TITLE STUDIES ON THE ENTRY OF RICIN SUBUNITS INTO CELLS

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STUDIES ON THE ENTRY OF RICIN SUBUNITS INTO CELLS

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For Mum, Dad and Karen.

'All things are poison and nothing is without poison.
The dose alone decides ...."

Paracelsus, a Swiss Physician of the early 16th Century.

CONTENTS

Summary

The potent cytotoxin ricin, obtained from the seeds of the castor oil plant Ricinus communis, is composed of two polypeptide subunits linked by a single disulphide bond. The binding of this molecule to the surface of eukaryotic cells is mediated via the sugar-binding activity of the B subunit. The exact nature of the ricin receptor(s) on the cell surface is unclear, but is most probably some glycoprotein or glycolipid containing exposed galactosyl residues. The ricin molecule becomes internalized by the cell and, by an unclear mechanism, ricin A chain escapes its endocytic vesicle entering the cell cytoplasm where it enzymatically and irreversibly inhibits eukaryotic ribosomes, bringing about the cessation of protein synthesis.

The toxicity of ricin A chain-containing immunotoxins can in many model systems be enhanced by the subsequent addition of ricin B chain. This apparent ricin B chain-mediated potentiation of cytotoxicity suggests that this subunit has some role in the mechanism(s) facilitating the entry of ricin A chain into the cell cytosol.

The work presented in this thesis has attempted to examine the potential of ricin B chain as a carrier of proteins into cells other than ricin A chain. In this example ricin A chain has been replaced with the type I ribosome inactivating protein, gelonin.

Further to these studies, preliminary work considering the possible importance of the hydrophobic C-terminus of ricin A chain in the translocation events, has been presented. To date this work has

demonstrated that it is possible to delete at least 30 amino acid residues from the C-terminus of the A chain and retain full ribosomal inactivation activity as judged by *in vitro* analysis. This truncated form of ricin A chain has been expressed in an *E. coli* expression system and a soluble and active recombinant protein has been partially purified. The implications of this work and possible future analysis of this mutant polypeptide have also been considered.

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Declaration

All the results presented in this thesis were obtained by the author and have not been used in any previous application for a degree. All sources of information have been specifically acknowledged by means of reference.

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Abbreviations

ADP	adenosine diphosphate
APS	ammonium persulphate
Bis	N'N'-methylene-bisacrylamide
BSA	bovine serum albumin
CIP	calf intestinal alkaline phosphatase
CsCl	caesium chloride
dH_2O	distilled water
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleoside triphosphates
DTT	dithiothreitol
E. coli	Escherichia coli
EDTA	diaminoethanetetra-acetic acid disodium salt
Endo H	endoglucosaminidase H
FCA	Freunds complete adjuvant
FICA	Freunds incomplete adjuvant
Fuc	fucose
GlcNac	N-acetylgalactosamine
HAc	acetic acid
HCl	hydrochloric acid
IPTG	isopropyl-β-D-thio galactopyranoside
Kd	Kilodalton
M	molar
Man	mannose
NAD	nicotinamide adenine dinucleotide
PAGE	polyacrylamide gel electrophoresis

PBS	phosphate buffered saline
PMSF	phenylmethylsulphonylfluoride
OD	optical density
PVP	polyvinylpyrollidone
RCA_I	Ricinus communis agglutinin
rATP	ribo adenosine triphosphate
RF	replicative form
RNA	ribonucleic acid
RNAase	ribonuclease
SDS	sodium dodecyl sulphate
SP6	Salmonella typhimurium phage
SPDP	N-succinimidyl-3-(2-pyridyldithio) proprionate
TCA	trichloroacetic acid
TEMED	NNN'N'-tetramethylethylenediamine
Tris HCl	tris (hydroxymethyl) aminomethane hydrochloride
v/v	volume/volume
w/v	weight/volume
X-gal	5-bromo-4-chloro-3-indolyl β galactose
Xyl	Xylose

1. Introduction

1:1:1 Overview.

The protein ricin is an extremely potent cytotoxin occurring with a number of other seed storage proteins within protein body organelles found in the seeds of the castor oil plant Ricinus communis (Tulley and Beevers, 1976; Youle and Huang, 1976). The toxic and medical properties associated with the seeds of this plant have been realised since ancient times; their use being recorded in classical Greek and Sanskrit medicine. More recently, in the nineteenth century, Paul Ehrlich established some of the basic principles of immunology using crude ricin preparations obtained from castor beans (Ehrlich, 1891). Ehrlich is of course the originator of the term "magic bullet", a term used to describe reagents designed for the targeted destruction of diseased cells. Nowadays this term frequently describes the construction of ricin-antibody conjugates, commonly referred to as immunotoxins.

An historical perspective of the scientific work relating to ricin has been made by Balint (1974) in his extensive review. In summary, Stillmark in 1887 was the first person to name the toxic component from the castor oil seed, ricin. However, its activity had been noted as early as 1878 by Ritthausen and later confirmed by Dixon in 1887. The first detailed description of ricin poisoning in man was published in 1899 by Muller. Early purification procedures were improved by Osborne (1905) and later by Kobert (1913) who introduced an ammonium sulphate precipitation stage. The major breakthrough however did not occur until 1965 when affinity chromatography using sepharose was first achieved by Dirheimer. This technique now forms the basis for most present day methods of ricin purification.

Ricin is a potent toxin towards eukaryotic cells. It has been estimated that 1 Kg of the purified toxin would be lethal to 3.6 million people, which equates to a lethal dose of 0.27 mg per person, (Balint, 1974). Sensitivity to the toxin varies from species to species; the horse and the guinea pig being the most sensitive. Indeed Ehrlich has calculated that a single gram of ricin would be enough to kill 1.5 million guinea pigs. At the other extreme, the domestic hen is the most resistant of the higher animals tested, and the frog apparently has an outstanding resistance to the toxin (Balint, 1974). Table 1:1 describes the sensitivity of different species to ricin intoxication (after Olsnes and Pihl, 1982).

Table 1:1

Animal	Lethal dose 50%
Mice	2.7 μg/Kg body weight
Rabbit	*50 ng/Kg
Dog	*1.25 μg/Kg
Human	*0.15 μg/Kg

*intravenous administration of abrin, a toxin closely related to ricin.

Inevitably throughout the ages ricin has been exploited for criminal purposes, with possibly the most famous case being the assassination of the Bulgarian expatriate Georgy Markov on Waterloo Bridge in London in 1978. Markov was shot in the leg with what is believed to be a ricin impregnated pellet, fired from a modified umbrella.

The use of ricin for medicinal purposes is, thankfully, far more common. Recently, attention has focused on ricin and associated toxins for

their potential as specific anti-tumour agents whereby the toxins are targeted to cancerous cells by being coupled to a suitable cell binding monoclonal antibody. These so-called immunotoxins have achieved reasonable efficacy in whole cell and animal model systems, but as yet only a limited response in clinical trials.

1;1;2 Structure of Ricin.

Ricin is a glycosylated, heterodimeric protein with an estimated molecular weight of 62,057 daltons (Olsnes and Pihl, 1982). It is composed of two subunits termed A chain and B chain. Both are N-glycosylated and have molecular weights of 30,625 daltons and 31,432 daltons respectively. The complete amino acid sequence of ricin A chain (Funatsu et al., 1978) and ricin B chain (Funatsu et al., 1979) has been established by peptide analysis and also deduced from the nucleotide sequence of cDNA clones coding for preproricin (Lamb et al., 1985). These latter studies showed preproricin to be composed of a 35 amino acid N-terminal sequence preceding the A chain (267 amino acids), which is joined to the B chain (262 amino acids) by a 12 amino acid linker region. In the mature protein the signal sequence and linker are removed by specific processing enzymes. The two subunits are covalently linked by a single disulphide bond between cysteine residues at position 259 in the A chain and position 4 in the B chain. However, it is apparent from X-ray crystallographic analysis of ricin that a more complex interaction occurs between the two subunits (Montfort et al., 1987). In addition to the interchain disulphide bond, ricin B chain contains 4 intrachain disulphide bonds. In contrast ricin A chain has no intrachain disulphide linkages. Both subunits possess two N-glycosylation sites, both of which are occupied in the B chain by high mannose type oligosaccharides (Foxwell et al., 1985). Ricin A chain may exist in two glycosylated forms, as the A_1 species with only one N-glycosylation site

occupied with a complex oligosaccharide containing xylose and fucose, or as the A_2 species in which the second N-glycosylation site is also occupied. A more detailed account of the oligosaccharide side chains of the ricin subunits is considered in the discussion section of Chapter 3.

1;1;3 Biological Activities of Ricin Subunits.

The structure and function of the respective subunits will be considered in more detail elsewhere. However, each subunit may be characterised through distinct biological activities. Ricin A chain, the effectomer, is known to enzymatically inactivate eukaryotic ribosomes resulting in a cessation of protein synthesis in the intoxicated cell. However, to inactivate the ribosomes ricin A chain must first become internalized into the cell, and then translocated into the cell cytoplasm. The biological activities of ricin B chain are believed to facilitate these processes. Ricin B chain, the haptomer, has a well-defined sugar-binding or lectin activity, exhibiting a specificity for galactosyl residues. This activity of the B chain mediates the binding of the whole toxin to the surfaces of cells, probably to galactosyl residues which occur on exposed glycoproteins and glycolipids on the cell surface. No specific receptors have been identified for ricin, rather its binding appears to be opportunistic towards a range of cell-surface molecules. The number of binding sites is therefore enormous. HeLa cells for example, possess 3 x 10^7 potential binding sites for ricin (Olsnes and Pihl, 1982). Together therefore, the two subunits constitute an extremely potent toxin which can bind, through the B chain, to any one of millions of sugar residues on a wide range of different cell types and become internalised resulting in the inactivation of susceptible ribosomes.

In addition to its sugar-binding role there exists indirect evidence that B chain has some role in promoting the entry of the A chain into the

cytosol. This effect has been observed in studies in which the cytotoxic activities of ricin A chain-containing immunotoxins has been potentiated in the presence of ricin B chain (Youle and Neville, 1982; McIntosh et al., 1983). This effect is apparently not a result of the binding of ricin B chain to galactosyl residues on the cell surface because it has also been demonstrated in the presence of high lactose concentrations which would ensure blockage of the binding sites.

1.1.4 The Cytotoxic Nature of Ricin.

The cytotoxic activity of ricin on eukaryotic cells may be considered in three stages. Stage I involves binding of the whole toxin to the cell surface. This is mediated through the sugar-binding sites of the B subunit. That ricin cytotoxicity can be abolished by the presence of free lactose (100 mM), suggests that (a) binding to the cell surface is a prerequisite for intoxication and (b) binding mediated through the lectin activity of ricin B chain represents the predominant means by which ricin interacts with (and ultimately kills) the cells. It remains unclear however, as to whether particular cell surface sugar residues on particular glycoproteins or glycolipids are more important than others with respect to the next stage, of toxin internalisation and ultimate delivery of ricin A chain into the cytosol. Studies by Simmons et al. (1986) have suggested that the mannose-rich oligosaccharide side chains of ricin may have some, possibly minor, role in the internalisation of ricin.

Stage II is concerned with the internalisation of ricin and translocation of ricin A chain into the cytosol. As it is of particular interest to the subject of this thesis, it has been considered in a separate section later in the Introduction.

Stage III involves the inactivation of eukaryotic ribosomes, leading to the inhibition of protein synthesis. This inhibition of protein

synthesis is brought about by the enzymatic inactivation of the large (60S) ribosomal subunit by ricin A chain (Olsnes and Pihl, 1976; Sperti et al. 1973). Cawley et al. (1978) have shown this inactivation to be catalytic in nature, requiring no cofactors. Kinetic experiments have indicated that a single ricin A chain molecule can inactivate salt-washed ribosomes at a rate of 1500 per minute ($Km = 1\text{-}2 \times 10^{-9}$ M), (Olsnes et al. , 1975). A more detailed account of ribosome inactivation has been given in the later section, Ricin A chain : Structure, Function and Evolution.

1:1:5 Biosynthesis of Ricin.

Ricin and the related castor bean lectin, RCA_I, are synthesised simultaneously in the endosperm cells of maturing castor bean seeds (Gifford et al., 1982). This synthesis occurs during and after testa formation when the organelles are being rapidly formed (Roberts and Lord, 1981a). The structural and functional homology between the two lectin species would suggest a similar biosynthetic pathway, indeed it seems likely that duplication of an ancestral gene may have evolved into the genes encoding for these two lectins. Work by Butterworth and Lord (1983) has shown that both the A and B subunits of ricin and the A' and B' subunits of RCA_I are derived from large precursors. The ricin precursor, preproricin, is composed of a 35 amino acid N-terminal signal peptide preceding the A chain (267 amino acids), which in turn is linked to the B chain (262 amino acids) by a 12 amino acid linker region (Lamb et al., 1985). The N-terminal signal peptide is cleaved during cotranslational translocation of the nascent precursor across the endoplasmic reticulum membranes at which stage core glycosylation occurs and the disulphide bonds are formed (Roberts and Lord, 1981b). The glycosylated precursor is then apparently directed via the Golgi to vesicles which fuse with the protein bodies. Transport is possibly directed by some kind of targeting

sequence. Within the protein bodies the precursor is processed to the mature form of ricin. Details surrounding these final processing events are presently being investigated.

The synthesis of ricin as a precursor molecule, its concomitant segregation within the endomembrane system and its transport to protein bodies prior to processing prevents enzymatic inactivation of castor bean endosperm ribosomes by ricin A chain. Thus if ever exposed to the ribosomes as a result of inefficient translocation, ricin A chain occurs as a precursor which is known to be biologically inactive (Lynne Roberts, Personal Communication).

1:2:1 The Structure, Evolution and Function of Ricin B Chain.

Ricin B chain is a lectin with an affinity for galactosyl residues. Earlier work by Zentz et al. (1978) and Houston and Dooley, (1982) established that ricin B chain binds two sugar molecules in a non-cooperative manner. These two authors disagree however as to the binding affinities of each of these sites; Zentz et al., suggesting that the sugar-binding domains can be differentiated as a low and a high affinity binding domain, whilst Houston and Dooley maintain that the binding affinities of each site are more or less equal.

Villafranca and Robertus (1981), have shown that ricin B chain is a gene duplication product, showing approximately 32% homology at the amino acid level between the two halves of the molecule. Each half of this molecule contains a sugar-binding domain. Work by Hise et al. (1986), has implicated the amino acid residue tyrosine 248 in the "strong" galactose binding site, and an undetermined tryptophan residue in the weak site (Hatakeyama et al., 1986). Each sugar-binding domain is proposed to contain two disulphide loops which show significant homology with residues 168-225 of discoidin I, a galactose binding peptide from the slime mould

Dictyostelium discoidaum (Robertus and Ready, 1984). These same authors have suggested that this peptide was an ancient galactose binding unit from which ricin B chain has evolved. More recently X-ray crystallographic analysis, revealing the three-dimensional structure of ricin at a resolution of 2.8 A, has confirmed that ricin B chian is composed of two globular regions, which although separate, share identical folding topologies. Each will bind a lactose molecule (Montfort et al., 1987). It is interesting to note however, that each sugar molecule binds to different sites within each domain.

A more detailed analysis of each domain, described by Rutenber et al. (1987), shows that each homologous domain can be divided into four peptide sub-domains. These authors have named these peptides λ-, α-, β- and γ-sub-domains. The λ-peptide of domain 1 (peptide 1λ, residues 1-16) is homologous to the λ peptide of domains 2 (peptide 2λ, residues 136-150). These two peptides are however not related to the other sub-domains within the molecule. At this point it is interesting to note that the 1λ sub-domain forms the N-terminal extension of ricin B chain which interacts with the carboxyl-terminal domain of ricin A chain (Montfort et al., 1987). The remaining sub-domains, the α, β and γ peptides are homologous with each other. These sub-domains, which comprise the main body of each domain have undergone considerable divergence but, despite this they still demonstrate a statistically significant homology.

In the model presented by Rutenber et al. (1987) the two sugar-binding domains, which are linked by the 2λ peptide, each bind a lactose molecule in a non-homologous fashion at the domain level. However, at the sub-domain level, the binding of lactose in each respective domain, to peptides 1α and 2γ is homologous (Rutenber et al., 1987). At both binding sites (in sub-domains 1α and 2γ) the lactose molecule is believed to lie in a pocket formed on one side by a kink in the peptide chain and on the other side an aromatic side chain. This kink formed by the residues Asp-Val-Arg is

conserved in both sub-domains, and the aromatic side chains belong to tryptophan residue 37 in 1α and tyrosine residue 248 in 2γ. These side chains however, serve only as a flat binding surface and apparently do not specifically interact with the sugar hydroxyl groups (Montfort et al., 1987). Specific non-covalent interactions in the form of hydrogen bonds do occur between the sugar and amino acid residues Asn 46 and Asn 255. These binding residues are only conserved in the sub-domains which still bind the sugar and have therefore been considered as prime targets for modification in attempts to abolish the sugar-binding activity of ricin B chain. Other highly conserved residues are believed to fulfil structural roles.

Rutenber et al. (1987), speculate that ricin B chain has evolved from an ancient galactose-binding peptide of approximately 40 residues which resembles the 1α domain found presently in the molecule. They further speculate that the ancient molecule existed as a trimer and that from gene duplication and fusion a $\alpha\beta\gamma$ molecule resembling a modern day B chain domain evolved. Such a fusion is thought to have abolished binding in the β subunit, leaving only two binding sites in the α and γ sub-domains. The addition of the λ peptide is expected to have stabilized the structure to form a $\lambda\alpha\beta\gamma$ unit which by a further duplication event would form the structure $(\lambda\alpha\beta\gamma)_2$, resembling modern ricin B chain. This final duplication event is thought to have blocked access of galactose to the 1λ unit. Sub-domain 2α appears to have also lost its binding residues at some point. This leaves two sugar-binding domains, 1α and 2γ which are 75 Å apart. Rutenber et al. (1987) speculate that the selection of two sugar-binding sites this distance apart reflects a requirement for cell surface binding and the triggering of endocytosis. The primitive "galactose-fold" speculated by these workers is consistent with structural analysis of the lectin discoidin I, extracted from the slime mould Dictyostelium discoideum, fueling the speculation that ricin B chain has evolved from a primitive sugar-binding protein (Robertus and Ready, 1984).

Throughout this analysis of the structure and function of ricin B chain it is interesting to note that no obvious candidate for a membrane penetrating domain, such as those observed in diphtheria toxin (Greenfield et al., 1987) and Pseudomonas aeruginosa exotoxin A (Allured et al., 1986) has been identified. Despite this, ricin B chain apparently possesses some ability to potentiate the cytotoxic activity of certain ricin A chain-containing immunotoxins (Youle and Neville, 1982; McIntosh et al., 1983). This activity, as mentioned earlier, is apparently independent of the sugar-binding activity of ricin B chain.

The possibility that ricin B chain interacts directly with lipid membranes has been examined by a number of workers (Ishida et al., 1983; Utsumi et al., 1984). In their experiments Ishida et al., 1983 used the Newcastle Disease Virus (NDV) as the target membrane for ricin and ricin subunits. The membrane of NDV is rich in the ganglioside GM_1 which provides an avid binding site for ricin and ricin B chain. In the case of both whole ricin and ricin B chain, the presence of galactose significantly reduced membrane interaction. In contrast the binding of ricin A chain to the virus membrane envelope was unaffected by the presence of galactose. These workers conclude that both subunits of ricin have the inherent ability to penetrate the lipid bilayer.

In similar experiments Utsumi et al. (1984) considered the interaction of ricin and ricin subunits with dipalmitoylphosphatidylcholine (DPPC) vesicles. They observed no association between these vesicles and the unreduced toxin, but considerable interaction when the purified ricin subunits were presented to the vesicles separately. In fact, their experiments indicated that ricin B chain demonstrated the greater lipid-protein association of the 2 subunits. However, these same authors observed a significantly greater perturbation of the DPPC bilayer after incubations with ricin A chain as compared with either whole ricin or ricin B chain. They concluded that although both subunits are able to form

strong associations with lipid membranes, only ricin A chain was able to achieve full penetration. This finding is in agreement with the speculation that the hydrophobic C-terminal region of the A chain is important in membrane penetration and entry of ricin A chain into the cell, (Uchida et al., 1980).

If, as has been suggested by these results, ricin B chain does not actively participate in membrane penetration, then the observed potentiation of A chain immunotoxins cytotoxic activity may be the result of some indirect or cooperative activity. Possibly ricin B chain acts to protect the A chain from proteolytic degradation, or perhaps being associated with ricin B chain acts to direct ricin A chain to an appropriate intracellular compartment for translocation into the cytosol. Observations by Montfort et al. (1987) and Lewis and Youle, (1986) have suggested that the ricin subunits form an intimate association. In particular Montfort et al. (1987) have speculated from their analysis of the three-dimensional structure of ricin, that the carboxyl-terminus of ricin A chain inserts between the two sugar-binding domains of the B chain. Possibly this association acts to maintain the hydrophobic C-terminus of the A chain in a protected conformation until intracellular conditions are optimum in facilitating subunit dissociation and the subsequent exposure of this protected region. For the mean time these suggestions remain untested speculation.

1;2;2 Structure, Function and Evolution of Ricin A Chain.

The X-ray crystallographic analysis of ricin carried out by Montfort et al. (1987) has divided ricin A chain into three arbitary domains. The first, comprising 117 amino-terminal residues, forms a flat domain at the base of the A chain, dominated by a five-stranded β-sheet structure. The second domain, comprising residues 118-210 is dominated by five α helices.

This domain is located above, and slightly to the left of the first domain. The third domain, comprising residues 211-267 forms a compact disc-like domain which, as well as interacting with the first two domains of the A chain, interacts with the ricin B chain. As mentioned in the previous section, this interaction results in the insertion of this C-terminal domain between the two sugar-binding domains of ricin B chain. The A chain is a more globular protein than the B chain. It is approximately 55 Å long, 45 Å wide and 35 Å thick. Of this, the C-terminal disc-like domain comprises of a region 25-30 Å across and 15-20 Å thick. In addition to its strong interaction with ricin B chain, this C-terminal domain represents a significantly hydrophobic region of the molecule which has been implicated in membrane associations (Uchida et al., 1980).

Ricin A chain is an enzyme whose substrate is 28S rRNA. Recently, work by Endo et al. (1987) has demonstrated a specific N-glycosidase activity associated with the A chain. The location of the active site however, remains unclear. Montfort et al. (1987) have identified a putative active site within the A chain structure, but readily acknowledge the need to carry out further studies. Interestingly this cleft, formed at the interface between the three domains, would be exposed to the aqueous media in the unreduced holotoxin, suggesting that upon reduction and dissociation from the B chain, ricin A chain undergoes some degree of conformational change to form the active molecule.

Ricin A chain inhibits protein synthesis by inactivating the function of the 60S subunit of eukaryotic ribosomes. (Sperti et al., 1973). This activity appears to be confined to eukaryotic ribosomes only. Ribosomes extracted from prokaryotic sources are totally insensitive when incubated with concentrations of ricin that result in complete inhibition in eukaryotic systems (Greco et al., 1974). Ribosomes extracted from higher plants have also been shown to be susceptible to ricin inactivation, although the concentration required to produce an IC_{50} in a cell-free

translation system was some 23,000 times higher than the concentration giving the same effect on mammalian ribosomes, (Harley and Beevers, 1982). These findings are in agreement with the result described in Figure 5:7 in which ricin A chain mRNA translated in a wheatgerm *in vitro* system does not apparently inactivate the ribosomes in that system. In contrast, when the same RNA is translated in a rabbit reticulocyte lysate system complete ribosome inactivation occurs within the duration of the experiment (see Figure 5:8). In addition the ribosomes from the protozoan, *Tetrahymena pyriformis* have also been reported to be resistant to ricin (Wilda *et al.*, 1979).

As mentioned earlier in this chapter, a single molecule of ricin A chain can inactivate a large number of ribosomes. Olsnes *et al.* (1975), have estimated that a single A chain molecule can inactivate 1500 salt-washed ribosomes per minute, which translates to a Km value between 1-2 x 10^{-9} M. The use of salt-washed ribosomes in these kinetic studies is important, since pre-bound elongation-factor 2 (EF-2) which is removed in the salt wash, has been shown to protect ribosomes against ricin inactivation. This suggests that ricin A chain may bind at or very close to the normal EF-2 binding site. Protection of ribosomes is also seen with amino acyl-tRNA (Fernandez-Puentes *et al.*, 1976). Furthermore ricin A chain treated ribosomes are apparently unable to bind EF-1, thereby blocking the binding of amino acyl-tRNA. All this data suggests that the action of ricin A chain is to somehow modify the binding sites for these accessory factors on the ribosomes. It is possible that EF-2 may bind to the same site or a site close to the EF-1 binding site on the ribosome. Interestingly ricin A chain has been shown to demonstrate some amino acid homology with hamster EF-2. The deletion of a contiguous stretch of six homologous residues from the ricin A chain sequence results in a molecule which displays no activity when tested in a ricin A chain sensitive *in vitro* translation system (May *et al.*, 1988). It is unclear however as to

whether this loss of activity has resulted from the inability of an otherwise active ricin A chain molecule to bind to the ribosomes, or the consequence of malfolding.

Evidence that ricin A chain might bring about ribosomal inactivation by inducing some subtle conformational change in the ribosome comes from the observations of Cawley et al. (1979) who were able to protect and even rescue ribosomes from the effects of ricin A chain. In their experiments, they found that high concentrations of Mg^{2+} ions reduced A chain inactivation and have speculated that this may be the result of an induced conformational change. More recently, work by Taroa et al. (1988) has demonstrated that the labelling of the ribosomal protein L-14 with either ^{3}H or ^{14}C labelled N-ethylmaleimide was reduced after treatment of the ribosome with ricin. This result suggests that ricin may alter the conformation of the ribosome in the vicinity of that protein. The same authors have speculated that these conformational changes are related to the specific N-glycosidase activity which is characteristic of ricin A chain action on eukaryotic ribosomes.

This N-glycosidase activity, discovered by Endo et al. (1987) produces an apparently minor, but highly specific, modification of the 28S rRNA. More precisely it catalyses the removal of an adenine residue from position 4324 in a highly conserved region of rat liver 28S rRNA. The ribosomal RNA backbone is not cleaved by the action of the A chain, but the phosphodiester linkages on either side of this residue become hypersensitive to cleavage using reagents such as aniline at low pH. This ability to cleave ricin A chain modified rRNA using simple chemicals is exploited in the RNA modification assay described in Section 2:11. Endo has also demonstrated that ricin A chain can catalytically modify naked rRNA extracted from both eukaryotic and prokaryotic ribosomes. This suggests that susceptible prokaryotic rRNA is somehow protected by the structure of the prokaryotic ribosome. From these studies, Endo has been

able to deduce the minimum requirement for the substrate of ricin A chain to be rRNA in a stem loop structure, having the sequence GA GA within the loop (Endo, Y., abstract from the International Symposium on immunotoxins, June 9-11, 1988, Sheraton University Centre, Durham, North Carolina, U.S.A.).

Considered together, this data would support a model in which ricin A chain binds at or near to the binding site for EF-2 on the ribosome. Ricin A chain then catalyses the removal of the adenine residue at position 4324 in the 28S rRNA. This adenine residue is located within a ribonucleotide sequence GA GA, situated in a stem/loop structure. This modification of 28S rRNA has been associated with conformational changes in the 60S ribosomal subunit (Terao et al., 1988) which are thought to result in inactivation of the whole ribosome. Interestingly this region of 28SrRNA, modified by the action of ricin A chain, is highly conserved in the large rRNA of both prokaryotic and eukaryotic ribosomes. The observation by Endo (Symposium abstract) that prokaryotic rRNA, when purified from associated ribosomal proteins, is also modified by ricin A chain suggests that prokaryotic ribosomes form a conformation which somehow protects this susceptible region.

Although these recent developments have contributed considerably to our understanding of ribosomal inactivation by ricin A chain, much work remains before the precise mechanism can be defined. Perhaps the greatest benefit to be gained from the efforts of Endo and co-workers is that it has focused the research on ricin A chain activity, which prior to this had consisted of an examination of a number of putative A chain associated phenomena. In order to avoid unnecessary confusion, these earlier observations which led to speculation regarding the mode of action of ricin A chain, such as dephosphorylation (Houston, 1978) or inhibition of ribosomal GTPase activity (Benson, 1977), have not been considered here.

Recently a number of authors have identified ribosomal RNA modification activity, identical to that found with ricin A chain, in a variety of other ribosome inactivating proteins, for example barley toxin, (Endo et al., 1988(c)), viscumin, a type II RIP from mistletoe (Endo et al., 1988(a)) gelonin, saporin, momordin, pokeweed antiviral proteins (PAP, PAP-II and PAP-S) (Endo et al., 1988(b)), recombinant ricin A chain, tritin, trichosanthin and dianthins (Stirpe et al., 1988). All these RIPs come from plant sources although from species that are taxonomically unrelated. The source and data describing the toxicity of these and many other RIPs have been extensively reviewed by Stirpe and Barbieri (1986). This diversity of RIP sources suggests that these proteins, and indeed ricin A chain, may have evolved from some primitive common ancestor. Furthermore, the observation that the cytotoxin from the bacterium Shigella dysenteriae (Shiga toxin) also catalytically inactivates ribosomes in a ricin A chain-like fashion supports this possibility (Endo and Tsurugi, 1987). The biology and biochemistry of Shiga and Shiga-like toxins have been reviewed by O'Brien and Holmes (1987), and their nucleotide sequence determined and compared with ricin (Calderwood et al., 1987). These workers conclude that the A subunit of Shiga-like toxin is homologous with the A chain of ricin. Analysis of the amino-terminal sequences of RIPs extracted from Phytolacca americana (PAP, PAPII, PAP-S), Phytolacca dodecandra (dodecandrin) and the A chains of ricin and modeccin, has indicated that all these proteins appear to be related (Ready et al., 1984). These same authors suggest that these proteins have diverged from a common ancestor, in some cases fusing with the genes for sugar-binding proteins, the predecessor to the modern B chains (type II RIPs). In other cases they remain as single chain molecules (type I RIPs). This type of analysis, together with the discovery of a common rRNA modification activity and the widespread, divergent occurrence of these proteins goes a long way to supporting this hypothesis.

The occurrence of hydrophobic regions within the ricin A chain sequence, in particular the significance of the hydrophobic C-terminal portion of this molecule have been considered briefly in the previous section. The possibility that this region may be involved in membrane interactions and its association with ricin B chain are considered more fully in the discussion section of Chapters 5 and 6.

1:3:1 Internalization and Intracellular Trafficking of Ricin.

For ricin A chain to bring about cessation of protein synthesis in eukaryotic cells it must gain access to its intracellular target, the 60S ribosomal subunit located in the cell cytosol. Access to this environment requires that ricin is internalized by the cell and that ricin A chain at least, is able to translocate the intracellular membrane and enter the cytosol.

The binding of ricin to the cell surface is mediated via sugar-binding sites within the B chain. As mentioned earlier, ricin is able to bind to a variety of cell surface molecules bearing terminal galactosyl residues, although it is unclear as to whether all these binding events result in the internalization of the toxin. Methods for detecting ricin on the surface of exposed cells, such as the use of horse-radish peroxidase (hrp) conjugates, or the use of immunoperoxidase cytochemistry, have indicated that ricin binds evenly over the cell surface, including regions of coated and uncoated pits. This is in contrast with similar studies carried out on transferrin-horse radish peroxidase complexes which are detected within or close to coated pit structures. This suggests that the receptors for transferrin are located only within this region (Sandvig et al., 1987). Interestingly, Shigella toxin - hrp, a toxin with an apparently identical ribosome inactivating activity to ricin binds to cells in a similar manner to transferrin, i.e. around or within coated pits, (Sandvig et al., in

preparation). It is important however, in any analysis of these protein labelling studies to determine the extent of any chemical modifications on the protein, induced by the presence of the label itself. For instance van Deurs et al. (1985) have reported that ricin-gold and polyvalent ricin-hrp could not be removed from the cell surface by lactose, whereas monovalent ricin-hrp could. If chemically altered, this begs the question - how physiological is the system under study? Such studies are further hampered by the apparently slow uptake of ricin by cells and the evidence that some internalized toxin becomes recycled to the cell surface (Sandvig and Olsnes, 1979). The apparent multiplicity of possible entry routes for bound ricin is a further indicator of the opportunistic nature of the cell binding step.

To date there is no firm understanding of the mechanism(s) by which ricin becomes internalized. Despite the limitations of the immunogold conjugate approach considered earlier, the association of ricin into coated pit regions, observed using this technique, suggests that at least some ricin becomes internalized by this route, i.e. the standard endocytic pathway. Equally this does not rule out the possibility of alternative, non-coated pit mediated endocytosis of ricin (van Deurs et al., 1988, in press).

Regardless of whether ricin becomes internalized via coated or smooth pits the endosome represents the first clearly distinct intracellular compartment which it encounters. Thus, internalized ricin enters an acidic environment. However, unlike other receptor-ligand complexes, only about 22% of ricin (initially bound at pH 7.0) was found to be released from its receptor at pH 5.0 (van Deurs, 1988, J. Cell Biol., in press). Studies by Sandvig and Olsnes (1979) and Sandvig et al. (1978) have indicated that the majority of toxin, which remains bound, becomes recycled to the cell surface. This recycling of the toxin apparently occurs via a fast and a slow route. In contrast to these findings no morphological evidence has

been presented supporting these observations. Some evidence exists that a proportion of internalized ricin molecules are transferred to the lysosomes where they are slowly degraded (Sandvig and Olsnes, 1979). This slow degradation may possibly result from the reported resistance of ricin to proteolytic enzymes (Olsnes et al., 1975) and if so would indicate that the ricin subunits had not become dissociated at this stage.

Unlike other toxins such as diphtheria toxin, modeccin and Pseudomonas aeruginosa exotoxin A, ricin and the related toxins abrin and viscumin do not require a low pH for translocation into the cytosol. Indeed if the pH of the intracellular vesicles is increased with 10 mM NH_4Cl the cells become sensitised to ricin (Sandvig et al., 1979), suggesting that ricin A chain translocation is not facilitated by low pH. Ca^{2+} ions appear to be important for ricin entry into the cytosol as their absence, or blockage of their transport, allows endocytosis but not translocation (Sandvig and Olsnes, 1982). Also the lag period observed after the initial uptake of ricin would suggest that translocation occurs from a compartment distal to the endosomes in the endocytic pathway. Furthermore this observed lag period argues against the possibility that ricin achieves direct entry into the cell cytosol across the plasma membrane.

