) (Engvall and Perlmann, 1971) where the basic principle may be divided into two operations. The first involves the reaction of antibody with corresponding antigen, with one of the immunoreactants being bound to an enzyme, while in the second phase, the catalytic conversion of substrate to a coloured product by the bound enzyme is used to monitor the degree of binding of antigen to antibody (Tijssen, 1985)

Since 1971, numerous ELISAs have been developed with an infinite number of variations on the central theme to encompass the diverse nature of the antigens being assayed. In the present studies, the ELISA developed for ATR is based on the competitive solid phase ELISA of Engvall and Perlmann (1971). In their method, pure antigen is bound to the solid phase while antibody is added in conjunction with free antigen (in the sample) causing the free and bound antigen to compete directly for antibody. The antibody bound to the coating antigen is then measured by the addition of a secondary antibody-enzyme conjugate (specific for the primary antibody) and appropriate substrate. The concentration of the product, which is monitored colorimetrically is, therefore, inversely proportional to the free antigen present in the standard or test sample (Tijssen, 1985).

Since there was no existing immunoassay for ATR, the present studies necessitated the sequential optimisation of every stage of the ELISA process, including selection of a suitable solid phase support, optimisation of the coating buffer pH and coating conditions, selection of a suitable blocking agent and application of the ELISA for the analysis of ATR in biological fluids. These aspects are reported in detail in separate subsections of this chapter, while the final optimised procedure is also detailed in section 3.5.2.

6.2 Selection of a solid phase support

6.2.1 Introduction and general considerations

In view of the fact that there was no known ELISA for the detection of ATR, it was first necessary to select a suitable solid support, since the success of a solid phase immunoassay is reliant on the stable binding of antigen or antibody to the solid phase. The coating antigen or antibody is most frequently bound to a polyvinyl or polystyrene solid phase, but cellulose nitrate, polyallommer centrifuge tubes, nylon and latex have also been employed (Herrmann and Collins, 1976; Cantarero *et al.*, 1980; Urbanek *et al.*, 1985). Cellulose nitrate is however, a poor solid phase support and thus cellulose nitrate containers are frequently employed for preparative work, since loss of immunoglobulins due to non-specific binding is minimal (Herrmann and Collins, 1976).

The binding of an antibody or antigen to a solid phase may either be by passive adsorption or active covalent linking. Passive adsorption involves exposure of the solid phase to the immunoreactant for a few hours, permitting hydrophobic interaction and bonding (Standefer, 1985). The process is divided into two distinct steps: the primary phase involving adsorption of the molecule to the solid phase, followed by the secondary stage which is associated with protein-protein interactions and development of multiple coating layers. It is the detachment of the secondary coating phase which is invariably responsible for random results (Cantarero *et al.*, 1980; Tijssen, 1985). Although these observations pertain to the coating of protein antigens, they are also of relevance to the ELISA for ATR, since this antigen was coated onto the solid phase as a protein-hapten conjugate. Active covalent linking of the immunoreactant to the solid phase involves pre-treatment of the support with, for example, 2% (v/v) glutaraldehyde prior to the addition of the immunoreactant (Standefer, 1985; Tijssen, 1985). Such pre-treatment, as opposed to merely adding the immunoreactant directly to the solid support, increases coating of the solid phase some 2-5 fold over a period of one hour at 37°C (Standefer, 1985). In the present

studies, the simpler, passive adsorption approach gave satisfactory results and was therefore the method of choice.

For convenience, ELISAs are invariably carried out in 96 well plastic microtitre plates which are available from a number of different manufacturers. In 1985 studies were carried out by Urbanek *et al.* (1985) to monitor the efficiency of various brands of microtitre plates in binding radio-labelled bee venom phospholipase A_2. The test plates included Dynatech Immunlon FM 129A, Dynatech Immunlon F removable well, Falcon polyvinylchloride number 3912 plates, Linbro polystyrene EIA plates and Nunc F immunoplates. As shown in Fig 6.1, Urbanek *et al.* (1985) found that the Nunc, followed by the Falcon plates consistently bind the most protein, while the Dynatech and Linbro plates bind the least. These workers also examined the ability of Nunc and Dynatech microtitre plates to bind a range of proteins. The results presented in Fig 6.2 once again show that Nunc plates are consistently better at binding a variety of proteins than are the Dynatech plates which also show a variable ability at attaching different proteins. Urbanek *et al.* (1985) were also able to demonstrate that the Nunc plates have the least desorption of coating protein under controlled incubation conditions both at 4°C and 37°C (9% compared to about 40% for other brands of microtitre plate).

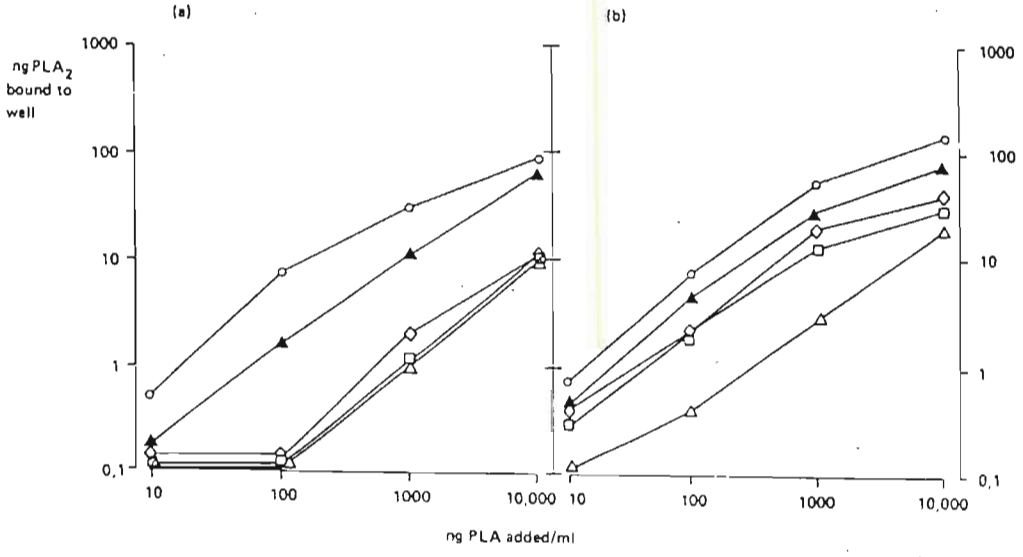


Fig 6.1 Comparison of the binding of bee venom phospholipase A_2 to different brands of microtitre plate at different concentrations (incubation volume 100 µl) at either (a) 4°C overnight or (b) under desiccating conditions at 37°C overnight (from the data of Urbanek *et al.*, 1985 and the graph of Kemeny and Challacombe, 1988). Dynatech Immunolon (◊) Dynatech Immunolon removable wells (□), Falcon (Δ), Linbro (▲) and Nunc (o)

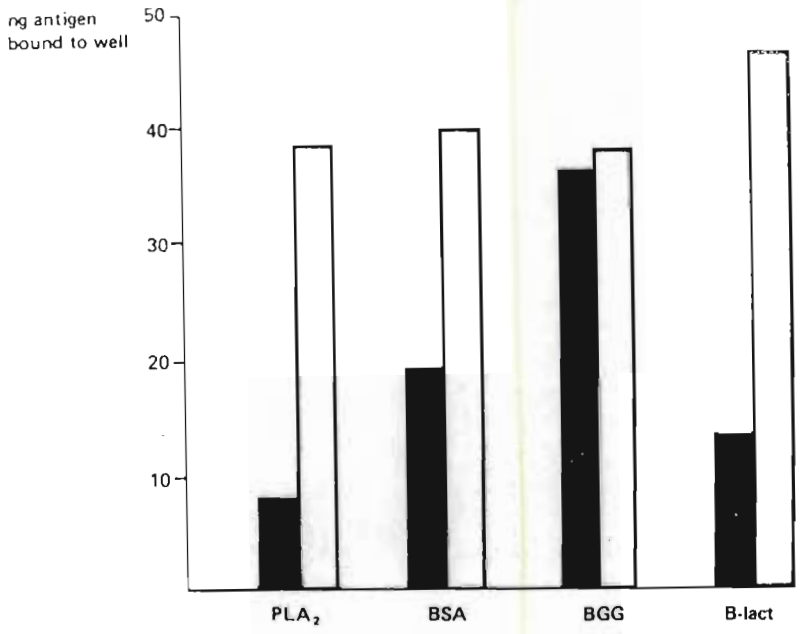


Fig 6.2 Comparison of the binding ability of bee venom phospholipase A_2 (PLA_2), bovine serum albumin (BSA), bovine γ-globulin (BGG) and β-lactogloblin (B-Lact) to Dynatech M129A (■) and Nunc Immuno-1 (❑) microtitre plates (Urbanek *et al.*, 1985)

Based on the above studies, the present author decided to employ Nunc plates for the development of an ELISA for ATR. What remained to be established, however, was which of two specific types of Nunc plates would be most suitable. Thus, the following experiment was designed to compare the ability of Nunc Immunoplate maxisorp F96 and Nunc microwell module U16 (immunoquality) to bind commercially prepared Goat anti-rabbit IgG-alkaline phosphatase.

6.2.2 Method

Microtitre wells (96) were coated with 200 µl/well of a 1:5 000 dilution of Goat anti-rabbit IgG-alkaline phosphatase, prepared in 0.05 M carbonate buffer, pH 9.6 (section 3.5.1). Plates were covered with cling film and incubated either at 4°C and 37°C overnight or at 37°C for 2 hours. Thereafter, the plates were washed thrice with saline-Tween (section 3.5.1). To each well was added p-nitrophenyl phosphate (1 mg/ml) in 0.1 M diethanolamine buffer, pH 9.6 (section 3.5.1) and all the plates were incubated for 1 hour at 37°C and the absorbance was monitored colorimetrically at 405 nm. The results were used to estimate the protein binding capabilities and edge effects associated with each of the two microtitre plates. Colour development was graded on a scale of 1 to 4, with 4 representing the greatest and 1 the least colour development and edge effects. Thus, the plate giving the highest colour development (4) and least edge effects (1) would be the plate of choice.

6.2.3 Results and conclusions

From the results reflected in Table 6.1, it can be concluded that the Nunc microwell module U16 plates (U16) have the highest overall protein binding and least edge effects when coated overnight at 4°C.

Table 6.1 The effect of temperature and incubation time on the binding of commercially prepared Goat anti-rabbit IgG-alkaline phosphatase to two types of Nunc microwells (F96 and U16). Protein binding and edge effects were rated on a scale of 1 (least effect) to 4 (highest effect) as outlined in section 6.2.2

Plate	Temperature	Time	Binding	Edge Effects
F96	37°C	Overnight	1	4
U16	37°C	Overnight	1	4
F 96	37°C	2 hours	3	2
U16	37°C	2 hours	4	2
F96	4°C	Overnight	3	2
U16	4°C	Overnight	4	1

Based on these results, the Nunc microwell U16 plates were used for all future immunoassays reported in this thesis. Selection of microtitre plates was proceeded by optimisation of the coating conditions, which are described in the next section.

6.3 Optimisation of coating conditions

6.3.1 Introduction and general considerations

While the choice of the solid phase is an important criterion, the binding of the antigen or antibody to that phase is also dependent upon an number of other parameters including the pH and ionic strength (μ) of the buffer, as well as incubation time and temperature, all of which are important considerations when developing a new immunoassay. These and other factors are evaluated in this section.

The binding of a molecule to a solid phase (coating) involves the meeting of the coating immunoreactant with the solid phase, which is proceeded by the hydrophobic binding of the molecule to the solid surface. This immobilization is mediated by a number of factors,

including the volume of immunoreactant added and the surface area available for binding (Løvborg, 1984). The amount of immunoreactant attached to the solid phase will be determined by the available surface area on the solid phase and therefore, the lower the volume of immunoreactant used, the greater the surface area to volume ratio and the more sensitive the assay (Kemeny and Challacombe, 1988). An optimal surface area to volume ratio exists for each assay and this ratio must be as high as possible, until an area size is reached which exhausts the solvent for molecules. Thereafter, a further increase in ratio has no positive effect on results (Løvborg, 1984). With regard to assay volume, Kemeny and Challacombe (1988) found that an ELISA in which volumes of 50 µl/well were used, is more sensitive than the same assay where the volume is increased to 200 µl/well. However, in the present study, for ease of pipetting and monitoring the final absorbance, it was decided that a standard volume of 200 µl/well would be used throughout the ATR immunoassay.

On consideration of the nature of the coating buffer, it is noted that in practice, a 0.05 M carbonate buffer, pH 9.6 is most commonly used (Catty and Raykundalia, 1988). It has been stated that the pH and µ of the coating buffer apparently have no effect on the degree of binding of a protein to the microtitre wells, as binding is a consequence of hydrophobic interactions (Tijssen, 1985; Kemeny and Challacombe, 1988). This is unexpected as clearly ionic strength (µ) and pH will influence protein conformation, which in turn is bound to influence hydrophobicity and consequently binding (Oreskes and Singer, 1961). In theory, a protein will most likely bind to the microtitre plates, when a net zero charge exists on the surface of the protein molecule. Thus, primary binding will be maximal at the protein's pI, resulting from reduced electrostatic repulsion between the proteins, while secondary binding will be minimal due to decreased protein-protein interactions (Standefer, 1985). Therefore, bearing the above reasoning in mind, a coating buffer should be selected with a pH close to the pI of the protein coat (for example, BSA has a pI of 4.90), but this is not applied in practice. Instead it is rather stressed, that once a buffer µ has been selected, that µ must be maintained when changing between coating, washing and incubation buffers as any alterations may disrupt hydrogen bonding, inducing detachment and assay variability (Løvborg, 1984). This concept was adhered to throughout the development of the ATR ELISA by the present author. Thus whereas in earlier work Bye *et al.* (1990) took the advice of Weiler (1986) in using a tap water wash, in subsequent assays this was replaced by a saline-Tween wash.

Despite the universal use of a carbonate coating buffer, pH 9.6 which is alluded to in the literature (Catty and Raykundalia, 1988), the present author felt it important that the pH

and nature of the coating buffer be optimised in the present studies, the results of which are presented below.

6.3.2 Methods

Microtitre wells (96) were coated with 200 µl/well of a 1:5 000 dilution of Goat anti-rabbit IgG-alkaline phosphatase, prepared in one of the following: 0.05 M carbonate buffer, pH 8.6 or 9.6, 0.05 M TBS buffer, pH 7.4 or 0.15 M citrate buffer, pH 5.0. Plates were covered with cling film and incubated overnight at 4°C. Thereafter, the plates were washed thrice with saline-Tween (section 3.5.1). To each well was added p-nitrophenyl phosphate (1 mg/ml) in 0.1 M diethanolamine buffer, pH 9.6 (section 3.5.1) and the plates were incubated for 1 hour at 37°C and the absorbance was monitored colorimetrically at 405 nm.

6.3.3 Results

The results presented in Table 6.2 below show no significant difference in the effect of pH and nature of the coating buffer on the degree of binding of the Goat anti-rabbit IgG-alkaline phosphatase to the solid phase, except in the case of the citrate buffer. Since the carbonate buffer, pH 9.6, afforded slightly better binding than the other buffer systems tested, this was used in all future immunoassays described in this thesis.

Table 6.2 Comparison of the effects of different buffers on the binding of commercially prepared Goat anti-rabbit IgG-alkaline phosphatase. The results are presented as the mean absorbance of 24 determinations. The experiment was performed as described in section 6.3.2

Buffer	Mean Absorbance	%CV
0.05 M carbonate buffer, pH 9.6	1.247	3.89
0.05 M carbonate buffer, pH 8.6	1.045	7.29
0.05 M TBS buffer, pH 7.4	0.976	5.90
0.15 M citrate buffer, pH 5.0	0.768	12.47

Based on these results and those from section 6.2, it was decided to bind ATR to the Nunc microwell module U16 plates, using a 0.05 M carbonate buffer, pH 9.6. Further optimisation was carried out based on these defined parameters and details and results of this development are given in the next section.

6.4 Determination of the binding of atractyloside to microtitre plates

6.4.1 Introduction

The rate limiting step in many solid phase immunoassays is related to the slow kinetics of macromolecular binding to the support, due to restricted diffusion and mass transfer across the liquid solid interface (Litman, 1985). Ultrasound has been demonstrated to accelerate coating of the solid phase and was investigated by the present author. Litman (1985) noted that nearly all the immunoreactive human chorion gonadotrophin (HCG) added to microtitre wells bound within the first 20 minutes in the presence of ultrasound, while in its absence, saturation was not achieved even after 150 hours exposure of the HCG to the solid phase. This binding was independent of the initial coating concentration and overall, ultrasound resulted in a 500-fold acceleration in the rate of HCG immobilization. This enhanced binding appears restricted to solid-liquid interfaces and is not demonstrated between antibodies binding to antigens in solution (Litman, 1985). It is thought that the microscopic mixing enhances diffusion and interfacial mass transfer, increasing the contact of the molecule to the support (Borisov and Stantnikov, 1966).

Any surface will bind a molecule to differing degrees, and if detachment occurs, exposed free sites may be blocked. The time required for a molecule to meet a surface and bind will be proportional to its size, taking into account that steric hindrance will also influence the degree of binding. Smaller molecules will bind to a greater extent than larger molecules, but there is a greater risk of detachment by these smaller molecules, since they show weaker binding. Furthermore, if an antibody is to bind to a compound of molecular mass < 1 000 Da (such as ATR), where the antigen exists as a monolayer on the surface of the solid support, full utilization of the coated antigen molecules will be impossible, as the large antibody molecules (> 150 000 Da) will sterically hinder each other. Only a few antibody molecules will be bound and therefore, despite the greater degree of binding by smaller molecules to solid phases, steric hindrance will result in inefficient use of the coated antigen. This may be overcome by either binding the hapten to spacer arms or by covalently coupling the small antigen to a large molecule such as albumin (Løvborg, 1984). It was proposed that the direct binding of ATR to the plate would result in the steric hindrance of the primary antibody and furthermore, it was anticipated that the negative charges on the ATR may cause repulsion of the toxin from the plate. This repulsion has also been noted with DNA (Tijssen, 1985), which carries a net negative charge and is poorly adsorbed to polystyrene. However, the binding of anionic antigens to microtitre wells has been reported by Engvall (1976) and Klotz (1982) to be significantly improved by pre-treating the microtitre plates with 1% (w/v) aqueous protamine sulphate.

In view of the above considerations it was decided to design a series of experiments to test the relative binding ability of free ATR and conjugated ATR to the solid support and the effects of ultrasound and pre-coating with protamine sulphate on such binding. The details of the methodology are given in 6.4.2 below while the results are presented in section 6.4.3.

6.4.2 Methods

Atractyloside (0.25 mg/ml in 0.05 M carbonate buffer, pH 9.6) and ATR-BSA (0.25 mg/ml in 0.05 M carbonate buffer, pH 9.6) were added directly to the microtitre wells (200 µl/well) and incubated overnight at 4°C. Another plate was pre-treated with a 0.1% aqueous solution of protamine sulphate for 90 minutes, and rinsed thrice with distilled water, prior to the addition of 200 µl/well ATR (0.25 mg/ml in 0.05 M carbonate buffer, pH 9.6). This plate was incubated overnight at 4°C. A further set of wells were prepared whereby ATR-BSA (0.25 mg/ml in 0.05 M carbonate buffer, pH 9.6) was added to each well (200 µl/well) and this plate was placed in an ultrasound bath (UM C2, 220 v, 50 Hz, Krugersdorp, South Africa) for 20 minutes. Following coating, the plates were incubated overnight at 4°C with a 1:300 dilution of primary antibody in TBS, then washed with saline-Tween and incubated with the secondary antibody-alkaline phosphatase conjugate (1: 5 000) for 2 hours at 37°C. Plates were washed and incubated with p-nitrophenyl phosphate (1 mg/ml) in 0.1 M diethanolamine buffer, pH 9.6 (section 3.5.1) then incubated for 1 hour at 37°C and the absorbance monitored colorimetrically at 405 nm. The mean results of the different coating conditions were calculated, together with the coefficient of variation (%CV). A %CV less than 10% is considered satisfactory (Feldkamp and Smith, 1987) although at Newmarket Horse Forensic Laboratory (Newmarket, UK, pers. comm., 1989) a %CV of less than 5% is deemed acceptable.

6.4.3 Results and conclusions

The results of the ATR binding experiment are given in Table 6.3 below. The direct binding of ATR to the plates gave very poor results as noted by the low absorbance value and very high %CV. This was probably due to the repulsion of the anionic ATR molecules which would have caused an unequal coating of the plates. It was hoped that this repulsion could be overcome by pre-coating the plates with protamine sulphate but as shown in Table 6.3, there was only marginal improvement in the degree of binding and also in the %CV. By contrast the binding of ATR to the plates as a toxin-BSA conjugate gave by far the highest absorbance value with the lowest %CV. Furthermore, coating of the same ATR-

conjugate, in the presence of ultrasound, also gave an acceptable level of binding and this method would have been considered for further assays, if the %CV had not been so high (14%). This latter method is however recommended when prompt results of an ELISA are required, but only if high accuracy is not important.

Table 6.3 A comparison of methods for the binding of ATR to Nunc microtitre wells. Results are expressed as the mean absorbance of each binding method (n = 24) with associated %CV

Binding procedure	Mean Absorbance	%CV
Pure ATR bound directly	0.060	48
Protamine sulphate + ATR	0.106	31
ATR-BSA	1.136	4
ATR-BSA + ultrasound	0.976	14

In conclusion, since the direct coating of ATR to microtitre wells was found to be ineffectual it was decided to use an ATR-BSA conjugate (prepared as described in section 3.4.2) for all subsequent assays. Having optimised the solid phase, coating buffer and the binding of ATR to the plate as a hapten-protein conjugate, attention was turned to determining the optimal ATR-BSA coating dilution and the primary and secondary antibody concentrations. These studies are presented in section 6.5 below.

6.5 Optimisation of the immunoassay conditions and relative concentrations of coating antigen, primary antibody and secondary antibody-enzyme conjugate

6.5.1 Introduction

The optimisation of the coating antigen and primary antibody concentrations may be determined using a so-called chequerboard ELISA (Catty and Raykundalia, 1988), whereby replicate serial dilutions of coating antigen running in vertical rows are incubated with serial dilutions of primary antibody (running in horizontal rows). Once the concentration of the primary antibody and coating antigen have been determined, a chequerboard may be carried out in the same manner to identify the optimal secondary antibody-enzyme conjugate dilution to be used in the assay. Thus, the optimal coating concentration, for a given primary and secondary antibody enzyme conjugate may be identified by the highest absorbance value, with the least background or non-specific binding (NSB). While performing this and other related experiments, it was noted that

incubation temperature for the primary antibody had a significant effect on the entire assay. Thus the aim of the present study was to optimise the incubation temperature and to use these optimal conditions to, in turn, optimise the relative concentrations of the coating antigen and the dilutions of the primary antibody and the secondary antibody-enzyme conjugate.

6.5.2 Method

Nunc microwell module U16 plates (referred hereafter as microtitre plates) were coated with either BSA (representing the control) or BSA-ATR conjugate (200 µl/well). The starting concentrate of coating antigen (prepared as described in section 3.4.2) was pre-filtered using Millex HV 0.45 µm filters, before being 10-fold serially diluted over the range 1 mg/ml to 1×10^{-7} mg/ml, in 0.05 M carbonate buffer, pH 9.6. Plates were covered with cling film and incubated overnight at 4°C. Based on the affinity assay results and characterisation tests (section 5.5) only antiserum from rabbit 4, raised against a naked bacteria-ATR conjugate was used. The antiserum was diluted in TBS (section 3.5.1) over the range 1:100 to 1:600 and to each well was added 200 µl of the appropriate antiserum, forming a chequerboard assay. Plates were incubated for 16 hours at 4°C or at 37°C for 2 hours (to monitor temperature effects), washed thrice in saline-Tween (section 3.5.1) and incubated for a further 2 hours at 37°C with 200 µl/well of a 1:5 000; 1:5 500; 1:6 000 or 1:7 000 dilution of Goat anti-rabbit IgG-alkaline phosphatase diluted in TBS. Plates were washed a further three times in saline-Tween and incubated for 30 minutes at 37°C with p-nitrophenyl phosphate (1 mg/ml) in 0.1 M diethanolamine buffer, pH 9.6 (section 3.5.1) (200 µl/well). The absorbance was measured at 405 nm. Final results were calculated as the mean absorbance of the test sample minus the absorbance value for a BSA control, at the same coating concentration and primary antibody dilution.

6.5.3 Results and conclusions

When in the above experiment, the primary antibody was incubated at 37°C for 2 hours and instead of 4°C for 16 hours, no colour development was observed on addition of the substrate. This was initially thought to be due to experimental error, but the result was reproducible on three successive attempts. Brief consideration was therefore given to the effects of temperature on the success of an immunoassay.

Temperature is an expression of molecular movement or velocity and at high temperatures there is more chance of a molecule meeting a surface, due to increased velocity (Løvborg, 1984). Since adsorption of molecules is directly related to time and temperature and double the binding occurs at 37°C than at 4°C (Cantarero *et al.*, 1980; Standefer, 1985), it would be feasible to maximise binding by incubating the immunoplates at 37°C. Unfortunately, however, edge effects are commonplace when plates are incubated at this temperature, due to the establishment of thermal gradients (Løvborg, 1984; Tijssen, 1985). It is nevertheless argued that since antibody-antigen reactions occur *in vivo* at body temperature, that all EIAs should be performed at 37°C. The use of this standard temperature may be a fallacy though as it is only a pre-requisite for precipitin, agglutination and complement fixation assays. So-called "cold-antibodies" have been identified, whereby binding of the antibody to its corresponding antigen is enhanced at room temperature or 4°C and observed to be depressed at 37°C (Tijssen, 1985).

"Cold-antibodies" are usually associated with auto-immune disease in humans, causing cold-haemolytic anaemias. Such antibodies do not associate with their corresponding antigen at 37°C, but do so at temperatures below 30°C. They are commonly IgM antibodies and their titre appears to fall off exponentially when incubation temperatures increase above 4°C (Dacie and Lewis, 1970). Since other current literature regarding these so called "cold-antibodies" pertains primarily to the clinical implications of producing such antibodies, no further comments can be made with regard to the apparent production of "cold-antibodies" against ATR. The fact remains, however, that at 4°C there was no loss of primary antibody activity, whereas at 37°C, no activity was recorded. From this result it was suggested that IgM antibodies were raised against ATR, and that class switch had not occurred, which could account for the apparent low titres of antibodies raised against this toxin (Chapter 5).

The results of the chequerboard assay are given in Fig 6.3 (a to c). In most cases, the highest absorbance was obtained with a coating concentration of 0.001 mg BSA-ATR/ml. When a primary antibody dilution of 1:100 was used (Fig 6.3a), the absorbance values were very low and frequently no change in absorbance was noted despite an alteration in the coat concentration. The trend for the 1:200 dilution of primary antibody, depicted in the same graph, was more favourable, but the 1:300 dilution (Fig 6.3b) gave even higher overall absorbance values. A similar pattern was observed with the 1:400 dilution of primary antibody, except that the absorbance value obtained for the coating concentration of 0.001 mg BSA-ATR/ml was slightly lower than that of the 1:300 primary antibody dilution.

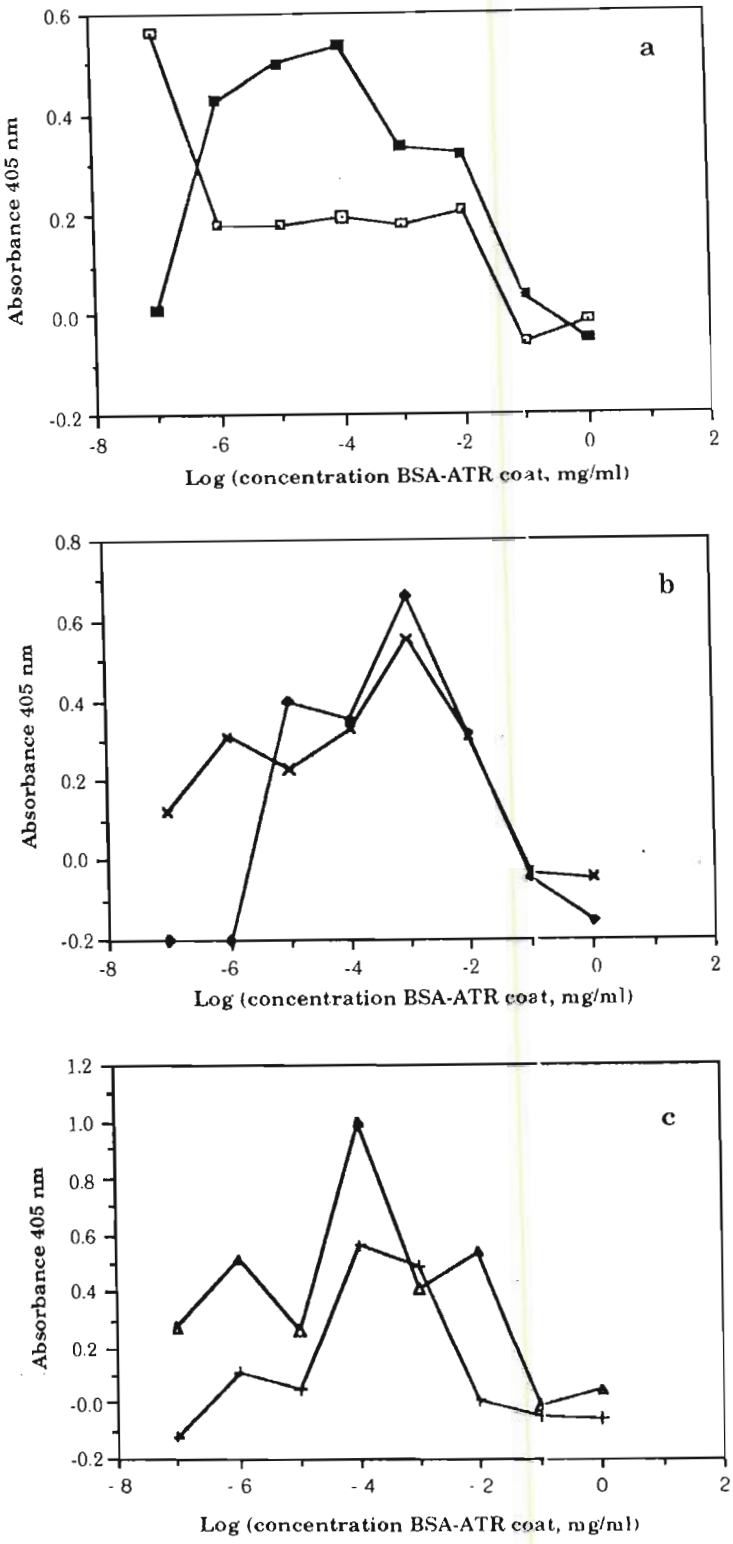


Fig 6.3 A comparison of the effect of various primary antibody dilutions on the coating ability of a range of concentrations of BSA-ATR. NUNC U16 plates were used for the coating process.
(primary antibody dilution: □ = 1:100; ■ = 1:200; ♦ = 1:300; × = 1:400; Δ = 1:500; + = 1:600)

The absorbance profile for the 1:500 dilution of primary antibody (Fig 6.3c) was greatest at a coating concentration of 0.0001 mg BSA-ATR/ml which was overall the overall highest absorbance value. However, the other coating concentrations, at the same primary antibody dilution, showed very little variation in absorbance values. The values obtained for the 1:600 dilution of primary antibody were lower than that of the 1:500 dilution (Fig 6.3c). Based on the results of this investigation, it was decided to use a coating concentration of 0.001 mg BSA-ATR/ml, in conjunction with an primary antiserum dilution of 1:300 for all future immunoassays reported in this thesis. This result constituted a considerable improvement on earlier work by the author Bye *et al.* (1990) in which the optimal working dilution was 1:40.

The results of the experiment designed to optimise the secondary antibody-enzyme conjugate dilution are shown in Table 6.4. From the table it is clear that a dilution of 1:5 500 gave the best result and was therefore incorporated into all future immunoassays.