Gonatas et al. (1977), were the first workers to show ricin-hrp conjugates associated with Golgi compartments in neuroblastoma cells and more recently Sandvig et al. (1986), have shown similar associations with Vero cells, the cell line used in the cytotoxicity assays described in this thesis. Experiments using secretory-Golgi pathway markers (van Deurs, 1988, J. Biol. Cell, in press) and immunoperoxidase cytochemistry studies have also demonstrated the presence of ricin in the Golgi compartments. However, as indicated before, such conjugate studies are limited by the influence of the coupled marker on the route of internalization. Thus van Deurs et al. (1986), observed that although ricin and monovalent ricin-hrp reached Golgi compartments, ricin-Au and polyvalent ricin-hrp did not. The

observations by van Deurs et al. (1988, in press) that ricin toxicity can be virtually abolished in cells incubated at 18°C has been associated with the fact that no ricin can be detected in the Golgi compartments at this temperature. Further evidence implicating the Golgi compartments in ricin A chain translocation comes from the observations that cells are sensitised to ricin after treatment with 0.1 μM monensin, (Sandvig and Olsnes, 1979). Treatment of cells with monensin has been shown to induce morphological changes in the Golgi (Tartakoff and Vassalli, 1979), although the same treatment has no measurable effect on the pH of endosomes and lysosomes (Sandvig and Olsnes, 1979).

Possibly the most original method employed associating ricin with the Golgi comes from the work of Youle and Columbatti (1987), who took hybridoma cells, synthesising monoclonal antibodies capable of blocking ricin toxicity and exposed these cells to extracellularly applied ricin. The ricin was internalized and yet these cells demonstrated a 50-300 fold resistance to ricin intoxication compared to control cells. As further controls, these workers eliminated the possibilities that either extracellular or surface bound antibodies acted to prevent ricin entry. They therefore concluded that intracellular monoclonal antibody synthesized and assembled de novo, blocked ricin toxicity. Thus these intracellular antibodies must meet the internalized ricin before it becomes translocated to the cytosol. This presumably occurs in the Golgi since this is a compartment common to both secretory and endocytic pathways. These observations suggest that ricin located within endosomes or lysosomes does not enter the cytosol. It is not clear at present whether ricin reaches the Golgi via the endosomal route. Youle and Columbatti (1987), suggest that ricin must reach the Golgi apparatus to find a neutral pH compartment from which it is able to enter the cytosol. In addition Youle and Columbatti (1987) have shown that a monoclonal antibody specific for a galactose binding site of ricin B chain also blocks the cytoplasmic entry

of ricin, suggesting that the sugar-binding activity of ricin is required intracellularly, possibly for the translocation of ricin A chain into the cytosol. These authors speculate that if efficient cytoplasmic entry requires that ricin interacts, via its sugar-binding sites, with a specific intracellular "receptor", this might account for the enhancement of cytotoxicity observed when ricin B chain is added to ricin A chain containing immunotoxins (McIntosh et al., 1983). If correct, this mechanism will have important implications in the design of immunotoxins incorporating ricin B chain with an abolished sugar-binding activity.

In conclusion therefore, it remains unclear as to how and from what compartment ricin A chain is able to translocate into the cell cytoplasm. Of all the possibilities considered there exists a body of evidence suggesting that cytosolic entry is achieved from part of the Golgi, possibly from within the trans-Golgi network (TGN).

1.6.1 A Comparison of Structure and Function between Ricin and Diphtheria Toxin.

Despite originating from quite different sources ricin and diphtheria toxin, from the bacterium Corynebacterium diphtheriae, show some striking similarities. These toxins have in common a dimeric polypeptide structure in which the two subunits are linked by a single disulphide bond. (Although diphtheria toxin is made as a single polypeptide, it is invariably nicked by proteases to generate two subunits or fragments linked covalently by a disulphide bond). In addition both toxins possess a cell surface binding capability and intoxicate susceptible eukaryotic cells by enzymatic inactivation of the protein synthesis machinery. These respective activities reside on one or other of the distinct polypeptide subunits. In both toxins, the A subunit enters the cell cytosol and brings about the enzymatic inactivation of protein synthesis within that cell.

The other subunit, the B subunit, is responsible for binding the toxin to sites on the cell surface. In addition there is evidence that the B subunit may function in the penetrative mechanism by which the enzymic A subunit enters the cell cytosol. With the B subunit of diphtheria toxin this role is fairly well defined. Hydrophobic regions of diphtheria toxin fragment B are actively involved in the penetration of fragment A into and across the intracellular membrane barrier into the cytosol. A more detailed analysis of these events has been considered by Boquet et al. (1976), Lambotte et al., 1980, and Sandvig and Olsnes, (1981). In contrast the B chain of ricin does not appear to interact with membranes in the same fashion although evidence exists suggesting ricin B chain may have some other role in promoting the entry of ricin A chain (Youle and Neville, 1982; McIntosh et al., 1983). Indeed with respect to the interaction of hydrophobic regions of the toxin with membranes, the C-terminus of ricin A chain appears equally likely to fulfil or participate in the membrane penetration step (Utsumi et al., 1984).

Despite these superficial similarities the more detailed comparison of the two toxins at the different stages of intoxication indicates that these two proteins demonstrate a parallel but never the less quite different series of activities.

1;4:2 Cell Surface Binding.

The lectin activity of ricin and the role of this activity during cell surface interactions and the intoxication process have been considered in detail earlier in this chapter. Briefly the lectin activity of ricin resides in the B subunit. Ricin B chain has a specificity for galactosyl residues and as a result is able to bind to a variety of cell surface markers, mostly cell surface glycoproteins or glycolipids. The binding of ricin to the cell surface is apparently a random event, and it is as yet

unclear as to whether specific cell surface residues are more important than others as functional binding sites.

In contrast the binding of diphtheria toxin to the cell surface occurs only at a limited number of sites on the cell surface. As yet it is unclear as to the exact nature of the diphtheria toxin receptor. Evidence exists that the receptor is a glycoconjugate, as neuraminidase treated cells demonstrate increased binding (Sandvig et al., 1978), whilst tunicamycin treatment reduced binding (Hranitzky et al., 1985). However, the possibility exists that these treatments may have induced cell surface alterations which may have in turn effected toxin binding. Other literature, associated with studies on the binding of diphtheria toxin to cell surfaces had been considered in a recent review by Olsnes and Sandvig (In: "Immunotoxins", A. E. Frankel, ed., Martinus Nijhoff Publishing, Boston, 1988).

1;4;3 Endocytosis and Intracellular Routing.

A detailed consideration of the internalization and intracellular routing of ricin has been made in the previous section. Despite considerable efforts, the events culminating in the translocation of ricin A chain into the cytosol remain vague.

In conjugate studies similar to those carried out with ricin, labelled diphtheria toxin has been shown to bind to the entire surface of Vero cells and then to subsequently migrate to coated pits (Morris et al., 1985). The toxin was then found to be internalized by coated vesicles. It became apparent from further experiments that toxin molecules internalized by this route resulted in intoxication of the cell, and if this specific internalization route was blocked, or diphtheria toxin was internalized from non-coated regions of the cell surface, then intoxication of the cells did not follow. Thus it would appear that receptor mediated endocytosis by

coated pit pathway is the major route by which diphtheria toxin is able to enter and subsequently intoxicate cells.

1:4:4 Transport to the Cytosol.

As before, studies relating to the translocation of ricin into the cytosol have been considered in the previous section. To date the exact mechanism and the roles, if any, of the ricin subunits during these events remains unclear.

With diphtheria toxin a critical step in cytosol penetration is the exposure of the receptor bound, internalized toxin to a pH of <5.3 (Draper and Simon, 1980; Sandvig and Olsnes, 1980). At pH 5.3 or lower a normally hiddan hydrophobic domain of the B fragment becomes exposed and the toxin structure opens up (Blewitt et al., 1985). As a result of this pH-facilitated exposure, the hydrophobic domain (and possibly other regions) become inserted into the membrane (Zalman and Wisniski, 1984; Hu and Holmes, 1984). This insertion then somehow facilitates the translocation of the A fragment into the cytosol. In contrast low pH does not facilitate the translocation of ricin A chain into the cytosol, nor is there evidence of any membrane penetrating mechanism analogous to that described for diphtheria toxin. A much fuller consideration of the transport of diphtheria toxin fragment A into the cytosol, including other conditions required for translocation are considered in the Olsnes and Sandvig review, mentioned earlier.

1:4:5 Enzymatic Inactivation of Protein Synthesis by Ricin and Diphtheria Toxin.

Despite the significant contribution of Endo and co-workers to our understanding of ricin A chain activity, the exact mechanism by which it

catalyses ribosome inactivation remains to be fully characterized. The most recent studies attempting to clarify this mechanism have been considered earlier in Section 1:2:2.

The inhibition of protein synthesis, catalysed by the activity of fragment A of diphtheria toxin occur as a result of a quite different inhibitory mechanism. Diphtheria toxin, and also the 26 Kd fragment of pseudomonas exotoxin A (from Pseudomonas aeruginosa) catalyse the process by which NAD is cleaved to yield ADP-ribose which then becomes covalently linked to the soluble ribosomal cofactor, elongation factor 2 (Pappenheimer, 1977). The process is known as ADP-ribosylation of elongation factor 2 (EF-2). The effect of this ADP-ribosylation is to severely reduce, but not completely abolish the normal activities of EF-2, i.e. GTP cleavage and peptide chain elongation. However these activities are so severely reduced that the cells are unable to maintain a level of protein synthesis adequate for survival.

Interestingly ADP-ribosylation occurs only at a particular amino acid, the diphthamide residue, of EF-2 (van Ness et al., 1980; Bodley et al., 1984). This particular amino acid is derived from a particular histidine residue in EF-2 which has been post-translationally modified so as to contain a 3-carboxyamido-3-(trimethyl-amino) propyl side chain (Moehring et al., 1984).

The turnover number for diphtheria toxin fragment A has been calculated at 2000 ADP-ribosylated EF-2 molecules per minute (Moynihan and Pappenheimer, 1981), with a K_m = 1.5 x 10^{-7} M for elongation factor 2 (Chung and Collier, 1977). This in effect means that in theory a single diphtheria toxin fragment A would be adequate to kill a cell (Yamaizumi et al., 1978). Thus despite achieving their inhibitory activities by different mechanisms, both ricin and diphtheria toxin are potent inhibitors of protein synthesis.

1:4:6 Concluding Remarks.

The realisation that potent toxins such as ricin might be suitable effector molecules in therapeutic agents constructed to be targeted to specific cell types, has resulted in considerable interest in these molecules. In the main, these cytotoxic proteins have been modified so as to facilitate coupling to cell specific monoclonal antibodies. These so-called immunotoxins combine the antibody's cell binding specificity with the toxin's potent cytotoxic activity and have demonstrated encouraging efficacy when tested in in vitro model systems. To date however these molecules have only demonstrated a limited efficacy in the clinical environment, as mentioned in Section 1:1:1. A significant obstacle to the engineering of a truly effective immunotoxin is the non-specific way that ricin binds to eukaryotic cells, a function mediated by the B subunit (see Section 1:1:2). One strategy to overcome this non-specific cell binding activity has been to construct immunotoxins from ricin A chain only, either biochemically purified from whole ricin (Gilland et al., 1980, Krolick et al., 1980, Blythman et al., 1981) or with recombinant ricin A chain (FitzGerald et al., 1987; Bjorn and Groetsema, 1987). In many instances these ricin A chain containing-immunotoxins show cell specific cytotoxic activity, a property determined by the specificity of the monoclonal antibody. However, this cytotoxic activity is usually reduced in comparison with equivalent immunotoxins made with whole ricin (Youle and Neville, 1980). This suggests that the presence of ricin B chain somehow acts to enhance cytotoxicity. This ricin B chain potentiation is apparently not a result of the sugar-binding activity of ricin B chain as it can be demonstrated in the presence of high concentrations of free lactose (Youle and Neville, 1980).

Currently a major research effort is being directed towards the design of immunotoxins possessing the potency of whole ricin in the absence of its characteristic, non-specific cell binding activity. As yet the precise role of ricin B chain (other than its sugar-binding activity) during the process of cellular intoxication is unclear. The work presented in this thesis has attempted to examine the function of ricin B chain by exploring the potential of using it as a carrier of a protein other than ricin A chain. In this case ricin A chain has been replaced with the type I ribosome inactivating protein (RIP), gelonin. In addition, the possible significance of regions of ricin A chain to the cytoplasmic entry process have been considered. Preliminary data presented here have demonstrated the maintenance of ribosome inactivating activity after the removal of 30 amino acid residues from the C-terminus of the polypeptide.

Coincident with this recent drug development has been the application of a wide variety of biochemical techniques which have gone some way to improving the understanding of these cytotoxins at both a cellular and molecular level. Yet despite these recent advances much work remains before the biological processes involved during ricin-mediated cytotoxicity can be fully understood. It is probable that an increased understanding of these processes will promote the design of improved immunotoxins.

2:0 Materials

Castor beans of mixed variety were obtained from Croda Premier Oils, Hull and Gelonin as a generous gift from the Institute of Cancer Research, or purchased from Pierce.

All chromatographic materials (Sepharose 4B, Sephacryl S-200, Polybuffer exchanger, polybuffer, PD10 columns, Blue sepharose, Cyanogen bromide activated Sepharose 4B) were obtained from Pharmacia fine chemicals. Galactose, N-acetylgalactosamine and asialofetuin were obtained from Sigma.

SDS-PAGE reagents were supplied by BDH except for acrylamine (Fisons), and bis-acrylamide (Kodak). Reagents for silver staining SDS-PAGE were obtained from Fisons or BDH, silver nitrate was supplied by Johnson Matthey Chemicals.

Nitrocellulose for Western blotting was supplied by Hybond, the Biotin-streptavidin kit by Amersham and the colour developing substrates from Sigma.

Phenylhydrazine, Heparin and Haemin required for the preparation of rabbit reticulocyte lysate were obtained from Sigma, as were the amino acids, creatine phosphate, creatine phosphokinase and spermidine components of the translation reaction mixes. Hepes buffer was supplied by BDH. All nucleotides (e.g. ATP, GTP, rATP, rGTP, rUTP and rCTP were obtained from Boehringer Mannheim. Cap was supplied by Pharmacia.

Phenol, formamide, formaldehyde, polyethylene glycol 6000 and caesium chloride were from Fisons, chloroform, Triton X100 and aniline (for RNA modification) from BDH and RNAase A, agarose, ethidium bromide, ampicillin, lysozyme, IPTG and X-gal from Sigma.

For isolation of DNA fragments, DE81 paper was obtained from BDH and low melting point agarose from BRL.

All components required for the maintenance of bacterial strains were supplied by Difco as was foetal calf serum required in eukaryotic cell media.

Restriction endonucleases were supplied from Amersham, BRL or NBL, T4 DNA ligase, polynucleotide kinase, "Klenow fragment" of DNA polymerase I and SP6 RNA polymerase were obtained through Amersham and calf intestinal alkaline phosphatase from Boehringer Mannheim.

The M13 cloning and sequencing kit came from Amersham as did all radionucleotides and radiolabelled amino acids.

Finally protein derivatisation reagents, SPDP and 2-iminothiolane were obtained through Sigma, Ellman's reagent from Aldrich.

2:1:1 Purification of castor bean lectins

(Nicholson and Blaustein, 1972)

The castor bean lectins, ricin and Ricinus communis agglutinin (RCA_I) were purified from mixed varieties of Ricinus communis seeds obtained from Croda Premier Oils, Hull, U.K.

Prior to grinding, whole seeds were washed, weighed and flash frozen in liquid nitrogen. The frozen seeds were then ground to a dry fine powder using an "atomix" blender and the powder resuspended in phosphate buffered saline (PBS) so as to form a light slurry. The slurry was stored at 4°C for 2-3 hours (with occasional stirring) before filtering through 6 layers of muslin to remove the larger debris. The filtrate from this step was centrifuged at 16,300 x g at 4°C for 10 minutes to pellet smaller debris. Floating fat, released from the seeds was removed at this stage and ammonium sulphate was added to the supernatant to 60% (w/v) at 4°C. Precipitated protein from this step was pelleted by centrifugation as before and the pellet resuspended in a small volume of PBS. The resuspended pellet was then extensively dialysed against PBS prior to loading onto an acid-treated Sepharose 4B column.

2:1:2 Affinity chromatography of castor bean lectins using Sepharose 4B.

Sepharose 4B (Pharmacia) pre-treated with 1 Molar (M) proprionic acid contains within its matrix, exposed sugar residues to which both ricin and RCA_I bind.

A column of Sepharose 4B (25 cm x 5 cm) was equilibrated in PBS at 4°C. Proteins from the prepared castor bean seeds (see Section 2:1:1) were applied directly to the column which was then flushed with PBS until all unbound protein had been eluted. Ricin and RCA_I were eluted together by passing 100 mM galactose in PBS through the column. If necessary the

lectin preparation was concentrated using an Amicon stirred cell ultrafiltration system.

2:1:3 Separation of castor bean lectins using gel filtration upon Sephacryl S-200.

Separation of the two lectins, ricin (60 Kilodaltons, Kd) and RCA_I (120 Kd), was achieved using gel filtration upon a Sephacryl S-200 (Pharmacia) column (80 cm x 3 cm).

The column was equilibrated with PBS at 4°C and the sample (volume < 10 ml) was applied. To achieve good separation of the two lectin species the column was run overnight at a flow rate of 0.7 ml min^{-1}. Fractions of approximately 10 ml were collected. Those fractions from each peak were pooled and concentrated where appropriate.

2:2:1 Purification of ricin subunits.

Ricin is composed of two dissimilar subunits, ricin A chain and ricin B chain. The two subunits, which have separate and distinct functions (see Section 1:1) are joined covalently by a single disulphide linkage. To successfully purify these subunits this disulphide linkage must be cleaved.

2:2:2 Reduction of interchain disulphide bond.

Ricin was incubated with 5% (v/v) 2-mercaptoethanol for 24-48 hours at 4°C in the presence of 100 mM lactose or galactose. The presence of the sugar helps prevent the denaturation and aggregation of the liberated B chain. (Olsnes and Pihl (1973)).

2:2:3 Separation of reduced ricin subunits using chromatofocusing.

The technique of chromatofocusing separates proteins on the basis of their differing isoelectric points (pIs). The widely differing pIs of the ricin subunits makes chromatofocusing a particularly powerful purification method. A sample of reduced ricin will contain both free subunits and a small proportion of non-reduced holotoxin. Using a chromatofocusing system with a pH range of 7-4, all three species can be resolved into separate peaks eluting from the column in order of their pIs viz: A chain pI = 7.5, whole ricin pI = 7.1 and B chain pI = 4.8.

A column of polybuffer exchanger (Pharmacia) (30 cm x 2 cm) was equilibrated in 25 mM degassed, Imidazole buffer (HCl) pH 7.4 at 4°C. The sample of reduced ricin was applied to the column and its components eluted according to their pIs using polybuffer 7-4 (Pharmacia) (HCl) pH 4.0. The addition of the polybuffer creates a pH gradient upon the column which facilitates the separation of proteins according to their pIs.

Optimum resolution of protein components was achieved by running the column overnight at a flow rate of approximately 1 ml min^{-1}. The eluting polybuffer 7-4 was made up with 100 mM lactose (see Section 2:2:2). Fractions from respective peaks were pooled and concentrated as appropriate after dialysis to remove polybuffer components.

2:2:4 Purification of ricin A chain by affinity chromatography using asialofetuin.

By treating the glycoprotein fetuin with neuraminidase it is possible to cleave terminal sialic acid residues from the oligosaccharide side chains creating a molecule with oligosaccharides terminating in galactose (Sandvig et al., (1978)). Asialofetuin is a suitable binding substrate for ricin B chain (K_a approximately 8 x 10^6 M^{-1} - 4.2 x 10^8 M^{-1} (Sandvig et

al., 1976; Baenziger, J. U. and Fiete, D., 1979). Asialofetuin (Sigma) was immobilized by covalent linkage to cyanogen bromide activated sepharose (Sigma), thus forming a column matrix.

Ricin A chain, greatly purified by chromatofocusing was passed through the asialofetuin column (22 cm x 0.5 cm) and the column washed with PBS until all unbound material (ricin A chain) had eluted. The process was repeated, providing a source of highly purified A chain. Any contaminating B chain or unreduced holotoxin remained bound tightly to the column and could not be subsequently eluted. The unbound material consisting of highly purified ricin A chain was pooled and concentrated where appropriate.

2;2;3 Storage of ricin and ricin subunits.

Whole ricin may be stored at -20°C for considerable periods of time without significant loss of biological activity. This activity may become reduced upon frequent freezing and thawing.

Purified ricin subunits are considerably less stable than whole ricin. Purified subunits were therefore never frozen but stored, filter sterile, at 4°C in the presence of 10-50 mM DTT. 100 mM lactose was also included in ricin B chain samples.

2;3;1 Sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE)

(Laemmli, U. K., 1970).

Mini gel system.

Two clean glass plates (one notched to receive a gel comb) were combined with suitable plastic spacers to form a mould for a polyacrylamide

slab gel. The complete mould was placed into a plastic trough containing melted 1% (w/v) Agarose (Sigma) which once set acted as a plug.

A 15% (w/v) polyacrylamide gel solution was prepared according to the recipe below.

Resolving gel.

		Final Concentration
Acrylamide/Bis-acrylamide (30%/0.8% (w/v))	10 ml	15%/0.4%
3 M Tris HCl pH 8.8	2.5 ml	375 mM
H_2O	7.25 ml	-
10% (w/v) SDS	0.2 ml	0.10%
10% (w/v) ammonium persulphate	83 µl	0.04%
TEMED	8.3 µl	

This solution was poured into the frame up to a level just below the position of the well forming comb. The resolving gel was overlaid with water saturated isobutanol and allowed to polymerise. Following polymerisation the isobutanol was removed by rinsing with H_2O and a stacking gel solution poured into the remaining space. A clean plastic well-former was inserted and the gel allowed to polymerise around it.

Stacking gel recipe.

		Final Concentration
Acrylamide/Bis-acrylamide 30%/0.8% (w/v)	2 ml	6%/0.16%
0.5 M Tris HCl pH 6.8	2.5 ml	125 mM
H_2O	5.0 ml	-
10% (w/v) SDS	0.1 ml	0.1 %
10% APS	50 µl	0.05%
TEMED	7.5 µl	

After complete polymerisation the comb was gently removed and any unpolymerised acrylamide solution washed from the wells. The gel was removed from the plastic trough, taking care to ensure the agarose plug remained in position and the gel was assembled into a gel tank. 500 ml of 1x running buffer (25 mM Tris HCl pH 8.8, 200 mM Glycine, 0.01% (w/v) SDS) was required to fill both upper and lower tank reservoirs. Samples for electrophoresis were prepared by diluting with an equal volume of 2x sample buffer, boiling for 2-3 minutes, and then loaded onto the gel. Electrophoresis was carried out at 26 mA constant current for 2-2½ hours.

Gel sample buffer (2x)		Final Concentration
0.5 M Tris HCl pH 6.8	2.5	125 mM
H_2O	0.5	-
Glycerol	2.0	20% (v/v)
10% (w/v) SDS	4.0	0.4% w/v
2-mercaptoethanol	1.0	10% (v/v)
Bromophenol blue	a few grains to colour	-

2:3:2 Non-reducing denaturing gels.

As in Section 2:3:1 except that the reducing gel sample buffer was replaced with a non-reducing buffer in which the reducing agent (2-mercaptoethanol) was replaced with water.

2:3:3 Sodium dodecyl sulphate - Polyacrylamide gel electrophoresis.

20 cm x 20 cm system.

The larger gel system has two principle advantages over the mini gel system (Section 2:3:1). Firstly, larger sample volumes may be loaded and

secondly greater resolution of protein bands may be achieved by electrophoresing proteins over a greater distance. It is, in contrast, less convenient and more time consuming.

Resolving gel

	10%	12%	15%	20%
30% Acrylamide 0.3% Bis-Acrylamide (w/v)	16	20	24	32
3 M Tris-HCl pH 8.8	6	6	6	6
H_2O	25.4	21.4	17.4	9.4
10% (w/v) SDS	0.48	0.48	0.48	0.48
10% (w/v) APS	200 µl	200 µl	200 µl	200 µl
TEMED	20 µl	20 µl	20 µl	20 µl

Units in mls unless otherwise stated.

Stacking gel.

10% Acrylamide 0.5% Bis-acrylamide (w/v)	5
0.5 M Tris HCl pH 6.8	2.4
H_2O	2.4
10% (w/v) SDS	0.1
10% (w/v) APS	100 µl
TEMED	5 µl

Sample buffer as in Sections 2:3:1 and 2:3:2.

Gel running buffer as in Section 2:3:1.

Gels were electrophoresed overnight at 8 mA for 15% resolving gel and for 6-8 hours; at 19 mA for 10% gels.

2:4 Acrylamide gel staining techniques.

2:4:1 Silver staining polyacrylamide protein gels.
(Merril et al., 1981)

Prior to staining, polyacrylamide gels were washed several times with 50% methanol to remove residual glycine absorbed from the gel running buffer during electrophoresis. To stain the gel, 0.8 g of silver nitrate was dissolved in 4 mls H_2O. This solution was added dropwise with rapid stirring to a solution of 21 mls 0.36% (w/v) NaOH and 1.4 ml 14.8 M ammonium hydroxide. The mixture was made up to 100 mls with distilled H_2O and applied directly to the gel for 15 minutes with constant shaking. After 15 minutes the gel was washed extensively with distilled H_2O to remove any excess silver ions before developing the staining reaction with 0.025% citric acid solution containing 0.25-0.5 ml 38% (v/v) formaldehyde solution. Bands develop between 5-10 minutes depending on the protein loadings of the gel. The reaction was stopped by placing the gel into destain solution comprising 10% Acetic acid, 45% methanol.

2:4:2 Coomassie Blue staining.

Stain solution.

0.5% (w/v) Coomassie Blue (Sigma) in 10% Acetic acid
45% Methanol

Immediately after electrophoresis gels were removed into the stain solution for 15-30 minutes with shaking.

Following staining the gels were removed into a destain solution of 10% Acetic acid and 45% methanol. Gels were destained until the background became clear.

2:4:3 Drying of SDS-PAGE gels.

Polyacrylamide gels were dried onto 3 MM filter paper using a vacuum drier. A piece of 3 MM paper cut to the size of the gel was placed in position on the drier. The paper was soaked with H_2O, and the gel placed upon the paper. Cling-film was placed over the gel, and a piece of card laid on top to prevent the gel from cracking. With the gel in position, vacuum and heat were applied for one hour. The dried gel was removed and stored flat.

2:4:4 Fluorography and autoradiography.

Radioactively labelled proteins were visualised by fluorography and autoradiography.

Gels containing radiolabelled proteins were washed extensively in destain solution (10% Acetic acid, 45% methanol) to (a) fix the proteins in the gel and (b) flush out any excess label not incorporated into the protein. Once fixed, the gel was placed in "Amplify" (Amersham) for 30 minutes and then dried as described in Section 3:4:4. Finally, the dried gel was affixed into a light-proof cassette and exposed to X-ray film (Fuji).

Films were developed under dark-room conditions using LX24 developer, (Kodak) and fixed with Unifix (Kodak), both diluted 1:4 with H_2O.

2:5 Sample preparation for SDS-PAGE.

2:5:1 Trichloroacetic acid protein precipitation.

To reduce sample volumes for convenient loading onto SDS-PAGE gels proteins were precipitated by adding trichloroacetic acid (TCA) at a final concentration of 10% (w/v). Samples were stood on ice for 30 minutes and precipitated proteins pelleted by a 15 minute spin at 13,000 rpm in a microcentrifuge. The supernatant was carefully discarded and the pellet washed in 100% acetone. The sample was recentrifuged, the acetone removed, and the pellet resuspended in sample buffer prior to loading onto the gel, as in Section 2:3:1.

2:5:2 Acetone protein precipitation.

Protein samples for precipitation were mixed with an equal volume of cold 100% acetone and stored at -20°C for a minimum of 2 hours. The precipitated proteins were then pelleted at 13,000 rpm for 15 minutes in a microfuge and all traces of acetone removed. The pellet was resuspended in sample buffer.

2:5:3 Treatment of proteins with Endo β-N-acetylglucosaminidase H (Endo H).

(Forwell et al., 1985; Forwell et al., 1987)

Proteins for Endo H treatment were TCA precipitated (see Section 2:5:1) and resuspended in 10 μl of 0.1 M sodium citrate buffer pH 5.6, containing 1% (w/v) SDS and 1 mM Phenylmethylsulphonylfluoride (PMSF). The samples were boiled for 2 minutes and then the SDS diluted 10 fold by adding Sodium citrate buffer pH 5.6, minus SDS. Each 100 μl sample was

divided into two, one remaining untreated the other incubated with 2.5 mU of Endo H (Miles Scientific). Samples were incubated at room temperature overnight and analysed by SDS-PAGE.

2:6 Western blotting.

2:6:1 Biotin-streptavidin method.

Proteins initially separated by SDS-PAGE were transfered onto nitrocellulose paper using the Bio-Rad transblotting apparatus. Transfer was achieved by sandwiching the polyacrylamide gel next to the nitrocellulose paper such that when an electrical current was applied, proteins would migrate from the gel and bind in the same relative positions upon the nitrocellulose paper. The transfer buffer was 20 mM Tris-HCl, 200 mM Glycine, 20% methanol pH 8.3 and blotting was carried out at 60 volts for 2 hours or 30 volts overnight.

Following blotting the gel and nitrocellulose sandwich was separated. The gel was placed into 50% methanol prior to silver staining (see Section 2:4:1), whilst the nitrocellulose filter was placed into PBS containing 1% "Marvel" milk product. Three 10 minute washes in the "Marvel" solution was adequate to prevent non-specific binding of antibodies to the filter. The filter was then probed with specific antibodies (a polyclonal preparation prepared from rabbit serum) raised against target proteins, for 2-4 hours at room temperature or overnight at 4°C. Following incubation the antibody solution was removed and stored at -20°C. The filter was washed 5 times in blocking solution and then incubated with a 1:300 dilution of biotinylated-protein A (Amersham) for 1 hour at room temperature. The filter was then washed as before and further incubated with a 1:300 dilution of Streptavidin-horseradish peroxidase complex (Amersham) for 30 minutes at room temperature. The filter was washed a further 5 times in blocking

solution prior to development.

2:6:2 Developing using diaminobenzidine substrate (DAB).

Following washing with blocking solution the filter was given a further two washes in PBS, before a final wash in 50 mM Tris-HCl pH 7.4, 0.9% (w/v) NaCl. For small filters, 10 mls of the final wash buffer was retained, into which 6 mg of DAB and 15 µl of 20% (v/v) hydrogen peroxide was added. The filter was developed in this solution which was scaled up approximately for larger filters. The reaction was stopped by air drying the filter.

2:6:3 Developing using 4 Chloro-1-Napthol (4ClN).

The filter was washed as described above except that the final wash was 0.5 mM Tris-HCl pH 7.4, 125 mM NaCl in a final volume of 50 mls. 30 mg of 4ClN was dissolved in 10 mls methanol together with 20 µl of 20% (v/v) hydrogen peroxide. The 4ClN solution was added to the final wash to develop the filter. The reaction was stopped by air drying the filter.

2:7 Electroelution.

2:7:1 Preparative SDS-PAGE.

Separated proteins were extracted from polyacrylamide gels by excising the appropriate gel bands and electroeluting the protein from the acrylamide into solution.

SDS-PAGE gels were prepared as Section 2:3:3 using a large single well forming comb. The gel was run as normal and then the edge of the wells trimmed and silver stained according to Section 2:4:1. Following staining,

the edge strips were used to identify the position of the appropriate band in the unstained region which was then excised from the gel prior to electroelution.

2:7:2 Electroelution of excised protein.

Strips of polyacrylamide gel containing the excised protein were placed into glass rods sealed at one end with an agarose plug. Sealed dialysis tubing containing a small volume of running buffer was placed over the end of each tube and the glass rods positioned so as to span the upper and lower buffer reservoir tanks of the electroelution equipment. Electrical connections were made so that the lower tank became the positive terminal and the protein was electroeluted overnight at 70 volts constant voltage. Immediately before collecting the sample from the dialysis tubing the direction of electrical current was reversed for 2 minutes to liberate any material adhering to the sides of the dialysis tubing. The collected samples was analysed by SDS-PAGE.

2:8:1 Spectroscopic determination of protein concentration.

Extinction coefficients for 1 mg/ml solutions at 280 nm wavelength

Ricin (1 mg/ml) - $E^{1\%}_{280}$ -	1.14
A chain	0.7
B chain	1.49
Gelonin	0.68

2:9 Biological Activity Assay Techniques.

2:9:1 Cell cytotoxicity assay.

Vero (African Green Monkey Kidney) Cells at 1 x 10^6 cells/ml were seeded (100 µl/well) into 96 well microtitre plates (Costar). The cells, growing in Glasgow's minimal essential media (GMEM), non-essential amino acids (NEAA) plus 5% (v/v) foetal calf serum (FCS), (Difco) and 4 mM glutamine were maintained at 37°C with 5% CO_2. Cells were allowed to form a stable monolayer before the media was removed, and replaced with 100 µl of toxin dilution in sterile PBS. The toxin was added directly onto the cells for 1 hour, then diluted by the addition of 100 µl of fresh media and incubated overnight. Following intoxication, the media bathing the cells was removed and replaced with 100 µl of sterile PBS containing (1 µCi/100 µl) [^{35}S] methionine 30 TBq/mmol (Amersham) for 2 hours at 37°C, 5% CO_2. Finally all label was removed, the cells washed twice with 5% (w/v) TCA and then dissolved in fresh 1 M KOH (200 µl/well). After 1-2 hours to dissolve the cells, the contents of each well were removed and counted in a liquid scintillation system in an LKB rackbeta 2010. Results were expressed as a percentage of no toxin controls. All assays were carried out in tripicate.

2:9:2 Toxin preparation for cell cytotoxicity assay.