Table 6.4 The effect of various dilutions of secondary antibody-alkaline phosphatase conjugate on its binding to the primary anti-ATR antibodies (n = 6)

Dilution	Absorbance (405 nm)	%CV
1:5 000	0.578	1.12
1:5 500	0.695	6.96
1:6 000	0.598	2.40
1:7 000	0.418	8.55

While carrying out the optimisation steps described above, suitable controls were included at all times and it was noted that the background absorbance readings were unacceptably high. This necessitated the selection of a suitable blocking agent to mask these extraneous sites of binding, the details of which are given in section 6.6.

6.6 Optimisation of blocking agents

6.6.1 Introduction

Non-specific binding (NSB) is a common source of error in any immunoassay and it is imperative that suitable controls are included to ensure that the results are valid. Thus a NSB control should include everything except the primary antibody. Then if the absorbance value obtained for this control is negligible, it is assumed that any association

of the primary antibody is regarded as specific (Petrusz, 1983). It is common practice that any non-specific binding may be minimised by "blocking" of such sites with albumins or casein (Petrusz, 1983). In view of the high background absorbance values obtained for non-specific binding of antibodies to free sites on the microtitre wells (section 6.5) it was decided to test the blocking efficacy of various selected proteins. The results are reported in 6.6.3 below.

6.6.2 Methods

Microtitre plates were coated with BSA (0.001 mg/ml; 200 µl/well) in 0.05 M carbonate buffer, pH 9.6. Plates were covered with cling film and incubated overnight at 4°C. Thereafter, 200 µl of a 0.1% (m/v) solution of BSA, casein, gelatin or buffer (as a control) was added to representative wells and plates were blocked for 1 hour at room temperature, whereupon the blocking reagent was discarded. The primary antibody solution was diluted 1:300 in TBS and 200 µl volumes were added to each well. Plates were incubated for 16 hours at 4°C, washed thrice in saline-Tween (section 3.5.1) and incubated for a further 2 hours at 37°C with 200 µl/well of a 1:5 500 dilution of Goat anti-rabbit IgG-alkaline phosphatase diluted in TBS. Plates were washed a further three times in saline-Tween and incubated for 30 minutes at 37°C with p-nitrophenyl phosphate (1 mg/ml) in 0.1 M diethanolamine buffer, pH 9.6 (section 3.5.1) (200 µl/well) and the absorbance was measured at 405 nm.

6.6.3 Results and conclusions

The results presented in Table 6.5 clearly show that the use of a blocking agent is imperative, since non-specific binding was very high in the case of the control experiment.

Table 6.5 A comparison of the ability of various proteins to block non-specific binding of the primary antibody to microtitre plates. The efficiency of 0.1% (m/v) solutions of BSA, casein, gelatin or buffer (as control) were used for blocking as described in section 6.6.2 (n = 24)

Blocking agent	Mean absorbance	%CV
BSA	0.078	3.91
Casein	0.253	34.48
Gelatin	0.071	2.56
Buffer (control)	1.456	14.75

Casein was non-soluble in the buffer, requiring hydrolysis and was, therefore, abandoned as a blocking agent. By contrast, gelatin proved to be the most efficient blocking agent, but concern was voiced as to the possible solidification of this protein during incubations at 4°C. Fortunately, BSA proved almost as efficient as gelatin and thus this protein was used as the standard blocking agent for all subsequent assays. This choice was also most appropriate in view of the earlier selection (section 6.4.3) of ATR-BSA as the coating conjugate, thus involving BSA in both instances. The advantage of this is that the antiserum used in the assay (from rabbit 4) would not cross-react with BSA (section 5.5.4).

Following selection of the solid phase, coating antigen and buffer, incubation times and temperatures as well as the concentration of the immunoreactants, the optimised ELISA for ATR was tested by application to various biological samples. The results are given in the next section.

6.7 Application of the ELISA to the detection of atractylosides in rat serum and urine

6.7.1 Introduction

In view of the high incidence of accidental ATR-related poisonings (Bhoola, 1983; Georgiou *et al.*, 1988), it was decided to develop a diagnostic assay for this intoxication. As outlined in Chapter 4, the development of an assay initially involved the optimisation of a HPLC method for the detection of ATR, but as this technique requires a skilled operator and expensive equipment, it was decided to also develop an immunoassay, that would be suitable for "in the field" screening of ATR poisoning.

Following the optimisation of the ELISA on pure ATR (sections 6.2 to 6.6), the aim was to test this method on authentic urine and serum samples from rats dosed with 0, 5, 10 and 25 mg ATR/kg bwt, collected at 24, 48, 72 and 96 hours post-dosing with the hope that this wolud also yield information as to the fate and excretion profile of ATR in rats.

A diagrammatical representation of the final optimised ELISA, for the detection of ATR, is depicted in Fig 6.4 below. Briefly, this ELISA constitutes four steps. The first involves the coating of the ATR-BSA conjugate to the plate with the subsequent addition of the standard toxin or the sample, together with the primary antiserum. A competition arises between the free and bound toxin for epitope binding sites on the primary antibody. Antibody bound to the ATR-BSA conjugate will be retained, while antibody bound to free ATR in the sample will be discarded.

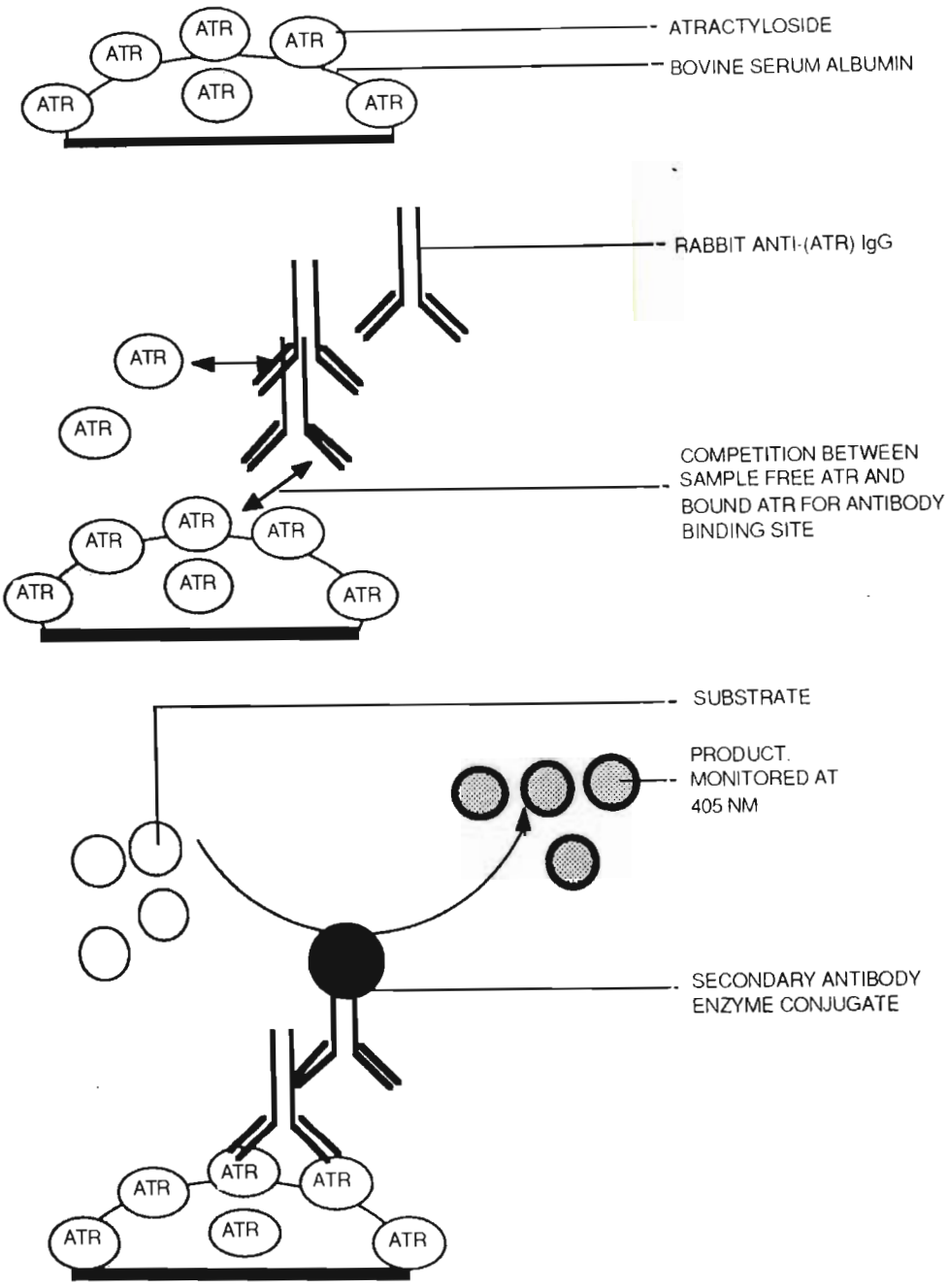


Fig 6.4 Diagrammatic representation of the competitive ELISA, developed for the detection of atractyloside in biological fluids

Thus the more free toxin there is in the sample, the less primary antibody will be bound to the ATR-BSA conjugate. In the third stage of the assay, a secondary antibody enzyme conjugate is added. Since the secondary antibody is specific for the primary antibody, it will in turn bind to the primary antibody bound to the ATR-BSA conjugate. Thus the less free ATR there is in the sample, the more primary antibody will bind to the ATR-BSA conjugate and hence the more secondary antibody enzyme conjugate will bind to the primary antibody. An appropriate substrate is added in the fourth stage, which is converted into a coloured product by the enzyme conjugate. The absorbance is monitored colorimetrically at 405 nm and thus the absorbance value obtained will be indirectly proportional to the amount of toxin present in the sample.

This ELISA was used for the detection of ATR in the biological samples collected from rats dosed with this diterpene glycoside (section 3.1), the results of which are presented in section 6.7.3 below.

6.7.2 Methods

Urine and serum samples from rats dosed with 0, 5, 10 and 25 mg ATR/kg bwt were collected as described in section 3.1. The serum and urine samples were extracted in a solution of methanolic hydrochloric acid (section 3.1.6), and then further diluted 1:10 and 1:100 in TBS (section 3.5.1) as suggested by staff at the Newmarket Horse Forensic Laboratory (Newmarket, UK, pers. comm., 1989). The ELISA was performed as described in section 3.5.2. Results were determined by calculating the mean of the replicates and comparing the mean absorbance of the 1:10 dilution with that of the 1:100 dilution. Theoretically, an increase in absorbance is expected with an increase in dilution, as less atractylosides would be present in the 1:100 dilution than in the 1:10 dilution. Thus in the former, more antibody would be available to bind to the coating antigen. The change in absorbance between the 1:10 and 1:100 dilution was calculated, and a 10% or greater increase in absorbance was classified as positive (+). Positive results noted for control samples were classified as "false positives" (FP). A corresponding blank of the methanolic hydrochloric acid was prepared to determine the effect of the extraction solvent on the immunoassay and the possible interference by glucose and ADP. The results of the ELISA on the urine samples were transposed into a bar graph by determining the percentage of positive results for each day.

A standard curve, for the determination of ATR, was constructed over the 10-fold dilution series of ATR from 1 mg/ml to 1 ng/ml. The mean absorbance values for each concentration were calculated and expressed as a percentage of the absorbance obtained for

a sample, in which the antibody was incubated in the absence of any free ATR (effectively 100% binding). Non-specific binding controls were also included, in which the test sample contained all the immunoreactants except the primary antibody, the latter being replaced with either pre-immune serum or buffer. In addition, each component of the immunoassay was sequentially omitted to validate the results of the ELISA on the biological specimens. The biological samples (urine and serum) from the dosed rats were assayed using the optimised ELISA procedure outlined in section 3.5.2 and the results are presented in section 6.7.3 below.

6.7.3 Results and discussion

A representative standard curve determined for ATR is given in Fig 6.5. From these results it can be seen that this immunoassay has a detection limit of about 10 ng ATR/ml, which was reproducible.

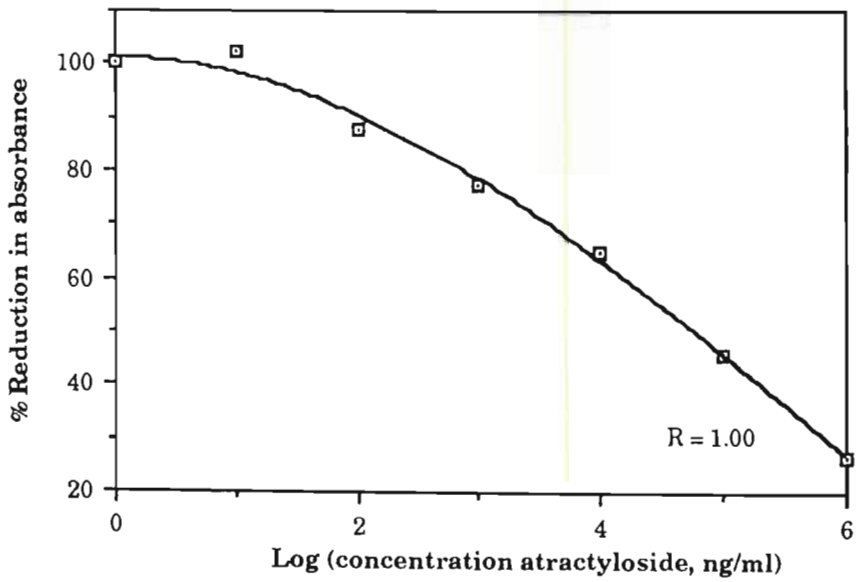


Fig 6.5 Representative standard curve for the enzyme linked immunosorbent determination of atractyloside over the concentration range 1 ng/ml to 1 mg/ml. The ELISA was performed as described in section 3.5.2 and the absorbance was monitored colorimetrically at 405 nm. Results are presented as the % absorbance of the sample compared to the absorbance given by a sample devoid of toxin (100%)

With regard to the level of precision of the assay, a coefficient of variation (CV) of less than 10% is considered satisfactory (Feldkamp and Smith, 1987), although at Newmarket Horse Forensic Laboratory (Newmarket, UK, pers. comm., 1989) a %CV of less than 5% is

deemed acceptable. For the present ATR immunoassay, the precision profile was determined by evaluating the known concentrations of standard ATR with their associated %CV values which enabled an assessment of the heteroscadasticity of the standard curve (Feldkamp and Smith, 1987). A mean %CV of 3.97% was obtained over the range 0.1 to 10 μg ATR/ml, indicating that this is the working range of the immunoassay. On either side of this working range, the %CV was unacceptably high, indicating that beyond these limits, the ATR immunoassay is inaccurate.

The results of the ELISA carried out on the rat urine and serum are summarized in Table 6.6. Whereas only a single known false positive was recorded for the control urine samples (control rat 4, day 4) there were six false positives for the control serum samples. Furthermore, the serum samples only gave six positive readings which were randomly distributed and this, together with the high incidence of false positive specimens, cast doubt on the validity of the serum results. Serum was therefore discounted as a useful biological fluid for the detection of ATR by ELISA, probably because the levels of ATR in the rat samples were too low to be detected by this method. The controls included in the assay, together with the work carried out on the charaterisation of the antiserum showed that the antibodies are specific for atractylosides. Likewise, the use of a methanolic hydrochloric acid extractant apparently did not affect the functioning of the assay. This is fortunate, since this extraction process was deemed vital to the success of the ELISA as, it was noted in the urinalysis studies (section 7.2) that, rats exposed to high doses of ATR release alkaline phosphatase (AP) into the urine.

Since antibodies directed towards certain epitopes present on an antigen may cross react with a number of antigens with common epitopes, the American Academy of Forensic Sciences, Toxicology Section issued a statement in 1986, calling for the reporting of immunoassay results to be restricted to positive or not detected, unless the results were confirmed by alternative analytical methods such as HPLC (Baselt, 1989). In the present studies, in view of the fact that the HPLC method (Chapter 4) did not detect ATR in all the urine and serum samples, that gave positive ELISA results, it was considered inappropriate to quantify the results obtained in this immunoassay.

Table 6.6 Results of the ELISA carried out on urine and sera of rats dosed with atractyloside at 0, 5, 10 and 25 mg/kg bwt, respectively*. Urine and serum samples were collected at 24, 48, 72 and 96 hours after dosing as described in section 3.1. The method for the ELISA is given in section 3.5. The increase in absorbance between the 1:10 and 1:100 dilution was calculated and a 10% or greater increase was classified as positive (+)

mg ATR/kg bwt		25 mg		10 mg		5 mg		0 mg	
Day	Sample	H (u)	H(s)	M (u)	M (s	L(u)	L (s)	C (u)	C(s)
0	1	-	FP	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-	-
	5	-	-	-	-	-	FP	-	-
1	1	+	-	+	-	+	-	-	FP
	2	+	-	+	-	-	-	-	-
	3	+	-	+	-	+	-	-	FP
	4	+	+	+	-	+	-	-	-
	5	-	-	+	-	+	-	-	-
2	1	-	-	+	-	-	-	X	-
	2	-	-	-	-	+	-	-	-
	3	-	-	+	-	+	-	-	X
	4	-	-	+	-	+	-	-	-
	5	-	+	+	-	-	-	-	FP
3	1	+	-	-	X	-	-	-	X
	2	-	-	-	+	+	-	-	-
	3	-	-	-	-	+	-	-	FP
	4	X	-	-	-	+	-	-	-
	5	-	X	-	-	+	+	-	-
4	1	-	+	-	-	+	-	-	-
	2	-	-	-	-	+	-	-	-
	3	-	-	-	-	+	-	-	-
	4	-	-	-	+	+	-	FP	FP
	5	-	-	-	-	+	-	-	-

* H=High dose ; M=Medium dose; L=Low dose ; C=Control; (u)=urine; (s)=serum;
– = atractylosides not detected; + = atractylosides detected; FP = false positive;
X = insufficient sample for analysis

The results presented in Table 6.6, therefore, indicate that urine is a suitable biological fluid to for screening by ELISA, provided endogenous AP activity is quenched. When the urine data from Table 6.6 was transposed into a bar graph, representing the daily percentage of positive samples per dose level, some clear trends were observed. The results are shown in Fig 6.6.

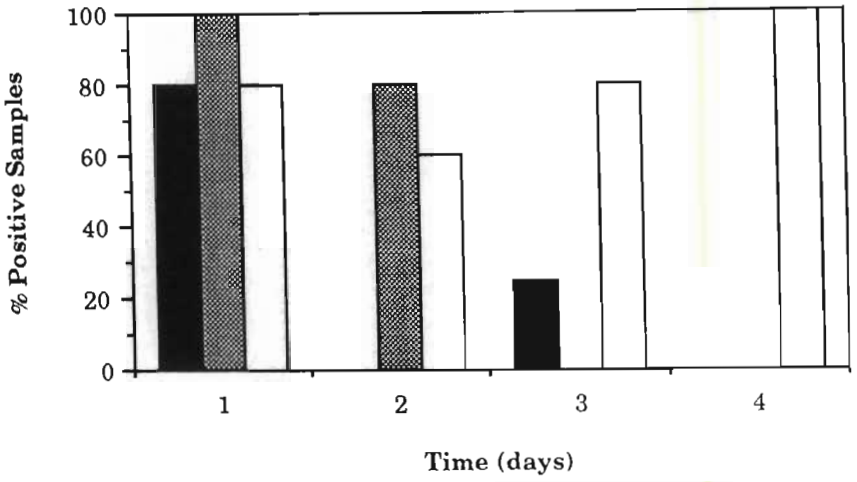


Fig 6.6 Daily percentage of atractyloside positive rat urine samples, monitored by ELISA. Eighteen hour representative samples were collected over a period of 4 days, following dosing with 5, 10 and 25 mg/kg bwt (low □, medium ▨ and high ■) respectively

The results presented in Fig 6.6 show that most urine samples were positive on day 1, post-dosing, regardless of the level of toxin administered, whereas only one urine sample tested positive for the high dose regime after day 1 (rat 1, day 3), suggesting that all the toxin was excreted within the first 24 hours after exposure. If residual toxin remained within the organism after this period, it is suggested that the ATR was "fixed" in the kidney of the animal (see Chapter 7). Rats dosed with the medium level of toxin, excreted ATR on days 1 and 2 and thereafter ATR was not detected. Thus, animals receiving the medium dose excreted all or most of the toxin within 48 hours, as monitored by ELISA. For the low dose regime, atractylosides were detected in the urine for 96 hours, at which point urine collection was terminated. It is unfortunate that at this stage, levels of ATR in the biological fluids are not quantifiable by ELISA as this would give some indication of the amounts of toxin excreted and whether residual ATR remained in the organism. This aspect was investigated by immunocytochemistry as outlined in Chapter 7, section 7.7.

It will be noted that the results of the ELISA do not strictly correlate with those of the HPLC outlined in Chapter 4 and although this will be extensively discussed in Chapter 8, briefly the ELISA was seen to detect atractylosides when the HPLC method did not and *vice versa*. Although nothing is known of the pharmokinetics of ATR, it is suggested that on days where the toxin was detected by ELISA and not HPLC, that ATR was sufficiently modified *in vivo* to alter its retention time on a reverse phase HPLC column (hence it was not detected chromatographically) but not enough to alter its epitope(s) so that the metabolite was still recognised by the anti-ATR antibodies. Furthermore, when ATR was detected by HPLC but not by ELISA it is suggested that the toxin was present in levels too low to be detected by the immunoassay.

The fact that both the ELISA and HPLC results showed that ATR (or its metabolite) is excreted into the urine over a time period inversely proportional to the dose, may be explained as follows. A drug may be "lost" once administered to an organism before reaching its receptor site, by binding to macromolecules. For instance, neutral compounds tend to associate with lipids, cations with ribonucleic acids or α_1 acid glycoprotein, while anions frequently bind to albumin. Acidic active substances (such as ATR) commonly bind to serum albumin by virtue of van der Waals forces between the tryptophan residue on the albumin and the aromatic moieties on the drug (Albert, 1985). Albumin is thought to act as a drug-depot (Albert, 1985) which can have serious repercussions if the compound is suddenly released into the system. The proportion of the active compound bound to the albumin is inversely proportional to the plasma concentration of the drug, as there are a limited number of binding sites on the albumin. Once the binding residues on the protein have been saturated, residual drug present in the plasma cannot associate with the albumin and is transported to its effective receptor or is excreted (Albert, 1985). If ATR binds to serum albumin, this would account for the almost immediate release of all the excretable toxin by the rats dosed with the high level of this compound. Conversely, the lower the dose, the higher the proportion of toxin bound to the albumin and consequently, the toxin will remain circulating in the organism, slowly being released and excreted. Unfortunately this explanation is only valid to a point, since if ATR associates with serum albumin, it should have been detected in the serum samples.

There are three possible explanations for this anomaly. Firstly, ATR was bound to the serum albumin, but during the extraction procedure in methanolic hydrochloric acid, was precipitated with the protein and therefore not detected by ELISA. Since, however, pilot studies on sera spiked with ATR and extracted with the acidic methanol, detected positive for ATR when monitored by TLC and HPLC (Chapter 4), this proposal is unlikely. Secondly, ATR may have associated with the serum albumin but was present in the serum

at a concentration which is too low to be detected, a possibility which is highly feasible, considering the relative high concentrations of serum albumin in the blood, which could have significantly diluted the ATR. Thirdly, ATR may not have associated with the serum albumin, nor did so transiently,but instead was transported directly to the target site i.e., the kidney, a possibility that is also feasible considering the hydrophilic nature of ATR, which could presumably be transported in the blood without the need of carrier protein. The kidney provides a major route for the excretion of hydrophilic drugs and hence this organ together with the liver, often contains high concentrations of drugs, as these organs are responsible for biotransformation and excretion of xenobiotics. It will be shown (Chapter 7, section 7.3) that despite being a potent inhibitor of oxidative phosphorylation, ATR is not hepatotoxic suggesting that the toxin proceeds directly to the kidneys and is excreted more rapidly into the urine.

Since the excretion of xenobiotics by the kidneys is influenced by urinary pH, the removal of organic acids, such as salicylates would be significantly reduced if the urine is acidic, whereas under alkaline conditions excretion would be promoted (Berndt, 1982; Barbieri, 1990). The average pH of human urine is pH 6.0, becoming more acidic under conditions of starvation, due to the presence of ketone bodies (Tietz, 1976). Since the rats dosed with ATR were fasted for the 18 hour period of urine collection it is conceivable that they would have also excreted acidic urine, thereby retarding the excretion of ATR. Diet and drugs can also influence urinary pH and, in retrospect, therefore, this parameter should have been monitored when the rat urine was collected. Furthermore, since nephrosis has been observed as early as 150 minutes after exposure to ATR (Carpenedo *et al.*, 1974) it is possible that the induced necrosis is accompanied by an alkalisation of the urine, in a dose responsive manner. Consequently, the higher the dose of ATR, the more kidney damage is observed and thus, the more alkaline the urine will be and the faster the toxin is excreted. Although highly speculative, this could account for the dose responsive excretionary rate of ATR by the rats.

Although the data and conclusions drawn from the ELISA may not be directly extrapolated to humans, it became apparent that the applicability of the ELISA as a diagnostic tool is dependent upon the dose. If it is assumed that the greatest proportion of ATR will be excreted within the first 24 hours after exposure, it is unlikely that the commercial development of this assay can be justified. As noted in the introduction, rural populations are forced to make use of alternative medical practices because of the shortage of western practitioners and if a patient is admitted to a western hospital, it is usually as a last resort. South Africa is a vast country and many of the people reside in isolated and inaccessible areas. Consequently, there can be a significant lag period between the decision to take a

person to hospital and the time of actual admission. It is a personal opinion, therefore, that the diagnostic potential of this ELISA is limited, but a definitive answer may only be obtained if hospitals can be encouraged to participate in a screening programme of all patients suspected of being poisoned by *Impila*.

The development of the ELISA and HPLC method, though possibly of limited usefulness from the clinical sense, gave considerable insight into the detoxification and excretion of ATR. Since the only previous work on the excretion of atractylosides was carried out on analogues isolated from coffee beans (Richter and Spiteller, 1978), and this is the first report that ATR *per se* is excreted into the urine. However, from a practical perspective it was noted by the present author, when visiting rural hospitals, that patients suspected of having *Impila* poisoning were usually babies in renal failure. Since in this situation, urine collection is hampered, the use of this biological fluid in the ELISA is not ideal. The fact that the period of ATR excretion is inversely proportional to the dose would also hamper an estimation of the severity of the poisoning. Furthermore, it is intimated that if the removal of this toxin is related to urinary pH, so physicians treating patients with suspected *Impila* poisoning would be advised to administer urinary alkalisers, to promote the rapid excretion of this toxin.

Since so little is known of the metabolic fate of ATR, many explanations for the observed phenomena are highly speculative and will remain so until tracer studies are carried out, using radio-labelled ATR. The availability of antibodies to this toxin, however, provided a probe for the subcellular localisation of ATR in tuber and rat kidney by immunocytochemistry. The results are presented in the next chapter together with studies on the localisation of the toxin by non-invasive methods, employing organ function enzymes and markers in the urine and serum.

CHAPTER 7

LOCALISATION OF THE TARGET SITES OF ATRACTYLOSIDE IN RATS

7.1 Introduction

Although the pharmacological action of ATR was first studied in 1958 (Santi, 1958) it wasn't until 1974 that Carpenedo *et al.* (1974) first reported that the kidney was the target organ for ATR. Subsequently it was shown by a number of workers that specifically the renal proximal tubules undergo gross changes in histology following exposure to ATR or plants containing this toxin (Carpenedo *et al.*, 1974; Stuart *et al.*, 1981; Hatch *et al.*, 1982; Bhoola, 1983; Martin *et al.*, 1986; Georgiou *et al.*, 1988). These findings were in part corroborated with organ function assays in both rats (Carpenedo *et al.*, 1974) or patients with suspected ATR poisoning (Bhoola, 1983; Georgiou *et al.*, 1988) but there is little information regarding further sites of action in the kidney or for that matter in the proximal tubule itself. In addition, it has been suggested that the liver may also be susceptible to this toxin but this is unconfirmed (Wainwright *et al.*, 1977; Georgiou *et al.*, 1988). Work on the precise subcellular site of action by this toxin has only been carried out on isolated organelles or organ homogenates (Klingenberg, 1978; Luciani *et al.*, 1978) but there has been no localisation of the target site of ATR *in vivo*.

The purpose of the study presented in this chapter was, therefore, to investigate whether the kidney or the liver is the primary target organs and which cells and organelles within these organs are specifically involved. The localisation of the primary target organs was carried out by dosing rats with ATR and collecting both urine and serum samples (section 3.1.2 and 3.1.3) which were then assayed for the presence of organ specific enzyme markers as well as urinary concentrations of albumin, glucose and calcium (section 7.2). As further confirmation of the target organ, *in vitro* tests were performed whereby amino acid incorporation into isolated viable cells from the target organ was monitored in the presence and absence of ATR (section 3.7), the results of which are presented in section 7.4. The urinalysis and *in vitro* studies were carried out at RIIEHS, University of Surrey, Guildford, under the direction of Dr Peter Bach.

Having identified the target organs for ATR, the availability of specific antibodies to ATR (developed as described in Chapter 5), enabled the subcellular localisation of ATR by

immunocytochemistry. As this is the first report of the immunocytochemical localisation of ATR, this procedure demanded a considerable amount of method development and optimisation. To assist this development, the immunocytochemical procedure was initially optimised on the plant tuber of *C. laureola,* since this material was known to contain significant amounts of the toxin (section 7.6). Subsequently, the optimised method was applied to the localisation of ATR in rat tissue, the results of which are presented in section 7.7 below. As in previous chapters, the developmental aspects of the methodology are given in the respective sections below, whereas the final optimised procedure is detailed in the Materials and Methods, Chapter 3.

7.2 The effects of atractyloside on the appearance of kidney function enzymes and markers in rat urine

7.2.1 Introduction

There is undisputed evidence in the literature that the accidental administration of the diterpene glycoside ATR induces kidney necrosis (Bhoola, 1983). Structurally, the kidney comprises two distinct organs; the cortex and the medulla, within which exist over 20 morphologically distinct cell types (Moffat, 1982; Bach and Kwizera, 1988). The kidney is teleologically designed to simultaneously perform a myriad of diverse and complex functions, such as filtration, concentration and excretion; thereby processing blood and removing waste products as urine (Bonner *et al.*, 1982, Fowler, 1982). The kidney, together with the liver, therefore, is paramount in mediating the biotransformation and excretion of xenobiotics.

The kidney is particularly susceptible to toxic insult and despite accounting for less than 1% of the total body mass, continually receives 20% of the total cardiac output (Roxe, 1975). Water-soluble drugs are freely filtered and reach concentrations within the tubular cells far exceeding levels found elsewhere in the body. Many nephrotoxins are highly specific, principally because the biochemical activity of a kidney cell predisposes it to the inhibitory action of toxins, or because the cell is capable of transporting and concentrating the xenobiotic. Furthermore, the metabolic activity of the kidney cell may render the now modified molecule more toxic (Roxe, 1975; Bach and Kwizera, 1988; Boogaard *et al.*, 1989a). Injury to the kidney may be the result of primary inhibition within target cells, or may manifest itself in adjacent cells, when the physiological modification in the target cell generates a degenerative cascade effect in the cells working in tandem (Bach and Kwizera, 1988; Bach, 1989).

The kidney comprises four compartments *viz.*, intravascular, intratubular, intracellular and interstitial. As shown in Fig 7.1, the nephrons of the kidney comprise the glomerulus, proximal tubule, loop of Henlé and the distal tubule which in turn is connected to the collecting duct. The glomerulus forms a barrier between vascular and tubular renal sections. The sialic acid and heparin sulphate residues on the glomerular basement membrane impart a net anionic state on the glomerular filter, facilitating cationic passage, while impeding anionic transfer (Moffat, 1982). Transfer of a molecule through the glomerulus is, therefore, dependent upon size, charge and shape, unless the compound is of low molecular mass (Berndt, 1982). Thus, the anionic nature of ATR would tend towards the molecule being retained by the glomerular filter, but its small size appears to negate this. Removal of the anionic residues on the glomerular bed membrane induces morphological changes in the epithelial cells of the foot-processs, altering the slit diaphragm and foot-process fusion, resulting in plasma proteins (mass > 70 000 Da) leaking into the proximal tubule. Ordinarily, by virtue of their size, these proteins are retarded by the glomerular filter and so the presence of albumin in the urine indicates compromise of glomerular permselectivity (Berndt, 1982; Moffat, 1982). Enzymes and proteins present in the urine, therefore, originate from the kidney unless there is glomerular damage (Fowler, 1982). Histological studies have shown that ATR does not affect the glomerulus (Carpenedo *et al.*, 1974; Bhoola, 1983) but this does not correlate with urinalysis studies as will be discussed later.