Stock solutions of proteins used in the cytotoxicity assay were diluted with sterile PBS to give a range of toxin concentrations from 1 x 10^{-11} M to 1 x 10^{-6} M

Subunit reassociation experiments involving the mixing of ricin B chain with either purified native ricin A chain or chemically derivatised (see Section 2:12) gelonin (a type I ribosome inactivating protein (RIP) were carried out as follows:-

Ricin A chain/gelonin dilutions were made in sterile PBS from 2 x 10^{-11} M to 2 x 10^{-8} M. Each dilution was then mixed with an equal volume of 2 x 10^{-8} M ricin B chain, and the mixture dialysed overnight against PBS at 4°C to remove any reducing agent or lactose from the preparation. Following dialysis, samples were applied to cells as outlined in the previous section.

2:10 Cell-free toxicity assay.

2:10:1 Preparation of rabbit reticulocyte lysate.

(Pelham and Jackson, 1976; Allen and Schweet, 1962; Clemens, 1984)

Rabbits were injected subcutaneously with 1.0 ml of 2.5% (v/v) phenylhydrazine (Sigma) daily for 5 days to induce anaemia. The rabbits were rested for 1 day and then bled by cardiac puncture. The blood was stored on ice in tubes containing heparin and then centrifuged at 2,500 x g for 15 minutes to pellet the cells. The plasma supernatant was discarded and the cells resuspended in washing buffer (0.13 M NaCl, 0.5 mM KCl, 7.5 mM $MgCl_2$) to a volume equal to the discarded plasma volume. The washing step was repeated a further two times and the cells then lysed by adding four times the packed cell volume of 2 mM $MgCl_2$ and stirring for 10 minutes on ice. One packed cell volume of 1.5 M sucrose containing 0.15 M KCl was added and stirring on ice continued for a further 10 minutes. The lysate solution was centrifuged at 15,000 x g for 10 minutes at 4°C. The supernatant from this step (the reticulocyte lysate) was aliquoted into 1 ml samples and stored in liquid nitrogen.

2;10;2 Preparation of reaction mix.

(Clemens, M. J., 1984).

L-amino acid mixtures

1 mM stock solutions of the following amino acids were prepared.

Amino acid.

Alanine	Histidine hydrate HCl	Tryptophan
Arganine HCl	Isoleucine	Tyrosine
Aspartic acid	Leucine*	
	Lysine HCl	Valine
Asparagine hydrate	Methionine*	Hydroxyproline
Cysteine	Phenylalanine	
Glycine	Proline	
Glutamic acid	Serine	
Glutamine	Threonine	

A 100 ml solution of 1 mM amino acids was prepared, neutralized to pH 7.4 (NaOH) and stored at -70°C.

*When using [^{14}C] leucine as a radiolabel an amino acid mix minus non-labelled leucine was used. When [^{35}S] Methionine was the radiolabelled amino acid the mix was prepared to include leucine, but non-labelled methionine was omitted.

Stock Solution A.

40 mM Magnesium acetate, 2 M Ammonium acetate, 200 mM Tris HCl pH 7.4 stored at 4°C.

Stock Solution B (made up frozen).

ATP	3.5 H_2O	(Sigma)	4.7×10^{-3} M
GTP	3.0 H_2O	(Sigma)	4.5×10^{-3} M

The rabbit reticulocyte assay mixture was made up as follows:

Amino acid mixture	1 ml
Solution A	1 ml
Solution B	0.1 ml
Creatine phosphate 5 H_2O (Sigma)	103 mg
Creatine kinase (Sigma)	1 mg
H_2O	510 µl
* α[^{14}C] leucine 11 GBq/mmol (Amersham)	600 µl

* For [^{35}S] methionine 30 TBq/mmol add 10 µCi (1 µl) per sample, fresh.

2;10;3 Cell-free toxicity assay using non-nuclease treated rabbit reticulocyte lysate.

The biological activity of free ricin A chain and other type I ribosome inactivating proteins (RIPs) was determined using a cell-free toxicity assay as follows:

A 20 ng/ml stock solution of the toxin was made in PBS/Bovine serum albumin (BSA) (2 mg/ml). Serial dilutions of 20, 10, 5, 2.5, 1.25 and 0.625 ng/ml were made giving final concentrations of 8, 4, 2, 1, 0.5 and 0.25 ng/ml in the total reaction mix.

25 µl aliquots of each toxin dilution were prepared in triplicate, including 3 x 25 µl H_2O controls for background radioactivity and 3 x 25 µl H_2O controls for 100% incorporation (no toxin controls). 12.5 µl of assay

mixture was added to each tube and allowed to equilibrate at 28°C. 1.0 ml of 0.1 M KOH was added to the three zero control tubes and then 25 µl of rabbit reticulocyte added to each tube at 15 second intervals. After 10 minutes the reaction was stopped by the addition of 1 ml 0.1 M KOH to each tube. 2 drops of 20% (v/v) H_2O_2 was added to each to bleach the reticulocyte mixture followed by 1 ml of 20% (w/v) trichloroacetic acid. The solution was mixed thoroughly and stood at 4°C for a minimum of 1 hour to precipitate any synthesized protein.

Precipitates were filtered onto Whatman glass microfibre filters grade GF/C (BDH) presoaked in H_2O. The filters were washed three times with 5% (w/v) TCA, dried and then counted using an LKB rackbeta liquid scintillation counter.

Results were corrected to background and expressed as a percentage of the 100% incorporation controls.

2:11 Ribosomal RNA modification.

(Endo et al., 1987)

2:11:1 Incubation of eukaryotic ribosomes with toxin.

Rabbit reticulocyte lysate was prepared as described in Section 2:10:1. 30 µl of non-nuclease treated lysate was mixed with 30 µl of toxin dilution and incubated at 30°C for 30 minutes. After incubation, lysate samples were diluted by the addition of 40 µl Kirby buffer (see Section 2:11:2), mixed and the total RNA extracted by two rounds of phenol/chloroform extraction. Phenol/chloroform extractions were carried out as follows: to the 100 µl sample, 50 µl of TE saturated phenol and 50 µl of chloroform were added. The solution was mixed, and the two phases separated by a brief centrifugation (2-3 minutes at 13,000 rpm in the bench centrifuge). The upper (aqueous) layer was removed and the procedure

repeated. RNA obtained from the aqueous phase of the second extraction was precipitated at -20°C by the addition of 0.1 x volume of 7 M Ammonium acetate and two volumes ethanol. RNA was pelleted by centrifugation (15 minutes at 13,000 rpm), rinsed in 70% ethanol, dried and resuspended in 10 µl sterile H_2O.

2:11:2 Kirby buffer.

Kirby buffer (Parish and Kirby, 1966) was added to aid the dissociation of RNA from protein thereby improving RNA yields.

1.2 g of 4 aminosalicylic acid (BDH) dissolved in 5 ml H_2O was mixed with 40 mM KCl, 10 mM Tris HCl pH 7.6 The cloudy solution formed was cleared by adding a few drops of phenol and the solution made up to 70 ml with H_2O.

2:11:3 Determination of RNA concentration.

RNA samples prepared in Section 2:11:1 were diluted 1 in 500 with H_2O and their absorbance measured at a wavelength of 260 nm.

Concentration of RNA (µg/ml) =

$$\frac{OD_{260} \times \text{Extinction coefficient} \times \text{dilution factor}}{1000}$$

$$= \frac{OD_{260} \times 40 \times 500}{1000}$$

$$= OD_{260} \times 20$$

2:11:4 Treatment of RNA with aniline.

4 μg of RNA was incubated with 20 μl aniline pH 4.5 at 60°C for 3 minutes. Immediately after incubation the samples were precipitated, in ethanol as described in 2:11:1, rinsed, dried and then resuspended in 20 μl of RNA sample buffer. Samples were heated to 65°C for 5 minutes, cooled on ice, mixed with 4 μl of dye and electrophoresed.

2:11:5 Electrophoresis of RNA samples.

1 x TEP - 36 mM Tris, 30 mM $NaH_2PO_4(2H_2O)$, 2 mM EDTA pH 8.0

RNA sample buffer - 60% formamide (deionized)
0.1% TEP

1.2% (w/v) Agarose formamide gel buffer 50% (v/v) formamide (deionized)
0.1% (v/v) TEP

Running buffer 0.1% (v/v) TEP

The 50% formamide - 1.2% (w/v) agarose gel was prepared using RNAase free gel former and comb. Once set, the gel was positioned into the gel tank and running buffer added level with but not over the gel. 20 μl of RNA sample buffer was placed into each well, then the samples loaded. The gel was electrophoresed at 20 mA constant current for 2-3 hours. Following electrophoresis the gel was stained with ethidium bromide for 15 minutes then destained in water prior to photography.

2:12 Protein-protein conjugation using heterobifunctional thiol linkers. (Carlsson et al., 1978; Thorpe and Ross, 1982; Cumber et al., 1985)

2:12:1 Protein derivatisation using N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP).

Heterobifunctional thiol linkers were used to introduce reactive thiol groups into proteins facilitating the formation of protein conjugates by disulphide exchange.

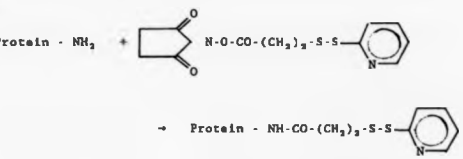
Protein for derivatisation was equilibrated in 50 mM boric acid, 290 mM NaCl buffer-NaOH pH 9.0 and adjusted to a concentration of 10 mg/ml. A pH of 9.0 favours the reaction of SPDP with amino side chains over the N-terminus of the protein. To introduce 1-1.5 SPDP residues/molecule of protein, SPDP was added in a 2.5 molar excess over the protein molarity.

Molecular weight SPDP = 314.2

$$\frac{\text{Amount of protein (mg)} \times \text{molar excess SPDP}}{\text{Molecular weight of protein}} = \frac{\text{Amount of SPDP}}{\text{Molecular weight SPDP}}$$

The appropriate amount of SPDP was dissolved in dry dimethylformamide, (10 µl per ml of protein solution) and added to the protein solution. The reaction took place at room temperature for 1 hour with stirring. Free SPDP was separated from derivatised protein using a PD10, G25M pre-poured column (Pharmacia) equilibrated in PBS. 500 µl fractions collected from the column were monitored at 280 nm and fractions containing the derivatised protein (first peak) were pooled.

2:12:2 Determination of the extent of derivatisation with SPDP.



The extent of derivatisation of a protein with SPDP can be measured by determining the release of S-pyridyl groups from the derivatised protein in the presence of a reducing agent such as dithiothreitol (Sigma).

500 µl of derivatised protein (OD_{280} > 0.2 OD units) was placed into a 1 ml cuvette. The optical density (O.D.) was measured at 450 nm to give a base-line zero, 343 nm to measure the release of free S-pyridyl groups and 280 nm to measure protein absorbance. Once the readings became steady 50 µl of 100 mM dithiothreitol (DTT) was added to the cuvette. The base-line was rezeroed at 450 nm and the absorbance at 343 nm and 280 nm measured.

The release of S-pyridyl groups was calculated as below

$$[\text{S-pyridyl}] = \frac{\text{Volume sample + DTT}}{\text{Volume sample}} \times \frac{\text{Change in } OD_{343}}{\text{Extinction coefficient of S-pyridyl}} = (A)\,M$$

Molar extinction coefficient of S-pyridyl groups at 343 nm = 8.08×10^3

The OD_{280} of the derivatised protein will have been increased by the presence of S-pyridyl groups. Therefore the correct OD_{280} was obtained by:

(A)M x molar extinction coefficient of S-pyridyl at 280 nm

and substracting this amount from the OD_{280} measured before the addition of DTT.

The concentration of protein was determined by:

$$\text{(B) M - [Protein]} = \frac{\text{correct } OD_{280}}{\text{Extinction coefficient for protein at 280 nm x molecular weight of protein}}$$

To determine the ratio of S-pyridyl groups to protein divide the value (A)M by the value B(M).

The appropriately derivatised protein was concentrated to 5-10 mg/ml using an Amicon stirred cell ultrafiltration system with a YM2 membrane (Amicon).

2:12:3 Protein derivatisation using 2-iminothiolane (Sigma).

An alternative method of introducing reactive thiol groups into proteins is to use 2-iminothiolane hydrochlorate (2-IT).

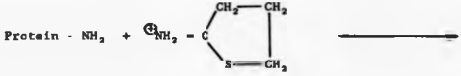
As with derivatisation with SPDP the protein sample was equilibrated in a borate buffer NaOH pH 9.0 and adjusted to a concentration of 10 mg/ml. To introduce a single 2-IT group into each protein molecule, 2-IT was reacted in a 10 fold molar excess over the protein concentration, calculated as below:

$$\frac{\text{Amount of protein (mg) x molar excess}}{\text{Molecular weight of protein}} = \frac{\text{Amount of 2-IT (mg)}}{\text{Molecular weight of 2-IT}}$$

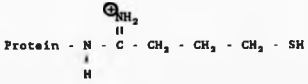
The 2-IT was dissolved in borate buffer pH 9.0 and the concentration adjusted so as to deliver the correct amount in 25 µl/ml of protein. The solution was reacted at room temperature for 1 hr. The reaction was stopped by adding 0.1 x volume of borate buffer containing 2.2 M glycine

and reacting for a further hour at room temperature. The derivatised protein was then treated for a further hour with 5'5' Dithiobis-(2-nitrobenzoic acid), Ellman's reagent (Aldrich). Ellman's reagent was added 50 µl/ml to give a final concentration of 2 mM. The addition of Ellman's was accompanied by the appearance of a bright yellow colour indicating the release of the ⅓ Ellman's group (see reaction).

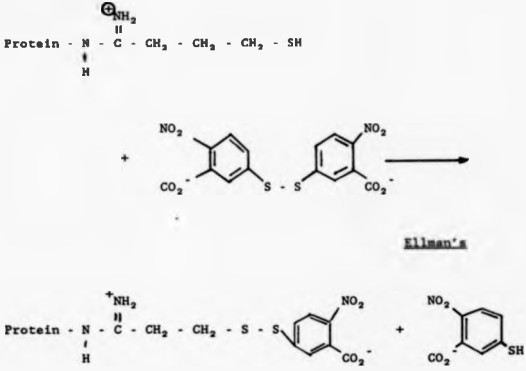
After the hour incubation with Ellman's reagent the derivatised protein was separated from unbound 2-IT using a PD10 column as described in Section 2:12:1.



2-iminothiolane



The -SH group from the 2-IT is now activated by the addition of Ellman's reagent.



Derivatised protein containing the protected thiol group from 2-IT.

2:12:6 **Determination of derivatisation with 2-iminothiolane.**

(Ellman et al., 1959)

The extent of derivatisation with 2-IT can be determined by following the release of 4 Ellman's following the reaction of the derivatised protein with a reducing agent such as dithiothreitol.

A 500 µl sample (OD_{280} > 0.2 units) was placed in a 1 ml quartz cuvette. The absorbance was determined at 600 nm, base-line zero, 412 nm, absorbance of 4 Ellman's and 280 nm absorbance of protein. Once the readings remained steady 50 µl of 100 mM DTT was added, the sample re base-lined at 600 nm and the change in absorbance recorded at 412 nm and 280 nm.

The extent of derivatisation was calculated as follows:-

$$[\text{½ Ellman's}] = \frac{\text{Volume of sample + DTT}}{\text{Volume of sample}} \times \frac{\text{Change in OD}_{412}}{\text{Extinction coefficient for ½ Ellman's}} = (A)M$$

$$[\text{Protein}] = \frac{\text{OD}_{280\text{nm before addition of DTT}}}{\text{Extinction coefficient at 280 nm of protein} \times \text{Molecular weight of protein}} = (B)\ M$$

The loading of 2-IT was determined by A/B.

If necessary the derivatised protein was concentrated and then stored filter sterile at 4°C.

2:13 Preparation of polyclonal antibodies raised against ricin subunits.

Highly purified ricin subunit preparations were used as antigens to raise antibodies in rabbits.

2:13:1 Innoculum preparation.

Prior to injection collect pre-immune serum.

50 µg of protein (in PBS) was mixed with 0.5 ml Freund's Complete Adjuvant (FCA) until the preparation became white and viscous. The innoculum was taken up in a 1 ml syringe and injected subcutaneously into New Zealand white rabbits. The rabbits were rested for two weeks and then given a booster injection of 100 µg protein in 0.5 ml Freund's Incomplete Adjuvant (FICA). After a further two weeks rest blood samples were collected by ear bleeds and the rabbit given a second booster injection.

2:13:2 **Preparation of antisera.**

Collected blood was "ringed" (detached from the edges of the glass collecting bottle) and allowed to form a clot overnight at 4°C. The sample was centrifuged at 6000 rpm in a benchtop centrifuge for 15 minutes and the serum collected. The volume of serum was measured, and ammonium sulphate added to form a 50% (w/v) saturated solution. Precipitated material was pelleted (12,000 rpm in the 8 x 50 rotor for 20 minutes) and then resuspend in PBS to a volume equal to half the original serum volume. The sample was dialysed against PBS and then divided into 500 µl aliquots for storage at -20°C.

2:14:1 **Growth and maintenance of Escherichia coli strains.**

Stock strains of *E. coli* were plated on L-agar media (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1.5% (w/v) Agar, 85 mM NaCl) and stored at 4°C.

Liquid cultures of *E. coli* were grown in L-Broth (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 170 mM NaCl). Liquid cultures were maintained at 37°C in orbital shakers at 200 rpm. Cells containing plasmids conferring antibiotic resistance were grown in, or plated on, media containing the appropriate antibiotic. Cells with plasmid conferred ampicillin resistance were grown in the presence of 0.1 mg/ml ampicillin.

2 : 13 : 3 ^{125}I labelling of proteins

Proteins requiring an ^{125}I label were treated as follows:

An aliquot, 0.5 - 1.0 mCi (5 - 10 µl) of $Na^{125}I$ was diluted with 10 µl 25 mM phosphate buffer pH 7.5.

Approximately 5 µg (10 µl) of protein was rapidly mixed with 50 µg (10 µl) of chloramine T solution and 120 µg (100 µl) of sodium metabisuphite solution (1.2 mg/ml). All solutions were made up with phosphate buffer as described earlier. The volume of this solution was made up to 1 ml with NaI solution (2.0 mg/ml). The NaI solution contained the $Na^{125}I$ component so that a measure of the total radioactivity in a known volume (1 ml) could be determined.

Labelled protein was separated from unreacted NaI using a prepacked PD10 (G25F) column. Fractions from this column containing the labelled protein were pooled and samples removed for counting.

An index of ^{125}I cpm/mg protein was determined.

2:16:2 Bacterial Strains.

(Carter et al., 1985)

Strain		Genotype
BMH	71.18	K12 Δ(lac-pro) supE thi/F' pro A'B' laq Iq lac Z ΔM15
BMH Mut L	71.18	As BMH 71.18 but Mut L :: Tn10

The above bacterial strains must be grown on minimal glucose plates. Mut L (Repair -) strains mutate at high frequency and should therefore be stored in glycerol at -20°C.

2:15:1 Preparation of competent cells.

E. coli cells were grown in a 50 ml culture of L-Broth at 37°C and 200 rpm to an optical density of 0.48-0.5 units at 550 nm. At this density the exponentially growing cells were removed from incubation and placed on ice. Cells were centrifuged a 3000 x g for 10 minutes, the supernatant carefully removed and the pellet gently resuspended in 25 ml (0.5 volume) of ice cold 50 mM $CaCl_2$. The suspension was stood on ice for 30 minutes and the cells repelleted as before. The pelleted cells from this centrifugation step were resuspended in 2.5 ml (0.05 volume) and stood on ice for a further 30 minutes after which the cells were judged as competent.

2:16:1 Transformation of competent cells.

100 µl of competent cells were mixed with dilutions of the DNA and stood on ice for 30-45 minutes. The cells were heat-shocked at 42°C for 90-120 seconds and then diluted to 1 ml with L-Broth. Cells were incubated for 1 hour at 37°C, pelleted by a brief centrifugation in a bench top microcentrifuge and then resuspended in 100 µl L-Broth prior to plating on appropriate L-agar plates for overnight incubation at 37°C.

2:17 Extraction and purification of plasmid DNA.

2:17:1 Mini preparation of plasmid DNA using the boiling method.
(Holmes and Quigley, 1981)

5 ml aliquots of L-Broth containing 0.1 mg/ml ampicillin were innoculated with individual colonies selected from L-Amp plates of transformed E. coli. The cultures were grown overnight at 37°C and 200 rpm and then harvested by centrifugation at 2000 rpm in a bench top centrifuge. Cell pellets were resuspended in 180 µl of 20% (w/v) Sucrose, 100 mM Tris HCl pH 8.0, 50 mM EDTA (SET) buffer and mixed with 180 µl of 4 mg/ml solution of lysozyme made up in the same buffer. Cells plus lysozyme were stood at room temperature for 5 minutes then mixed with 300 µl of 10% (v/v) Triton X-100 and boiled for 2 minutes before immediately returning to ice. The boiling and snap cooling acts to denature the chromosomal DNA which does not reanneal correctly, whereas the two DNA strands of the much smaller plasmid DNA correctly reanneals. The lysed bacterial cells were then centrifuged for 15 minutes in a microcentrifuge (13,000 rpm) and the pellet discarded. The supernatant was mixed with 300 µl of 7.5 M ammonium acetate and stood on ice for 20 minutes to precipitate any soluble protein, which was pelleted by bench top centrifugation as before. Finally, 630 µl

of propan-2-ol was added to the supernatant and the DNA precipitated by incubating the mixture at -20°C for 10 minutes.

The precipitated DNA was pelleted in a microcentrifuge for 10 minutes at 13,000 rpm, rinsed using 70% (v/v) ethanol, dried under vacuum and dissolved in 60 µl 10 mM Tris (HCl) pH 7.2, 10 mM EDTA pH 7.2 (TE) buffer.

2:17:2 Mini preparation of plasmid DNA using the Alkaline lysis method. (Birnboim and Doly, 1979)

Cells were grown and harvested as in Section 2:17:1. The pellet of cells from each 5 ml culture was resuspended in 100 µl of 20% (w/v) glucose, 100 mM Tris HCl pH 8.0, 50 mM EDTA (GET) buffer and then mixed with 100 µl of 5 mg/ml lysozyme made up in the same buffer. After 5 minutes at room temperature, 200 µl of lysis solution (1.0% (w/v) sodium dodecyl sulphate, 250 mM NaOH) was added and the mixture incubated on ice for a further 5 minutes. 150 µl of 3M sodium acetate (HAc) pH 4.8 was added, the solution vortexed, and stood on ice for 5 minutes after which it was centrifuged at 13,000 rpm for 10 minutes in a microcentrifuge. The supernatant was removed and extracted with TE saturated phenol/chloroform (50:50). The volume of the upper aqueous layer was measured and 0.1 volume of 7.5 M ammonium acetate and two volumes of 100% ethanol were added. The DNA was precipitated at -20°C for 30 minutes, then pelleted in a microfuge for 10 minutes, rinsed in 70% (v/v) ethanol, dried briefly and dissolved in 50 µl TE.

2:17:3 Large-scale preparation of plasmid DNA using the boiling method. (Holmes and Quigley, 1981)

400 ml cultures were innoculated with transformed *E. coli* cells and grown overnight at 37°C and 200 rpm. Cells were pelleted by centrifugation

(4000 x g for 5 minutes at 4°C) and resuspended in 7.2 ml SET, to which an equal volume of 4 mg/ml lysozyme was added. After 5 minutes at room temperture, 12 ml of 10% (v/v) Triton X-100 was added, and the mixture heated over a bunsen flame until the cell suspension became viscous. The lysed cells were immediately cooled on ice then transferred to 40 ml plastic Oakridge tubes. Cell debris and insoluble proteins were removed by centrifugation (48,000 x g for 25 minutes at 4°C), the supernatant removed and mixed with a 0.5 volume of 7.5 M ammonium acetate on ice for 20 minutes to precipitate soluble proteins. The precipitated proteins were removed by centrifugation (27,000 x g for 10 minutes at 4°C) and the supernatant mixed with 0.7 volume of propan-2-ol at -20°C for 10 minutes. Precipitated DNA was recovered by centrifugation (27,000 x g for 10 minutes) and the pellet drained.

2:17:4 Equilibrium density gradient centrifugation (Caesium Chloride Centrifugation).

The drained DNA pellet (from 2:17:3) was resuspended in EDTA TE buffer (10 mM Tris pH 8.0, 0.1 mM EDTA), mixed with 4.3 g CsCl and 0.5 ml (5 mg/ml) ethidium bromide. The dissolved solution was transferred to a 5 ml clearseal Beckman centrifugation tube, heat sealed and then centrifuged in a Beckman ultracentrifuge using a Vti 65.1 rotor for 7 hours at 55,000 rpm at 18°C. After ultracentrifugation the tubes were carefully removed, and the lower of the two horizontal bands apparent on the gradient removed using a hypodermic needle and syringe. The lower band represents plasmid DNA whilst the upper is chromosomal DNA.

The collected plasmid DNA was repeatedly mixed with an equal volume of isobutanol saturated with TE buffer containing excess CsCl, until all traces of ethidium bromide had been removed from the upper aqueous layer. The CsCl present was diluted by the addition of 4 volumes of TE and the DNA

precipitated by the addition of 0.1 volume of 3 M sodium acetate pH 6.0 and two volumes at 100% ethanol at -20°C for 30 minutes. Precipitated DNA was collected by centrifugation (27,000 x g for 10 minutes at 4°C, rinsed in 70% (v/v) ethanol and dissolved in 400 µl TE buffer.

2:18 Preparation of DNA from the single-stranded DNA bacteriophage M13. (Schrier and Cortese, 1979; Messing, 1983)

M13 is a filamentous, single-stranded bacteriophage ('phage) whose life cycle can be exploited for the production of single-stranded DNA template (Schrier and Cortese, (1979)).

The 'phage invades the host cell (E. coli F') via the F pillus. Once inside, the single-stranded DNA (of the uncoated 'phage particle) acts as a template for the synthesis of a complementary strand to produce the so-called replicative form (RF) which is double-stranded. DNA replication, exploiting the host cells replicative apparatus, produces a population of daughter RF molecules, (up to 100+ per cell) until a point is reached when replication becomes asymmetric such that new single-stranded DNA is synthesised. This occurs by the rolling circle method of replication. The DNA is packaged in viral coat proteins and mature phage particles are extruded from the host cell without causing cell lysis. Some 200 'phage particles per cell, per generation can be harvested as a source of pure single-stranded DNA.

The RF form of M13 being double-stranded is susceptible to enzymic manipulation and may therefore be used as a cloning vector. The Messing series of M13 mp vectors (Messing, J., 1983) have been specially modified to fulfil the role of cloning vehicle. They possess a cluster of convenient cloning sites (referred to as the polylinker or multiple cloning region) and a system allowing selection of recombinant over non-recombinant clones. This selection is based on the presence or absence of the enzyme

β-galactosidase. E. coli host cells infected with non-recombinant M13 (from the Messing series) will in the presence of IPTG (the lac operon inducer) produce a functional β-galactosidase which will hydrolyse the substrate X-gal to produce a blue dye and therefore blue plaques. Insertion of foreign DNA into the vector interferes with β-galactosidase production which under selection conditions produces colourless plaques.

2:18:1 **Preparation of single-stranded M13 DNA.**

The fact that double-stranded M13 DNA can also exist as single-stranded DNA depending on the natural infective cycle is important for two techniques where single-stranded DNA templates are required viz : dideoxy chain termination DNA sequencing (Sanger et al., 1977) and site directed mutagenesis (Hutchinson et al., 1978). Single-stranded M13 DNA was prepared as follows.

An appropriate volume of L-Broth was innoculated with a droplet of a host cell strain (E. coli F') and then aliquoted in 3 ml volumes into sterile narrow-necked universal bottles. Each aliquot was then infected with 'phage taken from plaques (areas of reduced growth rather than lysed cells) on B-agar (0.8% (w/v) bactotyptone, 100 mM NaCl, 2.0% (w/v) bactoagar) plates, see Section 2:23:5. The cultures were incubated at 37°C and 300 rpm for good aeration for 5½-6 hours. After incubation, 1.5 ml of culture was centrifuged for 5 minutes in a microcentrifuge to pellet the E. coli cells. The supernatant (containing the extruded virus) was then mixed with 200 µl 20% (w/v) polyethylene glycol (PEG)/2.5M NaCl and allowed to stand at room temperature for 20 minutes to precipitate the 'phage. The PEG/salt precipitate was pelleted for 10 minutes in a microcentrifuge and then all traces of the PEG/salt solution removed. The 'phage pellet was resuspended in 100 µl TE by vortexing and the protein coats were removed by phenol extraction. 100 µl TE saturated phenol was added to the solution,

vortexed and centrifuged. The aqueous layer was removed and extracted again this time using TE saturated phenol/chloroform (50:50). The last traces of phenol were removed from the second aqueous phase by adding 0.5 ml of ether. DNA in the final aqueous layer (i.e. the lower layer of the ether extraction) was precipitated by adding 0.1 x volume salt (3M Sodium acetate pH 6.0) and two volumes of 100% ethanol. The precipitated DNA was harvested by centrifugation, rinsed, dried and redissolved in 20 µl TE.

2:18:2 Preparation of RF DNA from M13.

10 ml phage cultures were prepared by innoculating L-Broth with a drop of a suitable culture of host E. coli and by infecting these cells with M13 mp 'phage picked from plaques on a B-agar plates (see Section 2:18:1). These cultures were grown overnight at 37°C, 200 rpm.

40 ml aliquots of sterile L-Broth were innoculated with a drop from a host E. coli culture and grown to a density of 0.5-0.6 OD units at 550 nm. These cultures were then infected with 100 µl of an appropriate 'phage overnight culture and incubated at 37°C for 4 hours at 300 rpm. Cells were harvested by centrifugation (3000 x g at 4°C for 10 minutes) and resuspended in 1.9 mls SET buffer. Fresh lysozyme (40 mg/ml) was added to a final concentration of 2 mg/ml and the solution stood on ice for exactly 15 minutes. Two volumes (4 mls) of freshly prepared lysis solution (0.2 N NaOH, 1% (w/v) SDS) was added and the mixture stood on ice for 10 minutes. A half volume (3 mls) of 3M sodium acetate HAc pH 4.8 was then added and the mixture returned to ice for a further 15 minutes. The dense precipitate formed during this procedure was centrifuged at 27,000 x g for 15 minutes at 4°C and discarded. The supernatant was retained and incubated with 5 µl RNAase A (10 mg/ml) for 15 minutes at 37°C. The preparation was extracted using TE saturated phenol/chloroform (50:50) twice and the DNA precipitated by adding 0.6 volume of isopropanol. The

precipitated DNA was harvested by centrifugation (27,000 x g at 4°C for 10 minutes) and the pellet redissolved in 2 mls of 6.5% (w/v) PEG/0.4 M NaCl on ice for one hour. The DNA was pelleted in a bench-top microcentrifuge for 10 minutes, the supernatant removed and the pellet rinsed, dried and redissolved in 40 µl TE buffer.

2:19:1 Use of restriction endonucleases.

Restriction enzymes were used according to the manufacturer's instructions. 10x core buffer (BRL) was used where possible, otherwise buffers were prepared from sterile stocks.

Stock restriction endonuclease buffer (10 mM Tris HCl pH 7.4, 10 mM $MgCl_2$, and 100 µg/ml Bovine serum albumin (BSA)) varied only in the NaCl concentration from 0 mM to 150 mM NaCl in 50 mM divisions. Incubations were carried out for 60 minutes at the optimum temperature for each enzyme, mostly 37°C. DNA concentrations varied from 10-100 ng/µl.

Digestion products were analysed as 1% (w/v) agarose gels containing 1 µg/ml ethidium bromide. Gels were made in Tris-Acetate-EDTA (TAE) running buffer (40 mM Tris HAc pH 8.2, 20 mM sodium acetate, 1 mM EDTA) and electrophoresed at 75-100 volts (constant voltage). Stained DNA was visualised under ultraviolet (u.v.) light and photographed using polaroid 667 film.

Any alteration to this protocol will be referred to in the appropriate results section.

2:20 Isolation of DNA fragments.

2:20:1 Isolation of DNA fragments using low melting point agarose.

Plasmid DNA, previously digested with appropriate restriction enzymes (see Section 2:19:1) was electrophoresed in 1% (w/v) low melting point agarose made up in TAE buffer. DNA was electrophoresed until stained fragments could be distinguished from each other under u.v. light and then the required fragment was excised from the gel, taking care to remove any excess agarose. The agarose slice containing the DNA fragment (typical volume approximately 50 µl) was placed into 150 µl of TE buffer at 65°C, a temperature at which low melting point (LMP) agarose dissolves. The solubilised DNA was extracted twice with TE saturated phenol (no chloroform) and the DNA from the second aqueous phase precipitated in ethanol, collected, rinsed and dried as described before. The dried DNA pellet was dissolved in 5 µl TE buffer.

2:20:2 Isolation of DNA fragments using DE81 paper.

Digested plasmid DNA was electrophoresed on 1% (w/v) agarose gels. At a position in front of the required fragment, a piece of DE81 paper (Whatman) was inserted into the gel. The surface of the gel was dried as much as possible and the DNA fragment electrophoresed onto the paper, which was then removed and incubated for 2 hours at 37°C with 400 µl of 1.5 M NaCl in TE buffer. The paper was pelleted (5 minutes in the microcentrifuge) and the DNA precipitated by adding 1 ml of 100% ethanol to the supernatant and incubating at -20°C as described earlier. The precipitated DNA was collected as normal and redissolved in 10 µl TE buffer.

DE81 paper was prepared by soaking in 2.5 M NaCl, washing extensively with H_2O and then storing in 1 mM EDTA pH 8.0 at 4°C.

2:21:1 Ligation of DNA fragments.

Plasmid or 'phage DNA and isolated DNA fragments were ligated for 2 hours at room temperature (or 15°C overnight) in 50 mM Tris HCl pH 7.5, 5 mM $MgCl_2$, 5mM DTT, 10 mM ATP (ligase buffer) with 0.1-0.3 units/µl of T4 DNA ligase (2.5 u/µl). Between 0.1 and 0.3 units of T4 DNA ligase was adequate to catalyse the reaction of 10-100 ng of total DNA.

After ligation competent E. coli cells of an appropriate strain were transformed as in Section 2:16:1.

2:22:1 Treatment of linearized plasmid DNA with Alkaline Phosphatase.