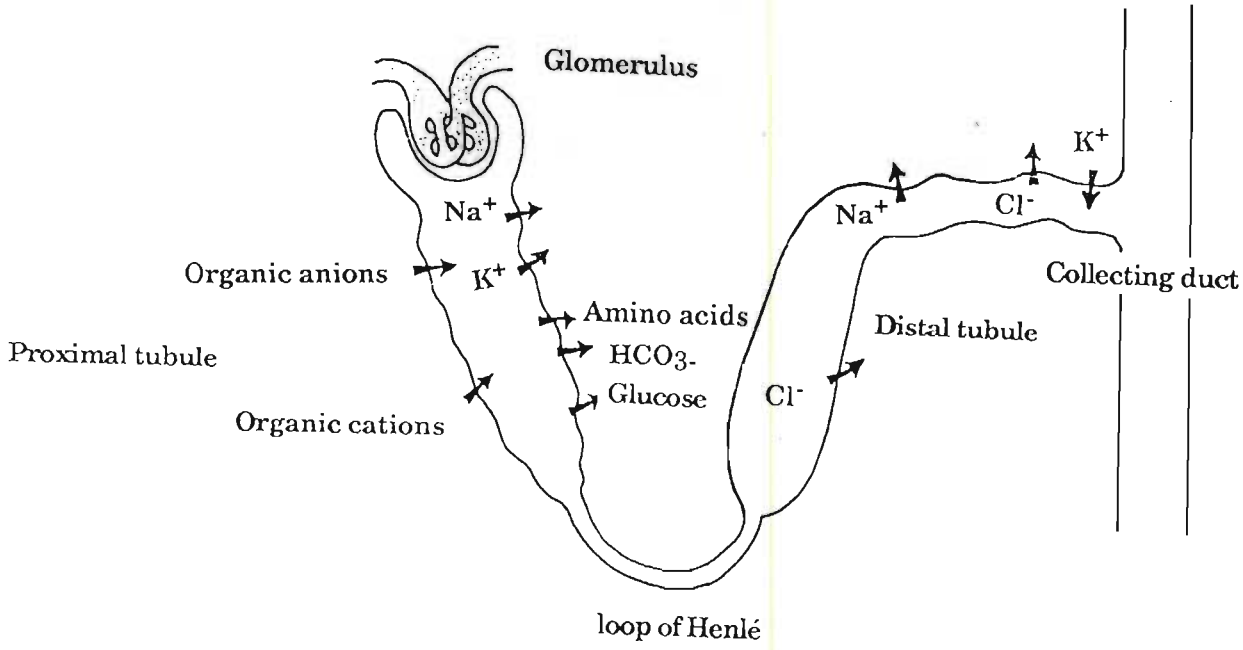


Fig 7.1 The structure of the nephron (after Berndt, 1982)

Proximal tubules are of primary interest from a nephrotoxicological perspective, as tubule cells are the first to be exposed to the glomerular ultrafiltrate. These cells are modified by virtue of anionic microvilli brush borders and basal invaginations which impart a large surface area to mass ratio to the cellular system. The cells isosmotically reabsorb solutes, salts, water and any protein which surmounts glomerular filtration (Fowler, 1982; Boogaard *et al.*, 1989a). Rich in mitochondria (the primary target site of ATR) (Berndt, 1982), the cells actively transport a variety of unrelated molecules and are capable of diverse metabolic activity (Boogaard *et al.*, 1989a,b). The proximal tubule is, therefore, highly susceptible to nephrotoxic insult. In general, a nephrotoxin may alter fluid states or interfere with tubular concentration, the latter dysfunction accounting for 60% of all renal injury by chemicals (Moseley *et al*, 1981; Boogaard *et al.*, 1989a,b). Atractyloside specifically causes proximal tubule necrosis, and morphological damage to these tubules has been noted as early as 150-180 minutes after dosing with 50 mg ATR/kg bwt (i.p.) in rats (Carpenedo *et al.*, 1974). There is however, a single allusion to the detrimental effect of atractylosides on the distal tubule (Hatch *et al.*, 1982), but this has yet to be substantiated and has been examined in section 7.2.3 below.

In the 1900s, renal disease was monitored by proteinuria (Piscator, 1989) and this was superseded by measuring serum parameters, which, proving both insensitive and ambiguous, were replaced by urine parameter assessment (Fowler, 1982). The monitoring of enzymes, total protein, carbohydrates and inorganic salts in the urine, has provided "biochemical biopsies" of the kidney (Dubach *et al.*, 1988). These compounds in the urine provide non-invasive markers of renal damage or disease, and diagnosis is facilitated by the fact that segments within the kidney are distinguishable on the basis of their specific enzymatic activity (Dubach *et al.*, 1988). Examples of some of the available markers are given in Table 7.1 below. Proteinuria can therefore provide a direct assessment of glomerular integrity, while evaluation of urinary enzyme excretion provides immediate and preliminary information regarding onset and site of nephrotoxicity (Smith and Hook, 1982).

Table 7.1 Site of injury and associated marker released into urine following insult or disease (after Dubach *et al.*, 1988; Smith and Hook, 1982)

Site of injury	Marker
Mitochondrial membrane	Calcium
Glomerular integrity	Proteinuria/albuminuria
Proximal tubule	Alkaline phosphatase
	γ-Glutamyl transferase
	Glucosuria
Brush border microvilli	Alkaline phosphatase
	γ-Glutamyltransferase
Distal tubule	Lactate dehydrogenase
Papillae	Alkaline phosphatase
Cytosol	Lactate dehydrogenase

The aim therefore of the present study, was to administer a single dose of ATR to male Wistar rats and monitor urine parameters for a period of four days post-dosing, as previous biochemical studies in this regard have only been extended to 12 hours post-dosing (Carpenedo *et al.*, 1974). In this manner, the site of action of ATR could be monitored using the urinary markers and in addition (as a corollary) an assessment made of the markers' potential as diagnostic tools in the evaluation of ATR poisoning.

7.2.2 Methods

Wistar rats were dosed i.p. with 0, 5, 10 and 25 mg/kg bwt ATR and 18 hour representative urine samples were collected, over ice, at 24, 48, 72 and 96 hours after dosing, as detailed in section 3.1.2. The levels of total protein, albumin, glucose, alkaline phosphatase (AP), lactate dehydrogenase (LDH), γ-glutamyl transferase (GGT) and calcium in the urine samples were determined using an automated CobasBio system, as described in section 3.6.2. The protein and albumin were monitored to investigate the effects of ATR on glomerular function, while AP, GGT and glucose determinations were carried out to establish the effect of ATR on the proximal tubule and the microvilli of the brush border. Lactate dehydrogenase was used to monitor the inhibitory effect of this toxin on the distal tubules, while calcium determination provided information regarding general nephrotoxicity.

7.2.3 Results

The first test performed on the ATR-treated rats was for the presence of proteinuria, which is a direct assessment of both glomerular and proximal tubule integrity (Berndt, 1982). Protein excretion was monitored in rats following i.p. injection of 0, 5, 10 and 25 mg ATR/kg bwt and the results are given in Fig 7.2 below.

From these results it can be seen that on day 1, the rats receiving the low dose of ATR showed no significant increase in urinary protein, whereas, in the animals receiving the medium and high dose regime, the levels of total protein increased some 1.6- and 3.7-fold respectively. Subsequently on day 2, the levels of total protein were raised in all three groups of rats exposed to ATR, although in the case of the medium and high dose regimen, these levels were lower than those obtained for day 1. On day 3, the rats receiving the low dose of toxin, excreted a level of total protein some 2-fold greater than that of the controls.

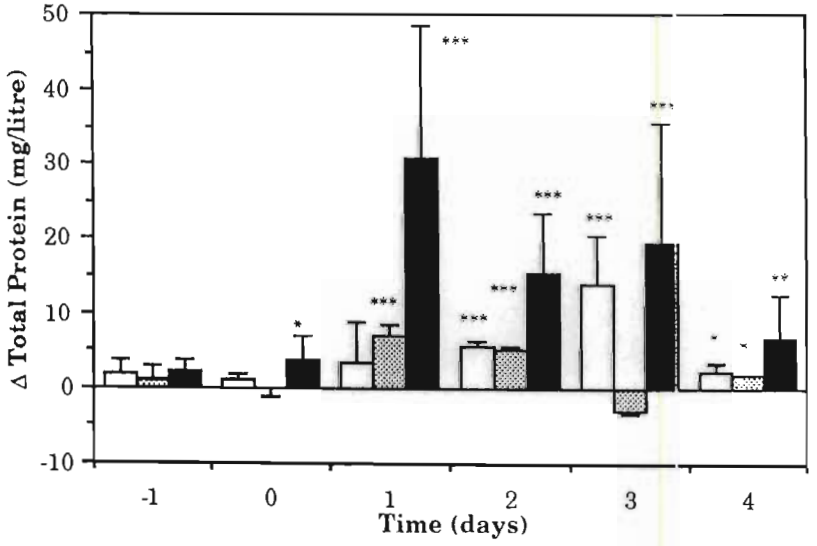


Fig 7.2 The release of total protein into the urine of rats given a single i.p. dose of ATR at the levels of 5, 10 or 25 mg ATR/kg bwt (low □, medium ▨ and high ■). Protein levels were monitored daily, two days prior to dosing and thereafter every 24 hours for a total of 96 hours (day 4). The results are expressed as the change (Δ) in excreted total protein over 18 hours by ATR-treated rats relative to that excreted by the untreated control rats. Values statistically significantly different from their controls are denoted by :-
* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ (n = 5)

By contrast the medium dose group excreted less protein than that of the controls, while the high dose group exhibited a further increase in the level of excreted total protein, when

compared to day 2. By day 4, when the trial was terminated, the levels of excreted protein had returned to normal in all the rats, except for the high dose group which still showed a measurable level above the controls. From these results it was concluded that the rats exposed to ATR had definitely suffered kidney damage and more specifically, possibly glomerular damage. It is of interest to note that proteinuria has also been observed in children accidently poisoned by *C. laureola* (Bhoola, 1983) and rats dosed with ATR, the response being evident within 12 hours of exposure (Carpenedo *et al.*, 1974). The possibility of glomerular damage was further investigated by monitoring the levels of albumin in the urine.

Excessive albumin excretion, or albuminuria, which is a good indicator of the loss of glomerular permselectivity integrity (Berndt, 1982) was found by Bhoola (1983) to occur in 80% of patients with suspected *Impila* poisoning. Thus, since alteration in the glomerulus by ATR has not yet been morphologically observed this is an area for further investigation (Carpenedo *et al.*, 1974). The results of the analysis carried out by the present author to monitor the levels of albumin in the rats dosed with 0, 5, 10 and 25 mg ATR/kg bwt, are shown in Fig 7.3 below.

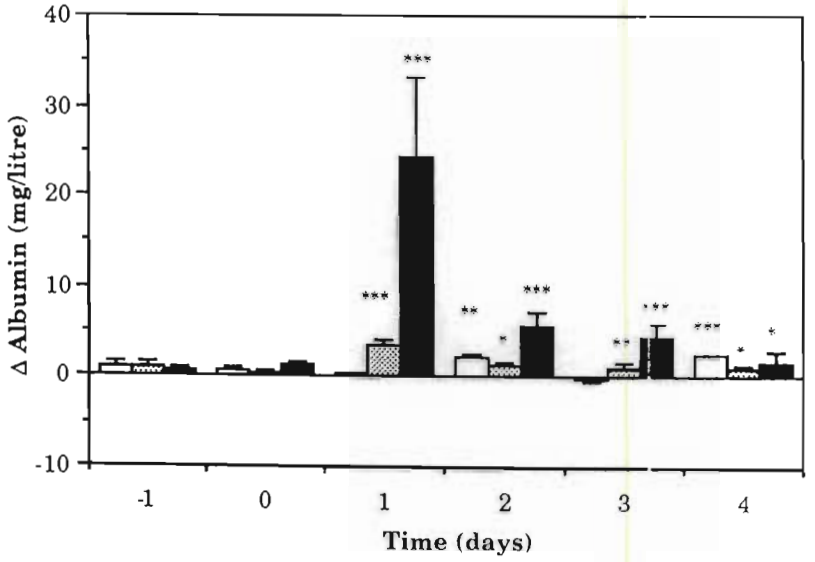


Fig 7.3 The release of albumin into the urine of rats given a single i.p. dose of ATR at the levels of 5, 10 or 25 mg ATR/kg bwt (low □, medium ▨ and high ■). Albumin levels were monitored daily, two days prior to dosing and thereafter every 24 hours for a total of 96 hours (day 4). The results are expressed as the change (Δ) in excreted albumin over 18 hours by ATR-treated rats relative to that excreted by the untreated control rats. Values statistically significantly different from their controls are denoted by:-

* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ (n = 5)

On day 1 post-dosing, the rats receiving the low dose of toxin exhibited no increase in albumin excretion, compared to the control, whereas those receiving the medium and high doses showed clear increases, with the high dose regimen demonstrating a 9.3-fold increase. On day 2, the levels of albumin in the urine from the rats dosed with either 10 or 25 mg ATR/kg bwt had decreased from those of day 1, but were still higher than that of the controls; the relative increase being dose responsive. The rats receiving the low dose of toxin showed an increase in the level of excreted albumin on day 2, but this decreased on day 3, only to increase again on day 4 when the trial was terminated. The medium group did not demonstrate an alteration in the level of urinary albumin on days 2 and 3, but this level decreased slightly on day 4. The trend in the rats administered 25 mg ATR/kg bwt was seen to be an overall decrease in the level of excreted albumin following the peak on day 1, post-dosing, although by day 4 when the trial was ended, the level of albumin in the urine was still higher than that of the controls. From these results it was concluded that ATR affects the glomerular permselectivity and that the glomerulus had still not recovered after 4 days. A different trend was observed in the low dose regimen, as glomerular alteration (noted by albuminuria) was delayed to day 2, post-dosing, whereupon albuminuria decreased on day 3, only to increase again on day 4. Although these results are statistically significant, as determined by the student *t*-test, it is proposed by the present author that this alteration may be the result of natural diurnal variation and not the result of ATR damage.

If however, the results of the low dose regimen are in fact indicative of ATR-damage, then this suggests that these animals are exposed to ATR on two separate occasions, causing compromise of the glomerulus twice, whereas in the case of the medium and high dose regimen, the kidney was exposed to a single release of toxin or alternatively a perpetual, but decreasing dose of ATR. Clearly, however, the highest dose of ATR was the only dose level to cause significant alteration to the glomerulus.

In addition to monitoring proteinuria and albuminuria, enzymuria has been used to facilitate identification of acute renal injury by non-invasive means (Dubach *et al.*, 1988). The enzyme γ-glutamyl transferase (GGT) is extrinsically present in proximal tubule microvilli brush border membranes (Sibernagl *et al.*, 1978) and the presence of this enzyme in the urine provides evidence of anatomical injury to proximal tubules (Dierickx, 1981; Gatta *et al.*, 1989). The results for the urinary excretion of GGT (Fig 7.4) show that on day 1, animals receiving the high dose of ATR (25 mg/kg bwt) demonstrated a 9.8-fold increase in the level of GGT. Clearly, this indicated that there was severe damage to the microvilli of the proximal tubule brush border following exposure to ATR. The low and medium dose regimen demonstrated a small increase in the level of GGT in the urine, but this was not directly proportional to the dose level. On day 2, post-dosing, the rats dosed with 25 mg/kg bwt of

ATR showed a decline in the level of GGT in the urine but, nevertheless, this was considerably higher than that of the control. The levels of GGT in the urine of the rats receiving the medium and low dose were slightly raised when compared to the controls, but this increase was not marked and a similar trend was noted throughout the remainder of the trial. By day 3, the levels of GGT in the urine of the rats receiving the high dose of toxin had decreased to below that of the controls rats (3.408 U/litre as opposed to 3.782 U/litre) and decreased further by day 4 to 2.745 U/litre when the trial was terminated. From these results it was concluded that ATR induces brush border damage to the proximal tubules and that this damage is induced within 24 hours of exposure to the toxin. Indeed, in support of this observation, morphological damage to the proximal tubules has been noted as early as 150-180 minutes after dosing i.p. with 50 mg ATR/kg bwt (Carpenedo *et al.*, 1974). However, this damage is not dose responsive, as rats receiving the low and medium dose of toxin did not demonstrate a similar response.

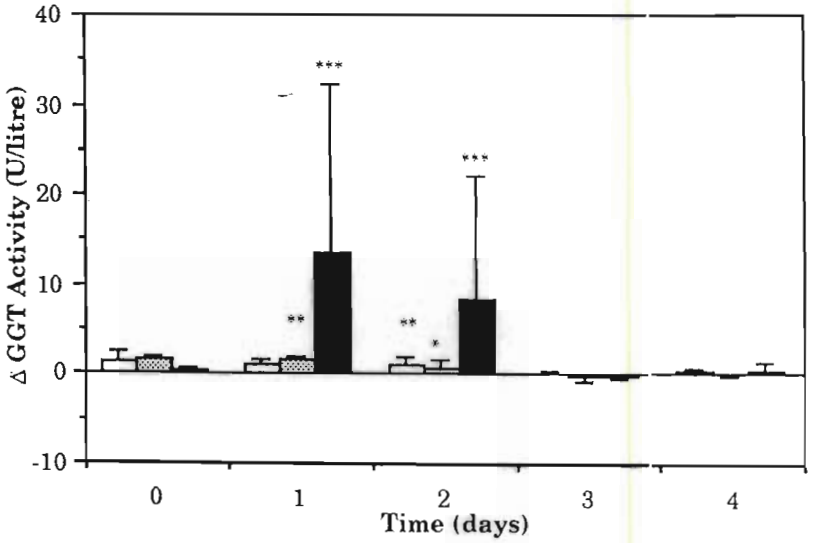


Fig 7.4 The release of γ-glutamyl transferase (GGT) into the urine of rats given a single i.p. dose of ATR at the levels of 5, 10 or 25 mg ATR/kg bwt (low □, medium ▩ and high ■). Enzyme levels were monitored daily, for one day prior to dosing and thereafter every 24 hours for a total of 96 hours (day 4). The results are expressed as the change (Δ) in excreted GGT over 18 hours by ATR-treated rats, relative to that excreted by the untreated, control rats. Values statistically significantly different from their controls are denoted by:- * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ ($n = 5$)

Alkaline phosphatase has also been used as a marker of proximal tubule and microvilli brush border damage (Dubach *et al.*, 1988) and thus it was decided to investigate the effect of ATR on the release of this enzyme into the urine. The rats receiving the high dose of toxin excreted a 13.5-fold higher level of AP into the urine than the control group of rats on day 1

(Fig 7.5). The medium dose group receiving 10 mg ATR/kg bwt demonstrated a 2-fold increase in excreted AP levels on day 1. The low dose regimen also responded to the toxin exposure with AP levels rising by 1.3-fold. On day 2, a decline in the release of AP was noted in the urine of the rats dosed with the high and medium levels of ATR, but the rats receiving the low dose of toxin demonstrated an increase in the levels of AP in the urine. This level exceeded that for the medium dose range at any one time. On day 3, the levels of AP in the urine of the rats dosed with the low and high levels of ATR decreased and were less than that of the controls. The rats receiving the medium dose range demonstrated a slight increase in the levels of AP, when compared to day 2, but nevertheless these levels were still lower than that of the control. On day 4, when the trial ended, the control, low and medium dosed rats excreted similar levels of AP into the urine, while that of the high dose regimen showed a slight increase in the excretion of AP.

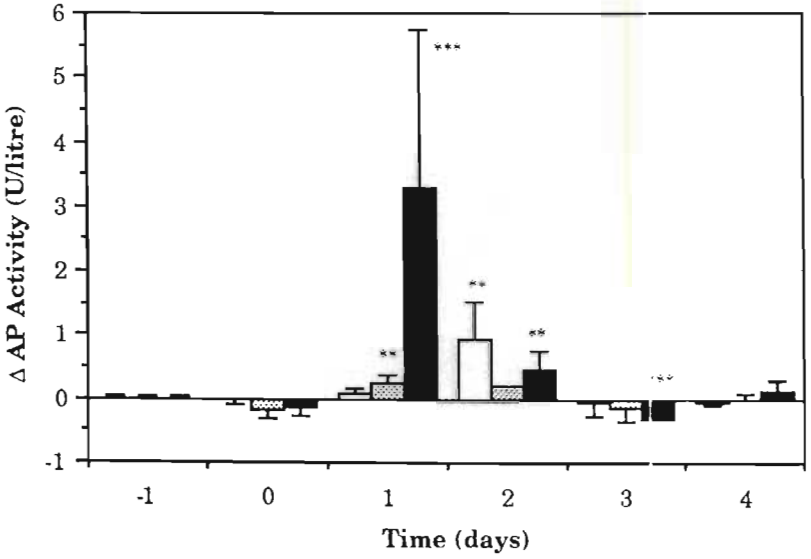


Fig 7.5 The release of alkaline phosphatase (AP) into the urine of rats given a single i.p. dose of ATR at the levels of 5, 10 or 25 mg ATR/kg bwt (low □, medium ▨ and high ■). Enzyme levels were monitored daily, two days prior to dosing and thereafter every 24 hours for a total of 96 hours (day 4). The results are expressed as the change (Δ) in excreted AP over 18 hours by ATR-treated rats, relative to that excreted by the untreated, control rats. Values statistically significantly different from their controls are denoted by:-
* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ (n = 5)

The presence of raised levels of AP in the urine is indicative of proximal tubule or microvilli brush border injury, as suggested by the GGT results. As an indicator of ATR poisoning however, AP would appear more sensitive than GGT as there were definite alterations in the excretion of AP at the low, medium and high dose levels. Alkaline phosphatase is relatively non-specific as a marker however, as release of AP into the urine can be caused by papillary

(Dubach *et al.*, 1988) and glomerular disease (Fowler, 1982) as well as tubular damage. Hence it is difficult to pin-point the precise site of damage from these results. Nevertheless, it is apparent that ATR induces renal damage at 5, 10 and 25 mg/kg bwt as noted by the excretion of AP into the urine. The release of AP into the urine on exposure to ATR-producing plants has previously been noted in feeder pigs given cocklebur (Stuart *et al.*, 1981), thus confirming the results obtained by the present author.

Tubular function may also be evaluated by glucose excretion (Berndt, 1976) since glucose is unimpeded by the glomerulus but passes directly into the proximal tubule where reabsorption, in part by the distal tubule and in the main by the proximal tubule, maintains the urine devoid of glucose (Berndt, 1982). Glycosuria indicates straight or convoluted proximal tubule damage, or more specifically a compromise of monosaccharide absorption (Fowler, 1982, Smith and Hook, 1982). This condition can also signify diabetes mellitis, but in this case there is a concomitant alteration in blood sugar levels. The results in Fig 7.6 show that levels of glucose in the urine increased in a dose responsive manner, with the glucose excretion by the high dose group being 100-fold greater than that of the controls on day 1.

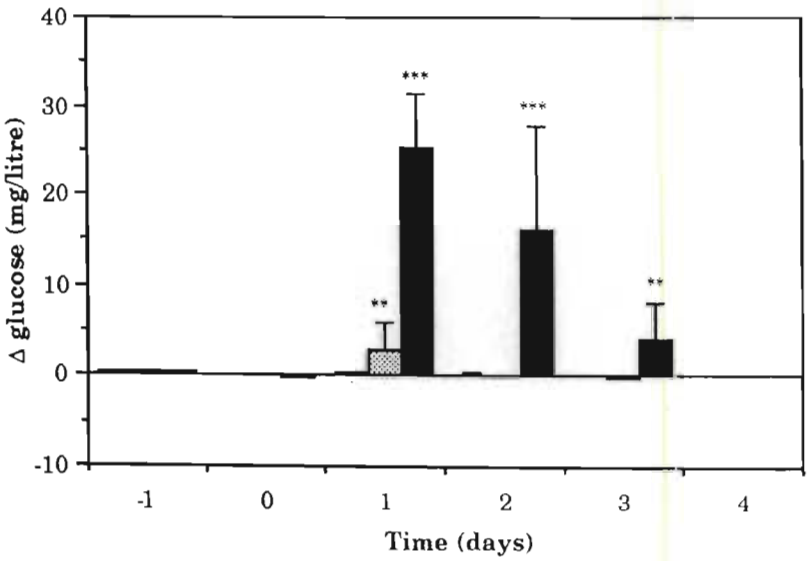


Fig 7.6 The release of glucose into the urine of rats given a single i.p. dose of ATR at the levels of 5, 10 or 25 mg ATR/kg bwt (low □, medium ▩ and high ■). Glucose levels were monitored daily, two days prior to dosing and thereafter every 24 hours for a total of 96 hours (day 4). The results are expressed as the change (Δ) in excreted glucose over 18 hours by ATR-treated rats, relative to that excreted by the untreated, control rats. Values statistically significantly different from their controls are denoted by:-

* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ ($n = 5$)

On day 2, the level of glucose in the urine of rats receiving the low dose of toxin remained stable, while that of the high and medium dose group decreased. Nevertheless, the glucose excreted by the high dose group was 43-fold greater than that of the controls. On day 3, the levels of glucose in the low and medium dose groups had slightly decreased to below that of the controls, and while the levels of glucose in the urine of the high dose regimen had decreased significantly by day 3, this concentration was still some 11 times greater than that of the control. By day 4 however, when the trial was ended, there was no evidence of glucosuria in any of the rats.

From the results on the determination of glucosuria, it was concluded that ATR targeted for the proximal tubule of the kidney and furthermore, that the microvilli of the brush border were particularly susceptible. Bhoola (1983) documented the results of the urinalysis carried out on 45 patients with suspected *Impila* poisoning, and of these patients tested, 33% demonstrated glucosuria (only one of the patients was suspected of suffering from diabetes mellitis). A similar response was noted in albino rats dosed i.p. with ATR (Carpenedo *et al.*, 1974), hence reaffirming the results of the urinalysis study. Having confirmed that the proximal tubule was particularly susceptible to the action of ATR, attention was turned to the effect of this toxin on the distal tubules.

Distal tubular damage is primarily assessed by LDH excretion in the urine (Fowler, 1982; Smith and Hook, 1982) and as alteration in distal tubules has previously been noted in rats (Hatch *et al.*, 1982) it was decided to monitor this marker in the urine of the rats dosed with 0, 5, 10 and 25 mg ATR/kg bwt respectively. The results are given in Fig 7.7 below. On day 1, the rats receiving the medium dose demonstrated a 6.5-fold increase in the excretion of LDH compared to the controls, while the rats administered the high dose of toxin demonstrated an approximate 90-fold increase in LDH urinary levels. By day 2, the response by the animals receiving the high dose had decreased sharply, but was still significantly higher than that of the controls. The animals receiving the low dose of toxin demonstrated unchanged levels of LDH in the urine, when compared to day 1. The medium regimen showed a slight increase in the excretion of LDH, when compared to that on day 1. By day 3, the LDH levels in all the rats had decreased and that of the low dose regimen was almost equal to the controls. A similar trend was noted in the control, low and medium dose regimen on day 4 but there was a slight increase in the levels of LDH excreted by the animals receiving the high dose of toxin, but at this point the trial was terminated.

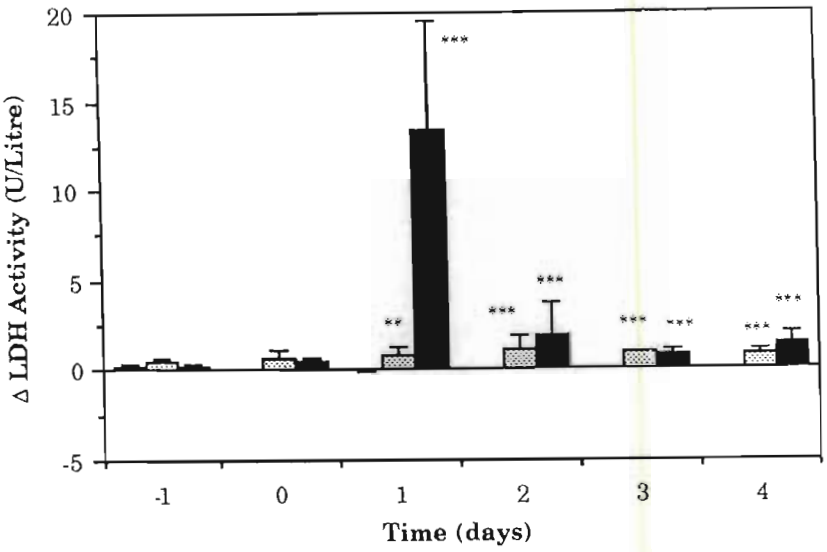


Fig 7.7 The release of lactate dehydrogenase (LDH) into the urine of rats given a single i.p. dose of ATR at the levels of 5, 10 or 25 mg ATR/kg bwt (low □, medium ▨ and high ■). Enzyme levels were monitored daily, two days prior to dosing and thereafter every 24 hours for a total of 96 hours (day 4). The results are expressed as the change (Δ) in excreted LDH over 18 hours by ATR-treated rats, relative to that excreted by the untreated, control rats. Values statistically significantly different from their controls are denoted by:- * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ ($n = 5$)

From the results presented in Fig 7.7 it is apparent that exposure to ATR induces release of LDH into the urine and this was most notable in animals dosed with the medium and high levels of ATR. The response by the animals receiving the low dose of toxin was not obvious. Since LDH is considered a fairly specific marker for distal tubule damage, these results indicate that ATR notably targets for the proximal tubule but also the distal tubule. There is a single reference to distal tubule damage associated with exposure to atractylosides (Hatch *et al.*, 1982) but historically proximal tubules have been identified as the primary target site of ATR (Carpenedo *et al.*, 1974; Stuart *et al.*, 1981; Bhoola, 1983; Georgiou *et al.*, 1988). The results of this study constitutes the first indication that ATR might act at more than one location in the kidney.

As pointed out in the literature review (Chapter 2), ATR induces spontaneous release of calcium from intramitochondrial stores and this release increases mitochondrial membrane permeability (Lê Quôc and Lê Quôc, 1988). It is not known if this permeability is confined to mitochondrial membranes or whether the cell membrane is also affected. If the latter is correct then, following exposure to ATR, calcium would then be released into the cytoplasm, cell lysis would occur and this divalent cation would be released into the urine. Thus, as a

general marker of cellular damage (Bach, pers. comm., 1990), the levels of calcium excreted into the urine by rats dosed with ATR at 0, 5, 10 and 25 mg/kg bwt, were measured, the results for which are shown in Fig 7.8.