Treatment of linearized vector with alkaline phosphate (calf intestinal) removes 5'-terminal phosphate groups thus preventing recircularisation of the vector during ligation. The addition of non-phosphatased insert provides one 5' terminal phosphate on one end of each strand between vector and insert. The unligated nick at the opposite end of each strand is repaired by host cell repair mechanisms following transformation into E. coli.

The phosphatase reaction was carried out as follows:-

Restricted vector (approximately 500 ng total DNA) was redissolved after ethanol precipitation in 50 mM Tris HCl pH 9.0, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 1 mM spermidine plus 50 mM glycine pH 9.4 and incubated with 1 unit of calf intestinal alkaline phosphatase (CIP). The reaction mix was incubated for 15 minutes at 37°C followed by 15 minutes at 56°C. A further 1 unit of CIP was added and the two stage incubation repeated.

The reaction was stopped by dilution with 20 μl H_2O and addition of 5 μl 10 x TNE (100 mM Tris HCl pH 8.0, 1 M NaCl, 10 mM EDTA), followed by heating at 68°C for 15 minutes. Finally the phosphatased DNA was extracted twice with phenol/chloroform (50:50) precipitated in ethanol and collected as described earlier.

2:23 Oligonucleotide site-directed mutagenesis.

(Hutchinson et al., 1978; Carter et al., 1985)

A BamHI fragment encoding the complete ricin A chain was gel purified from pRICA (O'Hare et al.,-1987) and ligated into M13mp19 restricted with BamHI (see Figure 2:1).

The construction of the BamHI fragment encoding ricin A chain has been described elsewhere (O'Hare et al., 1987), but briefly it consists of a remnant of the pUC18 polylinker encoding a region of 5 amino acid residues, a short stretch from the ricin leader sequence encoding the last four residues of the signal peptide, the complete A chain encoding sequence (for 267 residues) the linker region encoding twelve residues and the first 5 codons of ricin B chain to the BamHI site at base position +852 (base numbering as described by Lamb et al., 1985). A translation stop codon immediately after the A chain encoding sequence was introduced by oligonucleotide site-directed mutagenesis (O'Hare et al., 1987).

Figure 2:1

BamHI fragment encoding ricin A chain from pRICA

Base numbering after Lamb et al. (1985).

2:23:1 The mutagenic oligonucleotide.

The non-phosphorylated oligonucleotide was synthesised using an Applied Biosystems 380B DNA synthesiser and purified by High Pressure Liquid Chromatography (HPLC). It is complementary to bases 703-723 of the published ricin cDNA sequence (Lamb et al., 1985) and carries a two base mismatch at positions 713 and 714 indicated by the asterisks below;

5' - CGT AAT GGT TCC AAA TTC AGT - 3' Non-template DNA strand.
703 723

3' - GCA TTA CCA ACT TTT AAG TCA - 5' Mutagenic oligonucleotide
**

The two base mismatch encodes for a translation stop codon (TGA) at nucleotides 712-714.

2:23:2 Oligonucleotide phosphorylation.

The purfied oligonucleotide was diluted to 50 ng/μl with 50 mM Tris HCl pH 8.0, 10 mM $MgCl_2$ (Kinase buffer) in the presence of 5 mM dithiothreitol and 1 mM adenosine triphosphate and incubated with 5 units of polynucleotide kinase (Amersham) for 30 minutes at 37°C. The reaction was stopped by incubating at 65°C for 5 minutes. The reaction was scaled up as necessary.

2:23:3 Priming of single-stranded M13 template using mutagenic oligonucleotide (Annealing reaction).

Successful mutagenesis was achieved by reacting the mutagenic oligonucleotide with the M13mp19 template in the ratio 0.8:1.0.

400 ng of phosphorylated mutagenic oligonucleotide was reacted with 525 ng of single-stranded M13mp19 ricin A chain template (prepared as in Section 2:17:1), in 10 mM Tris HCl pH 8.0, 10 mM $MgCl_2$ buffer by heating at 80°C and then gradually cooling to room temperature. Heating the DNA at 80°C serves to denature the template DNA, opening up the structure so that the oligonucleotide can anneal in the correct place during the cooling step.

2:23:4 Preparation of double-stranded DNA in vitro (Extension reaction).

An extension reaction mixture containing 0.5 mM rATP, 0.5 mM deoxynucleotide triphosphates, 10 mM DTT, 1.0 unit of the Klenow fragment of DNA polymerase I 1.0 u/μl (Amersham) and 6 units of T4 DNA ligase (Amersham) was incubated with 10 μl (approximately 1 μg total DNA) of the annealing reaction at 15°C overnight. The reaction mix was diluted ten-

fold with TE buffer and stored at -20°C.

2:23:5 Transformation of 71.18 mut L *E. coli*.

71.18 mut L cells are an *E. coli* F' strain susceptible to infection with M13 'phage, and deficient in their DNA repair functions. These cells will therefore become infected with M13 'phage, but not correct the two base mismatch introduced into the ricin A chain encoding sequence by the mutagenic oligonucleotide (see Section 2:23:1).

71.18 mut L cells were rendered competent as described in Section 2:15:1 and mixed with dilutions from the annealing/extension reaction (e.g. 200 µl cells plus 0.005, 0.05, 0.1 and 0.2 volumes of the reaction mix). The cells were stood on ice for 30-45 minutes, heat shocked for 90 seconds and then plated onto B-agar plates as follows:-

3 ml aliquots of B top agar (0.8% (w/v) bactotryptone, 0.6% (w/v) bactoagar 100 mM NaCl) were prepared at 42°C and mixed with 40 µl 100 mM isopropyl-β-D-thio-galactopyranoside (IPTG, (Sigma)) and 20 µl 2% (w/v) 5-bromo-4-chloro-3-indoyl-β-galactoside (x gal) (see Section 2:18). 100 µl of expotentially growing 71.18 cells (not deficient in mismatch repair were added to each aliquot as cells to provide the bacterial lawn. The 200 µl aliquots of transformed competent cells were then added, mixed and quickly plated out evenly on top of dry B-agar plates. Once the B top agar mixture had set the plates were incubated overnight at 37°C.

All plaques (areas of retarded cell growth rather than of cell lysis) should be clear for clones of recombinant M13 'phage.

2:23:6 Colony hybridisation using mutagenic oligonucleotide.
(Wallace et al., 1980)

Since no attempt was made to purify closed circular double-stranded M13 from excess single-stranded template after the in vitro steps, it cannot be assumed that all the plaques obtained contain 'phage carrying the desired mutation. It is therefore necessary to screen the clones. This is achieved using the mutagenic oligonucleotide as a probe. This will anneal perfectly to DNA carrying the desired mutation, but imperfectly (i.e. incompletely) to DNA which remains wild type.

Individual plaques from the transformation step (Section 2:23:5) were toothpicked in duplicate onto L-agar plates in a pre-determined grid formation, and incubated overnight at 37°C so that the plaques can grow as infected colonies. One plate, serving as the master copy was stored at 4°C, whilst the second was used for colony hybridisation.

Nitrocellulose filters, 0.45 μM, 82 mm diameter (Schleicher and Schuell) were placed onto the surface of the L-agar plates and firmly pressed into place in order to lift the colonies evenly. Individual colonies, now transferred onto nitrocellulose, were lysed, and the DNA denatured, by placing the filters, colonies uppermost, onto 3 MM sheets soaked in 0.5 M NaOH. Each filter was given 3 x 1 minute exposures to the lysis solution after which the colonies should glisten indicating successful lysis. The pH was neutralised by 2 x 1 minute incubations on 3 MM filter paper soaked in 1 M Tris HCl pH 7.4 and the denatured DNA was fixed to the nitrocellulose by incubating as before with 0.5 M Tris HCl pH 7.4, 1.5 M NaCl for 5 minutes. After fixing the DNA filters were air dried and then baked in vacuo for 1 hour at 80°C.

The mutagenic oligonucleotide, phosphorylated using Adenosine 5'-[γ^{32}P] triphosphate (Amersham) served as a probe for correctly mutated template. Phosphorylation was carried out as detailed in Section 2:23:2,

replacing the 1 mM ATP with radioactively labelled ATP (approximately 30 μCi [γ^{32}P] ATP 185 TBq/mmol). After reacting for 30 minutes at 37°C the reaction was stopped and the labelled oligonucleotide diluted with 3 ml 6 x SSC (3 M NaCl 300 mM sodium citrate). The probe was filtered through a 0.2 μM millipore filter into a 30 ml petri dish and stored at -20°C.

The filter was pre-wet in 6 x SSC and prehybridized at 67°C for 5 minutes in 10 x Denharts (2% w/v BSA 2% (w/v) Ficoll 2% PVP) 6 X SSC and 0.2% (w/v) SDS to prevent non-specific binding of the probe. The filter was rinsed in 6 x SSC and then placed colony-side down into the probe solution for 1 hour at room temperature. After incubation the filter was removed and washed 3 times at room temperature in 6 x SSC, dried and then exposed to X-ray film for 1-2 hours. The probe solution was refiltered and stored at -20°C.

Applying the Wallace rules to the oligonucleotide (Wallace et al., 1980) (where 2°C is added for each A and T, and 4°C for each G and C) the dissociation temperature (Td) of the mutagenic oligonucleotide to the mutated DNA template was calculated. The filter was washed in again in 6 x SSC at a temperature 5°C below the Td. Washing was carried out for 3 x 1 minute periods, the filter dried and re-exposed to X-ray film.

Finally the washing procedure was repeated at the Td and the filter re-exposed. Any probe still bound should only be annealed to mutated template DNA. The series of autoradiographs (representing room temperature, Td-5°C and Td washes in 6 x SSC) give a graduating indication of those colonies carrying the directed mutation over those which remain wild type.

2;23;7 Plaque purification.

Putative positive colonies (i.e. those binding probe at the Td) were identified on the master plate and individually toothpicked into separate

1 ml aliquots of L-Broth. The culture was diluted 1000 fold and 1 µl from this dilution used to innoculate 200 µl of expotentially growing 71.18 feader cells. These cells were then mixed with soft agar and plated on B agar as in Section 2:23:5.

2:24 Didoxy chain termination DNA sequencing.
(Sanger et al., 1977)

The "Klenow fragment" of E. coli DNA polymerase I will accurately synthesise complementary DNA strands to single-stranded DNA templates (obtained as in Section 2:18:1) when provided with a primer possessing a 3'-hydroxyl group and all four nucleoside triphosphates (dNTPs).

Dideoxynucleoside triphosphates (ddNTPs) can also be incorporated into the growing complementary strand by DNA polymerase I activity, but, because dideoxynucleoside triphosphates lack the 3'-hydroxyl group required for formation of the next phosphodiester bond, the DNA chain terminates after incorporation of a ddNTP.

To determine the sequence of a single-stranded template four separate reactions are carried out. Each reaction is supplied with all four dNTPs, but only one ddNTP, for instance ddATP. As the enzyme moves along the template synthesising the complementary strand it will insert either dATP or ddATP at the appropriate position. If dATP is incorporated synthesis continues, but if ddATP is incorporated chain termination will result. By controlling the ratio of dNTP/ddNTP the incorporation of the ddNTP and therefore chain termination will occur randomly, resulting in a population of DNA fragments of different lengths.

2:24:1 Dideoxy chain termination DNA sequencing - The annealing reaction.
(Amersham M13 sequencing handbook)

Single-stranded M13mp19 template containing the BamHI fragment encoding ricin A chain (see Section 2:23) was obtained as outlined in Section 2:18:1.

The M13 universal primer is a single-stranded synthetic oligonucleotide:-

5' G T A A A A C G A C G G C C A G T 3'

The 17 mer is complementary to a sequence of + strand M13 DNA just down stream from the multiple cloning site present in all M13 mp vectors.

Annealing of single-stranded template to the M13 primer was carried out as described in the Amersham sequencing handbook, except that the reaction mixture was heated to 100°C for 5 minutes and then allowed to cool to room temperature. The rapid heating followed by the slow cooling opens up the M13 DNA allowing the M13 primer access to its complementary sequence.

2:24:2 Dideoxy chain termination DNA sequencing - The sequencing reaction.

All reagents required for the sequencing reaction were obtained in kit form from Amersham. Adenosine 5'-γ[^{35}S] thiotriphosphate(22 TBq/mmol)used as radiolabelled nucleotide in the reactions was also obtained from Amersham.

Deoxy- and dideoxynucleoside triphosphate working solutions were made up according to the Amersham handbook from the stock solutions provided in the Amersham Kit.

To the hybridisation mix (described in Section 2:24:1), 1 unit of "Klenow fragment" of DNA polymerase I, 2 μl $\gamma[^{35}S]$ ATP and 1 μl of 100 mM DTT (final concentration 5 mM) were added. 3.5 μl of this reaction mix was incubated with 2 μl of each dNTP/ddNTP mix for 20 minutes at 30°C. 1 μl of 0.5 mM dATP was added as chase and the reaction incubated for a further 20 minutes at 30°C.

4 μl of formamide dye mix (0.1% w/v Xylene cyanol FF, 0.1% (w/v) bromophenol blue, 20 mM Na_2 EDTA) was added to each reaction and then heated to 100°C for 5 minutes immediately before electrophoresis (see Section 2:24:3). The reaction mixes were loaded in a standard order, G, A, T, C on the polyacylamide gels.

2:24:2 Gel electrophoresis.

Samples from the sequencing reaction mixes were electrophoresed on 20 x 40 x 0.04 cm polyacrylamide slab gels for 1½-2 hours at 40 watts. The electrophoresis gel plates, spacers and comb were extensively cleaned and allowed to dry before assembly. The gel was poured, taking care to avoid the creation of air bubbles within the gel and allowed to polymerise for at least 1 hour. A slot was formed in the gel to accept the "sharks tooth" comb (0.4 mm thick, 5.7 mm point to point, BRL) prior to polymerisation.

Buffer gradient gels (Biggin et al., 1983) were used to analyse the sequence of the mutagenised ricin A chain clone as they produce more highly defined, evenly spaced band patterns than conventional gels.

Stock solutions for gradient gels were prepared as described in the Amersham sequencing handbook, the gradient being formed by mixing the two stock solutions in a 25 ml pipette by gently introducing air bubbles at the bottom. The gels were poured as described above.

Once polymerised the gels were assembled onto the tank apparatus. The slot made to accept the comb was rinsed to remove any unpolymerised

acrylamide, and the comb placed in position. To warm the gel, and maintain a denaturing environment the gel was pre-run at 40 watts for 20-30 minutes in 90 mM Tris HCl pH 8.3 890 mM boric acid 1 mM Na_2 EDTA $2H_2O$ (TBE) running buffer. Finally the wells formed by the comb were rinsed and the samples loaded.

2;2A;A 25% formamide buffer gradient gels.

To achieve a more denaturing environment and to eliminate the formation of secondary structures within the DNA sequence 25% (v/v) formamide buffer gradient gels were used. As with standard gradient gels two solutions of different ionic strength were mixed as described in the previous section.

Stock acrylamide solution

0.5X

40% acrylamide (19:1 acrylamide : bisacrylamide)	15 ml	6% (v/v)
10 x TBE	5 ml	5% (v/v)
Urea	46 g	46% (w/v)
Formamide (deionized)	25 ml	25% (v/v)

made up to 100 ml with H_2O and filtered through a 0.45 µM millipore filter and stored at 4°C in the dark.

2.5X

40% acrylamide (19:1 acrylamide : bisacrylamide)	15 ml	6% (v/v)
10 x TBE	25 ml	25% (v/v)
Urea	46 g	46% (w/v)
Formamide	25 ml	25% (v/v)
Sucrose	5 g	5% (w/v)
bromophenol blue	5 mg	To colour

made up to 100 ml with H_2O, filter and stored as above. 60 µl of 10% (w/v) APS and 5 µl TEMED were added per 10 ml acrylamide solution. The gel was prepared and electrophoresed as described in Section 2:24:3.

2:24:3 Autoradiography.

Following electrophoresis the gel was dismantled and (while still fixed to one gel plate) fixed in 5% (v/v) methanol, 5% (v/v) acetic acid for 20 minutes at room temperature. The gel was then mounted onto 3 MM filter paper (Whatman cut to size)and dried on a heated, vacuum block for 40 minutes. The dried gels were exposed to X-ray film overnight and developed as described in Section 2:4:4.

2:25 In vitro transcription and translation of the mutated ricin A chain clone (A stop).

To establish the efficiency of the premature stop codon introduced into the A chain sequence by site-directed mutagenesis and to ensure the fidelity of the retrieved A chain sequence (see Section 2:23:1), the mutated A chain sequence was ligated into an SP6 promoter driven, in vitro transcription vector, pSP64ΔBam.

2:25:1 Cloning into pSP64ΔBam from M13mp19.

RF DNA from M13mp19 containing the ricin A chain sequence, A stop, was prepared as detailed in Section 2:18:2. A fragment cut from the RF preparation with EcoRI and BglII was isolated from a low melting point agarose gel (see Section 2:20:1) and ligated (see Section 2:21:1) into the large EcoRI-BglII fragment from pUC8RA isolated in an identical manner. The resulting plasmid formed had the 5' end of the original ricin A chain cDNA up to the BglII site at position 589 and the 3' end derived from M13mp19 ricin A stop (Figure 2:2).

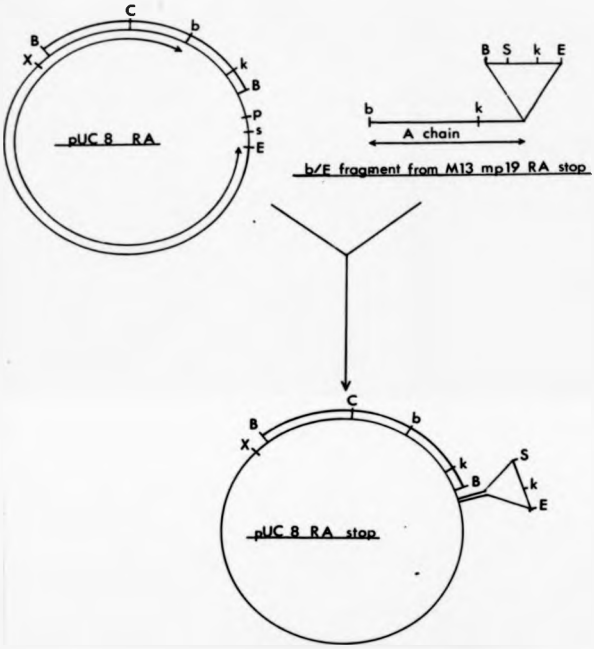
The integrity of the ricin A stop clone in pUC8 was established by limited restriction enzyme mapping (see Section 2:19:1) and a BamHI fragment of 893 base pairs isolated from this plasmid and ligated into BamHI restricted, phosphatased (see Section 2:21:2) pSP64ΔBam (see Figure 2:3).

Mini prep DNA was prepared as in Section 2:17:1 and correctly orientated fragments identified by restriction enzyme mapping. A large scale preparation of a correctly orientated BamHI fragment in the SP6 vector (pSP64ΔBam A stop) was made.

Abbreviations used for restriction endonucleases

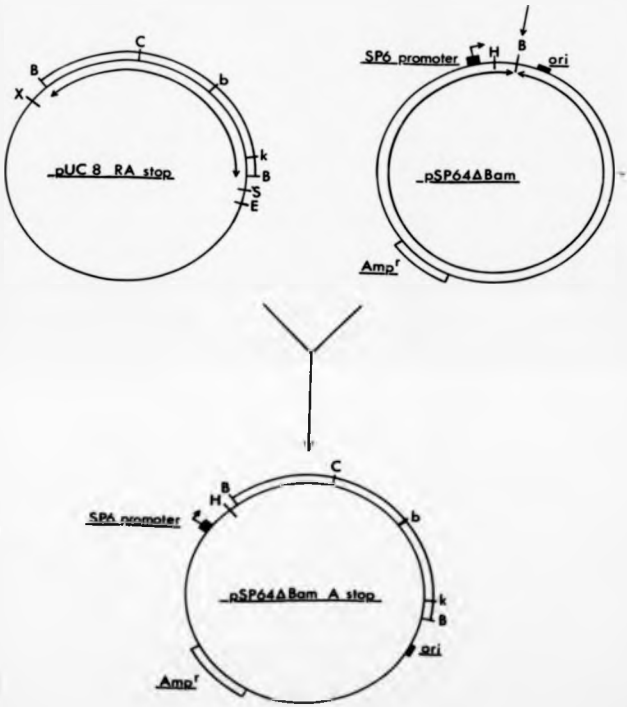
B	BamHI	s	SalI
X	XhoI	H	HindIII
C	ClaI		
b	BglII		
K	KpnI		
p	PstI		
E	EcoRI		
S	SmaI		

Figure 2:2 Cloning of BglII/EcoRI A stop fragment from M13mp19 RA stop into pUC8 RA.



pUC8 RA stop was differentiated from pUC8 RA by restriction endonuclease mapping using SmaI, PstI and SalI. pUC8 RA stop was linearized with SmaI, but remained uncut when incubated with either PstI or SalI.

Figure 2.3 Cloning of BamHI fragment from pUC8 RA stop into pSP64ΔBamHI



2:25:2 In vitro transcription of pSP64ΔBam A stop.

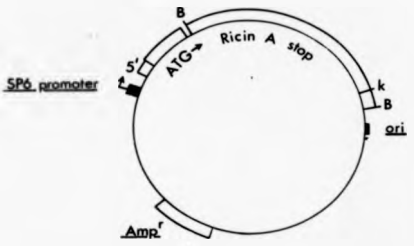
(Krieg and Melton, 1986)

The plasmid pSP64ΔBam contains a BamHI cloning site downstream from the bacteriophage SP6 promoter site. Between these two regions exists a sequence of 5' untranslated Xenopus β globin, an inframe translation start codon (ATG) and 8 codons of β globin (see Figure 2:4).

To act as a template for run-off RNA transcripts, pSP64ΔBam A stop was linearized by KpnI restriction endonuclease digestion (see Section 2:19:1). The digested DNA was extracted with TE saturated phenol/chloroform (50:50) and precipitated in ethanol as described in Section 2:18:1. Approximately 2 μg of linearized DNA was used in the transcription reaction as follows:-

2 μl of (1 μg/μl) linearized DNA was incubated with 12 μl transcription premix (40 mM Tris HCl pH 7.5, 6 mM, $MgCl_2$, 2 mM Spermidine, 10 mM DTT, 500 μM rATP, rCTP, rUTP, 50 μM rGTP (New England Nucleotides (NEN), 100 μg/ml BSA (nuclease free BRL), 1 unit/μl ribonuclease inhibitor (Promega) plus 2 μl of 100 units/μl SP6 RNA polymerase (Amersham). To produce capped transcripts (for increased translational activity and greater stability) 10 μl of G(5')ppp(5')G (monomethyl cap) was added to a final concentration of 500 μM (Pharmacia). The reaction mix was incubated at 40°C for 30 minutes and then a further 30 minutes after the addition of 2 μl 8 mM GTP (final concentration 0.7 mM). Reaction products were stored in liquid nitrogen.

Figure 2:4 pSP64ARam A stop



2:25:3 Translation of SP6 transcripts in a wheatgerm lysate system.

Wheatgerm lysate was prepared as described by (Roberts and Paterson, 1973) and (Anderson et al., 1983).

1 µl of transcription reaction mix containing newly transcribed RNA (Section 2:25:2) was incubated at 30°C for 1 hour with 3.75 µl wheatgerm lysate, 2.35 µl translation premix, 1 µl of [^{35}S] methionine 30 TBq/mmol (10 µCi/µl) made up to 12.5 µl with H_2O. The wheatgerm translation premix was composed of 20 mM Hepes KOH pH 7.6, 1 mM ATP, 8 mM creatine phosphate, 40 µg/ml creatine phosphokinase 30 µg/ml spermidine pH 7.0, 2 mM DTT, 20 µM GTP, 25 µM amino acid mix (from 5 mM stock excluding methionine), 2.5 mM magnesium acetate, 120 mM potassium acetate, final concentration (Anderson et al., 1983).

2 µl aliquots were spotted onto Whatman No. 1 (1 cm^2) paper and prepared for liquid scintillation as described in Section 20:10:3. Products from the wheatgerm lysate translation were separated by SDS-PAGE (see Section 2:3:1) and visualised by fluorography and autoradiography (see Section 2:4:4).

2:25:4 Translation of SP6 transcripts in a rabbit reticulocyte lysate system.

(Jagus, 1987; Jackson et al., 1979)

Rabbit reticulocyte lysate was prepared as described in Section 2:10:1. Prior to translation, endogenous mRNA (mainly α and β globin) was removed from the lysate by incubation with Ca^{2+}-dependent endonuclease (Jackson et al., 1976). Briefly to each 1 ml of lysate 10 μl of 100 mM $CaCl_2$, 20 μl of 1 mM haemin hydrochloride (Sigma) and 10 μl of 200 units/ml creatine phosphokinase were added. Nuclease S1 Ca^{2+} dependent from Staphylococcus aureus (Boehringer Mannheim) was added at an optimal concentration (usually between 25-100 units/ml) determined by assaying batch activity. The mixture was incubated at 20°C for 15 minutes and the nuclease then inactivated by the addition of 10 μl of 200 mM EGTA to chelate Ca^{2+}.

1.5 μl of transcribed RNA (see Section 2:25:2) was incubated for 30 minutes at 37°C with 2.5 mM Tris HCl pH 7.4, 3 mM Magnesium acetate, 250 mM potassium acetate, 150 μM amino acid mix (minus methionine), 10 mM phosphocreatine, 80 mU of creatine phosphokinase, 0.02 mM haemin with 1.2 μl [^{35}S] methionine 30 TBq/mmol (10 μCi/μl), 0.625 μl wheatgerm tRNA and 12.5 μl of mRNA dependent lysate (MDL) per reaction. In practise, a "master mix" of the above components was prepared and aliquoted for each separate translation reaction.

Products were analysed by SDS-PAGE or by scintillation counting as described in the previous section.

2:25:5 Toxicity analysis of rabbit reticulocyte translation products.

The biological activity of translated ricin A chain transcripts with respect to their ribosome inactivating activity could be assayed using a

rabbit reticulocyte lysate system as described in Section 2:25:4.

Ribosomes from rabbit reticulocytes are sensitive to inactivation by ricin A chain, therefore such a system will only support a limited amount of translation before the product concentration reaches a level where it inhibits protein synthesis. This feature can be exploited in the analysis of any mutated ricin A chains by firstly translating A chain mRNA for 30 minutes (as described in Section 2:25:4) and then adding a second mRNA for a non-inhibitory protein such as preproalpha factor (a yeast mating factor) or chicken lysozyme. If the first ricin A chain mRNA is translated to form biologically active protein then the second mRNA will not be translated. However, if the A chain mRNA translates to form a non-toxic protein then the second mRNA will be translated (May et al., 1988 submitted to EMBO Journal).

Translation conditions are described in Section 2:25:4. After 30 minutes translation with ricin A chain transcipts, the second mRNA encoding preproalpha factor was added and the system incubated for a further 30 minutes. A control using chicken lysozyme mRNA as the first transcript followed by preproalpha factor was introduced to show inhibition was specific to ricin A chain activity. Products from the translation reactions were analysed by SDS-PAGE as described previously.

2:26 Expression of truncated ricin A chain in Escherichia coli.

2:26:1 Construction of pDS3/3 A stop.

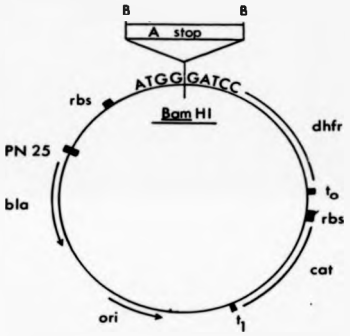
A BamHI fragment excised and isolated from pUC8 RA stop was ligated into the E. coli expression vector pDS5/3 as follows:-

10 μg of pUC8 RA stop was incubated for 1 hour at 37°C with the restriction endonuclease BamHI (see Section 2:19:1). An 893 base pair fragment was isolated from low melting point agarose (see Section 2:20:1)

and ligated (see Section 2:21:1) into a BamHI restricted, phosphatased (see Section 2:22:1) preparation of the E. coli expression vector pDS5/3 (O'Hare et al., 1987; Stueber et al., 1984). Correctly orientated fragments were identified by restriction mapping.

The vector pDS5/3 contains a strong coliphage T5 promoter fused to the E. coli lac operator with a ribosome binding site (RBS) and initiation codon immediately upstream from a unique BamHI cloning site (Stueber et al., 1984).

Figure 2:5 pDS5/3 A stop.



rbs	ribosome binding site
ori	origin of replication
t_1	terminator signal (rmB operon of E. coli)
to	terminator signal (phage λ)
PN25	fusion of coliphage T5 promoter with lac operator of E. coli
dnfr	dihydrofolate reductase gene
Cat	Chloroamphenicol acetyl transferase gene
bla	β lactamase gene

2:26:2 Transformation and induction of expressed A stop.

BMH 71.18 E. coli cells were made competent as described in Section 2:15:1, and transformed with dilutions from the ligated reaction between pDS5/3 and the BamHI fragment from pUC8 RA stop, described in Section 2:26:1. Transformed cells were plated on L-amp plates containing 0.1 mg/ml ampicillin and incubated overnight at 37°C.

Small colonies were selected, and mini prep DNA prepared as described in Section 2:17:2. Correctly orientated fragments were identified by restriction endonuclease mapping and a large-scale plasmid preparation made as described in Section 2:17:3.

DNA from the large-scale prep was used to transform competent BMH 71.18 cells and from transformed colonies, 10 ml overnight cultures of L-Broth (+ 0.1 mg/ml ampicillin) were prepared. Controls of cells transformed with full-length recombinant A chain (O'Hare et al., 1987) and pDS5/3 vector only were also prepared. From each overnight culture 100 µl cells were used to innoculate 10 mls of fresh L-Broth (+ ampicillin) to which 20 µl of 1 M IPTG (in TE buffer) was added (2 mM final concentration) and then incubated at 30°C for 5 hours. Following incubation, 100 µl aliquots were removed from each sample and analysed on 15% (w/v) SDS-PAGE under reducing conditions (see Section 2:3:1) and Western blotted (see Section 2:6:1) probing with polyclonal antibodies raised in rabbits against ricin A chain.

Note: All procedures relating to the expression of ricin A chain in E. coli were carried out under Category III containment conditions.

2:26:3 Scaled-up expression of truncated A chain and sample preparation.

500 ml cultures of L-Broth containing 0.1 mg/ml ampicillin, 1 mM IPTG were incubated with transformed cells containing pDS5/3 A stop and grown at

30°C, 200 rpm for 8 hours. Cells were harvested by centrifugation at 5000 rpm for 5 minutes using the 6 x 300 ml HSE 21 rotor at 4°C, and the pellets resuspended in 20-30 ml sterile PBS at 4°C. The cell suspension was divided into 0.75 ml aliquots containing 20 μg lysozyme and 10 μg PMSF. Each aliquot was sonicated on ice for three 20 seconds intervals and cell debris pelleted by centrifugation at 13,000 rpm in a microcentrifuge for 30 minutes at 4°C. The supernatant from this step was carefully decanted and immediately dialysed against 25 mM ethanolamine HAc pH 9.6 in preparation for chromatofocusing.

2:26:4 Purification of truncated A chain using chromatofocusing.

Material prepared in Section 2:26:3 was separated using a chromatofocusing system, pH range 9-6. Polybuffer exchanger 9-4 (Pharmacia) was equilibrated with 25 mM ethanolamine HAc pH 9.6 buffer at 4°C. Soluble E. coli proteins prepared as described above were applied to the chromatofocusing column (30 cm x 2 cm) and then sequentially eluted with polybuffer 96 HAc pH 6.0, overnight at a flow rate 0.8 ml min^{-1}. Once the eluent reached a pH of 6.0 the column was washed with 1 M NaCl to elute any protein still bound. Fractions were analysed by SDS-PAGE and Western blotting.

2:26:5 Determination of ricin A chain-specific activity in chromatofocused fractions.

Fractions from the chromatofocusing step judged to contain the expressed truncated ricin A chain were analysed for specific ricin A chain activity on RNA extracted from rabbit reticulocytes. The assay for ricin-specific modification of ribosomal RNA was carried out as described in Section 2:11, using 20 and 30 μl aliquots of chromatofocused fractions as

the toxin dilution. Ricin A chain specific modification was taken as indicative of soluble biologically-active recombinant truncated ricin A chain.

2:26:6 Use of Blue Sepharose affinity chromatography to purify truncated A chain.

Chromatofocused fractions containing the expressed truncated ricin A chain were dialysed against 50 mM sodium phosphate buffer pH 7.5. Blue Sepharose CL- 6B (Pharmacia) was swollen in the same buffer and poured to form a 0.5 x 24 cm column.. The sample was applied and the column washed with 50 mM sodium phosphate buffer pH 7.4 until all unbound material had eluted. The column was washed with one bed volume of 50 mM sodium phosphate buffer and bound protein then eluted with 0.5 M NaCl in the same buffer. The column flow rate was 0.15 ml min^{-1} and 1 ml fractions were monitored at A_{280} nm (Knowles and Thorpe, 1987). Fractions from both bound and unbound material were analysed for ricin A chain specific activity by the ribosomal RNA modification assay detailed in Section 2:11.

Blue Sepharose CL-6B is the dye, Cibacron Blue F_3G-A, covalently bound to Sepharose CL-6B (2 µmole dye ml^{-1} swollen gel). The structure of Cibacron Blue F_3G-A is such that it acts as a dinucleotide analogue binding a number of enzymes with a requirement for adenylyl-containing substances. It is via this type of interaction that ricin A chain is thought to bind to the dye (Appukuttan and Bachhawat, 1979).

3:1:1 Purification of lectins from Ricinus communis seeds.

The lectins Ricin (RCA_{II}) and Ricinus communis agglutinin (RCA_I) were purified from the seeds of the castor oil plant, Ricinus communis. Both ricin and RCA_I are found amongst the matrix proteins of protein bodies in the seed endosperm tissue (Youle and Huang, 1976; Tulley and Beevers, 1976), and are easily separated from other seed proteins by affinity chromatography using acid-treated sepharose 4B, as described in Sections 2:1:1 and 2:1:2. By binding to exposed β galactose residues in the sepharose column matrix, both ricin and RCA_I are retained whilst all other proteins pass straight through the column. Ricin and then RCA_I were sequentially eluted using PBS buffer containing 5 mM N-acetylgalactosamine and 100 mM galactose respectively (Nicholson et al., 1974). Figure 3:1 shows the protein elution profile from a typical lectin preparation.

An alternative and the preferred method for separating the two lectins from each other was to elute both together from the sepharose column, using PBS buffer containing 100 mM galactose and then to separate ricin from RCA_I by gel filtration upon a Sephacryl S-200 column as described in Section 2:1:3. A typical Sephacryl S-200 elution profile is shown in Figure 3:2, the large RCA_I (120 Kd) eluting first, followed by ricin (60 Kd). Material from each peak was analysed by SDS-PAGE under reducing conditions as shown in Figure 3:3. Under these conditions, ricin appears as two bands and RCA_I as three bands, (corresponding to the mobilities of their respective subunit components), enabling RCA_I-free ricin to be readily identified. The subunit composition of these two lectins is considered in more detail in Section 3:3.

Purified ricin was tested for its ability to inhibit protein synthesis in eukaryotic cells, as described in Sections 2:9:1. Figure 3:4 describes the inhibitory effect of ricin dilutions upon the ability of Vero cells to incorporate [^{35}S] methionine into TCA precipitatable proteins. The data

Figure 3:1 Protein elution profile : Ricinus communis lectin preparation by affinity chromatography using Sepharose 4B.

Figure 3:1 shows the isolation of Ricinus communis lectins from other seed storage proteins by affinity chromatography using Sepharose 4B, as described in Section 2:1:2. The elution of protein from the column was monitored by measuring the absorbance at 280 nm.

Peak I. represents material not binding to the sepharose column.

Peak II. represents material eluting with PBS buffer containing 5 mM galactosamine, predominantly ricin (60 Kd).

Peak III. represents material eluting with PBS buffer containing 100 mM galactose, predominantly RCA_I (120 Kd).

Figure 3: 1 Protein elution profile : Ricinus communis lectin preparation by affinity chromatography on a Sepharose 4B column.

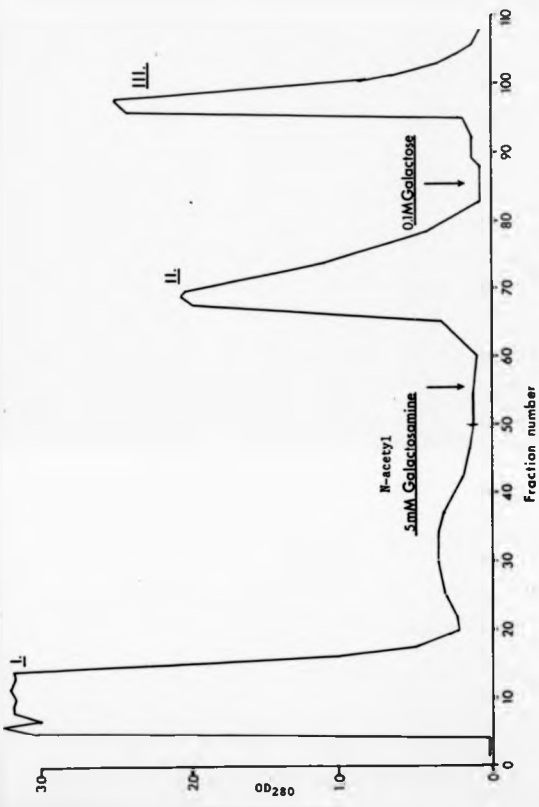


Figure 3:2 Protein elution profile : Separation of Ricinus communis lectins by gel filtration using a Sephacryl S-200 column.

Figure 3:2 shows the separation of Ricinus communis lectins by gel filtration, using a sephacryl S-200 column as described in Section 2:1:3.

Starting material, a mixture of ricin (60 Kd) and RCA_I (120 Kd) was applied to the column and the distinct lectin species sequentially eluted with PBS. The eluting protein was monitored by measuring the absorbance at 280 nm.

Peak I represents material eluting first from the column, composed of the larger 120 Kd RCA_I.

Peak II represents material eluting in the second peak from the column, composed of the smaller 60 Kd ricin.

Figure 3: 2 Protein elution profile : Separation of Ricinus communis lectins by gel filtration using a Sephacryl S-200 column.

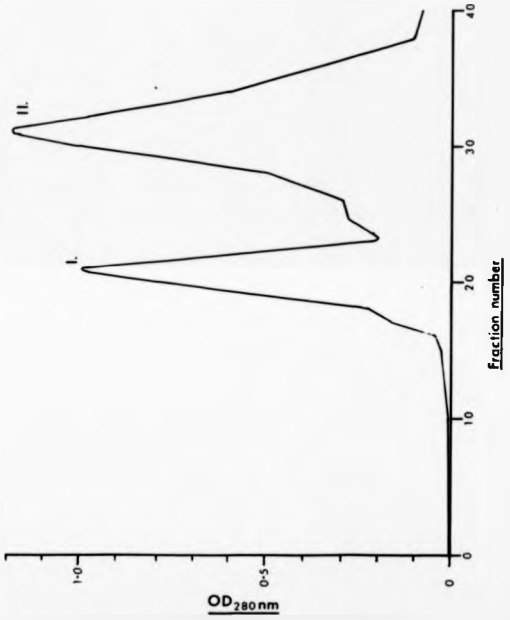
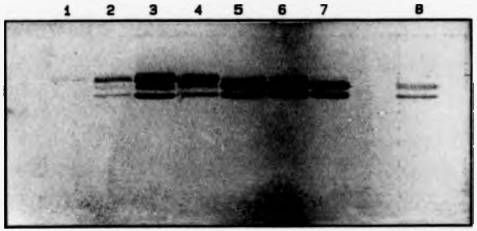


Figure 3: 3 SDS-PAGE analysis of fractions taken from Sephacryl S-200 gel filtration.



Silver-stained SDS-PAGE analysis, under reducing conditions of selected fractions from the Sephacryl S-200 gel filtration step. Tracks 1-4 represent samples from column fractions 15, 16, 19 and 24 from peak I (see figure 3: 2). Tracks 5-7 represent column fractions 28, 30 and 32 from peak II (see figure 3: 2). Track 8 is a purified ricin control.

Figure 3: 4 Cytotoxicity assay : Inhibition of protein synthesis by purified ricin concentrations.

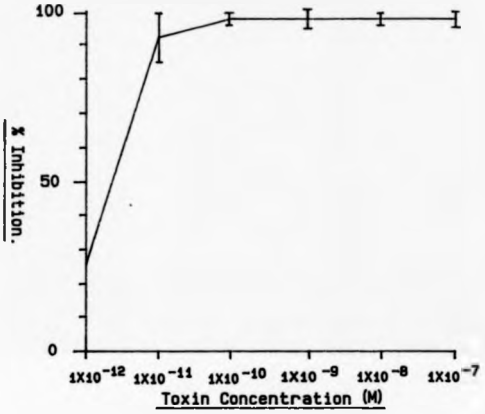


Figure 3: 4 shows the inhibitory effect of whole ricin dilutions upon protein synthesis in Vero cells as judged by the incorporation of 35-S methionine.

Results are expressed as % inhibition calculated from no-toxin control values.

have been expressed as percentage inhibition compared to the incorporation into no-toxin control. The concentration of ricin producing a 50% inhibition, (the inhibitory concentration 50% or $I.C._{50}$) was calculated at 5×10^{-12} M (0.31 ng/ml), in close agreement with published figures (Stirpe and Barbieri, 1986).

3:2 Purification of ricin subunits.

3:2:1 Chromatofocusing and SDS-PAGE analysis.

As a prerequisite for studying the properties of ricin subunits, a stringent purification and testing protocol was required. Earlier references to subunit purification (discussed in Section 3:3) describes methodology which achieves only partial purification of the subunits from one another. The protocol which consistently produced a high degree of subunit purification has been described in Sections 2:2:1 - 2:2:5, the key technique being the use of chromatofocusing which exploits the large differential in the pIs of the subunit components (Olsnes and Pihl, 1982). Once optimised, it was possible, using a chromatofocusing system with a pH range from 7-4 to separate A chain (pI = 7.5), unreduced holotoxin (pI = 7.1) and B chain (pI = 4.8) to homogeneity as judged by silver staining of polyacrylamide gels. Figure 3:5 shows a typical elution profile of chromatofocused ricin subunits, in which ricin A chain passes straight through the column without binding, followed by a small amount of unreduced holotoxin, (which elutes when the pH of the column equals 7.1) and then much later, ricin B chain, (which elutes at a column pH of 4.8). Silver stained SDS-PAGE analysis of B chain purified by this method is shown in Figure 3:6. Track 1 of this figure contains a sample of ricin, electrophoresed alongside two tracks containing chromatofocused ricin B chain (tracks 2 and 3), under reducing, denaturing conditions. This figure

Figure 3:5 Protein elution profile : Purification of ricin subunits by chromatofocusing.

Figure 3:5 shows the separation of reduced ricin subunits by chromatofocusing. Starting material, consisting of a preparation of ricin reduced by incubation with 2-mercaptoethanol (see Section 2:2:2) was applied to the chromatofocusing column pre-equilibrated to pH 7.4 with 25 mM imidazole buffer. The elution of the respective polypeptides was monitored by measuring the absorbance of each fraction at 280 nm.

Proteins eluted according to their pIs as the polybuffer 7-4 (HCl) pH 4.0 was applied, developing a pH gradient across the column, as indicated on the figure.

Peak I represents material not binding to the column, predominantly ricin A chain with a pI of 7.5. The high $OD_{280\,nm}$ of this peak is the result of the 2-mercaptoethanol which elutes from the column at this point.

Peak II represents material eluting from the column when the pH reaches 7.1, predominantly unreduced ricin with a pI of 7.1.

Peak III represents material eluting from the column when the pH reaches 4.8, predominantly ricin B chain with a pI of 4.8.

A pH meter was used to measure the pH of each fraction.

Figure 3:5 Protein elution profile : Purification of ricin subunits by chromatofocusing.

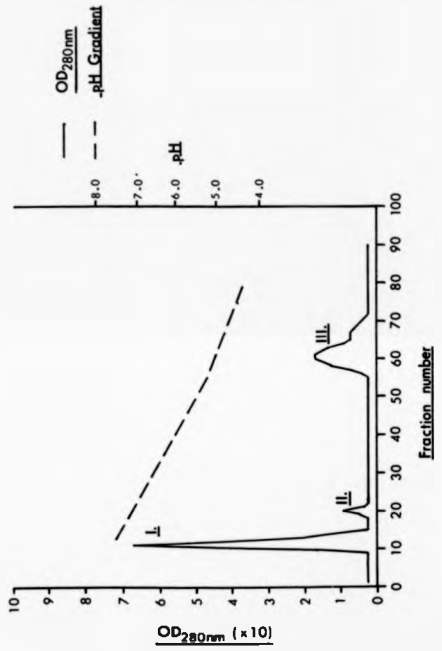


Figure 3; 6 SDS-PAGE analysis of chromatofocused ricin B chain.

Silver-stained SDS-PAGE analysis of ricin B chain purified by chromatofocusing. Track 2 and 3 represent 0.25ug and 0.5ug samples of chromatofocused ricin B chain respectively. Track 1 is a 0.5ug sample of purified ricin.

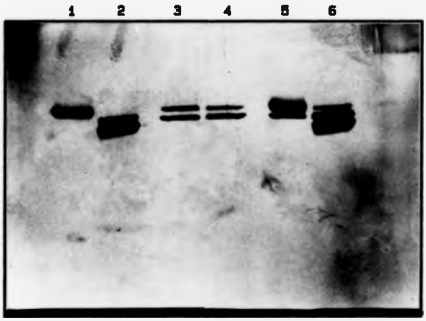
demonstrates the high degree of B chain purity obtained after the single chromatofocusing step, as no contaminating ricin A chain can be detected in tracks 2 and 3.

To determine the purity of the chromatofocused ricin A chain by SDS-PAGE analysis it was necessary to pre-incubate the samples with endoglucosaminidase H (endo H) as described in Section 2:5:3. This step was introduced to differentiate between ricin B chain and "heavy" ricin A chain, (A_2), which has two oligonucleotide side chains and which electrophoreses with the same mobility as B chain on SDS-PAGE under reducing conditions. B chain contains two oligosaccharide side chains, both of which are removed by endo H treatment, resulting in proteins with increased electrophoretic mobility, corresponding to either a single or a double deglycosylation event. The oligosaccharide side chains of A chain (either A_1 or A_2) however, are unaffected by endo H treatment. Thus, the presence of any contaminating B chain "hidden" by the "heavy" A chain will be revealed upon endo H treatment and subsequent SDS-PAGE analysis. Figure 3:7 shows a sample of chromatofocused A chain before (track 3) and after (track 4) endo H treatment. No B chain contamination is apparent in this analysis. On the same figure, control tracks of chromatofocused B chain, before (track 1) and after (track 2), and ricin, before (track 5) and after (track 6), endo H treatment demonstrate the deglycosylation activity of the endo H used. The deglycosylation of ricin subunits is discussed in more detail in Section 3:3.

3:2:2 A chain purification: Removal of contaminating B chain by affinity chromatography.

Chromatofocused ricin A chain was further purified by the removal of any remaining ricin B chain contamination, using affinity chromatography on asialofetuin columns as described in Section 2:2:4. Ricin A chain,

Figure 3.7 SDS-PAGE analysis of endo H treated ricin and ricin subunits.



Silver-stained SDS-PAGE analysis of endo H treated ricin and ricin subunits under reducing conditions.

Tracks 1 and 2 represent untreated and treated chromatofocused ricin B chain samples respectively.

Tracks 3 and 4 represent untreated and treated chromatofocused ricin A chain samples.

Tracks 5 and 6 represent untreated and treated whole ricin samples

purified by chromatofocusing, was able to pass straight through the column, whilst any contaminating B chain or unreduced holotoxin in the preparation bound almost irreversibly to the column and could not be eluted with 100 mM galactose buffer. Since chromatofocused ricin A chain after silver stained SDS-PAGE analysis was apparently free from contaminating B chain, post-asialofetuin treated ricin A chain was analysed for increased purity in cytotoxicity and cell-free assays (see Sections 2:9 and 2:10 respectively).

3:2:3 Cytotoxicity assay: Analysis of purified subunits.

Purified ricin subunits were analysed for their ability to inhibit protein synthesis in eukaryotic cells. In theory, completely pure subunits should be non-toxic to cells; A chain, because it lacks the binding and putative translocating functions of the B chain, and B chain because it lacks the ribosome-inactivating activity of the A chain. In practise, however, some toxicity was detectable when higher concentrations of subunits were applied to cells, which may be accounted for by slight contamination by the other subunit (thus reconstituting holotoxin activity) or by some innate toxicity associated with higher protein concentrations, i.e. not ricin-specific toxicity. These possibilities have been further tested with preparations of B chain as described later in Section 3:2:5.

Figure 3:8 shows the percentage inhibition of protein synthesis (as judged by [^{35}S] methionine incorporation) at the different stages of ricin A chain purification. After chromatofocusing the IC_{50} of approximately 7.0 x 10^{-9} M (0.21 μg/ml) is increased almost ten-fold by the subsequent asialofetuin step, to a value of approximately 5 x 10^{-8} M (1.5 μg/ml), and highly purified ricin A chain is shown to be non-toxic to cells at a concentration of 1 x 10^{-8} M (0.3 μg/ml). The decrease in toxicity after the asialofetuin treatment suggests that contaminating B chain or holotoxin has been removed by this step.

Figure 3:9 shows the inhibition of protein synthesis resulting from the application of ricin B chain dilutions from 1×10^{-11} M - 1×10^{-6} M onto Vero cells. The IC_{50} for chromatofocused ricin B chain was calculated at approximately 3×10^{-9} M (9.6 µg/ml) and B chain was non-toxic to these cells at concentrations up to 1×10^{-6} M.

To show that the respective biological functions of each subunit had not been significantly impaired during purification, purified ricin A and B chains were reassociated as described in Section 2:9:2 (after Olsnes et al., 1974) and then tested for their ability to inhibit protein synthesis in a standard cytotoxicity assay. Figure 3:10 shows the result from such an assay, in which the toxicity of purified A chain at the normally non-toxic concentration of 1×10^{-8} M is increased by the presence of 1×10^{-8} M B chain to give a 40% inhibition of protein synthesis. Above this concentration A chain itself is toxic, but this result demonstrates that both subunits are able to recombine to form an active toxin and is in agreement with the findings of other workers (McIntosh et al., 1988; Olsnes et al., 1974). Reassociated subunits were however, never as active as the same concentration of holotoxin on cells (also in agreement with the findings of McIntosh et al., 1988; and Olsnes et al., 1974). This might be the result of some denaturation during the purification process, or the result of inappropriate association. To examine the latter, samples of purified B chain, purified A chain and a sample of reassociated material (1×10^{-8} M A chain + 1×10^{-8} M B chain) were electrophoresed under non-reducing conditions on SDS-PAGE (see Figure 3:11). Track 1 of this figure shows ricin electrophoresed under non-reducing conditions with a mobility corresponding to 62 Kd protein. Tracks 2-4 show samples from the different stages of ricin A chain purification which are identical by SDS-PAGE analysis. The majority of the protein electrophoreses as the A_1 and A_2 monomers, but some material forms an approximately 62 Kd homodimer. Tracks 5 and 6 represent ricin B chain preparations, and again the majority of

Figure 3:8 Cytotoxicity assay : Inhibition of protein synthesis by ricin A chain at different stages of purification.

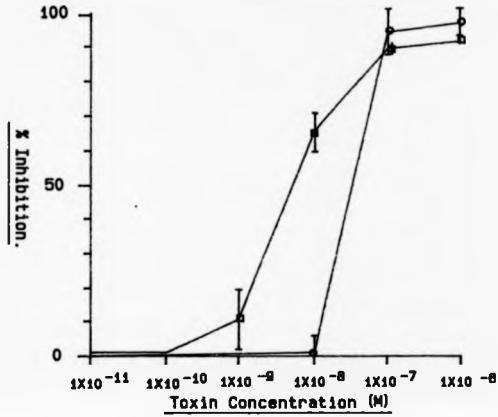


Figure 3:8 shows the inhibitory effect of purified ricin A chain upon protein synthesis in Vero cells, as judged by the incorporation of ^{35}S-S methionine.

o—o represents ricin A chain purified by chromatofocusing only

o—o represents ricin A chain purified by chromatofocusing and asialofetuin treatment.

Results are expressed as % inhibition calculated from no-toxin control values.

Figure 3.8 Cytotoxicity assay : Inhibition of protein synthesis by purified ricin B chain.

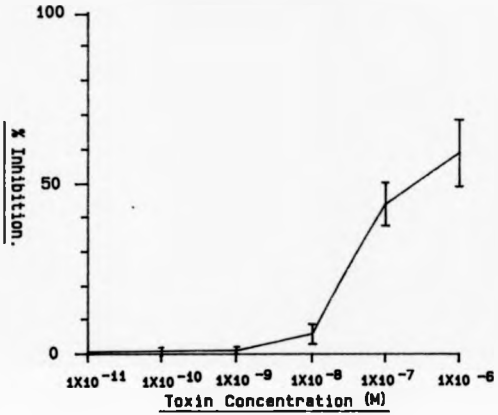


Figure 3.8 shows the inhibitory effect of purified ricin B chain upon protein synthesis in Vero cells, as judged by the incorporation of ^{35}S-S methionine.

Results are expressed as % inhibition calculated from no-toxin control values.

Figure 3.10 Cytotoxicity assay : Inhibition of protein synthesis by reassociated ricin subunits.

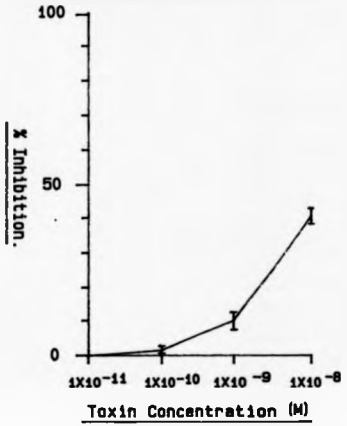


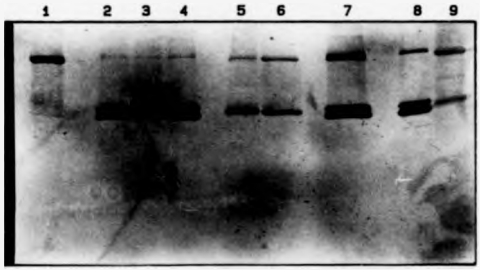
Figure 3.10 shows the inhibitory effect of reassociated ricin subunits upon protein synthesis in Vero cells, as judged by the incorporation of ^{35}S-S methionine.

Ricin B chain at a final concentration of $1X10^{-8}$ M was reassociated with ricin A chain concentrations from $1X10^{-11}$M - $1X10^{-8}$M and applied to the cells.

Note : Across the concentration range shown here both purified ricin subunits are non-toxic to Vero cells.

Results are expressed as % inhibition calculated from no-toxin control values.

Figure 3: ii SDS-PAGE analysis of ricin, purified ricin subunits and reassociated ricin subunits under denaturing, non-reducing conditions



Silver-stained SDS-PAGE analysis under non-reducing conditions.

Track 1 shows a sample of whole ricin. Tracks 2-4 show samples of chromatofocussed ricin A chain, post-asialofetuin treated A chain and post- antibody affinity purified ricin A chain respectively.

Tracks 5 and 6 show samples of chromatofocussed ricin B chain and post-antibody affinity purified ricin B chain.

Track 7 shows a sample from the reassociation of equimolar amounts of ricin subunits.

Tracks 8 and 9 show samples of purified ricin A chain and purified ricin B chain which were a gift from the Imperial Cancer Research Fund, drug targetting laboratory. These samples were purified by the method of Fulton et al., 1986.

protein exists in a monomeric form, but protein corresponding to the position of homodimers is clearly evident, which is in contrast to the findings of Olsnes *et al*. (1974) and Colmacher *et al*. (1987) who in their respective experiments did not observe homodimer formation. In track 7, which contains the reassociated A and B chains, an increase in the amount of material electrophoresing with a mobility corresponding to non-reduced ricin is increased, but a large proportion of material electrophoreses as subunit monomers. This apparently low efficiency of covalent reassociation may explain the reduced toxicity observed in Figure 3:10, but it should be realised that the data from track 7 in Figure 3:11 only shows material which has reassociated via a reducible linkage and that other interactions between the subunits may have been disrupted during sample preparation. The clear evidence of the formation of homodimers in both A and B chain preparations may also account for some loss of activity, with such material being unavailable to form holotoxin.

The observation from this data is that purified ricin subunits can be reassociated to form active holotoxin. The biological activity of the reconstituted holotoxin was shown to be less, compared to non-reduced holotoxin, which probably reflects inefficient covalent reassociation rather than denaturation of subunits. Therefore, ricin B chain, which is able to form heterodimers with ricin A chain, has apparently retained both its binding and putative internalization activity.

3:2:4 Cell-free Assay: Analysis of purified subunits.

Subunit dilutions (1×10^{-11} - 1×10^{-7} M) were tested for their inhibitory effect on the translation of endogenous α and β globin mRNAs in a rabbit reticulocyte lysate system, as described in Section 2:10. Figure 3:12 shows a representation of the results from such an assay in which the IC_{50} for purified ricin A chain is shown to be approximately 2.5×10^{-11} M

Figure 3: 12 Cell-free assay : Inhibition of protein synthesis by purified ricin subunits.

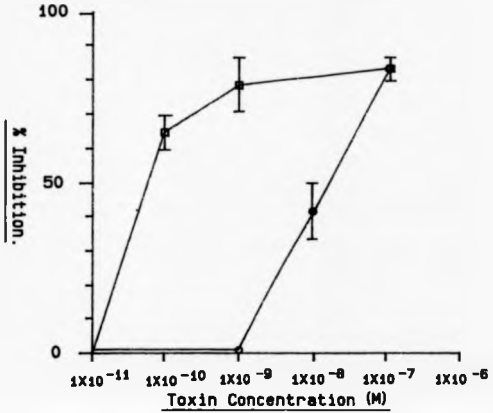


Figure 3: 12 shows the inhibition of protein synthesis in a rabbit reticulocyte lysate cell-free translation system after incubations with concentrations of purified ricin subunits, as judged by the incorporation of 35-S methionine.

□——□ represents the effect of ricin A chain concentrations upon protein synthesis in the system.

○——○ represents the effect of purified ricin B chain concentrations upon protein synthesis in the system.

Results are expressed as % inhibition calculated from no-toxin control values and have been corrected for background counts.

(0.8 ng/ml). This indicates that ricin A chain, purified by the methods described earlier and used in the reassociation experiments, has retained virtually full activity compared with the published IC_{50} value for ricin A chain of 1×10^{-10} M (Stirpe and Barbieri, 1986) in similar cell-free translation systems.

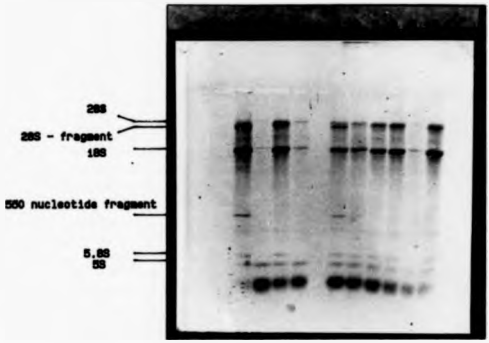
In contrast, ricin B chain purified by chromatofocusing and tested in the same system has an IC_{50} of approximately 8×10^{-8} M (0.27 µg/ml), as also shown in Figure 3:12. To determine whether the toxicity observed at the higher concentrations of B chain tested (10^{-8} and 10^{-7} M), was a result of contamination with ricin A chain, or the result of some other effect associated with ricin B chain by itself, similar dilutions of B chain (i.e. 1×10^{-11} M - 1×10^{-6} M) were tested on ribosomes, obtained from the rabbit reticulocyte lysate. These dilutions were tested for their ability to produce a ricin A chain-specific modification of 28S ribosomal RNA (after Endo et al., 1987).

3:2:3 Assessment of ribosome modifying activity of purified ricin B chain.

Dilutions of purified ricin B chain from 1×10^{-11} M - 1×10^{-6} M were incubated with aliquots of rabbit reticulocyte lysate, the RNA from these extracted, and analysed for the characteristic ricin A chain catalysed modification of 28S ribosomal RNA, (see Section 2:11).

Figure 3:13 shows the formamide gel of treated RNA extracted from each rabbit reticulocyte lysate incubation. Tracks 1 and 2 show ricin A chain treated RNA incubated with and without aniline respectively, whilst tracks 3 and 4 show the RNA extracted from ribosomes treated with buffer alone and then with and without aniline respectively. These four control lanes demonstrate that the characteristic 550 base RNA fragment indicated by the arrow in lane 1 is both ricin A chain specific and is only apparent after A chain modified RNA has been incubated with aniline. A more detailed

Figure 3: i3 RNA modification assay : Analysis of ricin B chain dilutions for ricin A chain specific activity.



Formamide gel of RNA samples extracted from rabbit reticulocyte lysate incubations.

Tracks 1 and 2 shows ricin A chain treated RNA incubated with and without aniline respectively.

Tracks 3 and 4 shows RNA extracted from reticulocyte lysate treated with buffer only and then incubated with and without aniline respectively.

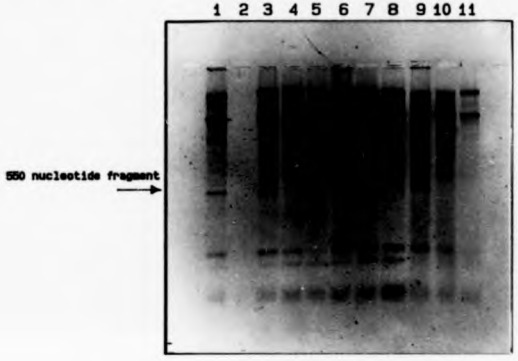
Tracks 5-10 show RNA extracted from reticulocyte lysates incubated with purified ricin B chain dilutions of $1x10^{-8}$ M to $1x10^{-13}$ M. All RNA samples in tracks 5-10 received subsequent aniline treatment.

analysis of the specific N-glycosidase activity of ricin A chain has been considered in the introduction section of this thesis. Tracks 5-10 of Figure 3:13 shows the RNA extracted from reticulocyte lysates incubated with dilutions of purified ricin B chain from 1×10^{-6} M - 1×10^{-11} M respectively. All the RNA samples in tracks 5-10 have been subsequently treated with aniline, but only at the highest B chain concentration (i.e. 1×10^{-6} M) is the ricin A chain specific RNA fragment apparent. This result indicates two important points; firstly that purified ricin B chain does have slight contamination with ricin A chain which can be detected only at a concentration of 1×10^{-6} M or higher, (but importantly, not at concentrations of 1×10^{-7} M or lower), and secondly, that the toxicity observed with ricin B chain concentrations of 1×10^{-7} M and 1×10^{-8} M in the cell-free assay analysis (shown in Figure 3.12) is unlikely to be caused by contaminating ricin A chain since no evidence of the specific A chain modification can be detected at these concentrations in the ribosome RNA modification assay (Figure 3:13, tracks 6 and 7). Therefore, it may be concluded from this data that the toxicity observed with purified ricin B chain preparations at concentrations of 1×10^{-7} M and below in both cytotoxicity and cell-free assay systems is unlikely to be the result of ricin A chain contamination, but rather a result of some other inhibitory effect of ricin B chain alone on these systems.

3:2:6 Quantitation of ricin A chain contamination in the ricin B chain preparation.

The specific RNA modifications observed at a B chain concentration of 1×10^{-6} M (Figure 3:13, track 5) is assumed to indicate ricin A chain contamination. After a further 10 fold dilution (i.e. a B chain concentration of 1×10^{-7} M), the modification of the ribosomal RNA was no longer detected. In order to quantify the extent of A chain contamination

Figure 3: i4 RNA modification assay: Determination of the lowest A-chain concentration to produce modification of 28S rRNA.



Formamide gel of RNA samples extracted from rabbit reticulocyte lysate incubations.
Tracks i-8 show 10-fold dilutions of purified ricin A chain from 0.ing/ul (3ng total protein) to 0.0000ipg/ul (0.0003pg total protein).Tracks 9 and 10 are ricin A chain treated RNA incubated with and without aniline respectively.Track ii is a -ve control, ie. no A chain, + aniline.

The lowest A chain concentration at which the diagnostic RNA fragment can be detected is ipg/ul (30pg total protein) as shown in track 3.

in the purified B chain preparation, dilutions of ricin A chain were tested for their ability to modify ribosomal RNA, until the lowest concentration of A chain producing the characteristic RNA fragment had been determined. It is important to stress here, that the experimental conditions and all reagents used in this analysis were the same as for the assay described in Section 3:2:5.

Figure 3:14 shows the RNA from rabbit reticulocyte lysate samples treated with ricin A chain dilutions. Tracks 9 and 10 show a control of ricin A chain treated lysate, with and without aniline treatment respectively. The RNA fragment released by treatment of ribosomes with A chain and aniline (indicated by the arrow) is apparent in track 9. Tracks 1-8 represent RNA extracted from ribosomes treated with 10 fold dilutions of ricin A chain from concentrations of 0.1 ng/μl (3 ng total protein) to 1 x 10^{-8} pg/μl (3 x 10^{-4} pg total protein). The last dilution at which the A chain specific modification can be detected is at 1 pg/μl (track 3). This dilution is equal to 30 pg of A chain. Therefore, from this data, a concentration of 1 x 10^{-6} M ricin B chain representing 0.96 μg of total protein is contaminated with approximately 30 pg of A chain. This represents purified B chain with only a 0.003% contamination after a single step purification from whole ricin.

3:3 Discussion.

3:3:1 Purification of ricin.

The aim of the chromatographic procedures detailed in Sections 3:1:1 to 3:2:6 was to establish a means of obtaining large quantities of highly purified, biologically active ricin subunits. The starting material for subunit purification, ricin, was purified together with *Ricinus communis* agglutinin (RCA_I) from the other seed proteins by utilising the sugar

binding activity of the two lectins, i.e. their affinity for β galactose residues exposed during the treatment of sepharose 4B beads with 1 M proprionic acid. The two lectins were eluted together from the sepharose column using 100 mM galactose in PBS, and then separated from each other by gel filtration on a Sephacryl S-200 column after the method of Nicholson and Blaustein (1972). The observation that ricin can be eluted from the sepharose column using 5 mM N-acetylgalactosamine (in PBS), without eluting RCA_I (Nicholson et al., 1974) provided a method by which ricin could be purified to homogeneity from seed material using a single chromatographic step. However, because ricin prepared by this method tended to vary with respect to contamination with RCA_I, the standard method described above was generally employed. Olsnes and Pihl (1973b) report the use of ion-exchange chromatography for separation of the two lectins, but this technique has not been widely employed and it was not necessary to include this method in the purification of ricin described in this chapter.

Purified ricin and RCA_I were analysed by SDS-PAGE under reducing conditions as shown in Figure 3:3, and seen to electrophorese as two and three protein bands respectively under these conditions. The lower band of the reduced ricin sample is composed of the A_1 species of ricin A chain, which only contains one N-linked oligosaccharide side chain, and which has a molecular weight of 30,625 daltons. The upper band is composed of both ricin B chain and the A_2 species of ricin A chain, which both contain two N-linked oligosaccharides, and which has a molecular weight of 31,423 daltons (Olsnes and Pihl, 1982). When RCA_I is electrophoresed on SDS-PAGE under reducing conditions it forms a pattern of three bands which have been identified by Cawley et al. (1978) as RCA_I B chain, and the two forms of RCA_I A chain. In order of increasing mobility, these bands have approximate molecular weights of 37 Kd, 33 Kd and 30 Kd respectively (Cawley et al., 1978).

Lord and Harley (1985) have reported that RCA_I B chain contains a fucosylated oligosaccharide side chain, which is not present on ricin B chain, and which accounts for the difference in molecular weight between the two. Roberts et al. (1985) report that the creation of a third possible N-glycosylation site in RCA_I has resulted from changes in amino acid residues of the B chain. Residue 79, which is aspartic acid in ricin B chain, is asparagine in RCA_I B chain, and amino acid residue 81, which is asparagine in ricin B chain, is changed to serine in the RCA_I B chain. This results in the sequence Asn-Cys-Ser in RCA_I B chain which conforms to the required Asn-X-Ser/Thr amino acid sequence required as a signal for N-glycosylation, where X can be any amino acid except proline. Therefore analysis of fractions eluted from the Saphacryl S-200 column on SDS-PAGE under reducing conditions readily identifies RCA_I-free ricin by the expected size differences of the glycosylated subunits (Figure 3:3).