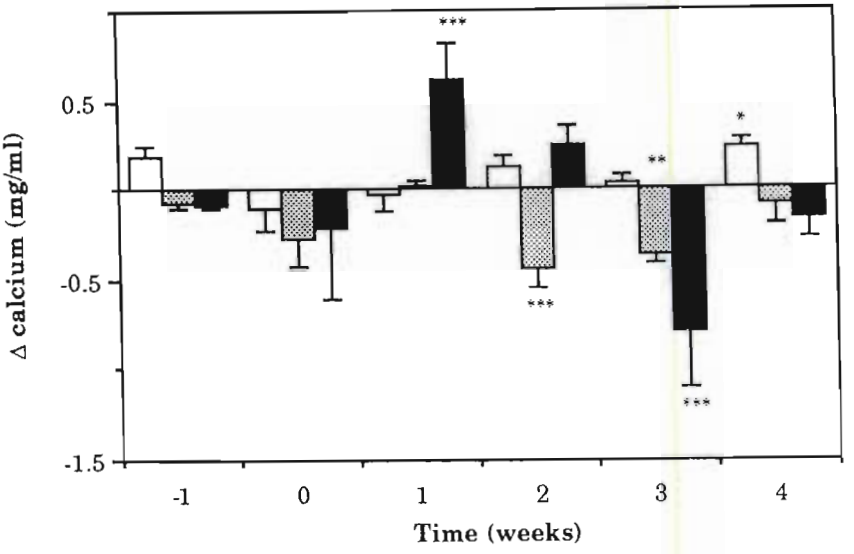


Fig 7.8 The release of calcium into the urine of rats given a single i.p. dose of ATR at the levels of 5, 10 or 25 mg ATR/kg bwt (low □, medium ▨ and high ■). Calcium levels were monitored daily, two days prior to dosing and thereafter every 24 hours for a total of 96 hours (day 4). The results are expressed as the change (Δ) in excreted calcium over 18 hours by ATR-treated rats, relative to that excreted by the untreated, control rats. Values statistically significantly different from their controls are denoted by:-

* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ (n = 5)

The rats receiving the high dose of ATR showed a transient increase in calcium levels in the urine on day 1 and thereafter, these levels decreased progressively over the next 2 days reaching a level significantly below that of the control by day 3, and increasing slightly on day 4. The rats receiving the medium dose of ATR excreted calcium into the urine at a level significantly lower than that of the controls and this trend was maintained throughout the trial. The rats given the low dose of toxin demonstrated a gradual increase in calcium excretion from day 2, decreasing slightly on day 3, only to peak on day 4 when the trial was terminated. Thus in summary, it is clear from Fig 7.8 that on day 3 and 4, the levels of calcium secreted by the medium dose regimen were significantly lower than that of the controls, while in the case of the high dose regimen, a sigmoidal response was noted, with the levels of excreted calcium decreasing significantly on day 4, only to increase again. It is proposed that this response is indicative of the effects of parathyroid hormones (PTH) on the excretion of calcium in the urine as, clearly, if calcium is excreted into the urine, ultimately serum calcium concentrations will decrease. In response, PTH secretion is stimulated,

resulting in the increased hydroxylation of 1,25-dihydroxy-cholecalciferol, which increases intestinal adsorption of calcium and also induces mobilisation of calcium from bone, thus re-establishing serum calcium levels. Following the increase in the serum calcium concentration, there is a resulting increase in the level of excreted calcium in the urine, to re-establish homeostasis (Ungar, 1986). Thus, the response noted in the high dose regimen is thought to be the result of PTH and 1,25-dihydroxycholecalciferol re-establishing homeostasis.

It is evident from the urinalysis that ATR induces proximal tubule and microvilli brush border injury and inhibits transport of glucose into the tubules, with possible glomerular functional compromise and distal tubule damage. Proximal tubule injury by ATR has been positively reaffirmed by the present author, but this is the first report of the inhibition of glomerular and distal tubule function, a finding which requires further confirmation. Collectively, the biochemical analyses carried out may aid diagnosis of ATR poisoning, but no single analysis may be deemed ideal, nor specifically indicative of ATR exposure or injury. Alternative, direct analyses for ATR, employing HPLC and ELISA (chapters 4 and 6) provides a more sensitive and specific diagnosis of poisoning of this nature. Following identification of the kidney as the target organ for ATR, attention was focused on the possibility that the liver may also be a target for ATR.

7.3 The effects of atractyloside on the appearance of liver function enzymes in rat serum

7.3.1 Introduction

The nature and extent of various lesions within the liver may be estimated using clinical enzymology. In 1954, increased serum aldolase activity was correlated with viral hepatitis infections and subsequently a number of other serum enzymes, including alcohol dehydrogenase, malic dehydrogenase and both alanine and aspartate transaminases (ALT and AST), became associated with liver disease (Hess, 1963). Serum transaminase activity increases with toxic hepatic damage, subsiding only on cessation of toxin administration and liver regeneration while most forms of histological damage to the liver is associated with elevated serum ALT levels and if centrilobular necrosis is present, AST levels also rise (Hess, 1963).

Since ATR has usually been presumed to be a hepatotoxin, most studies into the action of this toxin have been carried out on hepatocyte homogenates. In view of the inhibitory action of ATR and its effect on the kidney, it was conceivable that this toxin could also induce the

liver damage. This aim of this aspect of the work was to monitor the effects of ATR on serum transaminase activity, over a period of 96 hours and is, therefore, an extension of the study of Carpenedo *et al.* (1974), who monitored transaminase activity for only 12 hours after dosing. Thus the rationale for this extended study was provided by Wainwright *et al.* (1977) who observed liver alteration in rats only 24 hours after they were exposed to a semi-purified extract of ATR obtained from *C. laureola*, indicating that the investigation by Carpenedo *et al.* (1974) may have been prematurely terminated.

7.3.2 Methods

As a continuation of the study carried out in the United Kingdom, the experiment completed at the Roben's Institute (see section 7.2) was repeated at the BMRC, University of Durban-Westville, whereby male Wistar rats were dosed i.p. with 0, 5, 10 and 25 mg ATR/kg bwt, with serum instead of urine being collected over a period of 96 hours. At termination, the hepatotoxicity of the toxin was monitored in serum by assaying for AST and ALT levels by the methods described in sections 3.1.3 and 3.6 respectively.

7.3.3 Results and discussion

The results of the analyses on the rat sera are presented in Fig 7.9 and represent the mean of the AST and ALT levels for each of the five rats allocated to a dose level, for days 0 - 4. From the results it is evident that there was no significant alteration in the ALT and AST levels in any of the rats, and it may be concluded that ATR, over the dose range 0 to 25 mg/kg bwt, does not induce liver damage in rats.

By contrast, patients poisoned either by *C. laureola* or *A. gummifera* demonstrated elevated serum ALT and AST levels (sometimes 800-fold) indicating liver damage associated with the centrilobular area, and these clinical findings have been confirmed by histological investigation (Georgiou *et al.*, 1988; Bhoola, 1983). The nephrotoxicity of ATR has been well documented but the hepatotoxicity of this diterpene glycoside is not definitive. There is only one reference to the monitoring of serum transaminase activity in animals dosed with ATR (Carpenedo *et al.*, 1974). Serum samples were collected from rats at 4 hourly intervals for a period of 12 hours, and ALT and AST levels monitored but there was no apparent alteration in the levels of these enzymes in animals exposed to ATR. Although this was initially thought that the trial of Carpenedo *et al.* (1974) was terminated prematurely, the results of the present investigation disagree with this supposition.

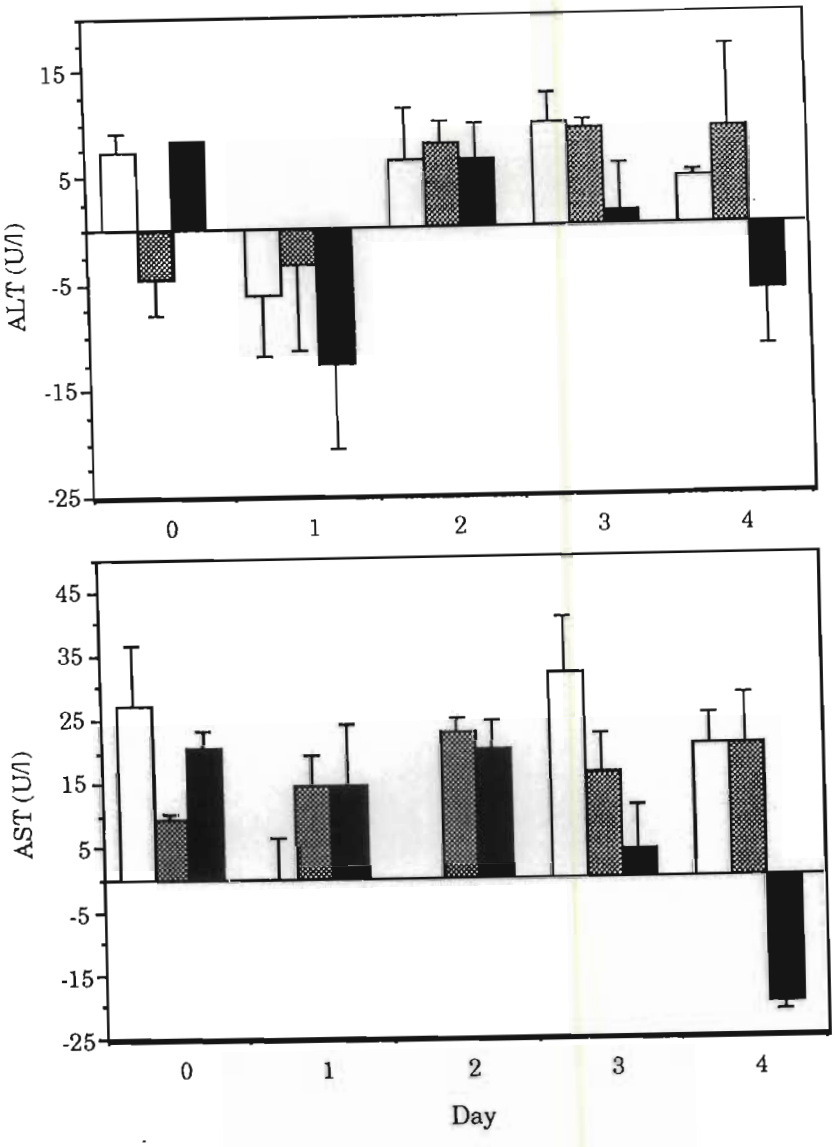


Fig 7.9 Results of the determination of serum aspartate and alanine transaminase (AST and ALT) monitored over a period of 4 days, at 24 hour intervals in rats dosed with atractyloside at 5, 10 and 25 mg/kg bwt (control, low □, medium ▨ and high ■, respectively). Results represent the mean of the levels of AST and ALT in the serum (n = 5) from which the representative control levels were subtracted

The nature of the hepatotoxic agent in plants biosynthesising ATR is still uncertain, but centrilobular necrosis has been associated with intoxication by *C. laureola*, *A. gummifera* and *X. strumarium* (Stuart *et al.*, 1981; Bhoola, 1983; Georgiou *et al.*, 1988) as summarized in Table 2.3, but to date there is but one reference to the induction of centrilobular liver necrosis on exposure to pure toxin. In this instance, however, rats were dosed with CATR and not ATR (Hatch *et al.*, 1982). The relationship between the rise in serum transaminase levels

and liver necrosis has been noted in patients and animals exposed to plants biosynthesising atractylosides (as outlined in Table 2.2 and 2.3) (Stuart *et al.*, 1981; Bhoola, 1983; Georgiou *et al.*, 1988) and this observation provided the rationale for this study. From the results of this investigation it is concluded that the identity of the hepatotoxin in Wainwright's ATR preparation remains unknown and forms an area for future research.

In conclusion from the results of the urinalysis and serum enzyme activity determination, it is clear that ATR affects the proximal tubule of the kidney, in particular the microvilli of the brush border (confirming previous findings) but in addition, this toxin appears to compromise glomerular permselectivity and distal tubules. The results of the serum studies suggest that ATR is not hepatotoxic, indicating that the effect of this toxin is localised to the kidney. Based on the results of this serum study and the urinalysis, it was decided to further pursue the nephrotoxicity of ATR by monitoring the effects of this toxin on the *in vitro* incorporation of proline into isolated rat proximal tubules. This is the first known report of the effects of ATR on *in vitro* amino acid incorporation in the kidney, the details of which are presented in the next section.

7.4 The effect of atractyloside on proline incorporation in proximal tubules

7.4.1 Introduction

The complexity of the kidney, hampers the study of its functions and susceptibility to nephrotoxins within defined areas of the intact organ, and thus the interpretation of *in vivo* nephrotoxicity becomes very complicated, especially as it is difficult to account for all the extraneous influences. The isolation of tissue and cell fractions has facilitated the study of each compartment in isolation, and for instance, the preparation of glomeruli and tubular fragments, which has been well documented (Ormstad, 1982) is now routinely carried out either by enzymatic or mechanical release of the tissue fragments (Boogaard *et al.*, 1989a,b).

A paramount function of the proximal tubules is the active vectorial transport of compounds from the glomerular filtrate to the blood and *vice versa* (Boogaard *et al.*, 1989a,b), and therefore the inhibition of such transport is frequently the first evidence of nephrotoxic insult. Thus, the *in vitro* isolation of rat proximal tubules has provided the means to give an early assessment of proximal tubular dysfunction in the presence of a xenobiotic with this system, and the transport of a number of different compounds may be monitored including glucose and various amino acids (Boogaard *et al.*, 1989a, b). Proline is actively incorporated into proximal tubules and from previous investigations it has been proposed that entry into

the cell by this amino acid is mediated by more than one transport system (Hillman and Rosenberg, 1969).

In view of the above finding it was decided to employ the *in vitro* incorporation of proline into viable rat proximal tubules, as a means of testing the toxic action of ATR on active transport in proximal tubules. Proline was also chosen for convenience as the methodology for this investigation has been optimised at RIIEHS and furthermore, it is unlikely that proline is radically metabolised after incorporation into the tubules, being a non-essential amino acid (Mehler, 1986). This is the first report of the investigation of the effect of ATR on amino acid transport in proximal tubules, the objective of which was to provide further information on the nephrotoxicity of this xenobiotic.

7.4.2 Methods

Proximal tubules were mechanically released from the kidneys of Wistar rats and incubated with [^{3}H]-L-proline, either in the presence or absence of ATR (1 to 6 μM). Control samples were collected at hourly intervals, from the tubules incubated in the absence of ATR, and protein biosynthesis and active transport were terminated by the addition of TCA. Thereafter, the tubules were washed and the incorporated [^{3}H]-L-proline was measured in a LKB 1219 liquid scintillation counter. The control was included to monitor the viability of the proximal tubules over the total incubation period. The tubules incubated with ATR were treated with TCA as described above, after a total of 3 hours, and the level of incorporated [^{3}H]-L-proline was determined as a percentage of the control, at time 3 hours. The detailed method for the isolation of the proximal tubules and the amino acid incorporation experiment is given in section 3.7, with a diagrammatical explanation depicted in Fig 3.2.

7.4.3 Results and discussion

The metabolic integrity of the isolated proximal tubules was assessed by their ability to incorporate [^{3}H]-L-proline, over a period of three hours. The results depicted in Fig 7.10 show that the incorporation of this amino acid was linear over the entire period monitored, which implies that the isolated proximal tubules were active over the full incubation period. Thus, any inhibition of amino acid incorporation into the proximal tubules, in the presence of ATR, would be solely due to inhibition by the toxin.

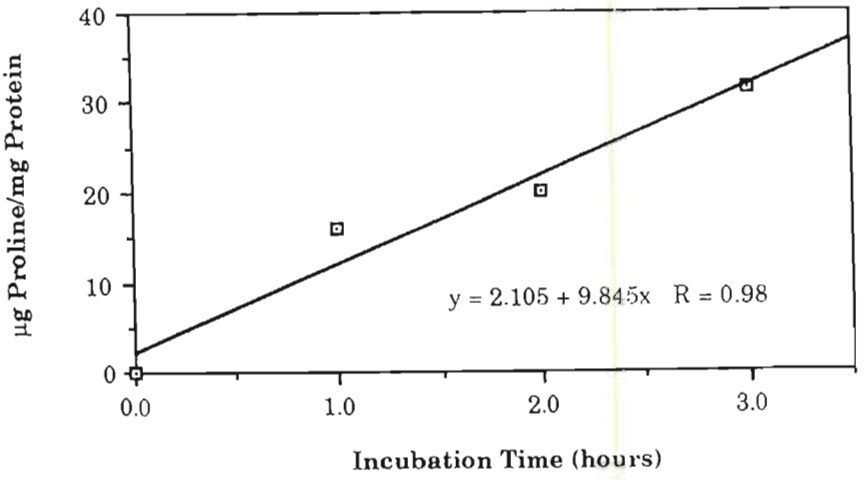


Fig 7.10 The incorporation of [^{3}H]-L-proline into the protein of isolated proximal tubules.Isolated proximal tubules were incubated in the presence of [^{3}H]-L-proline and samples were collected at hourly intervals, for a total period of 3 hours. Protein biosynthesis and amino acid transport was terminated by addition of TCA and the levels of [^{3}H]-L-proline were determined by scintillation counter as described in section 3.7

The results for the amino acid incorporation of proline in the presence of the ATR are depicted in Fig 7.11 below. These results clearly show a progressive increase in the inhibition of proline incorporation proportional to ATR concentration. Since this toxin inhibits amino acid transport in the tubules, it is highly likely that it also terminates protein biosynthesis in this tissue. The implications of this are clear, since a basal level of enzyme will always be excreted into the urine, if this excreted level drops significantly below that of the basal level after exposure to a xenobiotic, this would indicate that protein synthesis is being retarded. This could account for the apparent decrease in the levels of excreted AP and GGT, compared to the controls,particularly on days 3 and 4, post-dosing, observed in the urinalysis study described in section 7.2. Furthermore, since the IC_{50} for ATR was calculated to be as high as 1 µM ATR or 0.803 mg ATR, and considering that the rats receiving the high dose of ATR effectively were given about 5 mg of toxin, clearly this dose level would terminate protein biosynthesis in the proximal tubules. These results support the urinalysis findings reported in this chapter and confirm that ATR targets for the proximal tubules. The results of the experiments carried out on isolated glomeruli were inconclusive and are not included.

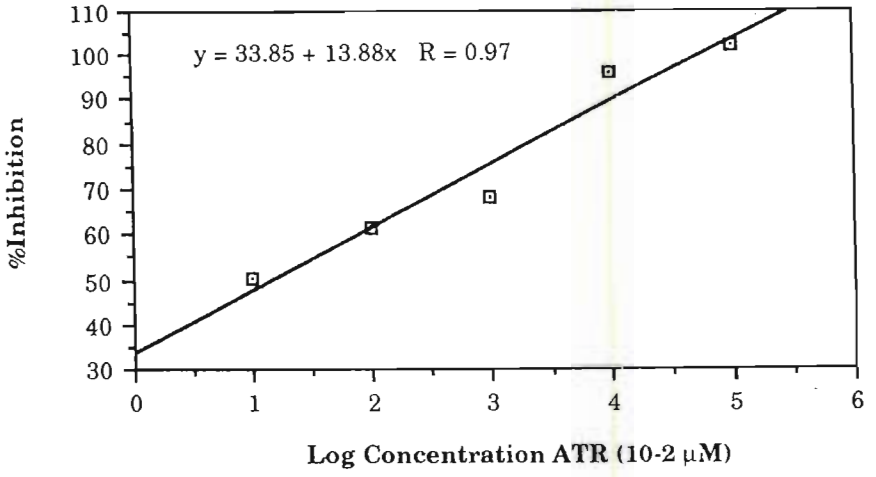


Fig 7.11 The effect of ATR (1-6 μM) on [^{3}H]-L-proline incorporation into isolated rat proximal tubules. The percentage inhibition was calculated by determining the relative incorporation of [^{3}H]-L-proline (z) by the sample as a percentage of the control z value at 3 hours. Details are given in section 3.7.5

From the results of the urinalysis, liver enzyme function tests and the *in vitro* amino acid incorporation studies, it was decided to attempt the immunocytochemical location of ATR *in vivo* using the rat kidney tissue only, as the results of the ALT and AST tests showed that ATR was not hepatotoxic. Therefore, only the kidney samples were used in the immunocytochemical study and in addition, the study was restricted to kidney tissue from rats dosed with the high level of ATR (25 mg/kg bwt) since it was this level that consistently showed the most marked effects on kidney function. Details of the optimisation of this procedure are given in section 7.5.

7.5 Development of an immunocytochemical protocol for atractyloside

7.5.1 Introduction and general considerations

The initial aim of this study was to develop a diagnostic assay for the detection of *Impila* poisoning and to investigate the target organs and organelles of ATR *in vivo*. Previously, the mitochondria have been identified as the subcellular targets of this toxin (Chappell and Crofts, 1965), but this observation has been based on work carried out on isolated organelles whereas to date there is no known evidence for the specific subcellular localisation of this

toxin *in vivo*. Concomitant to the collection of the urine specimens, liver and kidney samples were collected from the dosed rats and fixed in calcium formaldehyde at 4°C, to retain enzyme activity. Histochemical analysis was carried out on the representative samples and cursory examination by the present author demonstrated that there was severe renal necrosis at all dose levels and furthermore, there was no evidence of regeneration in the kidney even 96 hours after animals were exposed to the toxin. The liver superficially appeared to be unaffected by the ATR. The sections were retained in the United Kingdom for evaluation by a pathologist and unfortunately at the time of submission of this thesis, the results were unavailable. Nevertheless, since the results of the urinalysis, liver enzyme function tests and the *in vitro* amino acid incorporation studies pointed clearly to the kidney as the main target organ for ATR, it was decided to perform immunocytochemical location of ATR *in vivo* employing rat kidney tissue since the development of antibodies to ATR (Chapter 5) provided the means to visually locate this toxin. In addition, the study also afforded the opportunity to observe more detailed morphological changes.

Immunocytochemistry involves the localisation of a target antigen within a tissue or cell and the principle is based on the specificity of an antibody for an antigen. Electron-dense material, bound to the immunoreactants, permits the localisation of the antigen using the electron microscope. The success of this method is, however, dependent upon the preservation of both cellular ultrastructure and antigenicity although the retention of tissue integrity is often compromised in favour of preserving the epitope determinants on the antigen (Hayat, 1986).

The quality of the antisera used to locate the antigen is also important since it is vital that the antibody specifically targets for the antigen. Thus, monoclonal antibodies were thought to be ideally suited to this purpose, but since these antibodies recognise a single antigenic determinant, the chance of the antibody not recognising this solitary epitope, which may be lost or denatured during tissue processing, is far greater than if the antibody targeted a number of antigenic determinants, as is the case with polyclonal antibodies. The use of polyclonal antibodies therefore, increases the likelihood of the antigen being located in the tissue (Polak and Van Noorden, 1987). Unfortunately, the incidence of non-specific binding when using the polyclonal antibodies is increased and it is crucial, therefore, that the specificity of the antibodies are known in order to validate the immunolabelling. Firstly, the antiserum must target for the antigen of interest "to the exclusion of all others" and this may be verified by ELISA (Chapter 6). Secondly, one of the major concerns when developing the method for the immunocytochemical localisation of ATR, was the specific form of the toxin *in vivo*, as it was not certain if the molecule was biotransformed. The results of HPLC suggested that ATR is at least partly biotransformed *in vivo* but, nevertheless, it was

apparent from the ELISA (Chapter 6), that the antibodies raised against this toxin could detect the toxin, whether biotransformed or not, indicating that the major epitope of ATR remained intact.

The non-specific association of the primary antiserum with the tissue is considered the most likely source of false-positive labelling and this may be tested by substituting the primary antiserum with an inappropriate or pre-immune serum from the same species. If negligible binding occurs, this indicates that any association of the primary antibody may be regarded as specific. The specificity of the antibody-antigen reaction may also be tested by including an adsorption control. This involves the overnight incubation of primary antiserum, at 4°C, in the presence of excess pure antigen. The solution is centrifuged and the adsorbed antiserum is used for labelling. Theoretically , the antigen should bind to the antibody, thus blocking antibody binding sites and in this manner the specificity of the antiserum may be assessed (Petrusz, 1983). Another site of non-specific interaction of the primary antiserum is with free aldehydes on the tissue. These are by-products of glutaraldehyde fixation and it is not uncommon for antibodies to bind non-specifically to these aldehydes, causing non-specific endogenous labelling. This may be overcome by quenching the aldehydes by incorporating glycine into all the immunoreagents (Bendayan and Zollinger, 1983). In addition, non-specific binding sites on the tissue may also be minimised by "blocking" the tissue with albumins, gelatin or foetal calf serum.

Once the antibody has reacted with the tissue-bound antigen, the complex may be visualised by electron-dense labels, such as the frequently used particulate gold label (Horisberger, 1981). A diffuse enzyme-product label has also had application in the field of immunocytochemistry (Graf and Strauli, 1983) but for the purposes of this study, discussion of labels will be restricted to colloidal gold markers, as this was determined to be the label of choice by the present author. Colloidal gold particles are produced from the reduction of gold chlorides, their particle size being modulated by manipulation of the reaction conditions (Beesley, 1987). Non-covalent electrostatic adsorption of either a secondary antibody, directed against the primary antibody, or Protein A, to the colloidal particle provides an electron-dense immunoprobe. Bivalent Protein A is released from the cell walls of *Staphylococcus aureus* and has a strong binding affinity for the F_c portion of most classes of human IgG, as well as immunoglobulins from rabbits, pigs, dogs and guinea pigs (Langone, 1978). This binding is significantly depressed with immunoglobulins derived from bovine, murine and equine sources and is almost nonexistent with sheep, goat, rat and chicken immunoglobulins (Langone, 1982). Thus Protein A is ideally suited for work on rat tissue and when adsorbed to colloidal gold particles has proved to be a powerful immunoprobe for

immunocytochemical investigations. The principle for the localisation of tissue-bound antigens, using the Protein A-gold probe is depicted in Fig 7.12.

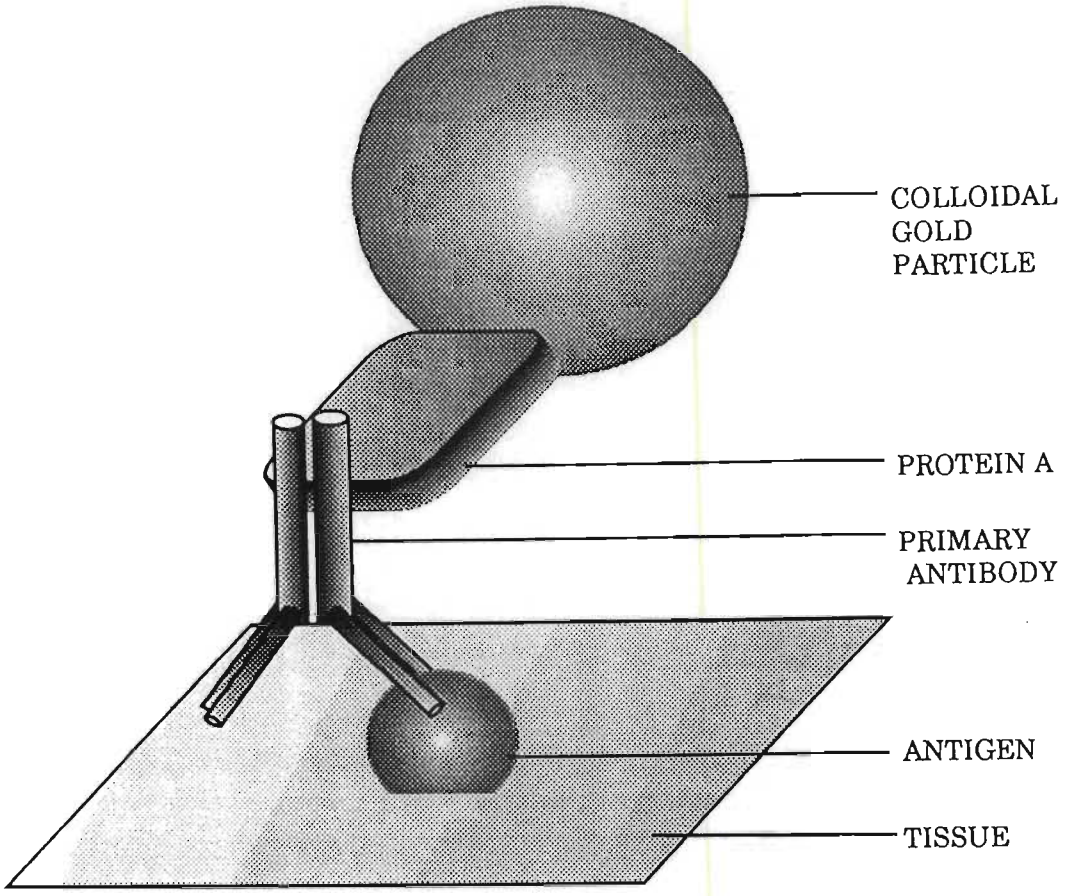


Fig 7.12 The principle of the immunocytochemical location of a tissue-bound antigen using a Protein A-colloidal gold particle

Unfortunately the use of a secondary label is another source of endogenous labelling, as this label may associate non-specifically with the tissue (Petrusz, 1983). The association of the secondary label with sites other than the primary antibody may be evaluated by carrying out a control experiment in which the primary antibody incubation step is omitted from the immunolabelling procedure. Any endogenous labelling detected may in part be overcome by blocking the tissue with foetal calf serum (Petrusz, 1983).

The aim of the study presented in this chapter was to immunocytochemically locate ATR in the kidney of rats at 24, 48 and 72 hours after exposure to this toxin. As the pharmacokinetics and metabolism of ATR in rats are unknown, it was decided to first optimise the immunocytochemical procedure in *C. laureola* tubers (known to contain substantial amounts of ATR), prior to working on mammalian tissue. Unfortunately, plant cells are not particularly predisposed to immunocytochemical investigation, by virtue of the

impermeable cell wall, which inhibits adequate tissue preservation and antibody penetration (Knox *et al.*, 1980). Thus, this necessitated considerable optimisation work before ATR could be immunocytochemically detected in the tuber.

Since this is the first report of the immunocytochemical localisation of ATR in tissue, a considerable amount method development was necessary, particularly as ATR, being a small molecule, is not readily accessible to its primary antibody. Consequently, the fixation of the tissue had to be optimised to ensure that not only was structural integrity maintained, but in addition that the epitope determinants on the toxin were not altered by the fixation procedure. Furthermore, the optimisation was hampered by the fact that the ATR was leached from the cell during the fixation and dehydration procedure, and thus, prior to preservation of ultrastructure, it was necessary to optimise the fixation of the toxin to subcellular proteins. This section therefore reports the optimisation of the entire protocol for the immunocytochemical detection of ATR.

7.5.2 Optimisation of tuber tissue fixation

7.5.2.1 Introduction

At the outset of the investigation, it was important to optimise fixation of the tuber for routine electron microscopy. This involved the testing of a variety of fixing agents and buffers while at the same time ensuring that the ultrastructural damage was kept to a minimum and there was no leaching out of ATR from the tuber tissue. Details of the various reagents tested are given in the materials and methods section below.

7.5.2.2 Materials and methods

In an attempt to develop a suitable fixing medium, the following protocols were tested in sequence:-

1. Tuber was fixed in 3% glutaraldehyde in 0.05 M cacodylate buffer, pH 6.96, osmicated, dehydrated and embedded in Epon resin;
2. Tuber was fixed in 3% glutaraldehyde in 0.05 M cacodylate buffer, pH 6.96, osmicated, dehydrated and embedded in Spurrs resin;
3. Tuber was fixed in 3% glutaraldehyde in 0.05 M cacodylate buffer, pH 6.96 containing caffeine (0.05%, w/v), dehydrated and embedded in Spurrs resin;
4. As in 3, sections being incubated with primary antiserum produced as described by Bye *et al.* (1990), with visualisation mediated by a secondary antibody-horse radish peroxidase;
5. As in 4, but colloidal gold instead of horseradish peroxidase;

6. Tuber was pre-treated with ACN prior to fixation in a 3% glutaraldehyde solution in 0.05 M cacodylate buffer, pH 6.96.

7.5.2.3 Results and discussion

When the tuber was fixed in glutaraldehyde and embedded in Epon resin (procedure 1, section 7.5.2.2), it was observed that inadequate tissue preservation and resin penetration had occurred. As an alternative therefore, fresh tuber was fixed, osmicated, dehydrated and embedded in Spurrs resin (procedure 2), but again there was evidence of poor resin penetration, possibly due to the presence of phenolics in the tuber which could be interfering with fixation. To counteract this problem, caffeine (0.05%, w/v) was added to the fixative and osmication was omitted (procedure 3) as this could make the tissue brittle (V. Bandu, pers. comm., 1989). Since this appeared to improve the tissue fixation, immunocytochemistry was carried out on this tissue, at the light microscope level employing visualization by a secondary antibody-horse radish peroxidase conjugate and precipitating substrate (3'3-diaminobenzenidine tetrahydrochloride) (procedure 4). Despite quenching of endogenous peroxidase in the tissue, by etching with 3% hydrogen peroxide, these results showed significant background staining confined to the cell wall. The method was therefore repeated with a secondary antibody-colloidal gold particle (procedure 5), with visualization being enhanced by means of silver amplification. The results obtained were difficult to interpret and suggested that the solvent-soluble ATR was being leached from the tissue during fixation and dehydration. Thus, the next task was to optimise the fixation of ATR *in vivo*.

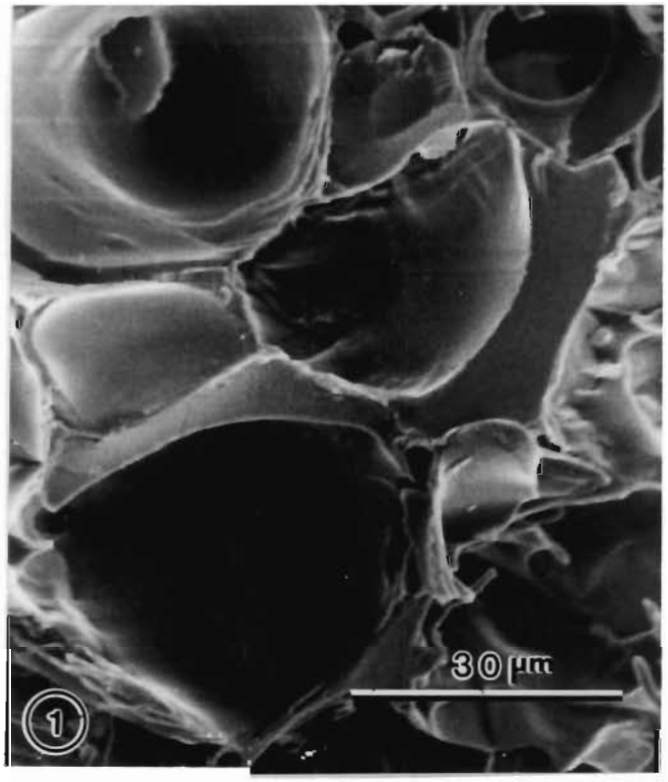
While attempting to detect ATR by HPLC, it was noted that ACN induced spontaneous and irreversible precipitation of the toxin. Since it was postulated that pre-treatment of the tuber tissue with ACN may prevent leaching of the toxin during subsequent fixation and dehydration, tuber was incubated with ACN (procedure 6) and the results were compared with untreated tuber, as monitored by scanning electron microscopy. The results are shown on plate 1, micrographs 1 and 2. Micrograph 1 shows untreated tuber tissue, where the cells are clearly visible. Some structural damage is noted to the cell wall and cell membrane, probably during specimen collection, but in general the tissue is intact. By contrast, the tuber tissue pre-treated with ACN (shown in micrograph 2) shows that this solvent induces precipitation of the cytoplasmic contents of the cell onto the sides of the cells. Clearly significant ultrastructural damage occurs in the presence of ACN and thus this method was abandoned as a possible means of preventing the leaching of ATR out of the tissue during fixation and dehydration.

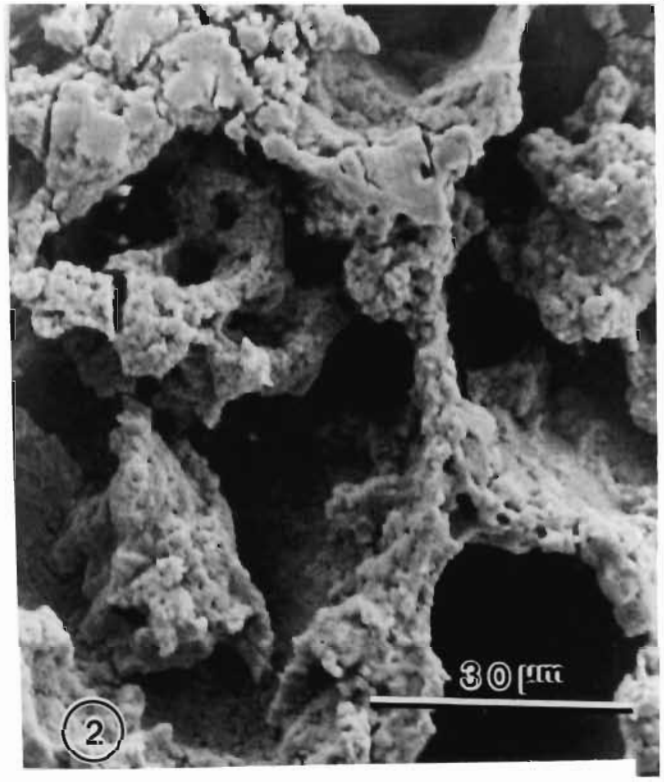
Plate I

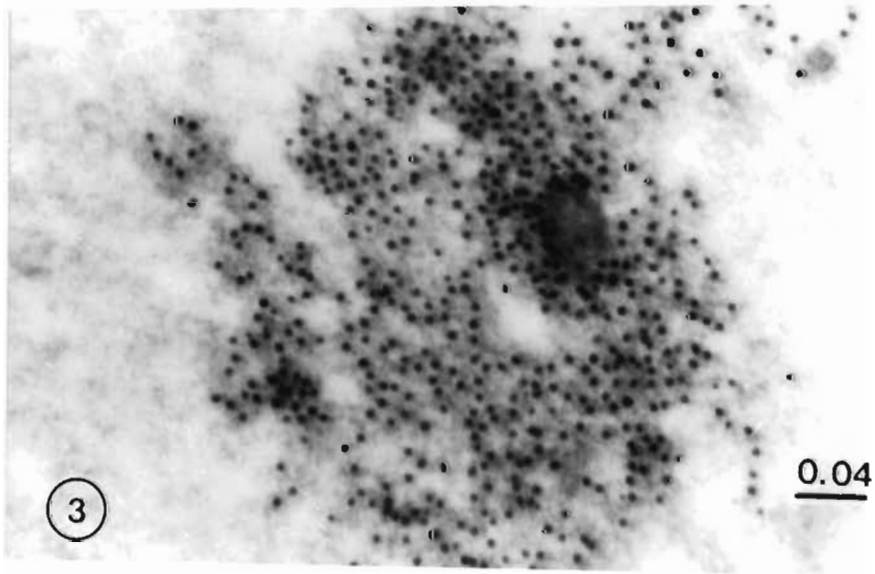
1. Control scanning electron micrograph of *Callilepis laureola* tuber showing cellular integrity. The methodology is detailed in section 7.5.2.2

2. Scanning electron micrograph of *Callilepis laureola* tuber, pre-treated with acetonitrile, showing encrustation and deposition of cellular contents on the cell walls. The method was carried out as described in section 7.5.2.2

3. *Callilepis laureola* tuber, pre-fixed with carbodiimide and embedded in Spurrs resin. Tuber was immunolabelled with protein A-colloidal gold particles of size 6 nm as detailed in section 7.6.2. Labelling is concentrated and may be enclosed in a vesicle


30 µm
1


30 µm
2


0.04 µm
3

To prevent the leaching of ATR, it was therefore decided to covalently couple it to intracellular components. Two methods were investigated, namely the periodate-lysine-paraformaldehyde (PLP) method of McClean and Nakane (1974) and the carbodiimide method of Sossountzov *et al.* (1988), the methodology and results of which are presented below in section 7.5.3.

7.5.3 Optimisation of the intracellular fixation of atractyloside

7.5.3.1 Introduction

The PLP method of McClean and Nakane (1974) is based on the oxidation of carbohydrate moieties to aldehyde groups by periodate. The aldehyde groups are in turn cross-linked by the divalent amine, lysine, while paraformaldehyde stabilises lipids and proteins. As ATR is a diterpene glycoside, it was suggested that the PLP method may fix the diffusible toxin *in vivo* and thus prevent it from being leached out by the fixation solvents. Since ATR may be conjugated to proteins by carbodiimide (Chapter 4, section 4.2.2.2) and this approach has been applied to the intracellular fixation of cytokinins (Sossountzov *et al.*, 1988) it was decided to also test this method for the fixation of the toxin. This section describes the evaluation of the degree of coupling by both methods employing an *in vitro* solid phase immunoassay which was adapted from the method of Sossountzov *et al.* (1988).

7.5.3.2 Materials and methods

Reagents

1. 0.02 M sodium periodate containing 0.25 M/ml ATR in 0.2 M phosphate buffer, pH 6.9
2. 2% (w/v) 1-ethylenediamine-3-(3-dimethylaminopropyl) carbodiimide containing 0.25 M/ml ATR in 0.2 M phosphate buffer, pH 6.9

Method

Plates were coated with 10 µg/ml ovalbumin (200 µl/well) in 0.05 M carbonate buffer, pH 9.6, for 2 hours at 37°C and rinsed thrice in distilled water. Two rows of wells were incubated with the above 2 reaction mixtures, devoid of ATR (representing the controls) while the remaining two rows were incubated with the complete reaction mixtures 1 and 2 (200 µl/well). Plates were rinsed thrice with tap water between each of the following incubation steps. The plate was incubated for 4 hours at 37°C, washed and incubated overnight at 4°C with a 1:200 dilution of rabbit anti-ATR antiserum (prepared as described by Bye *et al.*, 1990) followed by a sheep anti rabbit-horse radish peroxidase conjugate (diluted 1:650) for 2 hours at 37°C (diluted in TBS). Plates were washed and incubated with 2,2'-Azino-di-[3-

ethylbenzthiozolino-6-sulphate] (0.05%, w/v, in 0.15 M citrate buffer, pH 5.0 containing 0.015%, v/v, 30% hydrogen peroxide). The absorbance was monitored colorimetrically at 405 nm and the plates were photographed.

7.5.3.3 Results and discussion

The results for the *in vitro* binding of ATR to the ovalbumin coat are depicted in Fig 7.13 below. It will be noted that the intensity of the colour development of the controls is very low, indicating that there is little or no non-specific binding of the primary antibody to the ovalbumin coat (Fig 7.13, rows a and b).

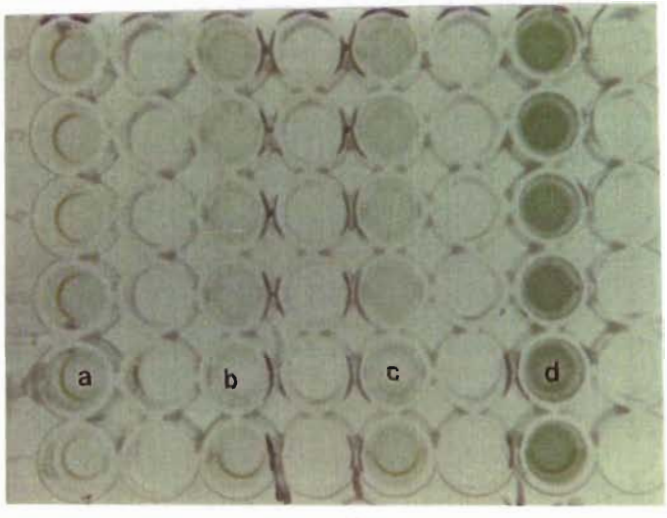


Fig 7.13 Determination of the ability of sodium metaperiodate and carbodiimide to couple atractyloside to ovalbumin, bound to a microtitre plate
Row a = periodate control (absorbance = 0.153(); row b = carbodiimide control (absorbance = 0.11); row c = sodium periodate + atractyloside (absorbance = 0.138); row d = carbodiimide + atractyloside (absorbance = 0.332), where absorbance values represent the mean absorbance per well of six replicates.

Furthermore, in the case of the carbodiimide control (row b), the results show no evidence of the presence of CAMOR antibodies. Row c depicts the degree of binding by ATR to the ovalbumin coat, as mediated by the PLP method of McClean and Nakane (1974). Clearly, the intensity of the colour development is less than that of the control (represented by row a), thus discounting the possibility of using this method for the fixation process. By contrast, however, the intensity of the colour development in row d, representing the coupling of ATR to the ovalbumin coat by the carbodiimide procedure, indicates that free ATR may be bound to endogenous protein by this method. Thus, from the results of the immunoassay it was concluded that 1-ethylenediamine-3-(3-dimethylaminopropyl) carbodiimide could be a suitable coupling agent for pre-fixing ATR to intracellular proteins. Consequently it was

decided incubate *C. laureola* tuber with carbodiimide, as described in section 3.8, prior to fixation and embedding in resin.

Prior to the immunolabelling of ATR in plant and mammalian tissue, however, it was necessary to ensure the recognition of the rabbit antiserum by the Protein A-gold probe and this was tested using an immuno-dot blot, as described in section 7.5.4.

7.5.4 Determination of the recognition of rabbit antisera by Protein A-gold probes as monitored by immuno-blotting

7.5.4.1 Introduction

The recognition of the rabbit antiserum by the immunogold probe was tested using an immuno-dot blot. Briefly, the rabbit serum was "dotted" onto a nitrocellulose membrane and incubated in the presence of the gold probe. The binding of Protein A-gold to the rabbit antibodies is indicated by the presence of a pink colour on the membrane, in the region of the "dotted" sample. Visualisation of this is facilitated by silver amplification. The procedure described below is based on the method of Moeremans *et al.* (1984) as modified by E. Elliott (pers. comm., 1989).

7.5.4.2 Materials and methods

Reagents

Buffer 1: 0.05 M Tris buffer, pH 7.4 containing 2.5% (w/v) sodium chloride and 0.05 % Tween 80

Buffer 2: 0.05 M Tris buffer, pH 8.2 containing 0.9% (w/v) sodium chloride

Buffer 3: Buffer 2 containing 1% (w/v) BSA

Physical silver developer:

All reagents were prepared in scrupulously clean glassware, which had been washed with detergent, rinsed in distilled water, acetone and finally air-dried. Citrate buffer of pH 3.5 (approximately) was prepared by dissolving trisodium citrate dihydrate (2.3 g) and citric acid (2.55 g) in distilled water (85 ml). Hydraquinone (0.85 g) was added, forming solution A. Solution B was prepared by dissolving silver lactate (0.11 g) in distilled water (15 ml). As silver lactate is photosensitive, all solutions were protected from light. Immediately before use, solutions A and B were mixed together.

Immuno-dot blot procedure

All incubations were carried out at room temperature in the dark. The nitrocellulose membrane (5 cm × 2 cm) was rinsed in distilled water (2 × 5 minutes) and air-dried. The rabbit antiserum was diluted 1:40 and 1:100 in buffer 2. Aliquots (5 µl) of each dilution as well as a control of buffer 2 only, were "dotted" onto the nitrocellulose membrane. The membrane was air-dried for 15 minutes, blocked with a solution of 5% (w/v) "Elite" milk powder in buffer 2 and washed in buffer 1 (2 × 5 minutes) followed by buffer 2 (1 × 5 minutes). The Protein A-gold was diluted to 1:20 in buffer 3. The dilution factor was determined spectrophotometrically by preparing a dilution series of the gold probe, in buffer 3 and monitoring the absorbance at 520 nm. The dilution factor giving an absorbance closest to 0.018, at this wavelength was the correct dilution for use in the immuno-dot blot (E.Elliott, pers. comm., 1990).

The appropriately diluted gold-probe (300 µl) was incubated with the membrane for 2 hours, rinsed in buffer 3 (3 ×10 minutes) and finally in distilled water (4 × 5 minutes). The membrane was incubated for 4 to 8 minutes in the freshly prepared silver developer, the time being dependent upon the degree of development after which the reaction was terminated by transferring the membrane to a solution of 5% (w/v) aqueous sodium thiosulphate for 2 minutes. Finally the membrane was washed in numerous changes of distilled water, air-dried and viewed. As the silver staining fades in the presence of light, care was taken to store the membrane in the dark.

7.5.4.3 Results and discussion

The results of the immuno-dot blot are shown in Fig 7.14 and demonstrate that the rabbit antiserum was indeed recognised by the Protein A-gold probe. Binding was most notable when the rabbit antiserum was diluted 1:40 (a) and only weakly discernible at a dilution of 1:100 (b) (although in the photograph the binding is not visible). As expected, there was no reaction with the buffer "dot" or control sample. When the procedure was repeated with fresh rabbit antiserum, (produced as described in section 5.3), the gold-probe also targeted for this antiserum. Based on these results the Protein A-gold probe was deemed suitable for use in the immunocytochemical location of ATR since the Protein A clearly recognised the rabbit antiserum.

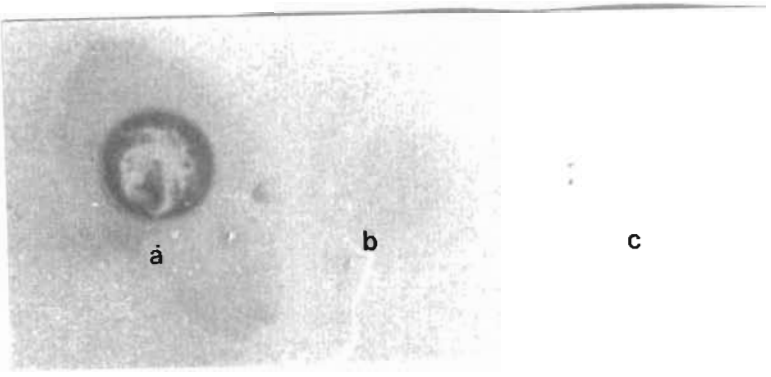


Fig 7.14 Photographic representation of the immunodot blot to test the recognition by the Protein A gold probe for the rabbit antiserum
a = primary antibody dilution 1:40, b = primary antibody dilution 1:100,
c = buffer control. All samples were incubated with protein A-colloidal gold and visualised with silver amplification as described in section 7.5.4.2

Having identified a suitable method for the fixation of ATR *in vivo* (section 7.5.3), ensured that the Protein A-gold label targets for the rabbit antiserum (section 7.5.4), optimised the ultrastructural fixation of the plant tuber (section 7.5.2) for electron microscopy, and characterised the antiserum (Chapters 5 and 6), this optimised protocol was then tested for its ability to immuno-localise ATR in the tuber of *C. laureola*.

7.6 Immunocytochemical localisation of atractyloside in the tuber of *Callilepis laureola*

7.6.1 Introduction

As mentioned earlier, the ultimate aim of this investigation was the localisation of ATR in mammalian tissue, but as there is no information regarding the pharmacokinetics of this compound, it was not known if the toxin would even be present in the kidney of rats exposed to this toxin. It was therefore decided to optimise the immunocytochemical procedure in tuber tissue, where it was certain that the toxin was present and non-biotransformed. Only if the immunocytochemistry on the plant material was successful would a similar attempt be made on the mammalian tissue. Theoretically therefore, the work on the plant tuber was included as a positive control. Immunocytochemistry has been used for the subcellular localisation of among others, the cytokinins, in plant material at different stages of cell differentiation (Sossountzov *et al.*, 1988). Clearly this approach has provided considerable insight into the involvement of such plant growth hormones *in vivo* and it was therefore hoped that the method developed in the present studies would prove equally informative in identifying the location of ATR in both plant and mammalian tissue.

7.6.2 Materials and methods

The protocol followed was that of Sossountzov *et al.* (1988) but included the modifications developed as described in section 7.5 above. The details of the final optimised fixation and immunolabelling procedure are given in section 3.8. Briefly though, sections were mounted on copper grids and etched for 1 minute in bromine vapours but unfortunately the bromine had a deleterious effect on the grids and thus subsequently, only nickel grids were used. Thereafter grids were sequentially incubated with primary antibody and the protein A-gold label, with the grids being copiously washed between all steps. Suitable controls were included throughout, namely, the primary antiserum was included at different dilutions to identify the dilution giving maximal binding with the least non-specific labelling, primary antiserum was replaced with pre-immune serum, the primary antiserum was omitted and an adsorption control was included. The control grids were observed under the electron microscope and photographed at a magnification of × 10 000. The photograph negatives were evaluated for the degree of non-specific labelling in the control sections using a Bell and Howell microfiche reader. The results of the immunolabelling on the plant material are given in section 7.6.3 below.

7.6.3 Results and discussion

Some difficulty was experienced with sectioning of the plant material, a phenomenon previously noted by other researchers using this method (Craig and Goodchild, 1982; Herman and Shannon, 1984) but the immunolabelling was nevertheless carried out on these sections. At the outset it should be noted that since the precise localisation of the gold-particles can be very difficult, areas of tissue were randomly selected, when viewed under the transmission electron microscope, and photographed at a magnification of about × 10 000. The negatives of these photographs were also viewed in a microfiche reader, which readily facilitates the resolution of the gold probes, as clearly demarcated white spheres against an essentially black background.

The results of the immunolabelling carried out on *C. laureola* tuber samples are shown in Plate I, micrograph 3. Since the tuber cells contain few organelles but only large areas of cytoplasm and vacuole, it was difficult to discern any form of cellular structure. However, there were areas of gold-probe "clumping" as shown in micrograph 3, which were absent in the control sections. The controls showed a very low degree of non-specific labelling and thus the specificity of the antiserum and the colloidal gold label was concluded to be very good. Tuber tissue fixed with PLP, was also included, but little or no labelling was noted, indicating the success and necessity of the carbodiimide fixation step. Since the tuber had

been treated with carbodiimide, concern was expressed at the endogenous labelling by CAMOR antibodies, but this was shown to be unfounded by the results of the characterisation assays detailed in section 5.5. Since the control sections showed only cytoplasm with little or no non-specific labelling, these micrographs are not included.

From these results it was concluded that the probe had located areas occupied by ATR which could be either for storage or processing. It was suggested by F. Dehrmann (pers. comm., 1990) that the ATR appeared encapsulated in vesicles and it was speculated that these vesicles may be involved in toxin processing or storage. The site of ATR biosynthesis and storage in the plant is unknown, but being water soluble it was suggested that the toxin is sequestrated to the vacuole. Storage of ATR in the vacuole would also be a way in which the plant could avoid the risk of self-intoxication. Such self intoxication of the plant is not unrealistic when one considers the reports of the inhibitory effects of ATR on mitochondria isolated from cauliflower or broccoli tissue (Jung and Hanson, 1973).

Thus, it was demonstrated that the immunocytochemical location of ATR in the plant is possible. While the development of this methodology provided a means of investigating the biosynthesis and storage of ATR in *C. laureola,* this was essentially performed as a means of optimising the detection of the toxin in mammalian tissue. Consequently, further work on the tuber was abandoned, although having laid the groundwork in this area, it would be relatively easy to continue an investigation into the biosynthesis and storage of the toxin in the plant, a possible future project. Information pertaining to the methods whereby the plant synthesises the toxin, would also facilitate the understanding of the functional role of this secondary metabolite in the plant. Furthermore, the methods the plant has for limiting self-intoxication may provide an antidote for *Impila* poisoning, unless of course the toxin is simply sequestrated to the vacuoles or vesicles. While this is a vast area for future research, the specific aims of this study were to investigate the site of the toxin in rat kidney and thus subsequent work concentrated on this aspect.

7.7 Immunocytochemical localisation of atractyloside in rat kidney

7.7.1 Introduction

The science of immunocytochemistry has provided insight into the subcellular localisation of antigens, in both plant and mammalian tissue. To date, most of the work has been carried out on protein antigens, although more recently work on the immunocytochemical detection of haptens has also been undertaken. Localisation of haptens, such as ATR, has however often been hampered by them being leached from the tissue during fixation and dehydration steps. As demonstrated in section 7.6, this problem was overcome in the case of ATR and thus based on the success of the immunocytochemical work on the tuber of *C. laureola*, it was decided to investigate the target sites of this toxin in mammalian tissue.

7.7.2 Materials and methods

Rats were dosed with 25 mg ATR/kg bwt and representative animals, euthanased at 24, 48 and 72 hours post-dosing and the kidney excised and fixed as described in section 3.8.2. The dosing, tissue collection and processing was carried out at the RIIEHS, University of Surrey, Guildford, UK, while the tissue sectioning and immunolabelling was carried out at the University of Natal, Pietermaritzburg. Details of the tissue collection and fixation, as well as the immunolabelling procedure are given in sections 3.1.4, 3.8.2 and 3.8.3 respectively and represent the final optimised procedure which was developed from the work reported in section 7.5, as well as the experience gained from the tuber work, section 7.6. As with the tuber investigation, control grids were observed under the electron microscope and photographed at a magnification of × 10 000. The photograph negatives were evaluated for the degree of non-specific labelling in the control sections using a Bell and Howell microfiche reader. The results of this immunocytochemical investigation are presented in section 7.7.3 below.

7.7.3 Results and discussion

As with the immunocytochemical work on the tuber, appropriate controls were included throughout, which were randomly photographed at low magnification and the degree of non-specific labelling estimated from the photograph negatives, as described in section 7.6.2. These included control kidney tissue (from a rat not exposed to the toxin) (Plate II, micrograph 4 and 5) and kidney tissue from a rat exposed to ATR, in which the primary antibody was replaced by pre-immune serum (Plate II, micrograph 7).

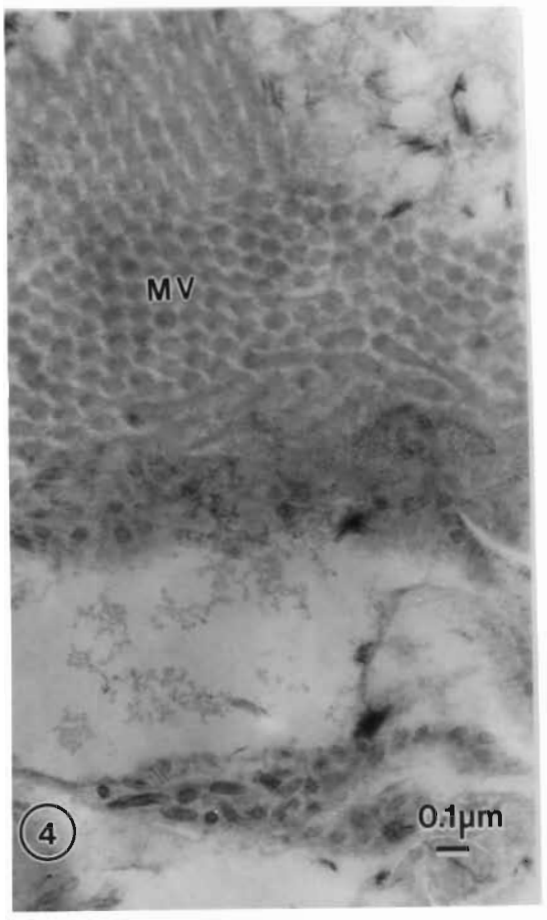
Plate II

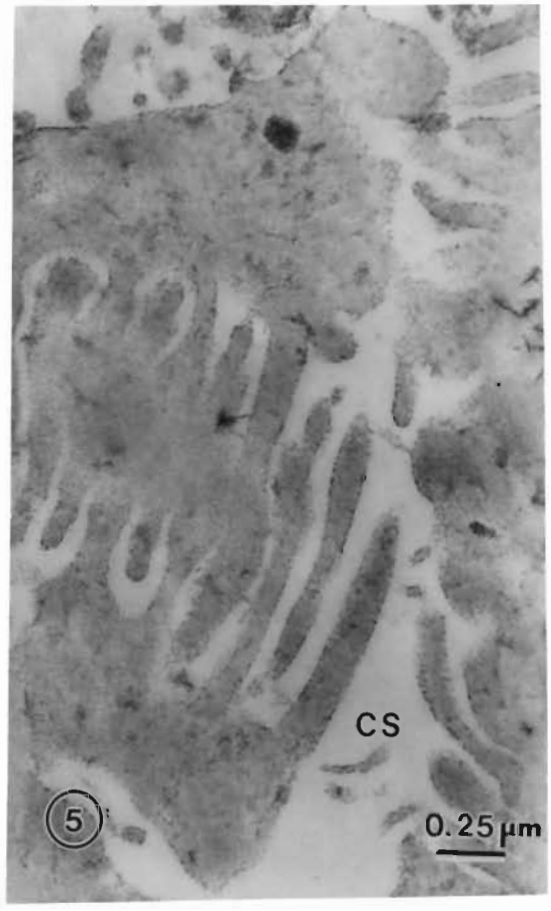
4. Control rat kidney tissue, pre-fixed with carbodiimide, embedded in Spurrs resin, as described in section 7.7.2. Microvilli (MV) of the proximal tubule brush border are clearly visible, indicating good retentionof cellular ultrastructure and stage 1 tissue according to Berndt (1976)

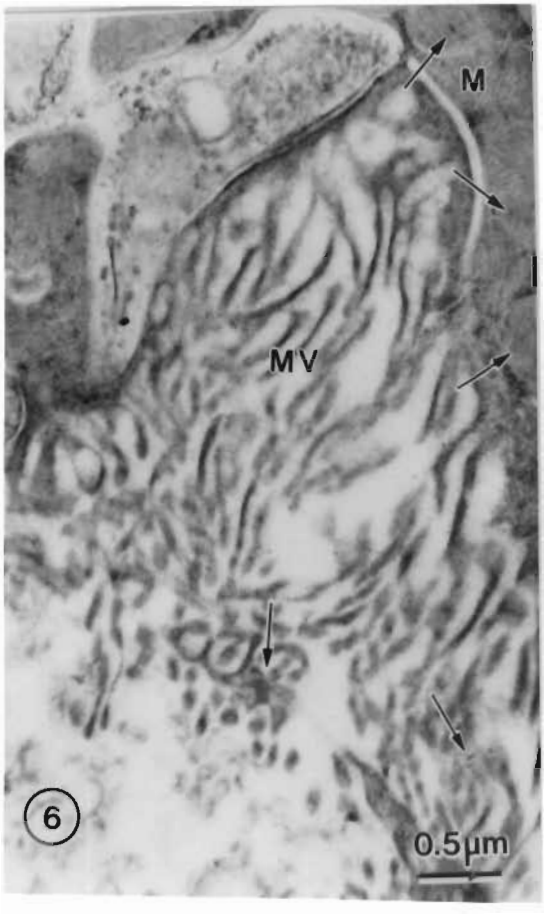
5. Control rat kidney tissue, pre-fixed with carbodiimide, embedded in Spurrs resin, as described in sections 3.8 and 7.7.2. Foot processes of the Bowman's capsule are clearly visible surrounded by the capsular space (CS), again indicating good retention of cellular ultrastructure under the optimised fixation procedure and stage 1 tissue according to Berndt (1976)

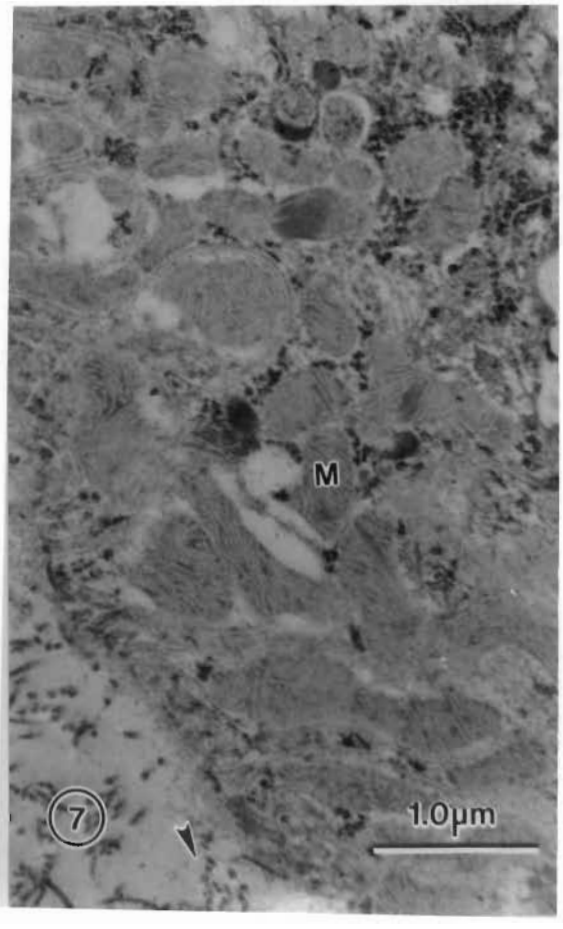
6 Kidney from rats dosed with 25 mg ATR/kg bwt, 24 hours after dosing. Tissue was pre-fixed with carbodiimide, embedded in Spurrs resin, incubated with a 1:300 dilution of primary antiserum and labelled with protein A-colloidal gold of particle size 3 nm as detailed in sections 3.8 and 7.7.2. Labelling is confined to microvilli (MV), which are disrupted and on the cristae of the mitochondria (indicated by arrows).