3:3:2 Purification of the ricin subunits.

The preparation of purified ricin subunits may be considered in three stages, I. the reduction of the interchain disulphide bond, II. the physical separation of the subunits and III. the analysis of subunit purity.

3:3:2:1 Reduction of the interchain disulphide bond.

Step I. Reduction of the interchain disulphide bond was achieved by incubating whole ricin with 5% (v/v) 2-mercaptoethanol at 4°C for 48 hours in the presence of 100 mM galactose. The use of the apparently high concentration of reducing agent is in agreement with the analysis of Lappi et al. (1978) who compared a number of regimes for reducing the interchain disulphide bond of ricin and concluded that 2-mercaptoethanol was required

at relatively high concentrations for complete reduction. Despite incubating ricin under these conditions, some unreduced material was always detected during chromatofocusing. The presence of galactose during the reduction of the holotoxin helped to reduce the precipitation of the liberated B chain (Olsnes and Pihl, 1973), and therefore 100 mM lactose was included in all buffers prepared for the separation of the two subunits.

3:3:2:2 Separation of the reduced ricin subunits.

Step II. Separation of the reduced subunits was achieved mainly by the use of chromatofocusing, a technique which utilises the differences in the pIs of the constituent proteins to achieve separation. By using a chromatofocusing system with a pH range of 7 to 4, the system was optimized for obtaining highly purified ricin B chain in a single step procedure. At the same time ricin A chain was obtained and judged pure by SDS-PAGE analysis (see Figure 3:7). The purity of this ricin A chain fraction could be further increased by removing any remaining B chain or unreduced holotoxin by affinity chromatography using an asialofetuin column. Production could be easily scaled up, e.g. 100 mgs of reduced ricin typically yielded approximately 20 mgs of highly purified B chain.

A consistent problem encountered when handling ricin B chain during any procedure was the lability of the polypeptide. This probably accounts for much of the material lost during chromatofocusing. To minimise such losses, B chain was maintained, whenever possible, in a reducing environment in the presence of lactose or galactose, at a protein concentration no higher than 1 mg/ml (see Section 2:2:5). Aliquots of B chain were filter sterilized and stored at 4°C. Subunit samples were never frozen.

3:3:2:3:1 Analysis of subunit purity by SDS-PAGE.

Step III. Analysis of the purity of the separated subunits began with SDS-PAGE analysis under reducing conditions. For ricin B chain, SDS-PAGE analysis was a straightforward examination of silver stained gels for the presence of any contaminating A chain present in samples from the chromatofocusing step, as described in Section 3:2:1 and shown in Figure 3:6. The analysis of chromatofocused A chain however, required the samples to be pre-incubated with endo H as described in Section 2:5:3. The differential sensitivity of ricin B chain and ricin A chain oligosaccharides to endoglycosidases has been used as a means of differentiating between ricin B chain and the A_2 species of ricin A chain, which electrophorese with the same mobility on SDS-PAGE (see Section 3:2:1). Analysis of the carbohydrate content of the isolated subunits and studies on the susceptibility of these oligosaccharides to cleavage with endo H, F, D or α mannosidase (Foxwell et al., 1985) have indicated the existence of two high mannose oligosaccharides on the B chain. Treatment of ricin B chain with endo H under non-denaturing conditions resulted in the removal of a single oligosaccharide, the second being removed by endo H only after denaturation of the protein (Butterworth and Lord, 1983; Foxwell et al., 1985). This finding suggests that in one of the high mannose oligosaccharides the glycosidic bond of the N,N'-diacetylchitobiose core structure adjacent to the asparagine residue and cleaved by endo H (Tarentino and Maley, 1974) is protected by the polypeptide structure, only becoming susceptible to cleavage when the polypeptide is denatured. In Figure 3:7 all ricin subunits treated with endo H were denatured with SDS. Track 2 shows the B chain control demonstrating the characteristic two stage deglycosylation of B chain after denaturation and endo H treatment.

The A_1 species of ricin A chain contains a single carbohydrate side chain containing $(GlcNac)_3$ $(Xyl)_1$ $(Fuc)_1$ $(Man)_{3-4}$ which is not susceptible

to endo H cleavage (Foxwell et al., 1985). It also lacks the trimannosidic core and therefore does not bind to concanavalin A (ConA). The second oligosaccharide side-chain of the A_2 species (which accounts for its reduced mobility on SDS-PAGE) does however bind to ConA (ConA(+)). If this ConA(+) material (predominantly A_2) is separated from the non-binding ConA(-) material (predominantly A_1) then it (A_2 chain) is apparently susceptible to endo H cleavage, comigrating with the A_1-chain. In unfractionated ricin A chain preparations however, both A_1 and A_2-chains were unaffected by endo H, even in the presence of SDS (Foxwell et al., 1985). This is in agreement with the data shown in Figure 3:7 (tracks 3 and 4). Foxwell et al. (1985), offer no explanation of this observation, but did note a slight decrease in the intensity of the A_2 band when unseparated A chain was endo H treated. The resistance of the A_1 chain oligosaccharide to deglycosylation is due to the presence of an internal fucose residue which prevents the enzyme from gaining access to the glycosidic bond between the innermost N-acetylglucosamines which is the bond cleaved by endo H. Analysis of the carbohydrate content of the A_2 chain indicates a slight increase in the amount of Xylose and fucose (compared to analysis of the A_1 chain), but is not sufficient for these sugars to necessarily be included in the second oligosaccharide side chain (Foxwell et al., 1985). The basis of endo H resistance of the second oligosaccharide side-chain of the A_2 species is therefore unclear, but is apparently dependant upon the presence of the A_1 chain.

3:3:2:3:2 Analysis of ricin A chain in a cytotoxicity assay.

As, after the chromatofocusing step, no contamination of the purified subunits could be detected by analysis of silver stained gels (see Figures 3:6 and 3:7), further analysis of purity was carried out indirectly by examining the inhibition or protein synthesis in cytotoxicity assays.

Analysis of purified A chain by this method (Figure 3:8) demonstrates an almost ten fold increase in the IC_{50} (and therefore an increase in purity) of chromatofocused A chain after subsequant treatment using an asialofetuin column. As asialofetuin strongly binds both B chain and holotoxin this enhanced purification is presumably the result of removal of this type of contaminating material. From this data, the IC_{50} of post-asialofetuin treated A chain is calculated at approximately 5×10^{-8} M (1.5 μg/ml) which is almost 20 fold more toxic than the 4 step procedure described by Fulton et al. (1986) for the purification of ricin A chain.

Briefly, this method involves binding whole ricin to a sepharose 4B column, eluting any unbound material, and then reducing the bound ricin on the column with a buffer containing 2-mercaptoethanol. After incubating with the reducing agent for 3 hours at 4°C, liberated A chain was eluted leaving most of the B chain still attached to the column. The collected A chain was separated into its A_1 and A_2-chains by CM cellulose ion-exchange chromatography and then applied to an asialofetuin column. The final step was the removal of any residual B chain by passing the A chain preparation through an anti-ricin B chain monoclonal antibody column. The most effective step in this procedure was the asialofetuin column stage which increased the IC_{50} from 0.61 μg/ml to 16.7 μg/ml - a 27 fold increase. Such an increase was not observed after chromatofocused A chain (IC_{50} = 0.21 μg/ml) had been passed down an asialofetuin column (IC_{50} = 1.5 μg/ml). Anti-ricin B chain monoclonal antibodies (kindly supplied by E. Vitetta) were at one time used here to produce antibody affinity columns. However, the use of such columns did not significantly improve the purity of the post-asialofetuin A chain and therefore this stage was not subsequently included in the purification protocol. These findings are in contrast to those of Fulton et al. (1986) who achieved a 1.7 fold increase in IC_{50} after an identical step. The high efficiency of purification demonstrated by Fulton et al. (1986) could not be reproduced despite following their

methodology exactly. In particular, only a 7 fold increase in the IC_{50} was achieved when chromatofocused ricin A chain (IC_{50} = 0.21 μg/ml) was treated with asialofetuin, compared with the 27 fold increase in the IC_{50} of post-CM cellulose ricin A chain (0.61 μg/ml) observed by Fulton et al. (1986) after an identical step.

3:1:2:3:3 Analysis of ricin A chain in a cell-free assay.

The biological activity of ricin A chain, with respect to its ability to inhibit protein synthesis, was fully retained in both purification regimes as judged by cell free assay (see Section 2:10). Figure 3:12 shows the inhibition of protein synthesis caused by ricin A chain, (purified by the methods described in the results section) in a rabbit reticulocyte cell-free system. The IC_{50} of 2.5×10^{-11} M (0.8 ng/ml) is in close agreement with published data (Stirpe and Barbieri, 1986).

3:1:2:3:4 Analysis of ricin B chain in a cytotoxicity assay.

The purity of the chromatofocused ricin B chain was also analysed in cytotoxicity assays producing an IC_{50} value of 3.0×10^{-7} M (9.6 μg/ml). This value is, however, 1.5 fold lower (i.e. 1.5 times more toxic) than that of B chain purified by the method of Fulton et al.. (1986). In their method, B chain was obtained by eluting it from the sepharose column after the reduction step and then employing two DEAE ion-exchange steps followed by a CM-cellulose ion exchange chromatography. Finally, an anti-ricin A chain antibody affinity chromatography step was used. B chain purified by this method had an IC_{50} of 14.4 μg/ml in cytotoxicity assays, which compares reasonably closely with the IC_{50} value of 9.6 μg/ml obtained with chromatofocused B chain.

3:3:2:3:5 Analysis of ricin B chain in a cell-free assay.

A much larger discrepancy was noted between the IC_{50} value for chromatofocused B chain (0.27 μg/ml) and that for B chain purified by Fulton et al., 1986 (> 1 mg/ml) obtained from rabbit reticulocyte cell-free assay systems. This initially suggests that the chromatofocused B chain has considerable A chain contamination, but subsequent analysis of RNA extracted from rabbit reticulocyte lysate ribosomes treated with a range of chromatofocused B chain, concentrations indicated the presence of A chain only at concentrations above 1×10^{-7} M (3.2 μg/ml), (Figure 3:13, track 5). This result therefore suggests that the toxicity observed with chromatofocused B chain concentrations 1×10^{-8} M and 1×10^{-7} M (Figure 3:12) is not the result of contaminating ricin A chain, as no concomitant modification of the extracted RNA was detected at these concentrations (Figure 3:13, tracks 6 and 7). This data is in contrast with Fulton et al. (1986) who did not detect any significant inhibition of protein synthesis in their cell-free assays up to B chain concentrations of 1×10^{-6} M. As an extension to these studies the extent of A chain contamination in the chromatofocused B chain preparation was determined as described in Section 3:2:6. Thus after a single-step purification procedure from whole ricin, purified B chain containing only 0.003% ricin A chain contamination was obtained.

3:3:2:3:6 Galactose-binding activity of purified ricin B chain.

To establish that binding activity had not been reduced during the purification procedure, aliquots of purified B chain were applied to sepharose columns, and then eluted with 100 mM lactose. Ricin B chain which eluted at a pH = 4.8 from the chromatofocusing column bound with high efficiency to the sepharose which suggested this material represented non-

denatured material. On occasions it was observed that some material remained bound to the chromatofocusing column after the pH had reached 4.0. This material was eluted with 1 M NaCl and was shown to be composed of ricin B chain by SDS-PAGE analysis. This material, thought to represent denatured B chain, was discarded.

3:3:2:3:7 Activity of reassociated subunits

B chain purified by chromatofocusing was also tested directly in cytotoxicity assays after reassociation with purified ricin A chain as described in Section 2:9:2. Under these conditions a concentration of ricin B chain of 1×10^{-8} M, which was non-toxic to cells (see Figure 3:9), was able to produce a significant cytotoxic effect when mixed with concentrations of ricin A chain which by themselves were non-toxic to cells (see Figure 3:10). This data was taken to indicate fully functional B chain in terms of its cell binding and putative translocation activity. The reduced cytotoxic activity observed with these reassociated subunits, compared to the holotoxin, probably reflects incomplete or incorrect association of the two subunits which is shown by SDS-PAGE analysis in Figure 3:11. This finding is in agreement with those of McIntosh et al. (1988), who also favour chromatofocusing as a method for purifying ricin B chain. Houston (1982) has also demonstrated cytotoxicity between reassociated ricin subunits, but his claim that reassociated subunits have a cytotoxicity almost equivalent to ricin holotoxin probably reflects the stringency of his subunit purification procedure which was based on the method of Olsnes and Pihl (1973), and therefore based on ion-exchange chromatography. In agreement with the findings of Fulton et al. (1986) these earlier procedures for subunit purification were found to be inadequate as means of obtaining highly purified material.

3:3:9 Conclusion.

The purity of subunits achieved by the methods described in this chapter is similar to that achieved using the more recent method of Fulton et al. (1986). In cytotoxicity studies, used for indirect analysis of subunit purity, subunits purified by either method give similar results, the slight discrepancies probably reflecting the use of different cells lines or assay conditions. The largest discrepancy between the two methods, comes in the analysis of purified B chain in the rabbit reticulocyte cell-free translation system. In their assay system, Fulton et al. (1986), observe no toxicity at concentrations of ricin B chain up to 1×10^{-8} M, whilst the chromatofocused B chain here produces 45% and 85% inhibition of protein synthesis at 1×10^{-8} and 1×10^{-9} M concentrations respectively. The possibility that this observed inhibition is the result of ricin A chain contamination is unlikely, since no concomitant modification of the ribosomal RNA could be detected at these B chain concentrations. The observation of inhibition in cell-free systems caused by some effect of ricin B chain is in contrast with the observations of Fulton et al. (1986). By a further application of the RNA modification it has been possible to make an accurate estimation of A chain contamination by assaying directly for a specific A chain modification, which for the first time allows a clear distinction to be made between ricin A chain specific inhibition of protein synthesis and inhibition resulting from some other undefined action.

4:1 Introduction of thiol linkers into gelonin using heterobifunctional reagents.

A number of workers have reported an enhancement of cell killing when A chain containing immunotoxins are provided with purified ricin B chain (see Discussion 4:9). In an attempt to examine this phenomenon and to assess the universality of the role of the B chain, ricin B chain was covalently linked via a reducible disulphide bond to the Type I ribosome inactivating protein, gelonin. The disulphide linkage between the two polypeptides was formed between the sulphydryl group at residue 4 (cysteine) of the B chain (Funatsu et al., 1979) and thiol groups introduced into the gelonin molecule by heterobifunctional reagents. The methodology and chemistry relating to the introduction of thiol groups using the commercially available linkers N-succinimidyl 3(2-pyridyldithio) proprionate (SPDP) and 2-iminothiolane (2-IT) has been detailed in Section 2:12.

Before committing large amounts of purified protein to such conjugations, preliminary experiments were carried out to determine (a) the appropriate reaction conditions for optimal derivatisation (ideally one introduced thiol group per protein molecule) and (b) the effect of derivatisation on the ribosome inactivating activity of gelonin. Section 4:1:1 and 4:1:2 give experimental details relating to the derivatisation of gelonin with SPDP and 2-IT respectively.

4:1:1 Derivatisation of gelonin using SPDP.

(i) Calculation of SPDP required.

0.4 mg of gelonin in 200 µl of borate buffer was reacted with a 2.5 fold Molar excess of SPDP.

$$\frac{0.4 \times 2.5}{29,000} = \frac{x}{314.2} = 1.08 \times 10^{-3} \text{ mgs of SPDP required}$$

where 0.4 mg = amount of gelonin

2.5 = molar excess of SPDP required

29,000 = molecular weight of gelonin

314.2 = molecular weight of SPDP

x = amount of SPDP required

0.3 mg of SPDP was carefully weighed and dissolved in 139 µl of dry dimethyl formamide so that 5 µl of this solution contained 1.08×10^{-3} mgs of SPDP. The SPDP and gelonin solutions were reacted at room temperature for 1 hour and then derivatised protein separated from free SPDP using a PD10 column as described in Section 2:12:1.

The extent of derivatisation was determined as follows:-

A 500 µl sample of derivatised protein taken from the PD10 column was placed into a quartz cuvette and the optical density determined at 450 nm (base line), 343 nm (the release of S-pyridyl groups) and at 280 nm (protein absorbance). 25 µl of 100 mM DTT was added, and the optical density at the three wavelengths redetermined.

To calculate the release of S-pyridyl groups the difference in OD_{343} before and after the addition of DTT was calculated.

Change in $OD_{343} = 0.216 - 0.059 = 0.157$

where $0.059 = OD_{343}$ before DTT

$0.216 = OD_{343}$ after DTT

To convert the change in OD_{343} into moles of S-pyridyl divide the change in OD_{343} by the molar extinction coefficient for S-pyridyl groups at 343 nm and correct for the volume change when the DTT was added.

(ii) Calculation of S-pyridyl released.

$$\text{Therefore } \frac{0.157}{8.08 \times 10^3} \times \frac{525}{500} = 2.04 \times 10^{-6} \text{ moles of S-pyridyl released (A)}$$

where 0.157 = change in OD_{343} (after DTT added).

8.08×10^3 = extinction coefficient for S-pyridyl groups at 343 nm.

525 = volume (μl) of sample + DTT.

500 = volume (μl) of sample.

To calculate the number of moles of protein, the observed OD_{280} was corrected to account for the affect of derivatising linker.

Therefore the actual OD_{280} = Observed OD_{280} - OD_{280} of attached SPDP groups.

OD_{280} of attached SPDP groups = moles of S-pyridyl x molar extinction coefficient S-pyridyl groups at 280 nm

$$= 2.04 \times 10^{-6} \times 5.1 \times 10^3 = 1.04 \times 10^{-1}$$

where

2.04×10^{-6} = moles of S-pyridyl groups released (from calculation)

5.1×10^3 = molar extinction coefficient S-pyridyl groups at 280 nm.

Thus the corrected OD_{280} for the protein is

$$0.362 - 0.104 = 0.258$$

where 0.362 = OD_{280} (before DTT)

0.104 = OD_{280} of S-pyridyl groups

(iii) Calculation of moles of protein.

To calculate the moles of protein

$$\frac{0.258}{0.68 \times 29{,}000} = 1.3 \times 10^{-4} \text{ moles of gelonin (B)}$$

where 0.258 = corrected OD_{280}

0.68 = extinction coefficient at 280 nm (= 1g/l)

29,000 = molecular weight of gelonin

(iv) Calculation of molar ratio.

Thus the molar ratio of derivatising residues to gelonin molecules was calculated by:-

$$A/B = \frac{\text{moles of S-pyridyl released}}{\text{moles of protein}}$$

$$= \frac{2.04 \times 10^{-4}}{1.3 \times 10^{-4}}$$

$$= 1.56 \text{ residue/gelonin molecule}$$

The conclusion from this experiment was that a 2.5 M excess of SPDP was appropriate.

4:1:2 Derivatisation of gelonin using 2-IT.

In this example 3.0 mgs of gelonin was derivatised with a 10 fold molar excess of 2-iminothiolane. The gelonin was in borate buffer pH 9.0.

(i) Calculation of 2-IT required.

$$\frac{3 \times 10}{29{,}000} = \frac{x}{137.6}$$

where 3.0 = amount of gelonin
10.0 = molar excess of 2-IT
29,000 = molecular weight of gelonin
137.6 = molecular weight of 2-IT
x = amount of 2-IT

x = 0.142 mgs of 2-IT to be added in a volume of 7.5 µl borate buffer.

Therefore 10 mgs 2-IT was dissolved in 526.3 µl of borate buffer such that 7.5 µl contained 0.142 mgs of 2-IT. The gelonin and 2-IT were reacted together at room temperature for 1 hour then the reaction stopped by adding 0.1 volume (31 µl) of 2.2 M glycine and reacting for a further hour.

To activate and protect the introduced thiol groups Ellman's reagent was added to a final concentration of 2 mM. In this example add 15 µl of a 21 mg/ml solution (in dry dimethyl formamide) was reacted for a further hour and the derivatised protein separated from free 2-IT by gel filtration using a PD10 column (see Figure 4:1).

Figure 4:1 Protein elution profile : Separation of derivatised gelonin from unreacted 2-iminothiolane.

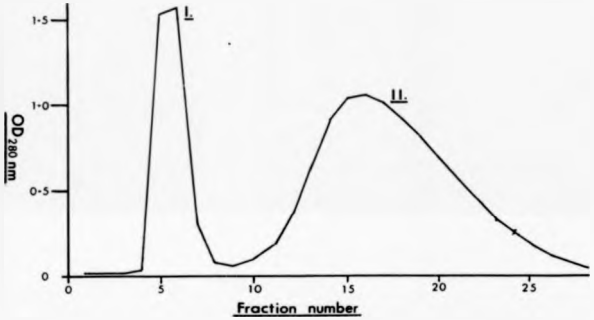
Figure 4:1 shows the protein elution profile for the separation of derivatised gelonin from unreacted 2-iminothiolane by gel filtration using a PD10 G25F pre-packed column as described in Section 2:12:1.

The elution of the derivatised protein was monitored by measuring the absorbance of each fraction at 280 nm.

Peak I represents the derivatised protein eluting as a tight peak from the column after the initial 2.5-3.0 ml void volume has passed throught the column.

Peak II represents the unreacted 2-iminothiolane derivatization reagent which elutes as a wider peak.

Figure 4: 1 Protein elution profile : Separation of derivatised gelonin from unreacted 2-iminothiolane.



As with the SPDP derivatised sample, the extent of derivatisation was determined spectroscopically.

A 500 µl sample was measured at OD_{650} (base line), 412 nm (the release of ½ Ellman's reagent) and 280 nm (protein absorbance). Once a steady state was achieved 50 µl of 100 mM DTT was added and the optical densities at the three wavelengths redetermined.

Difference in OD_{412} = 0.230 - 0.016 = 0.214

where 0.016 = OD_{412} before addition of DTT

0.230 = OD_{412} after addition of DTT

(ii) Calculation of ½ Ellman's released.

$$\frac{0.124}{1.359 \times 10^4} \times \frac{550}{500} = 1.732 \times 10^{-8} \text{ moles (A)}$$

where

0.214 = change in OD at 412 nm

1.359×10^4 = molar extinction coefficient for ½ Ellman's at 412 nm

550 = sample volume (µl) after DTT addition

500 = sample volume (µl)

(iii) Calculation of moles of gelonin.

$$\frac{0.336}{0.68 \times 29{,}000} = 1.703 \times 10^{-8} \text{ moles (B)}$$

where 0.336 = OD_{280} before addition of DTT

0.68 = extinction coefficient of gelonin at 280 nm (1g/l)

29,000 = molecular weight of gelonin.

(iv) Calculation of molar ratio.

The ratio of derivatising residues to gelonin molecules was calculated by

$$A/B = 1.732 \times 10^{-6}/1.703 \times 10^{-6} = 1.01/1$$

Therefore a 10 fold molar excess of 2-IT was appropriate for an almost 1:1 derivatisation of gelonin.

4:2 Determination of ribosome inactivating activity of derivatised gelonin.

Dilutions from 1×10^{-11} M to 1×10^{-6} M of free gelonin, gelonin derivatised using SPDP (gelonin-SPDP) and gelonin derivatised using 2-IT (gelonin 2-IT), were tested for their respective abilities to inhibit protein synthesis, as judged by the incorporation of radiolabelled amino acids, in a rabbit reticulocyte cell-free translation system (as described in Section 2:10).

The results, expressed as percentage inhibition of incorporated counts (compared with a no-toxin control) are shown in Figure 4:2. The IC_{50} for non-derivatised gelonin was calculated at approximately 1×10^{-10} M, equivalent to a final concentration of approximately 2.9 ng/ml. Derivatisation of gelonin with 2-IT results in an increase in the IC_{50} to 4.5×10^{-9} M (130.5 ng/ml), i.e. a 45 fold reduction in biological activity. The activity of gelonin is reduced even further after

Figure 4: 2 Cell-free assay : Inhibition of protein synthesis by gelonin, gelonin-SPDP and gelonin-2IT.

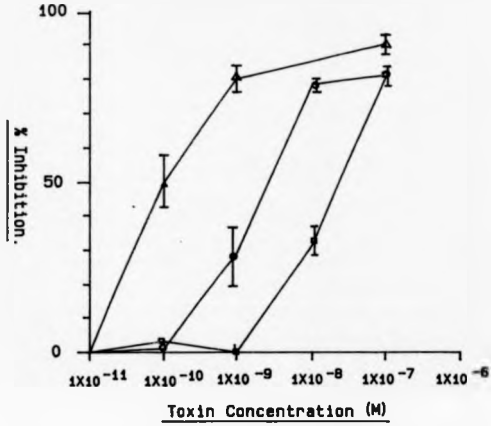


Figure 4:2 shows the inhibitory effect of gelonin, gelonin-SPDP and gelonin-2IT upon protein synthesis in a rabbit reticulocyte lysate cell-free translation system, as judged by the incorporation of ^{35}S-S methionine.

Δ——Δ represents the effect of native gelonin upon protein synthesis in the system.

o——o represents the effect of gelonin-2IT upon protein synthesis in the system

□——□ represents the effect of gelonin-SPDP upon protein synthesis in the system.

All results are expressed as % inhibition calculated from no-toxin control values and have been corrected for background counts.

derivatisation with SPDP, which has an IC_{50} of 3.5×10^{-8} M (1.015 μg/ml). This represents a 350 fold reduction activity when compared to the activity of non-derivatised gelonin.

The observation of reduced inhibitory activity with gelonin-SPDP is in agreement with the findings of Lambert et al. (1985). They observed up to 90% inactivation upon SPDP derivatisation. However these findings are in contrast to the findings of both Sivam et al. (1987) and McIntosh et al. (1988) who have successfully derivatised gelonin with SPDP under standard conditions (i.e. as described in Section 2:12) with no significant reduction in activity. Also Lambert et al. (1985) experienced no reduction in gelonin activity after derivatisation with 2-IT, which is in contrast with the data described here. However, this may simply reflect differences in experimental conditions. These workers carried out the derivatisation reaction at $0^{\circ}C$ under argon for 90 minutes, suggesting that these conditions may be required to maintain full protein synthesis inactivating activity of the gelonin.

From the results of the cell-free assay shown in Figure 4:2 it was concluded that 2-iminothiolane should be the reagent of choice for the derivatisation of gelonin despite the reduction in activity associated with its use. This reduced activity has been accounted for in the analysis of results from later experiments.

4:3 Reassociation experiment: Comparison of ricin B chain/ricin A chain and ricin B chain/derivatised gelonin in a cytotoxicity assay.

The inhibitory effect of derivatised gelonin upon protein synthesis in Vero cells, of a concentration range of 1×10^{-11} - 1×10^{-6} M is shown in Figure 4:3. Across this dilution range gelonin 2-IT exhibits no significant cytotoxicity, in contrast to the result observed when purified ricin A chain was tested in an identical assay (Figure 3:8). Here

Figure 4:3 Cytotoxicity assay : Inhibition of protein synthesis by the type I RIP gelonin.

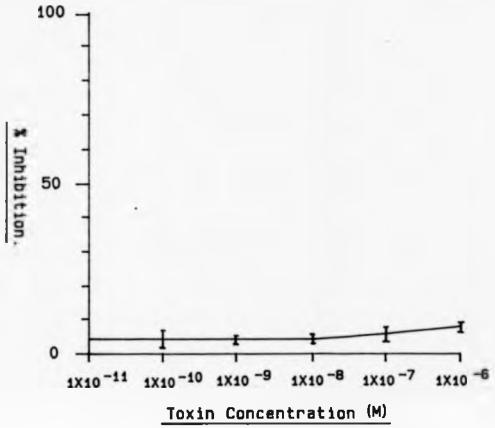


Figure 4:3 shows the effect of gelonin concentrations upon protein synthesis in Vero cells, as judged by the incorporation of ^{35}S-S methionine.

Across this concentration range, gelonin does not exhibit any significant inhibitory effect.

Results are expressed as % inhibition calculated from no toxin control values.

Figure 4.4 Cytotoxicity assay : Inhibition of protein synthesis in Vero cells by dilutions of gelonin 2IT mixed with purified ricin B chain ($1X10^{-9}M$).

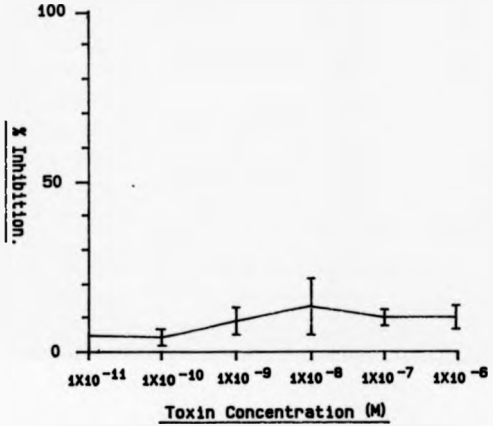


Figure 4.4 shows the effect on protein synthesis in Vero cells of the addition of gelonin 2IT concentrations with $1X10^{-9}M$ purified ricin B chain, as judged by the incorporation of ^{35}S-S methionine.

Across this range of gelonin 2IT concentrations no significant cytotoxicity was observed. At these concentrations both subunit components are non-toxic to cells when tested individually.

Results are expressed as % inhibition calculated from no toxin controls.

cytotoxicity was observed at ricin A chain concentrations of 1×10^{-7} M and 1×10^{-6} M. At concentrations of 1×10^{-8} M and below, ricin A chain was completely non-toxic to cells.

Work by Houston (1982) and data presented in the previous chapter (Figure 3:10), have demonstrated that the presence of purified ricin B chain (at a non-toxic concentration) can produce a cytotoxic effect when reassociated with normally non-cytotoxic ricin A chain concentrations. In an attempt to reproduce this result, ricin A chain dilutions were replaced with dilutions of gelonin 2-IT from 1×10^{-11} M to 1×10^{-6} M and reacted with ricin B chain (at a final concentration of 1×10^{-8} M) as described in Section 2:9:2. The cytotoxic effect of the reassociated B chain-gelonin dilutions is shown in Figure 4:4. No significant cytotoxic effect was detected over this range of gelonin concentrations which in contrast to the 40% inhibition of protein synthesis observed at a concentration of 1×10^{-8} M A chain (see Figure 3:10). In conclusion therefore, despite increasing the gelonin concentration 100 fold over the highest ricin A chain concentration tested (1×10^{-8} M), no cytotoxic activity was observed. This increase in the total gelonin concentration is, in reality, only a 55 fold increase in terms of the concentration of biologically active gelonin, taking into account the loss of activity resulting from 2-IT derivatisation.

These results suggest that the cell killing activity of ricin A chain in the presence of ricin B chain is chain-specific, and that when ricin A chain is replaced with equivalent concentrations of the Type I RIP, gelonin there is no equivalent reconstitution of cytotoxicity.

4:4 SDS-PAGE analysis of derivatised gelonin/ricin B chain conjugate

As an extension to the ricin B chain/gelonin reassociation experiments, derivatised gelonin was reacted with freshly reduced purified

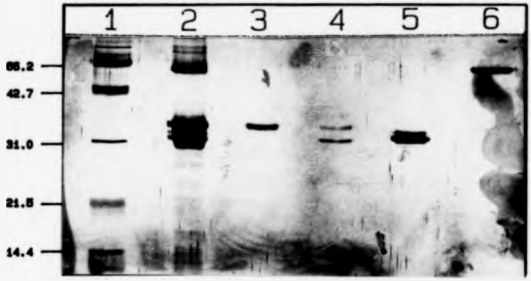
ricin B chain at 4°C under nitrogen for 36-48 hours in order to manufacture and ultimately purify ricin B chain - gelonin conjugates in an active form for analysis in cytotoxicity assays.

Prior to reacting with gelonin-2-IT, highly purified ricin B chain was incubated at 4°C with a final concentration of 10 mM DTT for 45 minutes. The freshly reduced polypeptide was then separated from the reducing agent by gel filtration using a PD10 column (as described in Section 2:12:1) and then added in a 1.5 molar excess to the derivatised gelonin and reacted as described above.

As a preliminary to any purification protocol, the reaction mix was analysed by SDS-PAGE, as shown in Figure 4:5. Under non-reducing conditions (track 2), a prominant band electrophoresing with a mobility equivalent to 60 Kd is apparent in the crude ricin B chain/gelonin reaction mix. This band is the expected size for a ricin B chain-gelonin conjugate. However, the situation is complicated by the possibility of both B chain and gelonin homodimers which, if formed, would electrophorese with almost the same mobility as the desired heterodimer. At a position approximately coinciding with the 31 Kd marker, a group of protein bands, probably representing unreacted B chain and gelonin monomers can be observed, indicating that a significant proportion of the conjugate components do not form dimers of any sort.

In order to analyse the content of the 60 Kd protein band, without risking the loss of material in unproven purification procedures, the pre-purified conjugate mix was electrophoresed under denaturing, non-reducing conditions and the band excised from the gel and electroeluted into electrophoresis buffer. Tracks 6 and 4 of Figure 4:5 show the excised protein electrophoresed under non-reducing and reducing conditions respectively. Tracks 3 and 5 show the mobility of reduced ricin B chain and reduced gelonin respectively. It can be seen that these proteins migrate with the same mobility as the upper and lower bands of the reduced

Figure 4.5 SDS-PAGE analysis of ricin B chain-gelonin reaction mixture



Silver-stained SDS-PAGE analysis of ricin B chain-gelonin reaction mixture.

Track 1 shows molecular weight size markers. Track 2 shows the pre-purified conjugate reaction mixture electrophoresed under non-reducing conditions. The 80Kd band from this mixture was excised from the gel and recovered by electroelution. Track 6 shows this excised band electrophoresed under non-reducing conditions. The same band, electrophoresed under reducing conditions is shown in track 4. Tracks 3 and 5 show ricin B chain and gelonin electrophoresed under reducing conditions respectively.

Figure 4:6 Western blot analysis of the electroeluted ricin B chain-gelonin conjugate.