7 Control kidney from rats dosed with 25 mg ATR/kg bwt, 24 hours after dosing. Tissue was pre-fixed with carbodiimide, embedded in Spurrs resin, incubated with pre-immune serum diluted 1:300 and labelled with protein A-colloidal gold of particle size 3 nm as detailed in sections 3.8 and 7.7.2. There was little evidence of non-specific labelling but brush border damage (arrow head) and mitochondrial swelling are apparent, indicating stage 4 damage according to the classification of Berndt (1976)


MV
4
0.1μm


CS
5
0.25μm


M
MV
6
0.5μm


M
7
1.0μm

An adsorption assay and a control in which the primary antiserum was omitted (Plate III, micrograph 11) were also included. The control sections showed little or no significant labelling such that, if labelling was present, the colloidal gold particles were in isolation and not clustered. The pre-immune control section (micrograph 7) showed only five randomly distributed particles. From these results, therefore, it was concluded that any immunocytochemical labelling of the non-control samples could be considered as specific for ATR or analogues of this toxin.

In 1976, Berndt reviewed the evaluation of proximal tubule necrosis and suggested that five stages of damage are recognisable. Briefly, stage 1 is depicted by control renal cortex in which the microvilli of the brush border remain organised and the mitochondria are well formed. Stage 2 is characterised by swelling of the ER and "rounding" of mitochondria. Mitochondria appear more condensed and the brush border is seen to be disrupted in stage 3 kidney damage. Stage 2 and 3 are apparently reversible (Berndt, 1976). Stage 4 damage is demonstrated by swelling of the mitochondria, although the matrix of these organelles remains organised. In stage 5 damage, there is loss of the mitochondrial internal structure, which appears to be granular (Berndt, 1976). Stage 1 of Berndt's classification is represented in the present work by micrographs 4 and 5 (Plate II) and are kidney sections taken from control rats, not exposed to ATR. As demonstrated in micrograph 4, the microvilli of the brush border are regimented and the exposure of the tissue to carbodiimide does not appear to have affected the ultrastructure. This ultrastructural preservation is verified in micrograph 5, which shows foot processes of the podocytes from the Bowman's capsule. It should be noted that the objective of the present author was to include a control section containing mitochondria, for comparative purposes, but unfortunately these structures were not readily identified.

The micrographs 6 and 7 (Plate II) represent sections of kidney taken from rats 24 hours after exposure to ATR. The section in micrograph 6 was labelled with primary antiserum and Protein A-gold, as described in section 3.8. From this micrograph it can be seen that the microvilli have been disrupted probably as a consequence of the exposure to ATR and not mechanical damage during excision and fixation, since this result was reproducible.

In addition, there is a considerable amount of labelling associated with these microvilli, indicating the localisation of ATR at this site of damage. Clusters of gold particles are indicated by arrows, but other solitary particles and pairs of label are also present. In addition, as indicated by the three arrows in the top right hand corner, clusters of colloidal gold particles are also present on the mitochondria and this labelling is highly specific for the cristae. Although the indicated labelling might appear to be very faint in the plates,

resolution was much better and clearly distinguishable when viewed by microfiche. This report therefore constitutes the first *in vivo*, visual localisation of ATR in mammalian tissue and confirms the findings of the *in vitro* work described in this thesis (section 7.2).

Micrograph 7 (Plate II) is included as a control, in which primary antibody was replaced by pre-immune serum, the results yielding little evidence of non-specific binding. This micrograph is also included to show brush border damage (denoted by the arrow head) and in addition, the significant alteration in the mitochondria (denoted as M). Based on the classification of Berndt (1976), this would represent stage 4 damage, as the microvilli of the brush border are disrupted (essentially, stage 3) but the mitochondria have become swollen. The fact that the mitochondrial matrix is still discernible denotes stage 4 and not stage 5 damage. The appearance of swollen mitochondria is not unexpected as Davidson and Halestrap (1987) noted that ATR induces mitochondrial swelling. This altered morphology is also characteristic of Reye's Syndrome (Martens *et al.*, 1986) and further corroborates the proposal of the present author that ATR may be associated with this Syndrome.

Micrographs 8 and 9 (Plate III), were collected from rats 48 hours after exposure to ATR. Although the resolution in micrograph 8 is not particularly good, as the section was very thick, the microvilli are nevertheless clearly visible. Unlike the brush border shown in micrographs 6 and 7, the microvilli depicted in this micrograph are not significantly disrupted, but the high labelling density on the microvilli reaffirms that ATR targets for the microvilli of the the proximal tubule brush border.

Micrograph 9 (Plate III) shows a number of sites of immunolabelling, with only the clusters of gold particles indicated by arrows. The arrow to the base of the micrograph denotes labelling associated with a membrane structure and although difficult to discern, this organelle is thought to be a mitochondrion despite the fact that the cristae are not clearly visible. This apparent loss of the clear definition of the cristae could, according to Berndt's classification, denote late stage 4 damage, or early stage 5 damage. What is of greater significance however, is the labelling at four sites on the membrane of the organelle located in centre field which is probably a Golgi vesicle with associated lipoprotein globule or secretory vesicle.

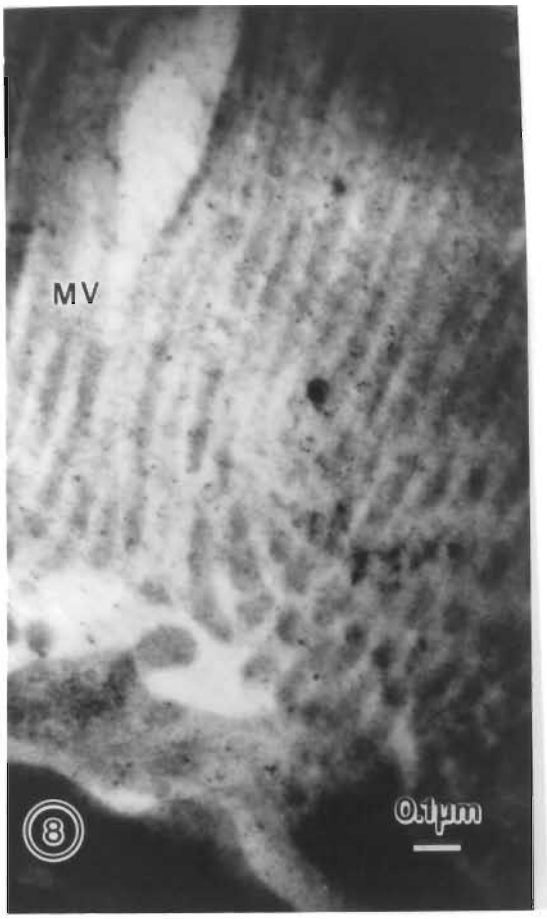
Plate III

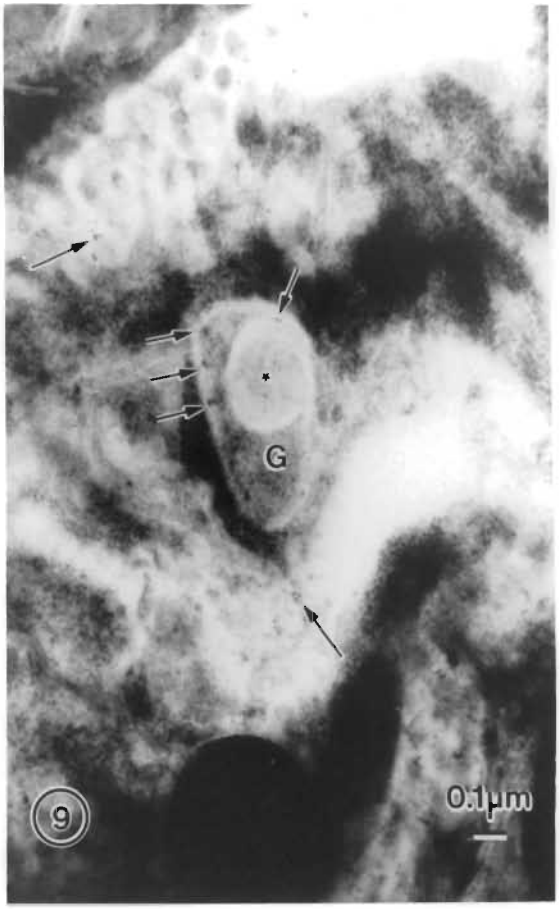
8 Kidney from rats dosed with 25 mg ATR/kg bwt, 48 hours after dosing. Tissue was pre-fixed with carbodiimide, embedded in Spurrs resin, incubated with a 1:300 dilution of primary antiserum and labelled with protein A-colloidal gold of particle size 3 nm as detailed in sections 3.8 and 7.7.2. Labelling is confined to the microvilli (MV) which show less disruption at 24 hours

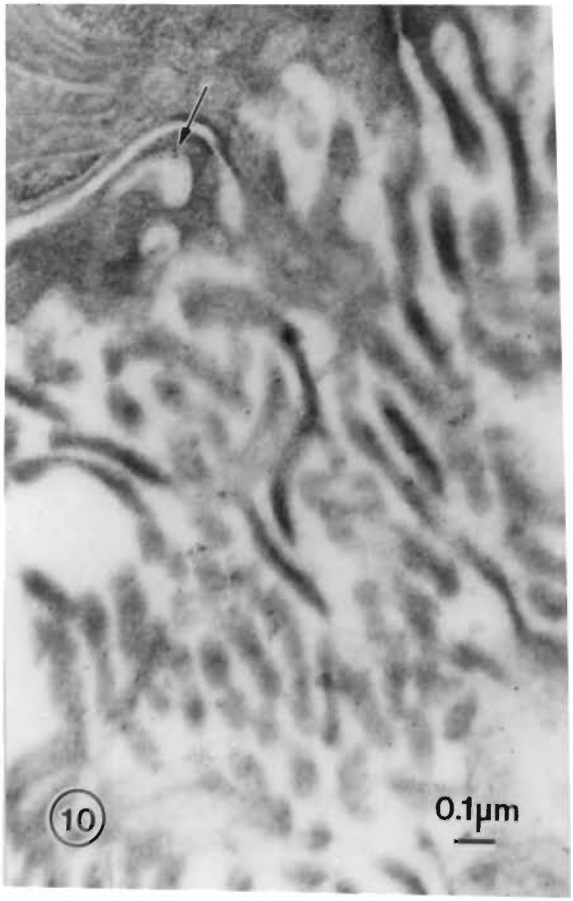
9 Kidney from rats dosed with 25 mg ATR/kg bwt, 48 hours after dosing. Tissue was pre-fixed with carbodiimide, embedded in Spurrs resin, incubated with a 1:300 dilution of primary antiserum and labelled with protein A-colloidal gold of particle size 3 nm as detailed in sections 3.8 and 7.7.2. Labelling is present at regular intervals on the membrane of the Golgi apparatus (G) with an associated lipoprotein globule or secretory vesicle denoted by an asterix (*). Labelling is also associated with mitochondrion, but cristae are not clearly visible, i.e., indicative of early stage 5 damage

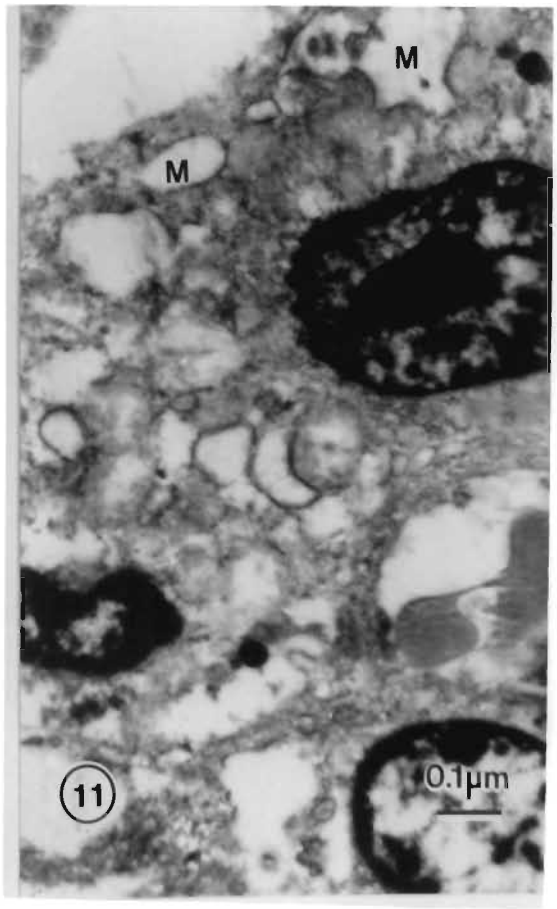
10 Kidney from rats dosed with 25 mg ATR/kg bwt, 72 hours after dosing. Tissue was pre-fixed with carbodiimide, embedded in Spurrs resin, incubated with a 1:300 dilution of primary antiserum and labelled with protein A-colloidal gold of particle size 3 nm as detailed in sections 3.8 and 7.7.2. Labelling is still associated with the disrupted microvilli

11 Control kidney from rats dosed with 25 mg ATR/kg bwt, 72 hours after dosing. Tissue was pre-fixed with carbodiimide, embedded in Spurrs resin, incubated with buffer instead of primary antiserum and labelled with protein A-colloidal gold of particle size 3 nm as detailed in sections 3.8 and 7.7.2. Non-specifc labelling was insignificant. Stage 5 damage is indicated as the mitochondrial matrix is disrupted, resembling a "flocculant precipitate"


MV
8
0.1μm


G
9
0.1μm


10
0.1μm


M
M
11
0.1μm

Since, both the GA and mitochondrion are associated with lipoprotein globules or secretory vesicles (Theron, 1979), an organelle such as that in centre field (micrograph 9) is not uncommon. This was initially presumed to be a mitochondrion, with an associated secretory vesicle (denoted in the micrograph with an asterix), however, careful study of the micrographs of Theron (1979) and Sandborn (1970) showed that this organelle was not a mitochondrion but a Golgi vesicle. From micrograph 9, it was clear that ATR associates with specific sites, at regular intervals along the membrane of the Golgi vesicle. It was pointed out in the literature review that *in vitro*, ATR and CATR inhibit the transport of PAPS across the Golgi membrane "possibly by interacting with the PAPS translocase which has structural features in common with the ADP/ATP antiporter" (Capasso and Hirschberg, 1984). Thus, from micrograph 9 it is clear that ATR does bind to specific sites on the GA membrane and this result is therefore of extreme significance.

Micrographs 10 and 11 (Plate III) are taken from the kidney of rats, 72 hours after exposure to ATR. Micrograph 10 shows that labelling is still associated with the disrupted microvilli (indicated by an arrow) and with other membrane bound structures, which are not readily identifiable. Micrograph 11 was included as a control, in which primary antibody was excluded. Clearly there was minimal labelling, reiterating the specificity of the immunocytochemical localisation of ATR. It was noted in this section, however, that the tissue showed stage 5 damage, as the matrix of the mitochondria (M) is disrupted and, in the words of Berndt (1976), resembles a "flocculant precipitate".

Thus from the micrographs it was concluded that the immunocytochemical labelling was highly specific and furthermore, ATR targets for the microvilli of the proximal tubule brush border, the mitochondria and the GA. In addition, it was also noted that, unlike the observations of Carpenedo *et al.* (1974) and Bhoola (1983), regeneration was not apparent 48 hours after exposure to the toxin. Berndt (1976) warns however, that the analysis of morphological changes is only of value if these alterations can be correlated with disruption in function (as monitored by organ function assays). This important corroboration of morphological changes, immunocytochemical location and organ function assessment will be presented in the general discussion (section 7.8) below.

7.8 General discussion

Up until the time of this study, details pertaining to the inhibitory effect of ATR had only been identified by histological observation or *in vitro* assays. Nevertheless it had been established that this toxin was nephrotoxic, targeting for the proximal tubules in rats, pigs and humans (Carpenedo *et al.*, 1974; Stuart *et al.*, 1981; Bhoola, 1983). These workers noted

regeneration 48 hours after exposure to ATR (Carpenedo *et al.*, 1974; Bhoola, 1983). The glomerulus was morphologically unaffected and distal tubular damage was only observed after exposure to CATR (Hatch *et al.*, 1982). Furthermore, it was established by *in vitro* assay that ATR inhibited the transfer of adenine nucleotides across the inner mitochondrial membrane, by binding to the ADP/ATP antiporter (Nicholls, 1982). This toxin also induced mitochondrial swelling (Davidson and Halestrap, 1987) and the release of intramitochondrial calcium stores into the cytoplasm (Asimakis and Sordahl, 1977; Lê Quôc and Lê Quôc, 1988). It was also noted, that ATR inhibited post-translational sulphation, probably by inhibiting the translocation of the sulphate donor, PAPS, across the GA membrane (Capasso and Hirschberg, 1984; Lee *et al.*, 1984). Based on these results it was decided by the present author to investigate whether these *in vitro* findings were applicable to the *in vivo* situation. To achieve this, the target sites of ATR were localised using organ function assays and immunocytochemistry. For the purposes of this general discussion, only the results of the high dose regimen (25 mg ATR/kg bwt) will be considered since they showed more significant tissue damage.

At the outset, organ function assays were carried out and these included monitoring the effects of ATR on the appearance of protein, albumin, AP, GGT, LDH, glucose and calcium in the urine of rats. Proteinuria which is not only indicative of renal damage *per se*, but more specifically of any compromise in glomerular permselectivity (Smith and Hook, 1982), has been noted in rats exposed to ATR as well as children accidently poisoned by *C. laureola* (Carpenedo *et al.*, 1974; Bhoola, 1983). Similar proteinuria was also observed in the present studies (Fig 7.2) as well as albuminuria (Fig 7.3) which has been used to assess glomerular integrity in the presence of ATR (Berndt, 1982). Bhoola (1983) also noted albuminuria in cases of suspected *Impila* poisoning and a similar response was noted in rats exposed to ATR (Carpenedo *et al.*, 1974). Thus, from the present reported results, as well as that of others it was concluded that ATR induces renal damage and affects glomerular permselectivity. While this renal damage was confirmed by the micrographs (Plates II and III), the effect of ATR on the glomerulus was not observed by the present author.

As a means of monitoring the effect of ATR on the proximal tubule and, more specifically, the microvilli of the brush border, the presence of AP and GGT in the urine of dosed rats was investigated. Alkaline phosphatase excretion into the urine has been associated with exposure to *X. strumarium* (Stuart et al., 1981) and in the present studies a similar response was noted (Fig 7.5). This response was evident up to 48 hours after exposure to ATR and thereafter, the level of excreted AP dropped below that of the control, suggesting some form of recovery. The marker GGT was also monitored as an indicator of proximal tubule and brush border damage and this enzyme was also detected in the urine of the intoxicated rats,

up to 48 hours after exposure (Fig 7.4). Thus, this enzymuria demonstrated that ATR induces damage to the microvilli of the proximal tubule brush border which was positively confirmed by the immunocytochemical investigations (micrographs 7, 8, 9 and 11). Apart from micrograph 7, the microvilli were clearly disrupted, but furthermore ATR was immunocytochemically located on these microvilli and not located in the surrounding milieu. Clearly, therefore, ATR targets for the proximal tubule, but the microvilli appear to be particularly susceptible, an observation not previously noted.

The effect of ATR on proximal tubule transport was monitored by glucosuria and proline incorporation. Glucosuria has been detected in patients who were accidently poisoned by *Impila*, and in rats dosed with ATR (Carpenedo *et al.*, 1974; Bhoola, 1983). These results were corroborated by the present author (Fig 7.6). In addition, ATR also inhibited the transport of proline in the proximal tubules, further suggesting that this toxin terminates active transport and seriously interferes with proximal tubule function.

Lactate dehydrogenase in the urine was monitored as a marker of distal tubule damage, and while this enzymuria has been noted in rats dosed with CATR (Hatch *et al.*, 1982), this was also observed for ATR in the present studies (Fig 7.7). By contrast this effect of ATR on the distal tubules was not readily apparent in the micrographs and therefore constitutes an area for future investigation.

Thus, apart from the LDH and albumin investigation, the results of the organ function assays were corroborated by the findings in the immunocytochemical investigations. Attention was therefore turned to the subcellular targeting of ATR. It has previously been established *in vitro*, that ATR inhibits the translocation of adenine nucleotides and PAPS across the inner mitochondrial and GA membranes respectively, by binding to the ADP/ATP antiporter and the PAPS translocase (Nicholls, 1982; Capasso and Hirschberg, 1984, Lee *et al.*, 1984). In addition, this toxin induces mitochondrial swelling and release of intramitochondrial calcium stores (Asimakis and Sordahl, 1977). From the present immunocytochemical studies, it was firstly observed that ATR induces mitochondrial swelling within 24 hours after exposure to this toxin (micrograph 7, Plate II) and furthermore, that this toxin binds to the cristae. Atractyloside was also observed to bind to the cytoplasmic side of the GA membrane, confirming the proposal of Capasso and Hirschberg (1984). Thus, the immunocytochemical investigation confirmed the previous findings on the subcellular localisation of ATR which, up until this time, had only been demonstrated *in vitro*.

It is hoped that these findings will significantly contribute to a better understanding of the effects of ATR and furthermore, that this work will warn researchers using ATR as a biochemical probe, that the interpretation of their results may not take into account all the effects of ATR on cellular function. As mentioned in the introduction, this study involved the development of suitable detection methods for ATR and the *in vivo* localisation of the toxin in rats with each investigation being designated to a separate chapter. Chapter 8 (General Discussion) will therefore serve as a means of collating these findings as well as providing suggestions for further areas of research and possible therapeutic agents which may prevent additional ATR-induced deaths.

CHAPTER 8

GENERAL DISCUSSION

The primary objectives of this study were to develop a diagnostic assay for ATR, the primary toxic component of *Impila* and furthermore, to localise the target sites of ATR at an organ, cellular and organelle level. In this regard, these studies succeeded in achieving the following original results:-

1. The development of an HPLC method for ATR and detection of this toxin in extracts of *C. laureola* tuber and urine from ATR-dosed rats;
2. Optimisation and production of antibodies to ATR for use in both an ELISA and immunocytochemical investigations;
3. Identification of the immunosuppressive action of ATR, probably on the T-lymphocytes;
4. Development of an ELISA and detection of ATR in rat urine and serum;
5. Provided preliminary evidence that this toxin is biotransformed *in vivo;*
6. Identified the microvilli of the proximal tubule brush border, glomerulus and distal tubules as target sites of this toxin, where previously only proximal tubule damage has been alluded to (Carpenedo *et al.*, 1974);
7. When administered i.p., ATR is not significantly hepatotoxic;
8. This toxin also targets for the cristae of the mitochondria and specific sites on the GA membrane.

The development of a suitable assay was approached both immunologically and chromatographically. Since antibodies had not previously been raised to ATR, this process necessitated a considerable amount of method development before they could be applied to an immunoassay. Atractyloside is categorised as a hapten and thus, as a first priority, to be rendered immunogenic, it had to be conjugated to a suitable carrier. Initially a modification of the method of Fuchs and Fuchs (1969) was used to couple ATR to either BSA or ovalbumin but when the resulting conjugates were used to immunize chickens, the antibody response was very poor. Subsequent antibody production was therefore carried out in rabbits, but despite the change in host species, there was no significant improvement in the antibody response. It was therefore suggested, that like other toxic haptens such as amanitin, T_2-toxin and tetrodotoxin (Faulstich *et al.*, 1975; Hunter *et al.*, 1985; Ragelis, 1985), ATR may be an immunosuppressant, probably inhibiting T-lymphocytes. Thus, it is proposed that ATR could inhibit T-cell activity by suppressing ATP synthesis. Furthermore, this toxin also induces the release of intramitochondrial calcium into the

cytoplasm, which may have a far more detrimental effect on the lymphocytes. The presence of calcium in the cytoplasm is thought to be an intracellular signal for inhibition of cell growth, and chronic levels of this cation in the cell are an early indicator of cell injury, particularly in immune cells (Lynn *et al.*, 1988). In this regard it has been noted that infection of T_4^+ lymphoid cells by the human immunodeficiency virus (HIV) (an immunosuppressant) is associated with enhanced permeability of the cell membrane, causing an influx of divalent ions, particularly calcium. The envelope protein (*env*) of the HIV or an *env*-gene product is thought to alter the membrane permselectivity, resulting in this influx of calcium ions. This increased concentration of cytoplasmic calcium causes cessation of growth and, ultimately, cell lysis (Lynn *et al.*, 1988). Immune suppression by HIV is well documented and the elevation of cytoplasmic calcium levels is thought to be involved in this inhibition (Lynn *et al.*, 1989). Since ATR alters mitochondrial membrane structure and thereby induces efflux of calcium from intramitochondrial stores into the cytoplasm, it is feasible that ATR may also affect T-cells in the same manner as HIV. Thus, by suppressing T-cells, B-cells would not produce antibodies and this would account for the poor immune response observed for the ATR-conjugates.

Any inherent immune toxicity of ATR which might exist, may be further enhanced by virtue of the high charge density associated with its two bisulphate esters, as this has previously been associated with immune suppression in such immunogens as protamine, histones and sialic acid (Absolom and van Oss, 1986). Similarly, an immunogen which is not readily metabolised *in vivo*, such as ATR, is also observed to suppress the immune response (Bach, 1982). Thus it can be concluded that the toxicity of ATR, its poor metabolism *in vivo*, its inherent charge density, as well as the too frequent immunization of the host species, may collectively account for the low quality of antibody produced.

Since the antibody response to a hapten may be improved by changing the coupling procedure (Weiler, 1981; Ohtani *et al.*, 1988), consideration was given to alternative conjugation procedures, carriers and dosing protocols. The site of conjugation was the first factor to be addressed and since the antibodies were required for use in both the immunoassay and immunocytochemical studies, the method for coupling ATR to the carrier was an important consideration. Landsteiner is reputed to have observed that an antibody is generally directed to the (hydrophobic) region of the hapten, furthest removed from the linkage (Tijssen, 1985), and thus it was speculated that anti-ATR antibodies would be directed towards the penhydro-region of the phenanthrenic ring of ATR (Fig 2.3), should the carboxyl moiety be used as a site of conjugation. The carboxyl group on the diterpene ring is crucial to the toxicity of the molecule, apparently associating directly with the ADP binding site on the antiporter (Nicholls, 1982), while the stereology of the

remaining portion of the molecule maintains ATR "locked" in this position (Vignais *et al.*, 1978). The success of an immunocytochemical investigation would, therefore, be dependent upon the antibody having access to an exposed portion of the ATR molecule, namely that furthest removed from the carboxyl portion. Based on this consideration, only the carbodiimide (P. Hoffman, pers. comm., 1989) and mixed anhydride methods were compared (Fuchs and Fuchs, 1969) as procedures for the conjugation of ATR to a carrier. It was found that the carbodiimide method yielded the most efficient binding of ATR to the protein carrier and would be the procedure of choice, particularly since ATR was observed to be more soluble in the DMF solvent, compared to 1,4 dioxan, promoting the reaction of the hapten with the coupling reagents.

The rabbit antiserum with the highest affinity and selectivity for ATR was identified by carrying out affinity assays and characterisation tests, employing both immunoassays and immuno-dot blots. From these results it was concluded that a naked bacterial carrier was most suited to the production of antibodies to ATR since, advantageously, the antiserum produced had minimal cross-reactivity with components used in the immunoassay, such as blocking and coating proteins. This novel carrier is therefore recommended for future antibody production against this toxin. By contrast, BSA is only recommended as an alternative in extreme cases, since evidence of "reciprocal cross reactions" (Bach, 1982) with such proteins as ovalbumin could result in false positive results. Since it was postulated that these "reciprocal cross reactions" may occur with a BSA carrier, an autologous carrier protein was also tested. Rabbit serum albumin (RSA) carriers, however, when injected into rabbits, have the potential to induce an autoimmune response (Bach, 1982; Roitt, 1988; Hudson and Hay, 1989) as was noted by an increase in the concentration of immune complexes in the sera after boosting with pure RSA, and should therefore be avoided.

Apart from identifying the carrier most suited for antibody production to ATR, namely naked bacteria, a number of other observations were made. Firstly, it was evident that considerable heterogeneity exists between the ability of different animals to produce antibodies to the same antigen, and this reiterates the importance of not pooling antiserum samples (Catty and Raykundalia, 1988). Secondly, and more importantly, it was also noted that the secondary response was not significantly greater than the primary response in any of the animals immunized with the ATR-carrier conjugates. Since the secondary antibody response is associated with an antibody class switch from IgM to IgG and, T-cells are thought to be imperative for this class switch to occur (Bach, 1982; Tijssen, 1985), any destruction of T-cells by ATR, could result in a persistent primary response and a predominant population of anti-ATR IgM antibodies. Characteristically, IgM antibodies

bind to their corresponding antigen at temperatures below 30°C, with binding being enhanced at room temperature or 4°C and depressed at 37°C (Dacie and Lewis, 1970; Tijssen, 1985). During the course of the development of the immunoassay by the present author, it was noted that activity was lost if the primary antibody was incubated at temperatures above 4°C, suggesting that IgM were indeed the predominant antibodies present in the rabbit serum. Since the class switch did not apparently occur, this reaffirms the proposal that ATR targets for the T-cells. Nevertheless, despite the problems associated with antibody production, a 5-fold improvement in antibody response was obtained with a second attempt at raising antibodies, in rabbits, to the ATR -naked bacterial conjugates.

The antibodies raised against ATR were used in the development of an ELISA for ATR which necessitated the selection of a suitable solid phase and coating buffer and the optimisation of primary and secondary antibody concentrations, the incubation conditions and most importantly, the manner in which ATR was bound to the microtitre plates. The resulting assay has a detection limit of 10 ng ATR/ml, and a sensitivity range of 0.1 to 10 µg ATR/ml. This is essentially 10 000-fold less sensitive than that for fenpropimorph (Jung *et al.*, 1989) but since these workers achieved a primary antibody working dilution of 1: 200 000, the sensitivity achieved in this assay is adequate for the titre of antibody produced. The inclusion of suitable controls verified that the assay was specific for ATR and no cross-reaction was observed with ADP or glucose. The assay was used for the detection of ATR in serum and urine samples, from rats dosed with ATR and the results for which are discussed further and evaluated against that of the HPLC study, later in this section.

As mentioned earlier, the development of a diagnostic assay for ATR and hence, *Impila* poisoning, was approached both immunologically and chromatographically. Previously, ATR has been detected by TLC (Brookes, 1979), but the detection limits of this method prevent its practical application as a diagnostic tool. The HPLC determination of this compound was hindered by the fact that ATR contains no chromophore and is amphipathic (Allman *et al.*, 1967), but it was proposed that these shortcomings could be overcome by forming dansyl derivatives of this toxin using the method of Boulay *et al.* (1983). Unfortunately, the reaction is dependent upon the maintenance of anhydrous conditions, limiting its application to biological samples. For this reason, ATR was derivatised with ADAM, a compound frequently used for the derivatisation of fatty acids for subsequent HPLC analysis (Matthees and Purdy, 1979). The advantages of this derivatisation reagent are that the reaction may be carried out under aqueous conditions, the derivative may be detected by both fluorescence or UV (the former being more sensitive, thus greatly improving detection limits), the derivative is stable and the reaction is reproducible

(Matthees and Purdy, 1979). Furthermore, it was established that the derivatisation of ATR by ADAM went to completion, as free toxin could not be detected by TLC after derivatisation. A suitable solvent combination was optimised, but the resolution of ATR in biological samples by HPLC was only achieved if the specimen was cleaned up both prior to derivatisation and again pre-HPLC analysis. A detection limit of 0.001 ng ATR/ml was determined, representing a detection limit of some 10 000-fold more sensitive than the immunoassay and is significantly more sensitive than the HPLC method for the detection of α–acetyldigoxin in *Digitalis lantana* of Ikeda *et al.* (1991), who quote a standard calibration curve over the range 1-12 μg α–acetyldigoxin/ml. Since α-acetyldigoxin is monitored using a UV detection system, when comparing the detection limits of this assay and the HPLC method developed in this study, clearly this reiterates the sensitivity of fluorescent detection. Atractyloside was positively detected in the extract of a tuber of *C. laureola* by HPLC at a level of approximately 6.7 μg/g of fresh tuber, and the presence of this toxin was confirmed by TLC. Thus, assuming that a person will consume an extract prepared from about 10 g of tuber (*Insangoma*, pers. comm., 1988), this represents a dose of 67 μg of ATR. This is significantly lower than that given to the rats used during the course of this study, but reiterates the toxicity of this compound to humans.