Figure 4:6, box I shows the SDS-PAGE analysis of ricin B chain-gelonin conjugate material and its components. Track 1 contains free derivatised gelonin electrophoresed under reducing conditions. Track 2 represents a sample of purified ricin B chain electrophoresed under reducing conditions. Track 3 represents the electroeluted ricin B chain-gelonin conjugate electrophoresed under reducing conditions and in track 4 the same conjugate sample electrophoresed under non-reducing conditions.

Box II shows the western blot analysis of this gel, probed with anti-gelonin antibodies. These antibodies react with the gelonin in track 1, the gelonin component of the reduced conjugate sample in track 3 and the 60 Kd unreduced conjugate sample in track 4.

Arrows have been used to indicate the positions of the reactive bands.

Box III contains the SDS-PAGE analysis of the ricin B chain-gelonin conjugate and its components similar to that shown in box I. In this box, track 5 contains the unreduced conjugate sample, track 6, the reduced conjugate sample, track 7 a sample of purified ricin B chain electrophoresed under reducing conditions, and track 8 a sample of derivatised gelonin electrophoresed under reducing conditions. Track 9 contains molecular weight size markers.

Box IV contains the western blot analysis of the electrophoresed proteins of Box III and probed with anti-ricin B chain antibodies. These antibodies react strongly with the reduced B chain sample in track 7 and weakly with the B chain component of the reduced conjugate sample, (track 6) and the non-reduced conjugate sample (track 5). Arrows have been used to indicate the position of these weakly reactive bands.

Figure 4.6 Western blot analysis of electroeluted ricin B chain gelonin-conjugate. I.

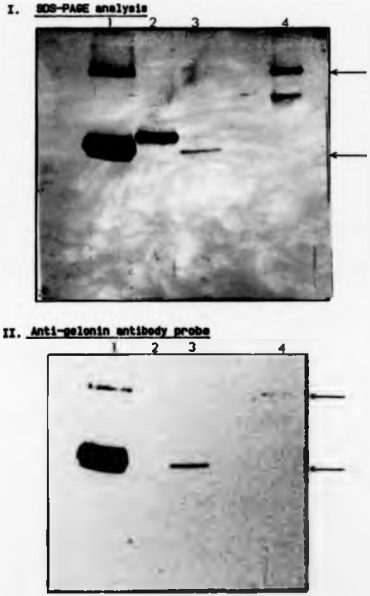
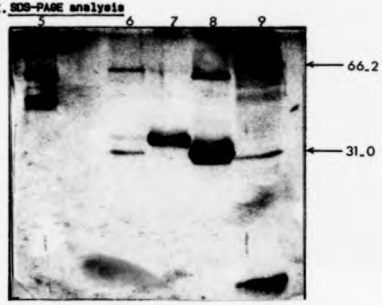
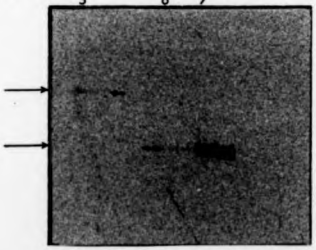


Figure 4: 6 Western blot analysis of electroeluted ricin B chain gelonin - conjugate. II.

III. SDS-PAGE analysis



IV. Anti-ricin B chain antibody probe.



60 Kd protein (track 4). This indicates that the electroeluted 60 Kd protein consists of a molecule of gelonin and a molecule of ricin B chain. Also, although not an absolute indication, the components of the 60 Kd protein when electrophoresed under reducing conditions appear in approximately equimolar amounts.

As additional confirmation, samples of the 60 Kd protein were electrophoresed under reducing and non-reducing conditions, Western blotted and then separate samples probed with either anti-gelonin or anti-ricin B chain polyclonal antibodies. In the non-reduced form, both antibody types reacted with the 60 Kd unreduced protein (tracks 4 and 5, Figure 4:6). However, anti-gelonin antibodies only bound to the gelonin component of the reduced 60 Kd protein (track 3). Equally the anti-ricin B chain antibodies only bound to the B chain component of the reduced 60 Kd protein (track 6). Anti-ricin B chain antibodies did not cross react with a gelonin only control (track 8), and anti-gelonin antibodies demonstrated only a slight cross reactivity with ricin B chain (track 2).

It was concluded from this data that the 60 Kd protein observed in non-reduced, pre-purified gelonin-B chain conjugate reaction mixtures, was composed of ricin B chain and gelonin in a 1:1 ratio.

4:3 The use of chromatofocusing in the purification of ricin B chain-gelonin conjugates.

The standard recommended technique for the purification of immunotoxins from their unreacted components is gel filtration using a Sephacryl S-200 matrix (Thorpe and Ross, 1982; Cumber et al., 1985). However, because of problems unique to the construction and purification of the ricin B chain- gelonin conjugate, such as the formation of homodimers (in particular B chain homodimers) of the same molecular size as the desired product, gel filtration proved an unsuccessful method for obtaining purified B chain-gelonin.

As an alternative to gel filtration, the pre-purified conjugate reaction mixture was chromatofocused using a pH range 9-6. The basis of this technique was to exploit differences in the pI's of the components, ricin B chain (pI = 4.8) and gelonin (pI = 8.5). It was assumed therefore that a conjugate composed of a single B chain component and a single gelonin component would have a pI somewhere between 8.5 and 4.8. With the exception of using a pH range of 9-6, the conjugate sample was chromatofocused in a manner identical to the purification of the ricin subunits (see Section 2:2:3). Figure 4:7 shows the protein elution profile at 280 nm from the chromatofocusing column. Also shown is the development of the pH gradient down the column and the elution profile for the [^{125}I] radiolabelled gelonin component. This trace of [^{125}I] labelled gelonin was added to the main gelonin stock prior to derivatisation with 2-IT, for use both as a tracer in subsequent purification studies, and as a means of calculating the concentration of the conjugate formed, (for which there would be no extinction coefficients).

Figure 4:7 indicates that no protein emerged over the pH range 9-6, but eluted as three peaks from the final salt wash. Only the first peak contained any [^{125}I] radioactive counts (and therefore gelonin). The other peaks were composed of unreacted ricin B chain as judged by SDS-PAGE (see Section 4:6). Two important observations may be made from this result. Firstly, no free gelonin (pI = 8.5) was detected, suggesting that all this material had reacted with the excess B chain added. Secondly, all the gelonin detected had eluted after the column had reached a pH of 6.0. This decrease in the apparent pI might be explained by the influence of the ricin B chain component (pI = 4:8) of the conjugate, which, when conjugated to gelonin, results in a product with a pI less than 6.0. Apparently the pI of any conjugate species is not necessarily the average of the pIs from the two components. Together these two observations indirectly suggest the formation of a B chain-gelonin conjugate. Fractions 78 and 79,

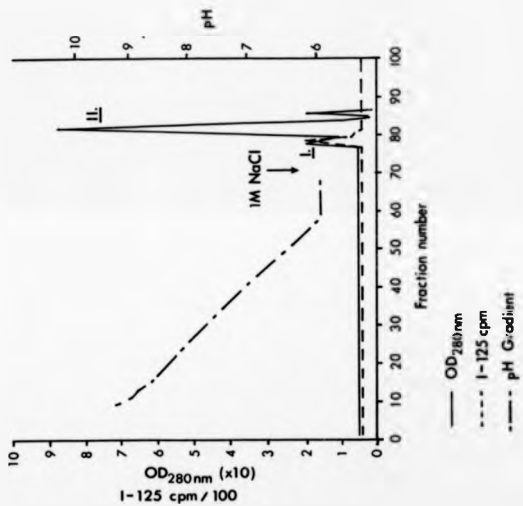
Figure 4:7 Protein elution profile : Partial purification of ricin B chain-gelonin conjugate by chromatofocusing.

Figure 4:7 shows the protein elution profile from the partial purification of ricin B chain-gelonin conjugate material using a chromatofocusing 9-6 pH range system.

Protein eluting from the column (solid line) was monitored by measuring the absorbance of the eluting material at 280 nm. Also indicated on the figure is the elution profile of ^{125}I cpm which indicates the position of the radiolabelled gelonin component (evenly broken line). The unevenly broken line represents the formation of the pH gradient generated as the polybuffer 9-6 elution buffer enters the column matrix.

Note: All material remained bound to the column of pH 6.0 and eluted in the final salt wash. Peak I, which contains all the ^{125}I counts, represents the partially purified conjugate material, whilst peak II contains free, excess ricin B chain.

Figure 4.7 Protein elution profile : Partial purification of ricin B chain - gelonin conjugate by chromatofocusing.



corresponding to the gelonin-containing peak were dialysed into 50 mM sodium phosphate buffer pH 7.5 prior to application onto a blue sepharose column. All peak fractions were analysed by SDS-PAGE (see Section 4:6).

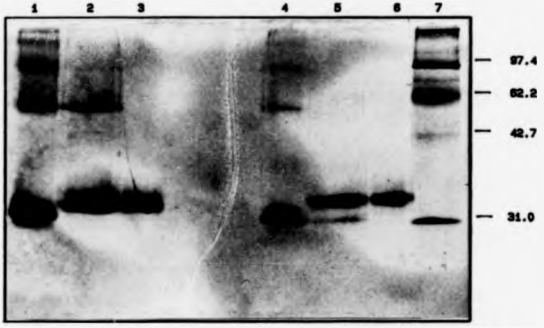
4:6 SDS-PAGE analysis of chromatofocused fractions.

Peak fractions from the chromatofocusing step described in Section 4:5 were analysed by SDS-PAGE under both reducing and non-reducing conditions. Only fractions 78 and 79 contained gelonin (as judged by [^{125}I] counts). The larger, second peak and smaller third peak appeared to be composed of excess, unreacted ricin B chain which, with a pI = 4.8, would have been expected to elute in the salt wash.

Figure 4:8 shows a 15% SDS-PAGE gel of fraction 78 electrophoresed under reducing and non-reducing conditions. Tracks 1-3 of Figure 4:8 contain gelonin, fraction 78 from the chromatofocusing step and ricin B chain electrophoresed under non-reducing conditions respectively. As considered earlier, the analysis of such data is complicated by the likely formation of homodimers which electrophorese with the same mobility as the desired heterodimer. Figure 4:8, track 1 of non-reduced gelonin-2-IT indicates that most of this material exists in a monomeric form (M_r approximately 30 Kd). However in this same track (track 1), material also exists with a mobility of 60 Kd which would correspond to the position of gelonin 2-IT homodimers. These putative homodimers are apparently in part, the result of a disulphide linkage as less 60 Kd band is apparent when gelonin 2-IT is electrophoresed under reducing conditions (track 4).

Track 2, containing non-reduced fraction 78, is composed of a sharp band at 60 Kd and a more diffuse band at around 30 Kd. This 30 Kd band electrophoreses with a mobility corresponding to that of ricin B chain (track 3 and 6), and is believed to represent free B chain which has coeluted with the gelonin-B chain conjugate fractions. Under reducing

Figure 4: 8 SDS-PAGE analysis of chromatofocused ricin B chain-gelonin conjugate material.



Silver-stained SDS-PAGE analysis of partially purified ricin B chain-gelonin conjugate fractions from chromatofocusing step.

Tracks 1-3 contain samples of gelonin, conjugate material, and ricin B chain respectively, all electrophoresed under non-reducing conditions.

Tracks 4-8 contain the same samples, in the same order, all electrophoresed under reducing conditions.

NB. The 62kd band apparent in the non-reducing conjugate sample (track 2) disappears upon reduction (track 6). The loss of this band coincides with the appearance of a smaller band with an electrophoretic mobility coincident with that of reduced gelonin 2-IT (track 4).

conditions, the non-reduced 60 Kd protein from fraction 78 disappears, whilst the intensity of the 30 Kd band (corresponding to the position of ricin B chain) increases and a new band, coinciding with the mobility of free gelonin monomers, appears (track 5). The conclusion from this data is that fraction 78 is composed of a ricin B chain-gelonin conjugate of 60 Kd which has some contamination with free monomeric ricin B chain.

The possibility that the 60 Kd protein from fraction 78 is composed of gelonin and/or ricin B chain dimers may be discounted since no gelonin monomers can be detected when fraction 78 is electrophoresed under non-reducing conditions (Figure 4:8, track 2). By comparison, when gelonin-2-IT is electrophoresed under identical conditions it comprises predominantly 30 Kd monomers with only a small proportion of dimers (Figure 4:8, track 1). Therefore the only possible way that gelonin can form a 60 Kd complex/without forming a homodimer) is to form a conjugate with ricin B chain in a 1:1 ratio. Further evidence of this association comes from the observed increase in the intensity of the ricin B chain monomer band when fraction 78 is electrophoresed under reducing conditions (Figure 4:8, track 5).

4:7 Use of Blue Sepharose CL 6B affinity chromatography to purify ricin B chain-gelonin conjugate.

Aliquots of fractions 78 and 79 from the chromatofocusing step were applied to a column of blue sepharose CL 6B as described by Knowles & Thorpe (1986). Figure 4:9 shows the elution profile from this step, together with the corresponding [^{125}I] cpm associated with the gelonin component. As expected, most [^{125}I] cpm became bound to the column and were eluted with 500 mM NaCl (fractions 50-53). A large proportion of material passed straight through the column. Both unbound and bound material was analysed by SDS-PAGE (Figure 4:10). Unbound material from

Figure 4:9 Protein elution profile : Partial purification of ricin B chain-gelonin conjugate by affinity chromatography using Blue Sepharose CL 6B

Figure 4:9 shows the protein elution profile from the partial purification of ricin B chain-gelonin by affinity chromatography using Blue Sepharose CL 6B.

Protein eluting from the column was monitored by measuring the absorbance at 280 nm (broken line).

Peak I represents non-binding material which passed straight through the column and was shown to be predominantly excess ricin B chain (Figure 4:10, track 4).

Peak II represents material binding to the blue sepharose, predominantly the partially purified ricin B chain-gelonin conjugate material. The elution of ^{125}I cpm represented by the solid line shows that the majority of radioactivity and therefore gelonin has bound to the column matrix.

Figure 4: 9 Protein elution profile : Partial purification of ricin B chain - gelonin conjugate by affinity chromatography using Blue Sepharose CL 6B.

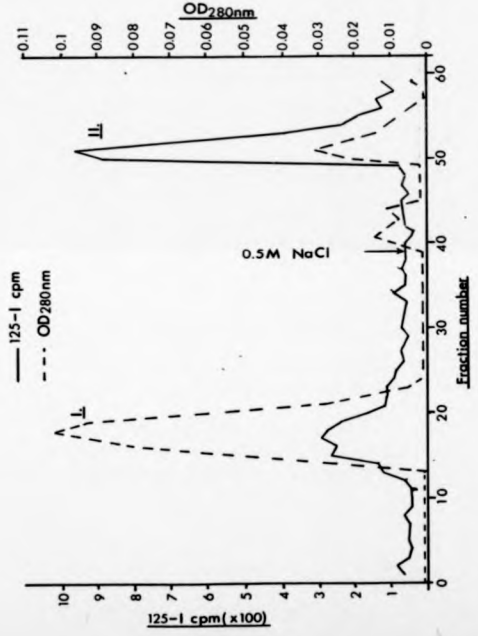
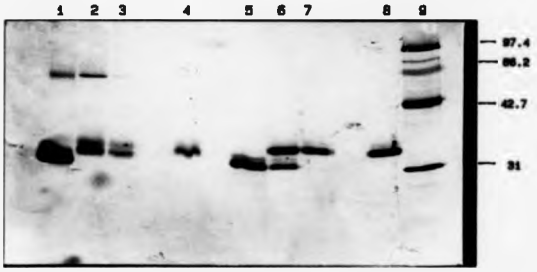


Figure 4: 10 SDS-PAGE analysis of protein fractions from Blue Sepharose CL 6B affinity chromatography step.



Silver-stained SDS-PAGE analysis of partially purified ricin B chain-gelonin conjugate fractions from blue sepharose CL 6B affinity chromatography step.

Tracks 1-4 contain samples of gelonin B-IT, conjugate fraction (bound material), ricin B chain and material not binding to the column respectively, all electrophoresed under non-reducing conditions.

Tracks 5-8 show the same samples, in the same order, electrophoresed under reducing conditions. Track 9 contains molecular weight markers.

NB. Non-binding material electrophoreses as a band with identical mobilities under both reducing and non-reducing conditions as ricin B chain.

fraction 18, when electrophoresed under non-reducing (Figure 4:10 track 4) or reducing (track 8) conditions, had a mobility which coincided with ricin B chain under the same conditions. Material from fraction 51 which bound to the blue sepharose column was composed of enriched conjugate with some contaminating B chain (track 2). The same material when electrophoresed under reducing conditions appeared as two bands with positions corresponding to free B chain and free gelonin (track 6). Tracks 1 and 5 of figure 4:10 show free gelonin-2-IT electrophoresed under non-reducing and reducing conditions respectively.

The use of the blue sepharose CL 4B affinity chromatography step aided the purification of conjugate by binding the gelonin component of the hybrid toxin. A large proportion of free B chain which contaminated the conjugate preparation after the chromatofocusing step passed straight through the column. Fractions 50 and 51, representing material which bound to the column, were dialysed into PBS containing 100 mM galactose at 4°C prior to analysis of their biological activities.

4:8 Analysis of the biological activity of partially purified conjugate, ricin B chain-gelonin.

At all stages during the purification procedures described in the previous sections, the protein samples were maintained at 4°C in buffers containing 100 mM lactose or galactose. This minimises the possibility of aggregation and biological inactivation. The biological activity of the partially purified conjugate was analysed in cell-free, RNA modification and cytotoxicity assays at the different stages of purification.

4:8:1 Cell-free assay: To determine the ribosome inactivating activity of the partially purified conjugate.

The concentration of the partially purified conjugate was determined by measuring the radioactivity associated with the gelonin component of each fraction, and then by comparing these values to the radioactivity produced by a gelonin standard of known concentration. Thus heterodimer concentrations were expressed as a function of their gelonin content. This made the assumption that all gelonin was in the form of a ricin B chain-gelonin complex, as indicated by SDS-PAGE analysis.

Figure 4:11 shows the results of a cell-free assay in which dilutions of gelonin-2-IT, fraction 79 from the chromatofocusing step and fraction 51 from the blue sepharose step were tested for their ability to inhibit protein synthesis. All three of these samples had almost identical inhibitory activity, i.e. IC_{50} values around 2.7×10^{-10} M (8 ng/ml). This result demonstrates that neither the association with ricin B chain, or the subsequent purification procedure has reduced the biological activity of the derivatised gelonin. A non-derivatised gelonin control with an IC_{50} value of 0.8 ng/ml has been included, indicating that the reduction in activity is a result of the initial derivatisation and not the result of storage or subsequent manipulations.

4:8:2 RNA modification assay: To determine a ricin A chain-type modification with a ricin B chain-gelonin conjugate.

The development of techniques to identify a RIP - specific modification of 28S rRNA from eukaryotic ribosomes has enabled the toxicity observed in the various assay systems to be associated with a specific ribosome modification event (Endo et al., 1987).

Figure 4.11 Cell-free assay : Inhibition of protein synthesis by samples from the different stages of purification of the ricin B chain - gelonin conjugate.

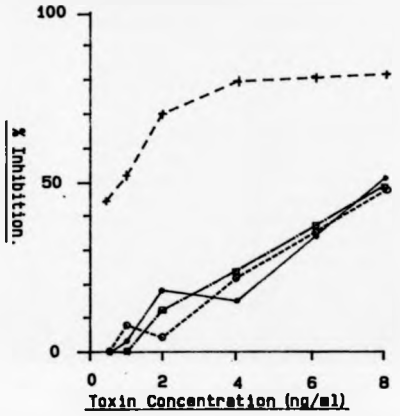


Figure 4.11 shows the inhibitory effect of gelonin, gelonin-DTT and purified ricin B chain conjugate concentrations upon protein synthesis in a rabbit reticulocyte lysate cell-free translation system, as judged by the incorporation of ^{35}S-S methionine.

- - - - represents the inhibitory effect of native gelonin.

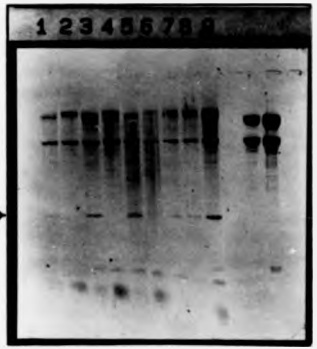
·——— represents the inhibitory effect of gelonin-DTT.

------ represents the inhibitory effect of ricin B chain-gelonin conjugate material (fraction 7H) from the chromatofocusing step, see figure 4.7.

—— represents the inhibitory effect of ricin B chain-gelonin conjugate material (fraction 8i) which bound to the blue sepharose column, see figure 4.8.

Results are expressed as % inhibition calculated from no-toxin control values and have been corrected for background counts.

Figure 4:12 RNA modification assay : Analysis of purified ricin B chain-gelonin conjugate fractions for RNA modification activity.



Formamide gel of RNA samples extracted from rabbit reticulocyte lysate incubations.

Tracks 1 and 2 shows ricin A chain treated RNA incubated with and without aniline respectively. Tracks 3 and 4 shows gelonin treated RNA incubated with and without aniline respectively. Tracks 5 and 6 shows gelonin S-XY treated RNA with and without aniline respectively, demonstrating the RNA modification activity of the gelonin has been retained after derivatisation.
Tracks 8 and 9 show RNA after incubation with reduced samples from chromatofocused fractions 7B and 7G, with subsequent aniline treatment. Track 7 shows RNA after incubation with a reduced sample of material binding to the blue sepharose column (fraction 8G), after aniline treatment.
All conjugate fractions were applied at concentrations equal to their IC_{50} as determined by cell-free assay (see figure 4:11).

The method (described in Section 2:11) has been employed to demonstrate that concentrations of conjugates shown to be toxic in the cell-free assay (Section 4:8:1) can produce the diagnostic RNA modification associated with gelonin activity (Figure 4:12). Tracks 1 and 3 from Figure 4:12 demonstrate the production of the diagnostic fragment (indicated by the arrow) when ribosomes are treated with ricin A chain and gelonin respectively followed by treatment with aniline. Without the subsequent aniline treatment, RNA from ribosome treated with either ricin A chain (track 2) or gelonin (track 4) does not show the characteristic breakdown fragment. This result strongly indicates that ricin A chain and gelonin exhibit identical RNA modification activity, and that the cleavage of the fragment from the modified RNA is dependent upon subsequent incubation with aniline.

Gelonin after derivatisation with 2-IT also exhibits an identical RNA modification activity (track 5) which is not apparent without aniline treatment (track 6). Conjugate material at a concentration equivalent to the IC_{50} (determined in Section 4:8:1) from fraction 51 of the blue sepharose step (track 7), and from fraction 79 of the chromatofocusing step (track 8), also produced the diagnostic RNA fragment after aniline treatment of toxin exposed ribosomes. This data shows that the inhibition of protein synthesis observed with these fractions is concomitant with specific modification of 28S rRNA which in turn, is associated with RIP activity.

4:8:3 Analysis of the galactose binding activity of ricin B-gelonin conjugate.

The binding activity of the various conjugate preparations were analysed in a modification of the standard asialofetuin radioimmunoassay (Vitetta, 1986). Briefly, 96 well microtitre plates were coated with 2

µg/well of asialofetuin and then blocked with a 1% "marvel" solution in PBS to prevent non-specific binding. The wells were extensively washed with PBS and the conjugates or ricin B chain then applied to defined wells. After 2 hours at 25°C the wells were washed with PBS and then probed with anti-ricin B chain antibodies which were detected with [^{125}I] radiolabelled protein A. The extent of binding was determined by comparing bound [^{125}I] cpm from a standard curve created using defined quantities of ricin B chain (Vitetta, 1986).

Since the major contaminant in the conjugate preparations was unreacted B chain, the lectin activity of the conjugate could not be analysed using anti-ricin B chain antibodies. Instead, bound samples were probed with anti-gelonin antibodies which were shown to have insignificant cross reactivity with ricin B chain. Thus the binding assay was modified to probe specifically for conjugate (B chain-gelonin) as it was assumed that gelonin would only be able to remain bound to the wells if coupled with ricin B chain. Prior to binding, lactose was removed from the samples by dialysis. Each sample was assayed in triplicate and counted three times using a Mini instruments type 6-20 γ counter to obtain a steady state reading. The results are shown in Table 4:1.

Ricin B chain (control 1) probed with anti-gelonin antibodies and the protein A shows that these antibodies do not react with ricin B chain. Therefore any counts detected when these antibodies are used to probe any conjugate fraction must be the result of binding to the gelonin component. The use of anti-gelonin antibodies is therefore a valid means of detecting bound conjugate, as it has been assumed that the gelonin component can only bind to the asialofetuin coated wells when conjugated to ricin B chain.

The second control comprises ricin B chain in which the sample has been probed with anti-B chain antibodies, but without the subsequent protein A step. This reveals the level of background counts detected. As the gelonin-2-IT itself was radiolabelled with ^{125}I it was necessary to

correct for these counts. This was achieved by omitting the antibody step (either α ricin B chain or α gelonin) and then determining bound counts. With all three fractions, when the antibody step was omitted a background count of about 850 cpm was detected.

When an anti-gelonin antibody step followed by [^{125}I] protain A was included, significant counts above background were detected in all three samples tested, indicating that some gelonin (and therefore conjugate) was bound to the wells. When anti-ricin B chain antibodies were used as the probe, the counts detected in chromatofocused fractions 78 and 79 were approximately 10 fold higher than the counts obtained when using anti-gelonin antibodies. This reflects ricin B chain contamination apparent upon SDS-PAGE analysis of the chromatofocused conjugate (Figure 4:8). After affinity chromatography using blue sepharose CL 6B the counts detected when material from fraction 51 is probed with anti-ricin B chain antibodies is significantly reduced compared to the chromatofocused fractions. This may indicate an increased conjugate purity after this step (not apparent by SDS-PAGE analysis, Figure 4:10).

Analysis of this data has to be confined to a qualitative analysis of conjugate binding. This is because it proved impossible to produce a quantitative curve of galactose binding activity directly since no absolutely pure ricin B chain-gelonin conjugate was available. Points that can be made from this data are:-

1. In this assay system, anti-gelonin antibodies did not significantly cross-react with ricin B chain and are therefore a valid probe for detecting conjugate (i.e. gelonin S-S linked to B chain which in turn is bound to asialofetuin).

Table 4:1 Analysis of galactose binding activity of ricin B chain-gelonin conjugate.

SAMPLE		PROBE: α B Chain	α Gelonin	^{125}I Prot. A	cpm
Control 1 ricin B chain			✓	✓	702
Control 2 ricin B chain		✓		x	633
20 ng	fraction 78			✓	880
20 ng	fraction 78		✓	✓	2538
20 ng	fraction 78	✓		✓	24660
20 ng	fraction 79			✓	837
20 ng	fraction 79		✓	✓	1439
20 ng	fraction 79	✓		✓	21624
60 ng	fraction 51			✓	845
60 ng	fraction 51		✓	✓	1326
60 ng	fraction 51	✓		✓	1237

2. When any of the three samples were probed with anti-gelonin antibodies, counts were detected above background indicating the presence of gelonin and therefore of bound conjugate.

3. When probed with anti-ricin B chain antibodies, fractions 78 and 79 demonstrated significantly increased binding which reflects the extent of ricin B chain contamination, also observed upon SDS-PAGE analysis of these fractions (see Figure 4:8).

4. Fraction 51, when probed with anti-ricin B chain antibodies, did not demonstrate the increased binding activity observed in fractions 78 and 79 which may reflect a purification enhancement after the blue sepharose step.

5. Because it was impossible to characterise a number of important parameters related to the binding assay, it was impossible to draw firm conclusions from this data. At best it suggests that at least some conjugate material is capable of binding to asialofetuin and that any free B chain contaminating the conjugate preparations, has retained its galactose activity despite undergoing various manipulations during both purification procedures and assays of activity.

4:8:4 Cytotoxicity assay: Analysis of the activity of partially purified ricin B chain-gelonin conjugate preparations.

Lactose-free dilutions of partially purified conjugate fractions were analysed for their ability to inhibit protein synthesis in standard cytotoxicity assays as described in Section 2:9:1. Protein concentrations were determined from the [^{125}I] content of each sample and indexed to an

Figure 4:13 Cytotoxicity assay. Inhibition of partially purified ricin B chain - gelonin conjugate.

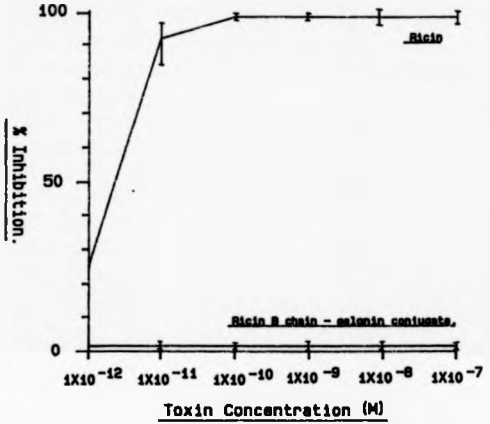


Figure 4:13 shows the inhibitory effect of the purified ricin B chain - gelonin conjugate (after blue sepharose) upon protein synthesis in Vero cells, as judged by the incorporation of ^{35}S-methionine.

Across this range of conjugate dilutions no significant inhibitory effect was observed.

A whole ricin control has been included as a comparison.

Results have been expressed as % inhibition, calculated from no-toxin control values.

[^{125}I] labelled gelonin standard as described in Section 4:8:1.

Figure 4:13 describes the results from the analysis of fraction 51 from the blue sepharose purified material. Even at the highest concentration, which contains 1 x 10^{-7} M gelonin, no cytotoxic activity was observed. Indeed a slight but consistent stimulation of radiolabelled amino acid incorporation was observed, although no substantiated explanation can be offered for this observation. No inhibitory activity was demonstrated when fractions 78 and 79 from the chromatofocusing step were applied to cells (data not shown), but because of the extensive contamination with free ricin B chain any toxic activity would have been difficult to interpret.

4:9 Discussion.

The series of experiments detailed in this chapter were designed to assess the feasibility of using ricin B chain as a vehicle for the binding and internalization of heterologous molecules, in this case gelonin, into eukaryotic cells. The observation of an increased cytotoxic activity of ricin A chain containing immunotoxins in the presence of free ricin B chain (Youle and Neville, 1982; McIntosh *et al*., 1983), or ricin B chain containing immunotoxins (Vitetta *et al*., 1983), has led to speculation that ricin B chain has some other role, in addition to its cell-binding role, that can aid or promote the entry of ricin A chain into the cell cytoplasm. This role of the B chain remains undefined both at the cellular and molecular level. It may, for instance merely aid toxicity upon internalization by directing or stimulating endocytosis to the correct environment from which the A chain can "escape" into the cell cytoplasm. Or, it may afford protection against proteases encountered *en route*. It is known for instance that whole ricin is much more resistant to proteases than individual subunits (Olsnes *et al*., 1975). Another possible role of

ricin B chain during internalization is some direct involvement in physically aiding the crucial membrane translocation step, which must occur to afford ricin A chain entry into the cell cytoplasm. It is not known whether the B chain gains access to the cytosol itself during this step, though the general feeling is that it does not.

Although work by Vitetta (1986) has suggested that the sugar binding activity of ricin B chain is operationally separated from its ability to enhance some intracellular aspect of toxicity, there has been no formal characterization of this. Thus, the observed enhancement of activity may not nessarily be a function unique to ricin B chain, but possibly the result of co-operation between the two subunits. This is a particularly attractive hypothesis, because ricin B chain does not appear to have an obvious membrane translocation domain of the type characterised in other toxins e.g. diphtheria toxin (Sandvig and Olsnes, 1981; Greenfield et al., 1987) and pseudomonas exotoxin A (Allured et al., 1986).

The Type I RIP, gelonin, because of its similar molecular weight (29 Kd) and its apparently identical mode of action to ricin A chain was considered a suitable molecule to study the putative translocation activity of ricin B chain. In addition, gelonin alone was shown to be virtually non-toxic to cells (Figure 4:3).

In order to achieve the formation of a disulphide bond between ricin B chain and gelonin it was necessary to introduce thiol groups into the gelonin component. Preliminary experiments established that a 10 molar excess of 2-iminothiolane over the protein concentration gave optimal derivatisation, i.e. one chemically introduced thiol group per protein molecule. The introduction of, on average, one thiol group per gelonin molecule using 2-iminothiolane did, however, result in a ten-fold loss of activity when the derivatised gelonin was tested for its ability to inhibit protein synthesis in cell-free assay systems. A considerably greater reduction in gelonin activity was observed when the heterobifunctional

linker SPDP was used to introduce the reactive thiol group (see Section 4:2). Therefore 2-IT became the choice of linker reagent in these experiments. The inactivation of gelonin observed after derivatisation with SPDP is in agreement with the findings of Lambert et al. (1985), but in contrast with the work of McIntosh et al. (1988) and Sivam et al. (1987) who detected no reduction in gelonin activity after SPDP derivatisation. It is possible that 2-IT and SPDP each react preferentially with different amino groups on the gelonin molecule which may influence resulting toxicity. Also the positive charge on amino groups is preserved upon reduction after treatment with 2-IT, and this may also account for the difference in observed activity between gelonin-2-IT and gelonin-SPDP (Lambert et al., 1985).

Having successfully introduced reactive thiol group into the gelonin molecules and characterised the activity of the derivatised protein in cell-free assays, these molecules were reacted with purified ricin B chain as described in Section 2:9:2. As a control, ricin A chain dilutions, normally non-toxic to cells alone, were reacted with a non-toxic concentration of ricin B chain and tested alongside the gelonin-2-IT/ricin B chain reaction mix in standard cytotoxicity assays (described in Section 2:9:2). Figure 3:10 from the previous chapter demonstrate the cytotoxic activity when dilutions of ricin A chain (upto 1×10^{-8} M) were reacted with 1×10^{-8} M ricin B chain. Individually, these concentrations of purified subunits are non-toxic in cytotoxicity assays, but when reacted together demonstrate a 40% inhibition compared to no toxin controls. No toxicity was observed when the gelonin-2-IT/ricin B chain reaction mixture was tested in an identical assay, despite increasing the concentration of active gelonin molecules by 55-fold (Figure 4:4). SDS-PAGE analysis of the reassociated ricin A chain/ricin B chain (Figure 3:11) and the gelonin-2-IT/ricin B chain mixtures (Figure 4:5) indicates that only a small proportion of the reactive components form disulphide-linked proteins with

electrophoretic mobilities corresponding to those expected for the reassociated or hybrid toxins. Despite this apparently poor reassociation between ricin B chain and either ricin A chain or gelonin-2-IT, the native ricin subunits are able to combine to produce a significant cytotoxic effect, whilst a gelonin-2-IT - ricin B chain conjugate is non-toxic under identical conditions.