It was noted on application of the HPLC method and ELISA to urine and serum samples from ATR-treated rats, that the results of the ELISA do not strictly correlate with those of the HPLC, since the ELISA was occasionally seen to detect atractylosides when the HPLC method did not and *vice versa*. In the case where ATR was detected by HPLC but not by ELISA, it is suggested that the toxin was present at a level too low to be detected by the immunoassay and as demonstrated in Table 4.1, the levels of ATR excreted into the urine, as detected by HPLC were in the order of 0.02 to 0.2 μg ATR/ml which are essentially less than the detection limits of the ELISA (0.1 to 10 μg/ml).

A comparative summary of the HPLC and ELISA results are presented in Table 8.1, together with those of the immunocytochemical investigation. As noted from Table 8.1, the ELISA method detected of ATR in more of the samples over the entire dose range than did the HPLC despite the greater sensitivity of the latter method. This was probably due to the altered structure of ATR whose metabolites would probably show different retention times on HPLC, but might retain the epitope(s) recognised by the primary antibody. If this reasoning is correct, then the ELISA would be the method of choice if it were immaterial whether ATR or its metabolites were detected. However, if a quantitative analysis was required, then HPLC would be most suitable. In the case of the high dose regimen, the immunocytochemical method is the most suitable method for the diagnosis of ATR-poisoning since from these results it evident that ATR remains "fixed" within the

organism, even 72 hours after exposure, at which time the other methods essentially gave negative results. However, it is unlikely that this method of diagnosis would ever be applied to routine analysis in hospitals, but may provide a very effective means of determining cause of death in suspected *Impila* poisonings, since urine or serum may not easily be withdrawn from cadavers.

Table 8.1 Comparison of the efficacy of the HPLC, ELISA and immunocytochemistry (IEM) methods for the detection of atractyloside in rat urine and kidney*

Method	HPLC of urine			ELISA of urine					IEM of
Day	1	2	3	3	1	2	3	4	kidney
High	-	+	-	+	+	-	+	-	Detected
	-	+	-	+	+	-	-	-	until 72 hours
	+	-	-	-	+	-	-	-	after dosing
	+	-	-	-	+	-	-	-	when trial
	+	-	-	-	-	-	-	-	terminated
Medium	-	-	+	-	+	+	-	-	ND
	-	-	-	-	+	-	-	-	ND
	-	-	-	-	+	+	-	-	ND
	-	-	+	-	+	+	-	-	ND
	-	-	+	+	+	+	-	-	ND
Low	-	-	-	-	+	-	-	+	ND
	-	-	-	-	-	+	+	+	ND
	-	-	-	-	+	+	+	+	ND
	-	-	-	-	+	+	+	+	ND
	-	-	-	-	+	-	+	+	ND

*HPLC = high performance liquid chromatography, ELISA = enzyme-linked immunosorbent assay, IEM = immunocytochemical electron microscopy, + = ATR detected, - = ATR not detected, ND = not done

In summary, therefore, from the results of the HPLC and ELISA, it is proposed that ATR is in part biotransformed *in vivo*. There is no set rule for the biotransformation of a molecule, which makes any prediction of the metabolic fate of a xenobiotic such as ATR, very difficult. Some drugs or xenobiotics are not metabolised at all (Albert, 1985), while others only undergo conjugation (Thayer, 1990). The elimination of most xenobiotics from the body is poorly elucidated, but appears to be dependent upon a number of factors including the sex, age and nutritional status of the host species and of course, the nature of

the drug or toxin. The metabolic fate of the xenobiotic in one species is also not strictly applicable to another species; thalidomide being a case in point (Albert, 1985).

It is well known that ATR induces kidney necrosis and, from the results of the ELISA and HPLC, is released into the urine. The body is designed to eliminate xenobiotics from the system and this may involve biotransformation to promote excretion. Such biochemical alterations occur primarily in the liver, but may also occur in the plasma, lungs or kidney. Ultimately, biotransformation increases the polarity of the molecule and by enhancing its hydrophilicity, promotes excretion (usually in the urine). Biotransformation reactions occur in two phases. The first involves the oxidation, reduction or hydrolysis of the xenobiotic which all promote hydrophilicity (Albert, 1985). Phase II reactions involve the conjugation of the compound to metabolites such as glucuronate, sulphate, glycine or glutathione (Barbieri, 1990). Coupling is mediated through amine, alcohols, phenols, amides or carboxylic acid moieties present on the xenobiotic (Albert, 1985; Barbieri, 1990; Thayer, 1990). In humans, glucuronate is most frequently conjugated to the xenobiotic, and this involves the activation of glucose-1-phosphate by uridine diphosphate in a series of reaction steps to ultimately produce glucuronate (Thayer, 1990). A microsomal glucuryl transferase catalyses the transfer of the glucuronate to a polar group on the xenobiotic, frequently a hydroxyl group (Thayer, 1990). Sulphate may also be conjugated to the xenobiotic, usually through a hydroxyl group, by the intermediate PAPS and catalysed by a cytoplasmic sulphotransferase. Sulphation is less common than glucuronidation, owing to the limited *in vivo* availability of inorganic sulphate (Thayer, 1990).

It is unclear from the literature precisely in which cellular compartment biotransformation occurs, except that most of the enzymes are cytoplasmic, although organic acids are conjugated to glycine in the mitochondria (Albert, 1985). The results of the HPLC analyses indicate that ATR is at least partially metabolised *in vivo*, but the question remains as to whether this occurs in the liver or kidney. The results of the liver-enzyme profile presented in section 7.4, show that ATR does not induce liver damage. This fact could be interpreted to mean that the toxin is not metabolised or biotransformed in the liver, otherwise some damage would have been expected to occur. Therefore, it may be speculated that biotransformation of the ATR, at least when administered i.p., does not occur in the liver, but rather elsewhere, possibly in the plasma or even in the kidney itself. It is apparent from the literature, however, that the hydroxyl group is the preferential site of attachment (Albert, 1985; Barbieri, 1990; Thayer, 1990). Although this moiety is in fact hydrophilic, should conjugation of a glucuronide or glycine molecule occur at this site, then this would serve to increase the hydrophilicity of the toxin, promoting excretion, while

still enabling derivatisation with ADAM. This alteration in the polarity of the toxin would change its k' on a reverse phase column, resulting in an altered retention time, which could account for the failure to detect the molecule in some of the representative urine and serum samples. Sulphation is also a feasible method of biotransforming the ATR, but sulphation is mediated by PAPS and since it was pointed out in the literature review (section 2.4.3) that ATR inhibits the translocation of PAPS into the GA (Capasso and Hirschberg, 1984), theoretically, ATR may inhibit its own biotransformation and therefore this method of alteration is considered to be unlikely.

From Fig 2.3, it will be noted that ATR contains a hydroxyl and carboxyl group on the diterpene ring structure as well as a sugar moiety and two bisulphate esters. As the goal of biotransformation is to increase hydrophilicity, it is proposed that ATR is unlikely to be deglycosylated to form the aglycone, atractyligenin, but rather is conjugated to a metabolite either through the carboxyl or hydroxyl moiety on the diterpene ring. This site of biotransformation is supported by the fact that the antibodies raised against this toxin still recognised a metabolic product. Tijssen (1985) states that antibodies raised against haptens bind to epitopes furthest away from the point of binding of the hapten to the carrier (in this case the carboxyl group was used). For instance, if glycine is bound to the hydroxyl group, this would not alter the the epitope determinants. Thus, while it was suggested that the aglycone may be the primary metabolic product of ATR biotransformation. This is considered unlikely, since the aglycone is essentially hydrophobic. The identity of the metabolite(s) of ATR is a subject for future work, which will involve radiotracer studies and chemical identification of the products using nuclear magnetic resonance spectroscopy.

Apart from the observation that ATR is excreted into the urine and is apparently biotransformed *in vivo*, the results of the HPLC and ELISA assays showed that the period over which ATR was excreted into the urine was inversely proportional to the initial level of toxin administered and this may be explained as follows. With a high dose of toxin, more tubular damage will occur, resulting in inhibition of tubular active transport and ultimately excretion of xenobiotics and metabolites will be uncontrolled. Thus, at high doses of toxin, excretion will occur more rapidly. With a low dose of ATR, less tubular damage will occur, active transport and filtration will remain functional, albeit it depressed, and consequently, the excretion of xenobiotics will be more controlled. This parallels the typical excretion profiles of protein and albumin (Figs 7.2 and 7.3) which show damage followed by recovery. Some ATR may in fact be re-adsorbed into the proximal tubules and then enter the hepatic portal system, where the toxin will be

transported to the liver. Once in the liver, the toxin may undergo biotransformation only to be returned to the kidney, for excretion.

Toxin was apparently not excreted all at once or over a period of several successive days, but it appeared to be "fixed" within the animal and then at different time points, was released. This phenomenon is apparently not uncommon, as drugs may bind to macromolecules, to a point where all their binding sites are saturated, after which any free, unbound xenobiotic will be excreted (Bradford, 1990). An equilibrium will therefore be established between bound and free compound; the latter being available for biotransformation and excretion. The bound fraction acts as a depot and is released once the free form of the xenobiotic is excreted, thus re-establishing equilibrium (Bradford, 1990). A similar situation could occur with ATR which could explain the differential rates of excretion of toxin into the rat urine. However, without further studies on the pharmacokinetics of ATR, this proposal will remain highly speculative.

Although xenobiotics frequently associate with serum albumins, the results of the immunocytochemical investigation demonstrated that even 72 hours after an initial exposure to ATR, toxin was resident in the kidney, indicating that this was the primary depot site. This site of binding may be related to the route of toxin administration, since as ATR was administered i.p. it will bypass the portal blood system and enter the general circulatory system, probably being transported almost directly to the kidney, in a manner analogous to that of rectal administration of toxins (Barbieri, 1990). It is proposed that, in the main, the toxin is not bound to serum albumins, but remains bound in the kidney, hence the failure to detect it in the serum. As with the association of the xenobiotics with serum albumins, it is proposed that an equilibrium is established between bound ATR in the kidney and toxin excreted into the urine. Thus, as a consequence of damage and non-specific dissociation, ATR is excreted into the urine in a dose responsive manner. This would account for the appearance of ATR in the urine over a period inversely proportional to the dose administered.

In conclusion, with reference to the diagnostic assays, it is proposed that both the HPLC method and ELISA may be used for the detection of ATR, but the HPLC method, being expensive, is best applied to research orientated investigations, including pharmokinetic studies and monitoring the levels of ATR in plant extracts. Alternatively, the immunoassay is more suited for use as a diagnostic tool for ATR and its metabolites and may be easily converted into a field-test kit such as that described by Chantler and Hurrell (1982) for the diagnosis of snake envenomation, which requires no expertise nor expensive equipment. As with all diagnostic assays, there is a limited time period after exposure

when the assay may be used to detect ATR poisoning and thereafter, false negative results are likely. In the case of the assays for ATR, this is proposed to be within the first 24-48 hours after exposure (i.e., the period of during which ATR is not bound within the kidney but is excreted) despite the fact that the immunocytochemical investigation demonstrated that ATR is still resident in the kidney, 72 hours after exposure.

The conclusions regarding this study are limited by the fact that so little is known of the pharmacokinetics of ATR *in vivo*. Nevertheless, use was made of urinalysis and immunocytochemistry as an indication of both the pharmacokinetics of ATR in the rat, as well as the location of the target sites of this toxin. The results of the urinalysis demonstrated that ATR compromises glomerular permselectivity and distal tubular function, but this observation was not confirmed histologically. Atractyloside related proteinuria and albuminuria have previously been alluded to in the literature (Bhoola, 1983), but this is the first correlation between the appearance of protein and albumin in the urine and a compromise of glomerular permselectivity. It is also apparent that ATR inhibits the transport of glucose and amino acids in the proximal tubule, as indicated by glucosuria and the *in vitro* study of proline incorporation into isolated proximal tubule fragments. Hence, ATR is also likely to inhibit protein biosynthesis in the proximal tubule as a consequence of the inhibition of amino acid transport; the mechanisms of which would be an interesting project.

The abnormal excretion by ATR-treated rats of elevated levels of GGT and AP suggest that ATR targets for the proximal tubule, specifically the microvilli of the brush border. Similarly, it was also evident that on day 3 post-dosing, the levels of excreted GGT in the high and medium dose regimen were lower than that of the control group. This was also exhibited on day 4 in the high dose regimen. Similarly, AP was excreted into the urine on day 3, at a concentration less than that of the control, by all the rats exposed to ATR, and on day 4 this phenomenon was only seen in the low dose regimen. This "undershoot" may be attributed to an inhibition of protein biosynthesis by ATR, as suggested by the *in vitro* test on proline incorporation. The subsequent increase in the levels of enzyme excretion on day 4 in some dose regimens is indicative of cellular and tissue regeneration and hence recovery. The proposed tissue regeneration in the rats employed in the present study was apparently delayed, compared to that reported by Carpenedo *et al.* (1974) and Bhoola (1983) who noted renal repair within 48 hours after exposure.

Clearly some form of recovery is occurring, as demonstrated by the levels of calcium excreted by the ATR-treated rats, particularly the high dose regimen (Fig 7.8), since these levels rise after initial exposure to the toxin, indicating cellular damage, and then are

seen to decrease to a minimum level significantly less than that of the controls on day 3. Homeostasis is re-established in the organism under the control of parathyroid hormones, which leach available calcium from such areas as the bone, in order to return the serum calcium levels to above acceptable limits (noted in Fig 7.8 on day 3, when the levels of excreted calcium are significantly lower than basal control levels). This over-compensation of calcium retention is rectified by the subsequent excretion of calcium into the urine within 12 hours (Ungar, 1986) which is clearly demonstrated in Fig 7.8, where on day 4, levels of excreted calcium were seen to rise again.

The results of the urinalysis were confirmed immunocytochemically. As immunocytochemistry had previously not been used for the determination of ATR *in vivo*, the method was optimised on *C. laureola* tuber, essentially providing a positive control. Unfortunately, the tuber cells contain few organelles and seemed to consist of large areas of cytoplasm or vacuole and thus it was difficult to discern any form of cellular structure. However, there were areas of gold-probe "clumping", and from this it was suggested that ATR was encapsulated in vesicles. The difficulty experienced in preserving good cellular ultrastructure of the tuber limited further discussion and speculation on the storage site of the toxin in the tuber, but nevertheless it was demonstrated that the immunocytochemical detection of ATR in the plant is possible. As this investigation constitutes a very new area of research, little comment may be added, but it is anticipated that immunocytochemistry will provide the means to investigate the metabolism of ATR and related metabolites in the plant. Hence if ATR is synthesised and stored in the tuber, it may be possible to identify sites of synthesis and if the enzymes involved in this reaction may be inhibited, it is possible that significantly less toxic strains of *C. laureola* may be cultivated for commercial use.

A number of problems were experienced during the optimisation of the immunocytochemical procedure, notwithstanding the leaching of the toxin during fixation and dehydration of the tissue. This necessitated the carbodiimide-mediated coupling of ATR to intracellular proteins. Provided this pre-fixation step was carried out, the immunocytochemical localisation of ATR rat kidney was possible and the incorporation of relevant controls (Petrusz, 1983) validated the results. Up until the time of this study, details pertaining to the inhibitory effect of ATR had only been identified by histological observation (Carpenedo *et al.*, 1974) or *in vitro* assays (Klingenberg, 1978). In the present studies, immunolabelling of rat kidney specimens, demonstrated for the first time that ATR directly induces disruption of the microvilli within 24 hours after exposure, and is closely associated with these structures. Furthermore, mitochondrial swelling was noted, with the simultaneous localisation of ATR on the mitochondrial cristae. Mitochondrial

swelling is frequently associated with toxic insult and has been identified as a characteristic alteration in Reye's syndrome (Martens *et al.*, 1986). It was mentioned in the literature review (Chapter 2), that there may be some connection between Reye's syndrome and ATR intoxication, since the two present very similar biochemical and subcellular alterations. This is however, an area for extensive research. At 48 hours after exposure to ATR, this toxin was still detected on the microvilli and furthermore, was found to be associated with the GA membrane, confirming the proposal of Capasso and Hirschberg (1984) that ATR binds to the PAPS translocation on the GA membrane and further reiterating that sulphation is unlikely to be the means by which ATR is biotransformed *in vivo*.

Thus, the immunocytochemical investigation provided indisputable evidence that ATR targets for the proximal tubule of the kidney, specifically the microvilli of the brush border and furthermore, also binds to mitochondria and GA, the latter of which had only been demonstrated *in vitro*, with isolated organelles (Klingenberg, 1978; Capasso and Hirschberg, 1984). Atractyloside has usually been presumed to be a hepatotoxin, but it was evident from the results of the AST and ALT study, that the hepatotoxicity of ATR is insignificant, if the toxin is administered i.p., but this may not be the case should the toxin be administered orally, since the ATR will proceed directly from the intestinal tract via the hepatic portal system to the liver (Barbieri, 1990). Thus it is proposed by the present author, that when *Impila* is administered orally, ATR is transported directly to the liver, where it causes some damage before being biotransformed and ATR is possibly rendered less toxic, whereupon, the metabolised molecule is excreted in the urine. However, if *Impila* is administered rectally, it is proposed that in avoiding the portal circulatory system, the ATR is transported directly to the kidney where necrosis occurs. Being an ADP analogue, ATR may be reabsorbed into the circulatory system, entering the portal system and transported to the liver. Here, biotransformation occurs, whereupon ATR is returned to the kidney and it is excreted. This explanation could account for the fact that the rectal administration is more toxic than the oral route. Thus, in conclusion, if *Impila* is administered orally it is likely to be less nephrotoxic than if administered as an enema. This proposal also still holds if ATR is not the primary hepatotoxic principle in *Impila*, but some other active compound. By administering ATR i.p., as was carried out in this study, it is proposed that the toxin will follow a similar route to that of ATR administered rectally and thus, it is possible that this could account for the failure of ATR to induce liver necrosis in the present study. Thus, ATR may be significantly more hepatotoxic if administered orally, than i.p., or rectally.

Statistically, more deaths occur in children than in adults, following administration of *Impila* (Bhoola, 1983) but whether this is a consequence of more children actually taking the herbal remedy than adults, is a contentious point. Nevertheless, from extensive consultation with *Insangomas* and herbalists, it is apparent that the remedy is most likely to be administered to children as an enema and to adults *per os* and this may be a significant observation.

The dangers associated with the use of *Impila* have been confirmed by this study and although the ultimate aim was to develop an assay for ATR, bearing in mind that the assay would have international application since ATR is present in a number of plant species (Debetto, 1978) and also to localise the target sites of ATR, during the course of the investigation thought was given to potential therapeutic agents for the treatment of *Impila* poisoning. The reversal of T_2-toxin inhibition has been observed following administration of anti-T_2-toxin monoclonal antibodies to T_2-toxin treated cells (Hunter *et al.*, 1990) and these authors have proposed that these antibodies may find clinical application. Thus, if anti-ATR monoclonal antibodies were prepared instead of polyclonal antibodies to ATR, these may provide a means to prevent further ATR-related deaths if administered in a similar manner to anti-snake venom antiserum. The limitations of this would be that the patient could only be treated once in this manner since repeated therapy of this nature would induce anaphylactic shock (Tijssen, 1985). While this suggestion is very much for long term consideration, in the short term, duramycin may provide a potential antidote to *Impila* poisoning (section 2.4.2), since duramycin and ATR act synergistically, inducing stabilisation of the mitochondrial membrane (Sokolove *et al.*, 1989). To reiterate, duramycin induces destabilisation of the mitochondrial membrane, resulting in the removal of sequestrated intramitochondrial calcium. This calcium is deposited in the cytoplasm where it ultimately causes cell death and lysis. It was noted by Sokolove *et al.* (1989) that restabilisation of the mitochondrial membrane occurs when ATR is added to a cell preparation previously treated with duramycin. Hence, the converse may also apply, namely that ATR-induced destabilisation of the mitochondrial membrane may be reverted in the presence of duramycin. In view of the deleterious effect of calcium on the cell, it is proposed that the effect of ATR on intracellular calcium levels may be of more significance than the fact that this toxin ultimately inhibits oxidative phosphorylation. In this regard it may be of benefit in future, to administer duramycin to rabbits in conjunction with the ATR-naked bacteria conjugate, as a means of preventing the deleterious effects of this toxin on lymphocytes and thus, improving antibody response. Furthermore, it is proposed that the administration of free radical scavengers could protect patients against ATR poisoning, as ATR-induced damage to the mitochondria is associated with the generation of free

radicals, which exert a deleterious effect on the cell, in particular cell membranes. Furthermore, and probably of more therapeutic benefit, would be the administration of the the non-caloric sweetener, stevioside (Ishii and Bracht, 1986). This analogue of ATR also inhibits adenine nucleotide transport in the mitochondria (but only in intact cells, since the molecule cannot traverse the cell membrane) but is significantly less toxic than ATR (Ishii and Bracht, 1986). If rat livers are perfused with ATR, the deleterious effects of this toxin are reversed when stevioside is also perfused into the liver (Ishii and Bracht, 1986), a fact which certainly warrants further investigation.

The above paragraph refers to possible therapeutic agents which may reverse the effects of ATR, but the adage of "prevention being better than cure" is thought to be more applicable in this instance. From extensive consultation with traditional healers and western physicians it became apparent that the cultural belief of the Zulus "runs too deep" to ever be able to totally eradicate the use of *Impila*. The accidental deaths resulting from the use of *Impila* appear more commonplace than in previous years, and this is in part attributed to the fact that the retail of traditional medicines has become a very lucrative enterprise (see review by Bye and Dutton, 1991) analogous to the peddling of drugs of abuse. Hence, many herbal retailers have only a rudimentary knowledge of the therapeutic benefits of the remedies, and are particularly ignorant of the correct dosage.

Thus, it is the personal opinion of the present author that the only means left for the prevention of indiscriminate herbal-mediated deaths is to educate the herbalists of the potential dangers of such herbs, particularly if the incorrect dosage is given or alternatively if the remedy is scientifically demonstrated to be too toxic for routine use, then it must be declared "illegal". Undoubtedly, many of the herbal remedies in daily use contain beneficial active ingredients, which, if the practice of traditional medicine is terminated would be lost forever. It is the task of scientists and traditional healers alike to somehow collate this to the benefit of all and to conclude, therefore, in the words of Paracelsus:-

> *"What is there that is not poison?*
> *All things are poison and nothing (is)*
> *without poison. Solely the dose*
> *determines that a thing is not a poison"*

From the translation by Deichmann *et al.* (1986)

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PUBLICATIONS

Appendix A:

Bye, S.N., Coetzer, T.H.T. and Dutton, M.F. (1990) An Enzyme Immunoassay for Atractyloside, the Nephrotoxin of *Callilepis laureola* (*Impila*) *Toxicon*, **28**, 997

Appendix B:

Bye, S.N. and Dutton, M.F. (1991) The inappropriate use of Traditional medicines in South Africa, *Jnl. Ethnopharm*, **34**, 253

Appedix C:

Dehrmann, F.M., Bye, S.N. and Dutton, M.F. (1991) The isolation of a storage organelle of atractyloside in Callilepis laureola, *Jnl. Ethnopharm*, **34**, 247

Submitted:

Thompson, M.C., Dutton, M.F., Bye, S.N. and Dehrmann, F.M. (1991) An investigation into the passage of selected natural toxins across the digestive tract wall using the everted sac technique, *Journal of Natural Toxins* -submitted

In preparation:

Bye, S.N., Berry, R.K., Anderson, T.R. and Dutton, M.F.
The HPLC determination of atractyloside in body fluids

Bye, S.N., Kruger, F., Thurman, G.D. and Dutton, M.F.
An improved method for the production of antibodies to atractyloside using a *Salmonella minnesota* carrier

Bye, S.N. and Dutton, M.F.
The detection of atractyloside in body fluids using ELISA

Bye, S.N., Delacruz, L., Moret, M. and Bach, P.H.
An investigation into the effect of atractyloside on kidney morphology and the appearance of urinary markers

Bye, S.N., Dehrmann, F., Elliott, E. and Dutton, M.F.
The immunocytochemical localisation of atractyloside in rat tissue

Toxicon Vol. 28, No. 8, pp. 997–1000, 1990.
Printed in Great Britain.

0041-0101/90 $3.00 + .00

AN ENZYME IMMUNOASSAY FOR ATRACTYLOSIDE, THE NEPHROTOXIN OF *CALLILEPIS LAUREOLA* (IMPILA)

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(*Accepted for publication* 6 *March* 1990)

S. N. BYE, T. H. T. COETZER and M. F. DUTTON. An enzyme immunoassay for atractyloside, the nephrotoxin of *Callilepis laureola* (Impila). *Toxicon* **28,** 997–1000, 1990.—Tubers of *Callilepis laureola,* a traditional remedy, contain an inhibitor of oxidative phosphorylation; atractyloside. A "competitive" ELISA was developed, using the antiserum produced to an atractyloside–protein conjugate. An ovalbumin–atractyloside conjugate was adsorbed to microtitre wells and plates incubated with sample (atractyloside or tuber extract) and antiserum. After successive incubation with secondary antibody–enzyme conjugate and substrate, the absorbance was read at 405 nm. Antibody working dilution was low, but results, confirmed by thin layer chromatography, indicate the immunoassay has diagnostic potential.

THE ZULUS and other African people regard Impila, the rootstock of *Callilepis laureola* as a powerful medicament. Infusions of the tuber, administered orally or rectally, are reputed to ward off evil spirits (WAINWRIGHT *et al.,* 1977). The tuber is, however, highly toxic (BRYANT, 1909) inducing both liver and renal necroses, with often fatal results (BHOOLA, 1983, Thesis, University of Natal). The hepatotoxin is unknown, but atractyloside (Fig. 1) has been identified as the nephrotoxin (CANDY *et al.,* 1977). This diterpene glycoside competitively inhibits the transport of ADP across the inner mitochondrial membrane, so terminating oxidative phosphorylation (SANTI, 1964). Atractyloside has been isolated from the rhizomes of the Mediterranean thistle, *Atractylis gummifera* (SANTI and LUCIANI, 1978) and coffee beans (RICHTER and SPITELLER, 1978). Consequently, this glycoside has been implicated as a mediator of pancreatic cancer in coffee consumers (PEGEL, 1981). Atractyloside has been detected in plant extracts, using thin layer chromatography (BROOKES, 1979, Thesis, University of Natal) but diagnosis of atractyloside poisoning has been restricted to histopathological means, with inconclusive results. The aim of this study was to develop an enzyme immunoassay specific for atractyloside. The specificity of this assay makes it suitable for diagnostic purposes and may assist in confirming atractyloside-mediated deaths.

Atractyloside (potassium salt; Sigma, St Louis, MO) was coupled to free amino groups on bovine serum albumin (BSA; Boehringer Mannheim, Johannesburg, RSA) and ovalbumin (Grade III; Sigma, St Louis, MO) respectively, by a mixed anhydride reaction,

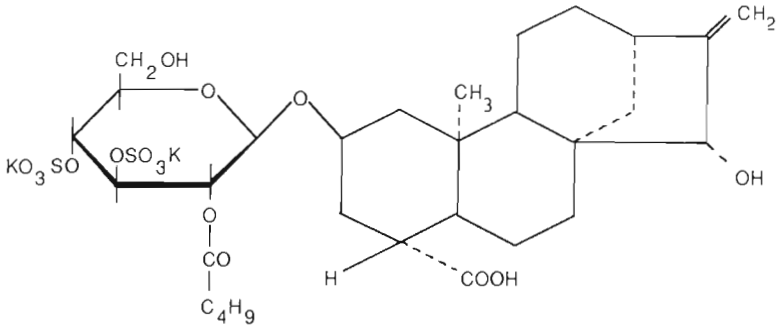

FIG. 1. THE STRUCTURE OF ATRACTYLOSIDE.

using the procedure of FUCHS and FUCHS (1969). Atractyloside (125 mg) was dissolved in dioxane (1.5 ml) and N,N′-dicyclohexylcarbodiimide (30 mg) added. The solution was stirred for 30 min at room temperature and centrifuged. The supernatant contains the acid anhydride and this was added dropwise to an ice-cold solution of protein (100 mg) in 0.1 M borate buffer, pH 8.5 (7 ml) and stirred overnight at 4°C. The conjugate was purified by dialysis for 16 hr against 0.1 M borate, pH 8.0 and 5 days against daily changes of distilled water (WEILER and WEICZOREK, 1981). The non-diffusible material was lyophilized.

Rabbits were immunized s.c. (1.0 ml) with a stable emulsion of atractyloside-BSA conjugate (2 mg/ml) triturated with equal volumes of physiological saline and Freund's adjuvant (Difco, MI). Boosters were administered a week later, twice fortnightly and thereafter monthly. Blood was collected from the marginal ear vein and immunoglobulin fractions were prepared by the method of POLSON (1977).

The "competitive" immunoassay was carried out with procedures modified from those of ROBINS (1986) and WEILER (1986). An atractyloside–ovalbumin conjugate (0.01 mg/ml) in 0.05 M carbonate buffer, pH 9.6, was adsorbed to NUNC microtitre wells (Maxisorp; Weil, Johannesburg, R.S.A.) for 16 hr at 37°C. Between all subsequent steps, plates were washed thrice with tap water (WEILER, 1986). Pure atractyloside, in incubation buffer (0.02 M Tris buffered saline, pH 7.4 containing 0.1% Difco-Bacto gelatin; w/v, 100 μl/well) and an equal volume of rabbit antiserum, diluted 1:10 in incubation buffer, were added to each well. Plates were incubated for 1 hr at 37°C, washed and incubated with a 1:3000 dilution of the goat anti-(rabbit IgG) IgG-alkaline phosphatase (EC 3.1.3.1) (Sigma, St Louis, MO) prepared in incubation buffer (200 μl/well) for 1 hr at 37°C. Freshly prepared enzyme substrate (1 mg/ml *p*-nitrophenyl phosphate; Boehringer Mannheim, Johannesburg, R.S.A.; in 0.1 M glycine buffer, pH 10.4, containing 0.001 M $MgCl_2$ and $ZnCl_2$) was added (200 μl/well) and the plates incubated for 1 hr at 37°C. The reaction was stopped by addition of 6 M KOH (50 μl/well) and the absorbance read at 405 nm, using a Biotek spectrophotometer.

Working dilution of primary antibody was notably low, but this phenomenon has been noted with toxic haptens. HUNTER *et al.* (1985) attribute the low working dilution of antiserum to T_2-toxin, to be the result of immunotoxicity. Using a Marbrook Chamber they demonstrated that T_2-toxin was released from a freshly dialysed T_2-toxin-BSA conjugate and inhibited protein biosynthesis in tissue cultures. Although atractyloside has a different inhibitory action, a similar situation may exist and is an area for future consideration.

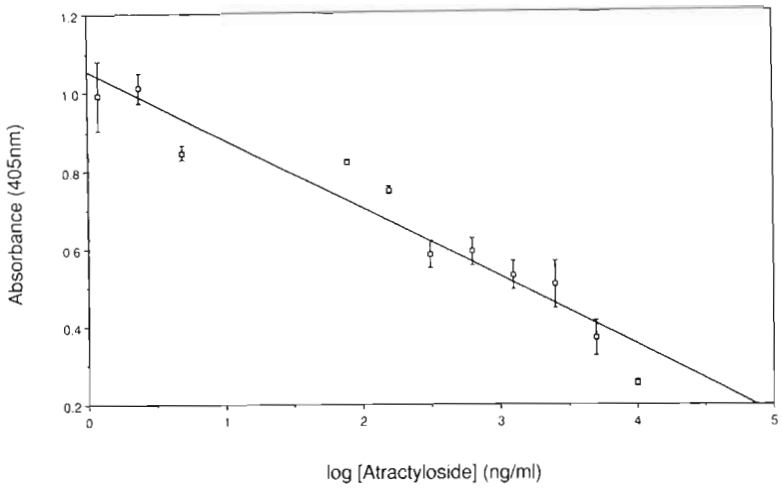

FIG. 2. STANDARD CURVE FOR THE DETERMINATION OF ATRACTYLOSIDE BY "COMPETITIVE" ELISA. Plates were coated with 0.01 mg/ml ovalbumin–atractyloside conjugate. To this was added the primary antibody and atractyloside. The correlation coefficient (r) was 0.97. Results represent the mean of three determinations on a single ELISA microtitre plate. Error bars represent the standard error. The coefficients of variations ranged from 3.1–13.4%. Toxin concentrations on the X axis are in log scale.

Throughout the procedure, suitable controls were included. Each step in the immunoassay was successively omitted, to determine non-specific binding and presence of endogenous phosphatase activity which could contribute to the final absorbance reading. In addition, the adsorbed conjugate was substituted with ovalbumin to determine non-specific binding by both primary and secondary antibodies. In all instances, readings were negligible, confirming the specificity of the assay. Antiserum was replaced with both pre-immune and inappropriate antiserum, but evidence of cross-reactivity was not apparent. The possible presence of coupling agent-modified residue antibodies was investigated (BRIAND *et al.*, 1985). Ovalbumin and conjugation reagents, excluding the hapten, were reacted and adsorbed onto microtitre plates. The immunoassay was carried out and absence of colour formation on addition of substrate, excluded the presence of coupling agent-modified residue antibodies. Furthermore, as atractyloside contains an ent-kaurene backbone (kaurenoic acid being a precursor to the gibberellin plant growth hormones) gibberellin GA_3 was tested, but cross-reactivity was negligible. From the results obtained it was concluded that the antiserum raised against atractyloside was specific for this compound. The results of the standard enzyme immunoassay are given in Fig. 2. The reactivity of the antibody examined by the immunoassay correlated with concentrations of atractyloside ranging from 10–10,000 ng/well. The assay was repeated on crude methanol extracts of the tuber. Atractyloside or related compounds were detected in the methanol extract and results were confirmed by thin layer chromatography. The presence of methanol did not appear to interfere with the assay. No attempt was made to quantify toxin levels in the samples. It has been suggested that the quantitative reporting of immunoassay results is inappropriate unless the assay has been verified by another method (BASELT, 1989). Investigations are underway to develop an alternative assay for atractyloside and to optimize detection of atractyloside in body fluids.

To our knowledge, this is the first report of the production of antibodies to atractyloside and although the immunoassay requires further development, the assay shows poten-

tial for diagnostic purposes and immunocytochemical investigations. The latter should reveal sites of storage of toxin in the tuber and the pathophysiology of the glycoside in humans.

Acknowledgements—The authors thank Mr R. K. Berry, Mr L. Jackson and Steve Wozniak for their advice and assistance.

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Journal of Ethnopharmacology, 34 (1991) 253—259
Elsevier Scientific Publishers Ireland Ltd.

The inappropriate use of traditional medicines in South Africa

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Traditional medicines are culturally very important to the Zulu population of Southern Africa. The inappropriate use of herbs has however, resulted in numerous fatalities, invariably in children. This paper briefly summarises the belief of the Zulu population in traditional remedies, together with an outline of the problems at present being experienced in South Africa. A further note on the personal impressions and experiences of the authors and description of one such toxic herbal remedy, including use, toxic action and research carried out to date, are also given.

Key words: herbalism; Zulu medicine; atractyloside, nephrotoxin.

Introduction

Western ideology has largely underestimated the inordinate belief in traditional medicines by many people, particularly those from developing countries. The reliance of the Zulu population on traditional remedies is no exception (Wainwright et al., 1977; Ellis, 1986).

Geographically, the Zulus are concentrated in the north-east of South Africa, namely the Natal/KwaZulu region. Culturally, the Zulus believe disease to be a manifestation of disharmony between a person and the ancestors (Bryant, 1909). Sorcery and medicine are hence intrinsically linked and it is the function of the traditional healer, regarded as the protector of society, to act as diagnostician, apothecary and diviner (Krige, 1981).

The *Isangoma*, or diviner, is the diagnostician and augur of society and consults with the spirits to identify the source of an affliction. The herbalist, or *Inyanga*, claims no affiliation to the spirits, however, but functions as an apothecary, preparing and dispensing various herbal remedies (Kiernan, 1978). Conceivably, the link between magic and medicine has resulted in two distinct categories of traditional medicine. White medicine *(Umuthi omhlophe)* is used for the amelioration of physical afflictions, while black medicine *(Umuthi omynama)* is thought to exorcise evil spirits (Krige, 1981).

Despite the westernization of the Zulus through urbanization and education, the belief in traditional remedies and healers remains firm. A dire shortage of western doctors in the rural areas, estimated in 1982 to be in the ratio of one medical practitioner for every 17,500 people (Savage, 1985) has forced rural inhabitants to consult with traditional healers, if not by choice, then certainly by necessity. With the exponential population growth rate, the demand for traditional remedies is increasing at an insatiable rate, placing severe strain on already depleted natural resources (Cunningham, 1988).

Efforts are in progress to rationalise conservation policies regarding the gathering of herbs for medicinal purposes. Such local organizations as the KwaZulu Bureau of Natural Resources, Natal Parks Board, and the Durban Municipality, in conjunction with the Institute of Natural

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Resources (University of Natal, Pietermaritzburg), are investigating methods to overcome exploitation of flora and fauna for medicinal purposes, without inhibiting cultural practices. Commercial cultivation and inclusion of medicinal herbs as part of an annual crop by subsistence farmers, have been suggested as means of counteracting this problem.

The demand for herbs with the simultaneous decline in availability has served to increase the price of traditional remedies dramatically (Cunningham, 1988). Retail of these medicaments is, therefore, very lucrative, but some store proprietors claim to be ignorant of the beneficial properties of the herbs, rather relying on the knowledge of informed employees (Hutchings and Terblanche, 1989). The interior of a typical traditional remedy shop is shown in Fig. 1 (a and b).

By law all herbalists in KwaZulu are required to be registered, a prerequisite being that the person is "skilled in herbalism" (Zulu Law, Act 6 of 1981). To be registered as a herbalist, certain conditions must be met. The applicant must be black, have served an apprenticeship for seven years with a registered herbalist and be in possession of character references from the local Magistrate, Headman and a teacher or police officer. Application is then made to the Secretary for Health, KwaZulu, who in turn, notifies the Herbalist Association (Nyanga National Association, NNA). Officials from the NNA examine the applicant's knowledge of herbs and herbalism and if successful, the applicant pays a nominal fee for a permit, allowing the herbalist to practice his trade (Xaba, pers. commun., 1989). Registration, appears, however, to be relatively unsuccessful (Cunningham, 1988).

What then are the implications of ignorant people dispensing tribal medicaments? Despite routine use of some 400 herbs, the pharmacological properties are essentially unknown and many contain highly toxic principles (Gundidza, 1985). Undoubtedly, the use of herbal remedies has had fatal consequences, but the precise number of herb-induced fatalities is unknown. Suspected herbal-mediated deaths, primarily in infants, have done little to improve communications between doctors, traditional healers and patients alike (Hutchings and Terblanche, 1989). Many people are reticent to admit either using or administering traditional remedies. Some doctors, particularly paediatricians, express anger at the parents, who through ignorance, administer herbal remedies to healthy babies, resulting in the child's death.

The breakdown in communication is further aggravated by the fact that when the child enters the hospital, it is usually too late to respond to treatment and invariably dies. Therefore, the parents view western medical practices with suspicion, believing the doctors and not the herb potentiated the child's death. It was recounted that on one occasion a child was admitted to a rural hospital suffering from hepatitis and after intensive care treatment, the now fully recovered baby was discharged. A week later the child died as a consequence of the administration of a herbal remedy. The feeling of impotence was highlighted by the comment that the effort put into saving the child was in fact wasted.

Health officials working in these rural hospitals are all too aware of the problems of herbal intoxications, but at present are unable to alleviate the situation. It is indeed most sobering when visiting these rural hospitals to be greeted by a delegation of nursing sisters, who thank one in advance for coming and telling them "how to save the children", especially when you know that there are no answers at this time and the work you are covering is but the "tip of the iceberg". One is supposed to remain unemotional and clinical in science, but it is extremely difficult not to be affected when you are presented with such a situation.

Personally, one of the major hurdles to be crossed is convincing higher authorities of the problems. At present post mortems are not being carried out in some of these hospitals, unless unnatural cause of death is suspected. Herbal intoxications are not considered to be an unnatural cause of death, as if they were to be so, a murder docket has to be opened and a full police investigation mounted. Here one enters an ill-defined area, as can one be convicted of believing in one's tribal customs? This matter will remain unanswered, until such time as the precise number of herb-induced fatalities are known. With this information, authorities will have to take cognisance of these

Fig. 1. Interior of a typical herbal medicine shop. (a) Window view, demonstrating the use of mixtures of milled plant material, stored in jars for distribution (b) Interior of store, demonstrating use of both animal and plant material for medicinal purposes (note: skins in background and bundles of reeds and tuberous plants in foreground).

deaths and decisions regarding the legal implications debated.

Precise analytical procedures will have to be developed to identify the type of poisoning and this will be concomittant to investigating the toxicity of popular medicinal herbs. In this manner it will be possible to accumulate data regarding the incidence of herbal intoxiciations in South Africa and hopefully identify which herbs are responsible. It has also been suggested that these toxic herbs be scheduled, on a system akin to that used for western pharmaceuticals (Cunningham, 1989). This information could be used to educate herbalists and rural inhabitants alike, and here cognisance will have to be taken of the cultural beliefs of the people. In addition, it may be possible to cultivate non-toxic forms of the plant and this will most likely present the most beneficial method. The depletion of natural resources may for once be a blessing in disguise, as only non-toxic strains may be cultivated by choice in commercial ventures.

These are obviously long term plans and the primary objectives at this time are to identify the toxic species and the toxic components. Suitable diagnostic assays will have to be developed, the toxicological action of these compounds investigated and an attempt made to devise antidotes for the compounds. At the same time the incidence of herbal poisonings can be obtained. With at least 88 toxic species to cover, this will require a great deal of time, energy and finance. Unfortunately, the latter is slow in forthcoming, but unless research of this nature is initiated, the indiscriminant deaths will continue unabated.

With this in mind, over the past three years, work has been initiated on one such toxic herb, namely *Callilepis laureola,* the idea being to perfect methodology on one such plant and then apply this to other toxic species. A member of the family Compositae, *C. laureola* is commonly known as the ox-eye daisy or *Impila* (Zulu = health). This perennial bears a tuberous root, likened to a sweet-potato (Wainwright et al., 1977, Brookes et al., 1983) and flowers from August to November, bearing solitary creamy-white flowers with a purple disc (Dyer, 1975; Hilliard, 1977; Palmer, 1985).

The Nguni people (Zulu, Bantu, Sotho) regard *Impila* as a valuable *muthi* (medicine) (Dlamini, pers. commun., 1987). The tuberous rootstock is used by the Zulus to prepare the potion (Debetto, 1978), although a leaf infusion is reputed to have limited curative properties (Bryant, 1909). The tuber is harvested in winter, dried and crushed (Bryant, 1909; Ellis, 1986). The resultant pulp is boiled for 30 min in a suitable volume of water and the decoction administered either orally or rectally (Insangoma, pers. commun., 1987).

Essentially a multi-functional *muthi, Impila* is taken to ameliorate stomach complaints, tapeworm infestations (Bryant, 1909), to induce fertility (Debetto, 1978), as a cough expectorant and whooping cough remedy (Maberley, 1906, cited by Watt and Breyer-Brandwyk, 1962). Undoubtedly though, the "protective powers" of this plant are its greatest attribute. A tuber buried in the vicinity of one's home is thought to intercept any evil directed towards the household (Dlamini, pers. commun., 1987), while an *Impila* tincture consumed prior to festivals is believed to offer protection from "those harboring evil intent" (Wainwright et al., 1977). Independent surveys indicate that the popularity of this herb cannot be underestimated (Bhoola, 1983; Ellis, 1986).

Why the scientific interest in *Impila*, when this herb is but one of 400 used and is by no means the most popular? In the mid-1970s, the high incidence of centrilobular liver necrosis in blacks residing in the Natal/KwaZulu regions, was cause for concern (Wainwright and Schonland, 1977). Invariably accompanied by renal necrosis, this hepatic damage accounted for 2% of all deaths at the King Edward VIII Hospital, Durban, over the period 1958—1977 (Bhoola, 1983). *Impila* was identified as the primary causative agent (Wainwright et al., 1977; Bhoola, 1983) and to date some 260 deaths, a third of these being in children of less than five years of age, have been attributed to use of this herb (Brookes, 1979; Bhoola, 1983). In view of the popularity of this herb and the unavailability of a specific assay to determine *Impila* poisoning, these figures are deemed conservative (Brookes, 1979).

The reluctance of the black population to admit using herbal remedies is highlighted by the fact that in only 20% of suspected herbal intoxications was use of traditional remedies conclusively pro-

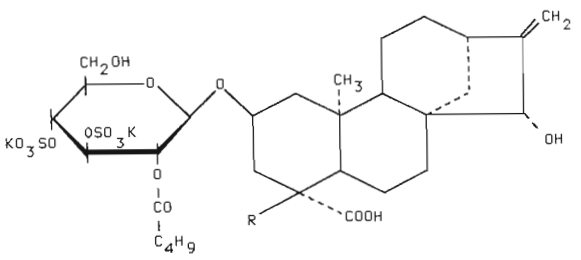

Fig. 2. The structure of atractyloside (R = H) and carboxy-atractyloside (R = COOH).

ven. Nevertheless, significant recurrent clinical and biochemical changes were noted in patients exhibiting centrilobular liver necrosis and of these, 59% were suspected to be *Impila* poisonings. Vomiting, jaundice, abdominal pain and convulsions were commonly seen. Hypoglycaemia, uraemia and evidence of disturbed liver function were presented. Clinical records showed that the duration of the illness was short and in 91% of cases, resulted in death. In summary, patients with suspected *Impila* poisoning demonstrated renal and hepatic necrosis, with associated hypoglycaemia, which usually culminated in death (Bhoola, 1983).

The major toxic principles, extracted from the tuber of *C. laureola* are atractyloside (ATR) and carboxy-atractyloside (CATR) shown in Fig. 2 (Brookes, 1979). Atractyloside was first isolated from the Mediterranean thistle *Atractylis gummifera.* This plant is still used for the treatment of syphilitic ulcers, for inducing abortions, and for bleaching teeth, but intoxication by this plant is not uncommon (Georgiou et al., 1988). Hydrolysis products of ATR consist of 1 mol of glucose, 2 mol sulphuric acid and 1 mol each of isovaleric acid and atractyligenin, respectively. Atractyligenin is an acidic diterpene with a basic 18-nor(−)kaurene skeleton (Ghisalberti et al., 1968) the latter being a precursor to the gibberillins (Takahashi et al., 1986). Subsequently, CATR was extracted from *A. gummifera* tubers. Identical to ATR, apart from a second carboxyl group on C_4 of the diterpene ring, CATR is restricted to fresh tubers and thermally decomposes to ATR (Bombardelli et al., 1972).

Compounds related to ATR are not restricted to *A. gummifera* and *C. laureola,* since an ATR-analogue has been isolated from coffee beans. In this derivative, the glucose moiety is replaced by glucuronic acid and is devoid of the sulphate and isovaleric esters. This compound is excreted in the urine of habitual coffee drinkers at the levels of 0.001 mg/ml (20—40 mg/day) (Obermann and Spiteller, 1976). It has been suggested that the sub-lethal but chronic administration of this compound may mediate pancreatic cancer in coffee drinkers (Pegel, 1981). As no suitable analytical test is available, levels of this toxin in coffee beverages are not being monitored and implications of this could be far reaching.

Atractyloside is the nephrotoxic agent (Bhoola, 1983) and its renal toxicity is well documented (Carpenedo et al., 1974). Furthermore, ATR is a potent hypoglycaemic agent (Luciani et al., 1978). Carboxyatractyloside, though inherently more toxic than ATR, is devoid of nephrotoxicity. This is either because CATR induces death before cellular damage is visible, or the increased polarity of CATR impedes access of the molecule to tubular cells. While the renal toxin of *Impila* has been conclusively identified, the hepatotoxin is as yet unknown.

Mitochondria have been identified as the target organelles of ATR (Vignais et al., 1962) and ATR has been conclusively demonstrated to inhibit oxidative phosphorylation and thus energy transduction (Santi, 1964; Bruni et al., 1964). The most active intramitochondrial reaction is ATP synthesis, whereby ADP is phosphorylated by ATP synthase, to produce energy in the form of ATP. By virtue of the high turnover rate of this reaction, transport of substrates such as ADP, P_i and ATP, associated with oxidative phosphorylation, must be both active and efficient (Klingenberg, 1978). These compounds are, however, hydrophilic and cannot traverse the inner mitochondrial membrane, unless facilitated by protein carriers. Two carriers are involved, one transporting P_i and the other, a protein translocase, importing ADP and exporting ATP to and from the matrix (Nicholls, 1982). Atractyloside competitively inhibits the transport of endogenous (cytosolic) ADP across the inner mitochondrial membrane, by binding to

the ADP-binding site on the intermembrane side of the protein translocase. This prevents passage of ADP across the inner membrane, ATP cannot be synthesized and without usable energy, the cell dies (Chappell and Crofts, 1965).

The toxicity of *Impila* cannot be refuted, and it was the initial aim to develop a diagnostic assay for use in both hospitals and forensic laboratories. Thereafter, it was decided to investigate storage sites of ATR in the plant and target sites of the toxin in mammals. Antibodies were raised to ATR, for use in both immunoassays (Bye et al., 1989) and immunocytochemical studies. In this manner it will be possible to detect ATR and ATR-analogues in biological fluids, both plant and animal. In addition, the availability of anti-(ATR) antibodies will provide visual evidence of storage sites of the toxin in the plant, target mammalian organs and organelles and hence subcellular binding sites of the toxin in animal tissue. Immunocytochemical location of the toxin in the tuber has been carried out and though requiring further refinement, may be applied to mammalian systems.

We have been accused of merely presenting the "bad side of the coin" and certainly, all the herbal remedies which are used with success are not seen by either ourselves or the doctors. Undoubtedly many traditional remedies are beneficial and do have a definite role to play in rural health, but what is of concern is that many of the potentially toxic herbs are in fact used for their "magical properties" and are doing little to prevent disease. The purpose of this paper was to give a brief resumé of work being carried out on toxic Zulu herbal medicaments. No attempts have been made to delve into the pharmacological potential of the plethora of herbs used by the Zulus and nor has the work being carried out in other scientific institutions been considered. The objective of the research being carried out at this University is, as mentioned, to develop assays for the toxic herbs and accumulate data regarding the incidence and type of medicinal herbs being used. In addition, this data can be used to inform both authorities and rural inhabitants alike of the dangers of these remedies and attempt to terminate the senseless deaths of black children.

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Journal of Ethnopharmacology, 34 (1991) 247–251
Elsevier Scientific Publishers Ireland Ltd.

The isolation of a storage organelle of atractyloside in *Callilepis laureola*

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(Accepted May 28, 1991)

A method has been developed for the preparation of protoplasts from both the leaves and tubers of *Callilepis laureola*, a plant used extensively as a medicament by black people in South Africa. The cellular vacuoles from these protoplasts were isolated and tested for the presence of the nephrotoxic substance, atractyloside, by thin layer chromatography and immunoassay. Both methods indicate that the vacuole of *C. laureola* is the primary site of storage for atractyloside in the cells of the tuber.

Key words: protoplasts; vacuoles; nephrotoxin; hepatotoxin; ELISA.

Introduction

The herbaceous plant *Callilepis laureola* is used extensively by the black tribes of South Africa as a herbal medicament. Its toxicity, however, was initially documented at the turn of the century (Bryant, 1909). An extraction prepared from the tuber of this plant is called *Impila* by the black population and, although it has use as a vermifuge and decongestant, the herb is primarily administered to ward off evil (Wainright et al., 1977). Infusions of the tuber, administered orally or rectally, induce both liver and renal necroses, with associated hypoglycaemia (Watson et al., 1979; Bhoola, 1983).

The diterpene glycoside, atractyloside, extracted from the tuber, has been identified as the nephrotoxin (Candy et al., 1977) but the hepatotoxin remains undetermined. Atractyloside (ATR) (Fig. 1) was first isolated from the rhizomes of the Mediterranean thistle, *Atractylis gummifera* (Santi and Luciani, 1978) and an associated metabolite has been extracted from coffee beans (Richter and Spiteller, 1978).

Atractyloside inhibits the transport of adenine nucleosides across the inner mitochondrial membrane and this inhibitory action is not restricted to mammalian cells but has also been demonstrated in cauliflower (Jung and Hanson, 1973). This suggests that *C. laureola* must possess an efficient transport and storage system, whereby intoxication of the plant is avoided. Atractyloside contains an ent-kaurene moiety, which is known to be the precursor to the gibberellins. As these growth hormones are stored in the vacuole and this organelle is responsible for their metabolic sequestration and storage (Wagner, 1982) it may well also store ATR.

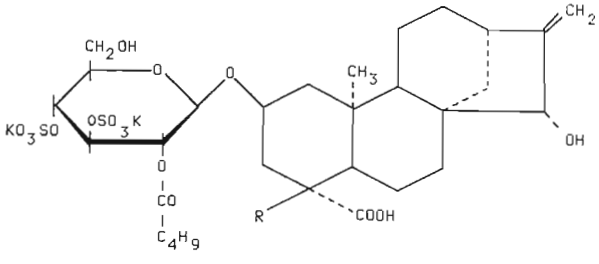

Fig. 1. The structure of atractyloside.

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0378-8741/$03.50

The aim of the work reported here was to establish and optimise a protocol for the isolation of vacuoles from the tubers of *C. laureola*, via the intermediate step of forming protoplasts; and to identify the vacuole as a possible storage organelle of ATR in this plant.

Materials and Methods

Materials

C. laureola specimens were collected from Eshowe, Natal. The pectinase Macerozyme and cellulase Onozuka RS enzymes were purchased from Yakult Honsha (Japan). Mannitol, ficoll, goat anti-(rabbit IgG) IgG-alkaline phosphatase conjugate and ATR (potassium salt) were obtained from Sigma Chemical Co, St Louis, U.S.A. Rabbit anti-atractyloside antibodies were prepared as described by Bye et al. (1990). Anisaldehyde was purchased from Merck (R.S.A.) and NUNC microtitre plates were obtained from Weil (Johannesburg, R.S.A.). All other reagents were of analytical grade.

Protoplast production

Fresh *C. laureola* tuber (1 g) was chopped (± 2 mm^3) and surface sterilized in sodium hypochlorite and Tween 80 (1% and 0.1%, respectively) solution for 10 min at room temperature. Sterilized tissue pieces were washed thoroughly with basal medium (0.5 M mannitol, 1 mM each of magnesium chloride and potassium dihydrogen phosphate and 0.002 g/ml each of citric acid and ascorbic acid, pH 5.6) to remove all traces of sterilants. The washed tissue was incubated for 6 h at 26°C at 70 strokes/min in 20 ml of basal medium containing 2% Macerozyme and 4% Onozuka RS (w/v), respectively. The solution was filtered through a 220-μm mesh filter and pooled with the initial filtrates. Aliquots (0.5 ml) of this suspension were added to equal volumes of Evan's blue dye (2% w/v in basal medium) and average yields determined by counting on an Improved Neaubauer Haemocytometer. Centrifugation of the filtrate at 100 × *g* for 10 min caused viable protoplasts and cellular debris to sediment. The supernatant was discarded, the pellet resuspended in basal medium and overlayed onto a 0.6 M sorbitol solution containing 25 mM potassium dihydrogen phosphate and titrated to pH 5.5 with citric acid. Centrifugation at 100 × *g* for 3 min allowed intact protoplasts to band at the interface and purity was evaluated microscopically.

Vacuole isolation

Various protocols were tested but that of Grandstedt and Huffaker (1982) proved most reproducible. Purified protoplasts from the tuber were removed from the interface with a pasteur pipette and resuspended in 6 equal volumes of 12% ficoll (w/v) containing 60 mM potassium dihydrogen phosphate (pH 8.0) and 1 mM dithiothreitol (DTT). Repeated inversions of the tube for 5 min caused protoplasts to lyse and the solution was overlayed with 2 volumes of 0.6 M mannitol solution with 50 mM potassium dihydrogen phosphate (pH 7.0) and 1 mM EDTA (sodium salt). Centrifugation at 1000 × *g* for 10 min caused vacuoles to migrate to the top 1—2 volumes (0% ficoll fraction) while cellular debris sedimented. Vacuoles were visualised with acidophilic neutral red dye.

The 0% ficoll phase, interphase and pellet fractions were removed for analysis by thin layer chromatography and immunoassay.

Thin layer chromatography

Samples of the resuspended pellet, interface and 0% ficoll fractions (20 μl) were spotted onto the origins of two-dimensional thin layer chromatography (TLC) plates (10 × 10 cm aluminium backed silica gel G 60, Merck). Suitable controls, i.e., a standard ATR solution (1 mg/ml), and a standard sugar solution containing equal amounts of basal medium, sorbitol solution and vacuole isolating medium, were also subjected to the same TLC system. All the chromatograms were developed in chloroform/methanol (3:2 v/v) in the first dimension and butanol/acetic acid/water/chloroform (6:2:2:1 v/v/v/v) in the second dimension. The chromatograms were air dried, sprayed with anisaldehyde reagent (Brookes, 1979) and heated at 120°C for 3 min.

Enzyme immunoassay

A competitive immunoassay was carried out, as

described by Bye et al. (1990). Briefly an ovalbumin-atractyloside conjugate (0.01 mg/ml in 0.05 M carbonate buffer, pH 9.6) was adsorbed on microtitre wells for 16 h at 37°C. The plates were washed three times with tap water and pellet, interface and vacuole fractions (undiluted; 1:10; 1:100; and 1:1000, diluted in 0.02 M tris buffered saline, pH 7.4 with 0.1% Difco-Bacto gelatin) were added to wells (100 μl) followed by an equal volume of rabbit anti-atractyloside-IgG, diluted 1:20. Plates were incubated for 3 h at 37°C, washed and the secondary antibody-enzyme conjugate (diluted 1:3000) added (200 μl/well). The plates were incubated for 1 h at 37°C, washed and enzyme substrate added (1 mg/ml *p*-nitrophenyl phosphate, in 0.1 M glycine buffer, pH 10.4 containing 0.001 M magnesium chloride and zinc chloride). The plates were incubated for 1 h at

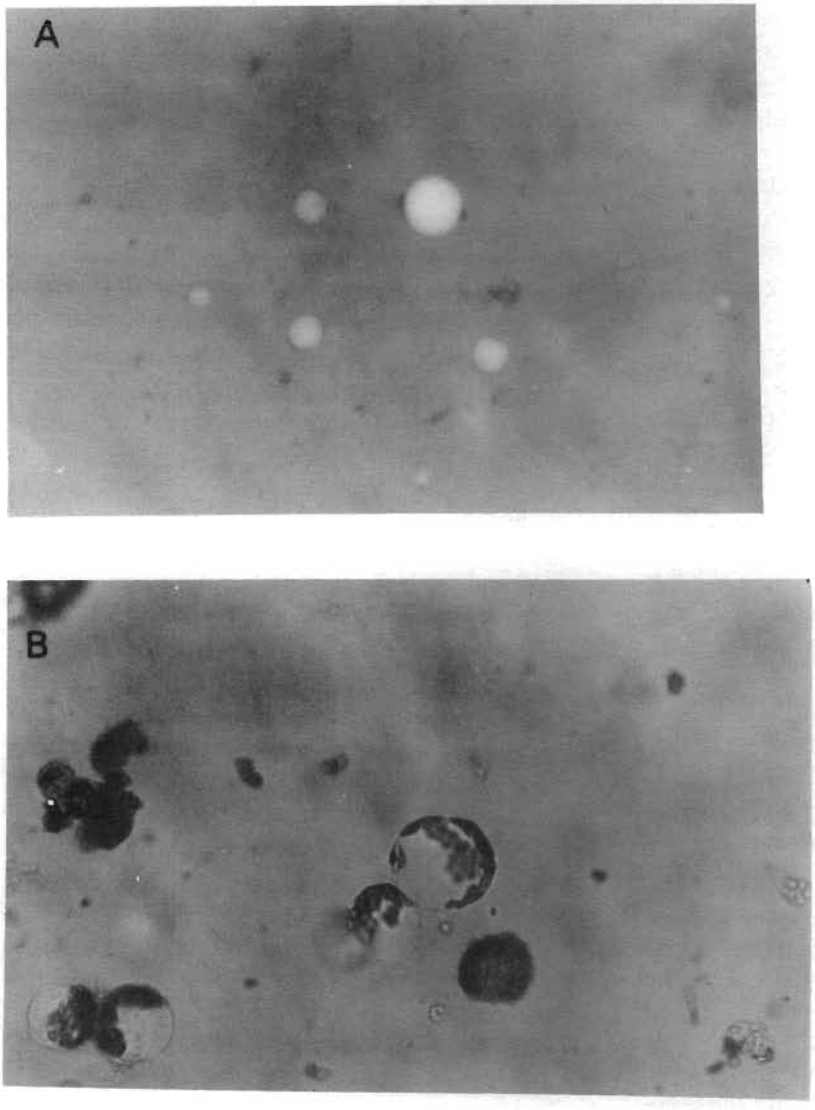

Fig. 2. (A) Protoplasts derived from the tuber of *Callilepis laureola* contrasted by staining the background with Evan's blue. (B) Protoplasts dervived from leaf tissue of *Callilepis laureola*.

37°C and the reaction was stopped by the addition of 6 M potassium hydroxide (50 μl/well) and the absorbance read at 405 nm, using a Biotek spectrophotometer.

Results and Discussion

Optimisation of protoplast production

Protoplasts were isolated from the tuber of *C. laureola* (Fig. 2A) but initial yields were low (4×10^3 to 4×10^4 protoplasts/ml). This in part was attributed to the presence of plant phenolics and to negate the effects of these, citric and ascorbic acids (0.002 g/ml) were added. This, together with the omission of the purification step using the sorbitol gradient, resulted in improved yields of 1—2 $\times 10^6$ protoplasts/ml.

An additional optimisation involved the determination of the most effective sterilant concentration, which was found to be 1% sodium hypochlorite and 0.1% Tween 80 and became the treatment of choice.

Reduction of Onozuka RS (cellulase) from 4% to 2% (w/v) using the same protocol as applied to tuber tissue, enabled the production of protoplasts from leaf tissue at a level of 4—5 $\times 10^6$ protoplasts/ml (Fig. 2B).

Subcellular fractionation and vacuole isolation

Samples of the vacuole fraction stained with equal volumes of neutral red solution, enable visualisation of vacuoles as bodies stained dark red. Microscopic examination revealed that the vacuoles were free from other plant material and debris and, therefore, constituted a pure fraction. Yields were low (3—4 $\times 10^2$ vacuoles/ml) due in part to the passage of neutral red dye into the vacuole upsetting the osmotic balance and causing the vacuole to collapse.

Location of atractyloside in the vacuole

Examination of the various fractions by TLC, showed that the vacuole fraction gave a pink spot with the anisaldehyde reagent at R_f values of 0.36 and 0.74 in the first and second dimensions, respectively. These values coincided with those obtained for standard ATR and were not observed on any of the other control or pellet and interface fraction chromatograms.

The results from the immunoassay indicated that ATR was present in all three fractions, i.e., vacuole, interface and pellet. Although the immunoassay is only semi-quantitative it did show, however, that the levels of ATR in the vacuole were much higher than in the other two fractions. This result was expected, as the immunoassay is much more sensitive than the TLC-spray reagent and ATR must be present in the other fractions, if only from vacuoles ruptured in the isolation procedure. It can be concluded that ATR is present in far higher concentrations in the vacuole than other parts of the cell and this, therefore, appears to act as the storage organelle in the plant.

This is the first report of the determination of the sub-cellular localisation of ATR in the vacuole of *Callilepis laureola*. Although more work is required to optimise the isolation of viable vacuoles in higher yields, their production from tubers and, in particular, leaves of the plant is reproducible and should, therefore, allow the mechanism of concentration of the toxin in the organelle to be elucidated.

Acknowledgements

The authors wish to thank Dr P. Watt and Mr R. Berry, University of Natal for their assistance and time; and the University of Natal for funds to support the work.

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