A consistent problem encountered during the analysis of hybrid toxin formation between ricin B chain and derivatised gelonin was the formation of homodimers. Samples of both free ricin A chain (Figure 3:11 tracks 2-4) and free ricin B chain (tracks 5 and 6) indicate the formation of homodimers in the absence of reducing agent. This observation of homodimer formation is in agreement with Cushley et al., 1984, but in contrast with similar studies in which no homodimer formation was reported (Olsnes et al., 1974), or where their possible formation has not been considered (Goldmacher et al., 1987; Foxwell et al., 1987; Ovadia et al., 1988). Since the electrophoretic mobilities of both gelonin-2-IT/ricin B chain heterodimers and homodimer species made them indistinguishable by SDS-PAGE analysis, it was impossible to accurately estimate the percentage of hybrid toxin formed by simple visual means, as described by Olsnes et al. (1974) in their study of ricin/abrin hybrid toxins, and Sundan et al. (1982) in their study of diphtheria/ricin hybrid toxins. Instead, gelonin-2-IT and ricin B chain were reacted together as described in Section 4:4 and then applied to a purification procedure with the aim of obtaining pure heterodimers free from both monomeric and dimeric forms of the constituent polypeptide components.

The first purification steps was to apply the conjugate reaction mixture to a chromatofocusing column with a pH range 9-6. Over this pH range it was hypothesised that monomers and dimers of gelonin would elute according to their pI of about 8.5, whilst ricin B chain monomers and dimers would remain bound to the chromatofocusing column until the final

salt wash. Any genuine hybrid molecules composed of one ricin B chain and one gelonin-2-IT molecule should therefore elute with a net pI somewhere between the pIs of the components. Gelonin has a pI = 8.5 and ricin B chain has a pI = 4.8. The actual elution profile of the chromatofocused conjugate mixture is shown in Figure 4:7 and described in Section 4:5. Briefly, this technique achieved a partial purification of the conjugate material, although it remained contaminated with free ricin B chain which had been added in excess to ensure all free thiol groups on the gelonin molecules were reacted. In retrospect it might have been advantageous from the point of view of purification, to have reacted an excess of gelonin-2-IT with ricin B chain (as described by Goldmacher et al., 1987). In theory such a reaction mixture would not contain any free B chain, as it would all react with the excess gelonin 2-IT. It would therefore have been easier to separate conjugate gelonin 2-IT - ricin B chain from free gelonin by using a sepharose 4B affinity chromatography column. However, this method would not separate any ricin B chain homodimers from the gelonin-B chain hybrid if they formed.

In an attempt to improve the purity of the chromatofocused conjugate material, aliquots were applied to a blue sepharose CL 6B column, the aim being to bind any conjugate formed via its gelonin moiety and to wash any unbound material (i.e. free B chain) from the column. Material eluting from the column with a 500 mM NaCl buffer (see Figure 4:9) contained the [^{125}I] labelled gelonin component conjugated to ricin B chain (see Figure 4:10 track 2) but still carried contaminating B chain. Because of the limiting amount of starting material for these purification procedures, and the losses encountered when material was applied to each respective column, it was only possible to attempt small scale purification. At each stage material was retained for analysis of biological activity, and throughout all procedures of coupling to ricin B chain, purification and storage, no reduction in the activity of the derivatised gelonin was detected, (see

Section 4:8:1, Figure 4:11). This inhibitory activity observed in cell-free assay systems was found to be concomitant with a modification of the 28S rRNA from eukaryotic ribosomes. This has been judged as specific to ricin A chain and other RIPs (see Section 4:8:2, Figure 4:12).

Analysis of the galactose-binding activity of the purified conjugate was carried out using a standard asialofetuin radioimmunoassay (Vitetta, 1986), modified so as to probe the material bound to the asialofetuin-coated wells with anti-gelonin antibodies. Anti-gelonin antibodies which did not cross react with ricin B chain were used as a probe specific for the conjugate and demonstrated that some conjugate material had bound to the coated wells. This bound conjugate material could not however, be accurately quantified as the required binding curve control, specific for conjugate could not be produced (see Section 4:8:3 for a more detailed analysis of this data).

The final product obtained after the chromatographic procedures was an apparently enriched gelonin-ricin B chain conjugate linked via a disulphide linkage. Because this material was contaminated with unreacted ricin B chain a complete analysis of the galactose binding activity could not be achieved, but the protein synthesis inhibiting activity of the gelonin moiety was found to be no different from the activity of free derivatised gelonin. This conjugate however, failed to exhibit any cytotoxic activity when tested on Vero cells (see Figure 4:13). It was therefore concluded that from both this analysis of partially purified gelonin-ricin B chain and the reassociation experiments described in Section 4:3, that ricin B chain does not mediate the entry of gelonin into cells so that it can act to inhibit protein synthesis.

The conclusion from the data described in this chapter, that ricin B chain does not mediate the internalization and/or translocation of gelonin, are in agreement with the findings of McIntosh et al. (1988). In their experiments they found that pre-treatment of cells with either ricin B

chain or abrin B chain, sensitized those cells to treatment with either free abrin or ricin A chains and to immunotoxins containing either abrin or ricin A chain. Furthermore, they observed the greatest potentiation with either B chain species when the interacting species contained the homologous A chain. In particular the weakest potentiation was observed with ricin B chain and either abrin A or abrin A chain containing immunotoxins. They were however, unable to sensitize cells with any B chain pre-treatment to either free gelonin or gelonin-containing immunotoxins.

Further evidence of the limited ability of ricin B chain to reassociate with heterologous A chains comes from the observations of Olsnes et al. (1974) who noted that only between 10 and 30% of abrin A/ricin B subunits reacted together to form a hybrid toxin, in contrast to the reaction between abrin B/ricin A which was almost complete after 24 hours. Further to this, Sundan et al. (1982) were able to produce a toxic hybrid molecule from diphtheria fragment B and ricin A chain, but a hybrid protein between ricin B chain and diphtheria fragment A was shown to be non-toxic despite being able to bind to cells. Thus, a body of evidence exists in the literature which suggests that ricin B chain is unable to potentiate the entry of all but what are speculated to be the most closely related heterologous A chains (e.g. abrin A chain). The best results in terms of cytotoxicity are with reassociated ricin A chain, although this to is reduced as compared to whole ricin. In contrast Foxwell et al. (1987), claim to have reassociated 70% intact toxin from deglycosylated ricin A and B chains, as judged by SDS-PAGE analysis. They have not however, in their analysis, made any attempt to account for the formation of ricin B chain homodimers which would be indistinguishable from reassociated ricin A and B chains upon SDS-PAGE under non-reducing conditions.

The poor interaction between ricin B chain and heterologous A chains, including in this case the Type I RIP gelonin, makes the purification of

such hybrid toxins difficult as only a small amount of product can be obtained from an already limited amount of starting material. To avoid the problems associated with purifying such small amounts of material Olsnes et al. (1974) and Sundan et al. (1982) estimated the extent of reassociation from SDS-PAGE analysis and corrected their results accordingly. In these earlier reassociation experiments however, it is unlikely that the constituent toxin subunits were purified to the high stringency now possible using more recent technology. Therefore it is possible that the complete toxicity observed in the Olsnes et al. (1974) abrin/ricin reassociation experiments reflects the incomplete purification of the constituent subunits. In contrast McIntosh et al. (1988), who describe a more stringent subunit purification methodology, found a reduced cytotoxicity from reassociated abrin/ricin subunits compared to the respective holotoxins.

It is apparent from the experiments described in this chapter, and from the work of others described earlier, that the reassociation of ricin B chain either to ricin A chain, or some other RIP requires more than just the simple formation of a disulphide bond between polypeptides. With respect to the association of the ricin subunits, a body of evidence in the literature suggests that the two ricin subunits are held together by non-polar forces (Lewis and Youle, 1986; Houston, 1980). Further to this, Lewis and Youle (1986) have suggested that the disulphide linkage between the two subunits is not critical for toxicity, and only functions to maintain protein-protein interactions at low ricin concentrations. These findings are in contrast with those of Lappi et al. (1978) and Wright and Robertus (1987) whose results implicate the interchain disulphide bond in the transport of the A chain into the cytoplasm. Analysis of the three-dimensional structure of ricin has indicated a close interaction between a carboxyl-terminal region of the A chain and the amino terminal region of the B chain (Montfort et al., 1987). The interaction between the two ricin

subunits will be considered in more detail in the following chapter, but such observations fuel speculation as to a more complex interaction between the two subunits than just the formation of a disulphide bond. Indeed a more complex subunit interaction, and the possibility of conformation changes in the subunits associated with reduction of the disulphide bond (Montfort et al., 1987) may go some way to explaining (a) the poor reassociation of ricin B chain with heterologous A chains and related RIPs and (b) the reduced cytotoxic activity of reassociated ricin subunits.

In contrast to these previous observations and the overall conclusion from the data presented in this chapter, Goldmacher et al. (1987) has described the preparation of a ricin B chain-gelonin conjugate with cytotoxic activity against the human B-cell line, Namalwa. In their experiments this conjugate together with ricin, diphtheria toxin, and a series of ricin A chain, or gelonin based immunotoxins (reactive towards Namalwa cells) were tested for their cytotoxicity against wild type, or toxin resistant Namalwa cell lines. A direct comparison of the cytotoxic activities of the various conjugates upon each cell line is not possible as the assaying technique varied for the constructs tested. However, a direct comparison can be made between the cytotoxic activity of the ricin B chain-gelonin conjugate and ricin, upon wild type Namalwa cells. These experiments show the ID_{50} for the ricin B chain-gelonin conjugate to be 1000-fold higher than that for whole ricin i.e. the conjugate is 1000-fold less toxic than ricin. Further to this Goldmacher and co-workers do not disclose the source, or purification procedure for obtaining ricin B chain used in these experiments, nor do they describe any analysis of the toxicity of this component alone to the target cells. Without this essential control therefore, it is impossible to associate the toxicity observed with this conjugate to a genuine ricin B chain-mediated entry of entry of gelonin into Namalwa cells.

In a similar study by Ovadia et al. (1988), barley RIP was crosslinked to ricin B chain, purified by gel filtration and affinity chromatography and then assayed for cytotoxic activity against a K562 cell line derived from a patient with chronic myelogenous leukemia. In these assays the ID_{50} for the conjugate was calculated between 0.15-0.25 μg/ml (2.3 nM-3.9 nM) which is some 2000 fold less toxic than the published ID_{50} for whole ricin on HeLa cells (Stirpe and Barbieri, 1986). No data for the cytotoxic activity of whole ricin against K562 cells has been presented. Despite this reduced cytotoxicity compared to whole ricin, this barley RIP-ricin B chain conjugate does apparently exhibit some ricin B chain mediated activity, which can be blocked by the addition of 50 mM lactose.

In all these hybrid toxin studies, the absolute test of genuine RIP-mediated inhibition of protein synthesis would involve the analysis of ribosomal RNA extracted from intoxicated cells. Because no cytotoxic ricin B chain-gelonin conjugate was produced by the procedures described earlier, this method could not be employed appropriately. It would however, enable a distinction to be made between any possible non-specific cytotoxicity and that caused by the successful translocation of a ribosome inactivating protein into the cell cytoplasm and ultimately to its target, the ribosomes.

As more information has become available regarding the biochemistry and mode of action of these ribosome inactivating proteins it now seems likely that these proteins, which are widespread throughout the Plant Kingdom, have originated from some common ancestor. A more detailed consideration of the evolution of plant toxins has been made in the introduction to this thesis and therefore only an outline of these perspectives, as they relate to the material in this chapter, are discussed below.

The B chains of Type II RIPs may have originated from a common ancestor (Ready et al., 1984) as has been proposed for the A chains of Type

I and Type II RIPs (see introduction). The body of evidence supporting this makes the cytotoxicity observed with some hybrid toxins less surprising. However, to fully understand the interrelationships of the various hybrid toxins it would be necessary to perform detailed sequence and structure comparisons. Unfortunately because only the sequence and structure of ricin B chain has been analysed in any detail no comparison between the B chains of Type II heterodimeric plant toxins has been made. Studies on the structure of ricin have shown it to be composed of two sugar-binding domains (Villafranca and Robertus, 1981) which exhibit considerable homology, each binding a galactose residue in a non-cooperative fashion (Zentz et al., 1978; Houston and Dooley, 1982). Although two putative galactose binding sites have been identified from the X-ray structure (Montfort et al., 1987), no other functional domains have been correlated with the three dimensional structure of B chain. Supposing that the B chain does have a role in membrane translocation, then a translocating domain (usually a region of significant hydrophobicity) should be apparent in the amino acid sequence. However, detailed sequence and structural analysis of ricin B chain have not identified, or even implicated, a domain likely to become embedded within a lipid bilayer during the membrane translocation of ricin A chain. For this reason it is perhaps important not to consider the membrane translocation of ricin A chain as a function solely confined to ricin B chain, but possibly the result of some synergistic interaction between the two subunits. Additionally, some workers now feel that the potentiation of toxicity observed during the intracellular phase of intoxication in the presence of ricin B chain is the result of a specific interaction between the two subunits rather than some generalised non-specific effect of the B chain upon the cellular membranes. McIntosh et al. (1988) have described experiments which indirectly support such a theory. They argue that because abrin and ricin B chain enhance the cytotoxicity of their own, or

each others A chains when reassociated, but do not enhance the cytotoxicity of gelonin under the same conditions, this potentiation is the result of specific interactions between closely related subunits (Funatsu et al., 1988) and not some generalised effect on the membranes caused by the B chain. Work carried out by Houston (1982) and McIntosh et al. (1988) suggests that this interaction occurs prior to endocytosis.

Perhaps the strongest evidence suggesting that the cytoplasmic entry of ricin A chain is not a function confined to ricin B chain comes from the observation that various ricin A chain (or Type I RIP) conjugates with cell surface specific antibodies or other ligands are capable of considerable cytotoxic activity, against appropriate target cells. Oeltmann and Heath (1979) have prepared a specifically cytotoxic hybrid molecule from purified ricin A chain and the β subunit of human chorionic gonadotrophin and in more recent studies, Cawley et al. (1980) produced an epidermal growth factor-ricin A chain conjugate with specific cytotoxic activity towards its target cells. Although many other examples of ricin A chain immunotoxins with specific and potent cytotoxic activity may be quoted the possibility of contaminating ricin B chain enhancing their activity could never be ruled out. However potent, specific cytotoxins constructed from recombinant ricin A chain and antibodies raised against murine transferrin receptor had similar activities to immunotoxins constructed from biochemically purified ricin A chain (Fitzgerald et al., 1987). Furthermore, immunotoxins prepared using gelonin (a Type I RIP) have also demonstrated considerable cytotoxic activity (Lambert et al., 1985; Thorpe et al., 1981). This raises two questions. Firstly do the A chains (or Type I RIPs) used in these conjugates possess some inherent ability by which they promote their own translocation into the cell cytoplasm upon endocytosis? And secondly, can they in any way influence the route to their intracellular destination, or is this directed by the ligand to which the A chains are bound? The diversity of ligands might suggest that the A

chains can promote their own translocation, and at least in these examples cited are not adversely influenced by the ligand to which they are conjugated. However, no direct evidence for such a mechanism exists.

A review of the current literature discussed here strongly suggests that the presence of ricin B chain somehow promotes the entry of ricin A chain into the cell cytoplasm (McIntosh et al., 1988; Youle and Neville, 1982; Houston, 1982). Whilst some authors, e.g. Vitetta (1986), have suggested that ricin B chain possesses some translocation activity distinct from its galactose binding activity, no direct evidence for such a function has been forthcoming. Indeed more recent studies of the structure of ricin B chain (Montfort et al., 1987) have failed to identify or even imply any putative translocation domain on the B chain. Indirectly in agreement with these findings, the results presented in this chapter suggest that the potentiation of ricin A chain when reacted with ricin B chain is a specific interaction, and does not extend to the potentiation of the Type I RIP gelonin. The agreement in favour of a specific interaction between ricin subunits has been further strengthened by the observations of poor or non-existent associations with either abrin A chain (Olsnes et al., 1984) or diphtheria fragment A (Sundan et al., 1982) respectively. In contrast to the results described in this chapter, Goldmacher et al. (1987) and Ovadia et al. (1985) both describe hybrid toxins constructed from purified ricin B chain and Type I RIPs which exhibit cytotoxicity. In both cases the activity of the hybrid toxins is more than 1000 fold less than that for whole ricin which would give these hybrid toxins IC_{50} values comparable to the same RIP coupled to a cell specific antibody. Furthermore the observations of McIntosh et al. (1988) that neither abrin or ricin B chain could potentiate the activity of either free gelonin or more significantly gelonin immunotoxins strongly suggests that the nature of the subunits is vital in the reconstitution of potent cytotoxins. This possibility is considered in much greater detail in the following chapter.

5:1 Introduction of a premature translation termination codon into ricin A chain cDNA by oligonucleotide site-directed mutagenesis.

The introduction of a translation stop codon at nucleotides +712-714 of the published ricin cDNA sequence (Lamb *et al*., 1985) was achieved by the application of oligonucleotide site-directed mutagenesis as described in Section 2:23. The aim of this mutagenesis procedure was to ultimately produce a truncated form of ricin A chain devoid of the final 30 amino acids. This C-terminal region forms a significantly hydrophobic domain (see Figure 5:1) which is believed to interact with regions of ricin B chain and may therefore be important in ricin subunit interactions (Montfort *et al*., 1987). Furthermore this C-terminal hydrophobic domain has been shown to have an affinity for lipids, which may indicate some membrane penetration function associated with this region (Uchida *et al*., 1980). By successfully deleting this C-terminal region it was hoped to establish its importance, if any, during the translocation of ricin A chain into the cell cytoplasm.

5:2 Colony hybridization using the mutagenic oligonucleotide as a probe.

Details of the mutagenesis of the ricin A chain-encoding sequence, prior to the colony screening step, have been considered in Sections 2:23:1-2:23:5. After the transformation of competent *E. coli* 71.18 mut L cells with the oligonucleotide primed extension reaction, plaques (representing areas of retarded bacterial growth due to M13 'phage infection) were picked onto duplicate L-agar plates in a predetermined grid formation as described in Section 2:23:6. After incubation, one of the duplicate plates was stored at 4°C, whilst the other was used for the colony hybridization. The colonies were transferred onto nitrocellulose, lysed, neutralized, fixed and baked and then probed with the mutagenic

Figure 5:1 Hydrophobicity plot of the C-terminus of ricin A chain.

Figure 5:1 shows a diagrammatic representation of the hydrophobicity of the C-terminal region of ricin A chain.

Indicated on the figure are the 30 C-terminal amino acids deleted as a consequence of the introduced translation termination signal introduced into the DNA sequence.

As a result of this deletion, the cysteine residue at position 259 which is normally involved in the interchain disulphide linkage has been removed. Also this deletion has disrupted the N-glycosylation site at residues 236-238 in the A chain, as indicated on the figure.

Figure 8: i Hydrophobicity plot of the C-terminus of ricin A chain

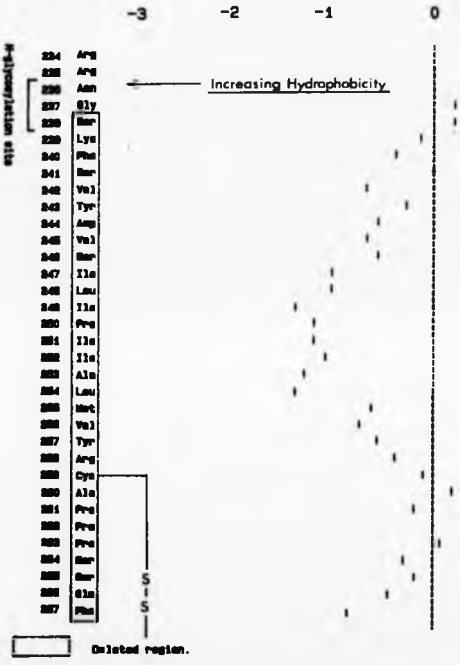
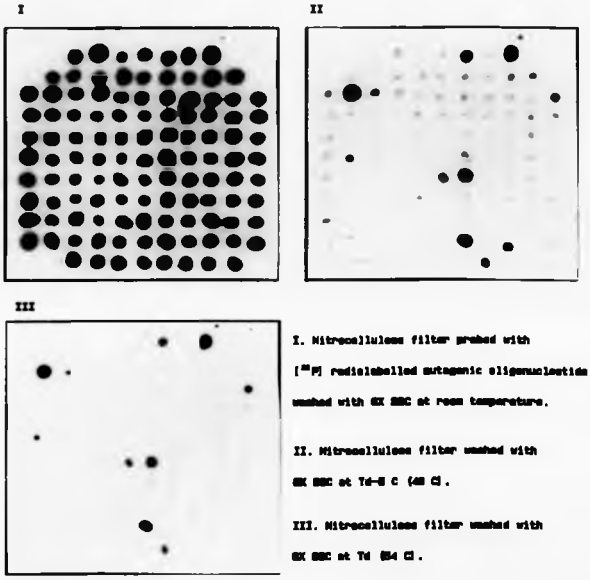


Figure 8: 2 Colony hybridization screen of mutagenised clones probed with the radiolabelled mutagenic oligonucleotide.



I. Nitrocellulose filter probed with [^{32}P] radiolabelled mutagenic oligonucleotide washed with 6X SSC at room temperature.

II. Nitrocellulose filter washed with 6X SSC at Td-8 C (48 C).

III. Nitrocellulose filter washed with 6X SSC at Td (54 C).

oligonucleotide which had been end-labelled using [γ^{32}P], as described in Section 2:23:6.

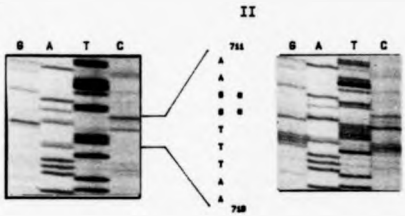
By applying the Wallace rules (Wallace et al., 1980) the dissociation temperature of the mutagenic oligonucleotide from the mutated ricin A chain sequence was calculated at 54°C. Figure 5:2 shows a series of autoradiographs representing 6 x SSC washings of the nitrocellulose filters at room temperature, the dissociation temperature -5°C and the dissociation temperature for mutagenic oligonucleotide from the mutated ricin A chain DNA sequence. At room temperature, as expected, the [γ^{32}P] labelled oligonucleotide probe binds to every colony. When the temperature of the wash was increased to 49°C a significant reduction in bound probe was observed on all but six colonies. Finally the filter was washed at 54°C, the dissociation temperature. The autoradiograph of the filter after this wash shows that the probe has remained bound to these six colonies at the higher temperature.

From this colony hybridization step, six putative positive colonies were identified on the master plate and plaque purified as described in Section 2:23:7. Single-stranded M13mp19 template containing the BamHI fragment encoding ricin A chain was prepared from the plaque purfied material as described in Section 2:18:1 and its sequence analysed by dideoxy-chain termination DNA sequencing, see Sections 2:24:1 to 2:24:5.

5:3 Analysis of mutated DNA sequence encoding for ricin A chain.

Of the six putative positive colonies identified from the plaque purification step only one, colony 26, possessed the mutated sequence. Figure 5:3 shows the sequence analysis of single-stranded template obtained from colony 26 on standard buffer gradient polyacrylamide gels (Biggin et al., 1983), compared to unmutated ricin A chain template. On all gels the order of nucleotide tracks is GATC from left to right. As can be seen from

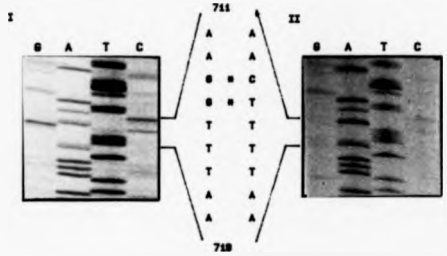
Figure 5:3 DNA sequence analysis of mutagenised ricin A chain clones on standard buffer gradient gels.



I. Non-mutated ricin A chain sequence analysed on a standard buffer gradient DNA sequencing gel (Biggin et al., 1983). Sequence complimentary to bases 711-718 of the published ricin DNA sequence (Lamb et al., 1985) has been indicated. The asterisks indicate the positions of the oligonucleotide directed two base mismatch in the mutated ricin A chain sequence.

II. Mutated ricin A chain sequence analysed on standard buffer gradient DNA sequencing gels. The two base changes at positions 713 and 714 in the ricin A chain sequence resulted in secondary structure formation around the mutated region. To verify that this secondary structure formation was the result of the directed mutagenesis, the mutated sequence was analysed under more denaturing conditions (see figure 5:4).

Figure 5: 4 Analysis of mutated ricin A chain DNA sequence on 25% (V/V) formamide buffer gradient gels.



I. Non-mutated ricin A chain sequence analysed on a standard buffer gradient sequencing gel , as described in figure 5: 3.

II. Mutated ricin A chain sequence analysed on a 25% (v/v) formamide buffer gradient gel. The nucleotide sequence complimentary to bases 711-718 from the published ricin sequence (Lamb et al., 1985) is shown. The two base changes have been indicated by asterisks.

this figure the sequence becomes unreadable around the position of the mismatch in the colony 26 single-stranded template, although it can still be clearly read over the same region in the unmutated template sequence. To overcome this problem, template from colony 26 was analysed on 25% formamide buffer gradient gels (see Section 2:24:4). Using this type of gel creates a more denaturing environment in which to electrophorese the DNA fragments and in this example was successful in eliminating any secondary structure, see Figure 5:4. Unfortunately in using these 25% formamide gels the resolution of the individual bands is reduced compared to the standard buffer gels. However, it was possible in this example to establish the existence of the mutated sequence, as shown in Figure 5:4. At certain positions, notably in the C track, the bands are extremely faint and because of this the position of some bands has been estimated from the relative positions of adjacent bands. What can be clearly observed in Figure 5:4 however, is the existence of the modified bases at positions 713-714 in the ricin A chain nucleotide sequence (Lamb et al., 1985). These two base changes have created a codon, TGA, which is a translation termination signal (stop codon). The existence of a functional stop codon at this position in the ricin A chain sequence was later verified by in vitro transcription/translation analysis, discussed in Section 5:6.

5:4 Analysis of pUC8RA stop by limited restriction endonuclease mapping.

During the sequence analysis of the modified ricin A chain sequence in M13mp19 it was observed that sequence upstream of the BglII site at position 589 no longer encoded for ricin A chain. In order to reconstitute the complete ricin A chain sequence and include the introduced stop codon, a BglII/EcoRI fragment isolated from double-stranded RF M13mp19RA stop was ligated into the large fragment isolated from a BglII/EcoRI digest of pUC8RA, as described in Section 2:25:1 and shown in Figure 2:2. The new

Figure 5:5 Restriction endonuclease mapping of pUC 8 RA "stop"

pUC 8 RA "stop" was digested with the following restriction endonucleases.

Track 1 Xho I / Eco RI

Track 2 Xho I / Bam I

Track 3 Xho I / Kpn I

Track 4 Xho I / Bal II

Track 5 Xho I/ Cla I

Track 6 Bam HI / Bal II

Track 7 Bam HI / Cla I

Track 8 Eco RI / Bal II

Track 9 Eco RI / Cla I

Track 10 pBR322 Hin fI size markers

Table 5:1 Limited restriction endonuclease mapping of pUC8RA stop.

TRACK NO.	RESTRICTION ENDONUCLEASES	FRAGMENT SIZES
1	pUC8RA stop XhoI/EcoRI	949, 2678
2	pUC8RA stop XhoI/SmaI	944, 2683
3	pUC8RA stop XhoI/KonI	901, 2726
4	pUC8RA stop XhoI/BglII	705, 2922
5	pUC8RA stop XhoI/ClaI	455, 3172
6	pUC8RA stop BamHI/BglII	630, 263, 2734
7	pUC8RA stop BamHI/ClaI	380, 513, 2734
8	pUC8RA stop EcoRI/BglII	285, 3342
9	pUC8RA stop EcoRI/ClaI	535, 3092
10	pBR322 HinfI	1631, 517, 506, 396, 344, 298, 221, 220, 154, 75

plasmid, pUC8RA stop contains the complete ricin A chain encoding sequence composed of the 5' end of the clone to the BglII site at position +589 from pUC8RA and the remaining 3' end from M13mp19 A stop. The reconstituted ricin A chain clone was analysed by limited restriction endonuclease mapping as shown in Figure 5:5. Table 5:1 shows the expected fragment sizes from each digest, including the HinfI digested pBR322 marker track. The actual sizes of the digested fragments from pUC8RA stop corresponds with the expected sizes shown in Table 5:1.

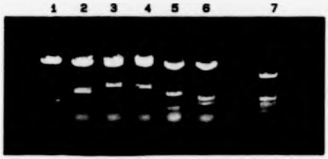
Evidence that the modified ricin A chain sequence has been introduced into the pUC8 ricin A chain encoding plasmid is shown in track 2. In this track, the plasmid has been digested with XhoI and SmaI, to give a 944 base pair fragment. This SmaI site has been derived from the M13mp19 polylinker region and as no SmaI site exists in pUC8RA its presence in the plasmid pUC8RA stop confirms the introduction of the mutated A chain sequence. Also by replacing the BglII/EcoRI fragment from the 3' end of the ricin A chain sequence in pUC8RA with the mutated ricin A chain sequence from the BglII/EcoRI fragment from M13mp19RA stop a SalI and a PstI site are lost. Thus when pUC8RA stop was incubated with either of these enzymes no cleavage of the DNA occurred (data not shown).

The creation of pUC8RA stop as described in Section 2:25:1, provided a working stock of modified ricin A chain sequence with which to carry out further manipulations. The next stage in the analysis of ricin A stop was to establish the effectiveness of the introduced stop codon in an *in vitro* transcription/translation system. To achieve this the modified ricin A chain clone was ligated into a suitable *in vitro* transcription vector as described in Section 2:25:1.

5:5 Analysis of pSP64ΔBam A stop by limited restriction mapping.

pSP64ΔBam A stop was created by ligating the 893 base pair BamHI

Figure 5:6 Restriction endonuclease mapping of pSP64 Bam A "stop"



pSP64Δ Bam A "stop" was digested with the following restriction endonucleases

Track 1 Hind III / Cla I

Track 2 Hind III / Bal II

Track 3 Hind III / Kpn I

Track 4 Bam HI / Kpn I

Track 5 Bam HI / Bal II

Track 6 Bam HI / Cla I

Track 7 pBR322 Hin fI size markers

fragment, encoding the complete, modified ricin A chain, from pUC8RA stop into BamHI restricted, phosphatased pSP64ΔBam (Figure 2:3). Figure 5:6 shows the limited restriction endonuclease mapping of this clone. Table 5:2 shows the expected fragment sizes from each digest including the pBR322 HinfI markers (track 7). All fragments corresponded to the expected sizes as shown in Table 5:2. Track 3, the HindIII/KonI digest of pSP64ΔBam A stop, demonstrates that the BamHI fragment derived from pUC8RA stop has ligated into the SP6 vector in the correct orientation.

In vitro transcription of the modified ricin A chain sequence in pSP64ΔBam A stop was carried out as described in Section 2:25:2.

Table 5:2 Limited restriction endonuclease mapping of pSP64ΔBam A stop.

TRACK NO.	RESTRICTION ENDONUCLEASES	FRAGMENT SIZES
1	HindIII/ClaI pSP64ΔBam A stop	423, 3477
2	HindIII/BglII pSP64ΔBam A stop	673, 3227
3	HindIII/KonI pSP64ΔBam A stop	910, 2990
4	BamHI/KonI pSP64ΔBam A stop	867, 26, 3007
5	BamHI/BglII pSP64ΔBam A stop	630, 263, 3007
6	BamHI/ClaI pSP64ΔBam A stop	380, 513, 3007
7	HinfI pBR322	1631, 517, 506, 396 344, 298, 221, 220 154, 75

5:6 Analysis of pSP64ΔBam A stop transcripts in a wheatgerm lysate system.

Newly transcribed RNA from the transcription of pSP64ΔBam A stop (see Section 2:25:2) was translated in a wheatgerm lysate system as described in Section 2:25:3. The products of this wheatgerm translation were separated by SDS-PAGE under reducing conditions, and visualised by fluorography and autoradiography (see Figure 5:7). Track 1 of this figure shows the translation product from non-modified ricin A chain mRNA, which electrophoreses with an apparent molecular mass of 30 Kd. The transcription product of the ricin A stop transcripts, shown in track 2, clearly has an increased mobility, which corresponds to the expected decrease in molecular mass resulting from the introduced stop codon into the ricin A chain sequence. Track 3 is the translation product of a non-related ricin A chain deletion not directly relevant to the work discussed in this chapter. As a control to demonstrate that the wheatgerm translation system was functioning, and as a molecular weight marker, prepro-alpha factor mRNA was also translated (track 4). This protein electrophoreses with a mobility corresponding to a molecular mass of 18.6 Kd.

From the result shown in Figure 5:7 it is apparent that the premature stop codon introduced into the ricin A chain encoding DNA sequence, is functioning to produce a truncated product. The removal of 30 amino acids from the C-terminus of ricin A chain would be expected to reduce the molecular mass of ricin A chain by approximately 3.3 Kds. The observed electrophoretic mobility of the truncated A chain product agrees with this predicted size reduction, see Figure 5:7 tracks 1 and 2. Having established that the introduced stop codon was functioning to direct the production of a truncated A chain, the next stage was to establish the effect, if any, of the modification upon the ribosome-inactivating activity of the translated products. Because the ribosomes in a wheatgerm system

Figure 5:7 Analysis of ricin A chain transcripts in a wheatgerm in vitro translation system.

Autoradiograph of ricin A chain transcripts translated in a wheatgerm in vitro translation system and analysed on 15% SDS-PAGE under reducing conditions.

Track 1 contains a sample of the translation product from the addition of complete ricin A chain mRNA.

Track 2 contains the translation product from the addition of the truncated ricin A chain mRNA, with the introduced translation stop codon at amino acid residue 238.

Track 3 contains an A chain translation product not related to this work.

Track 4 contains the translation product from the addition of preproalpha factor mRNA.

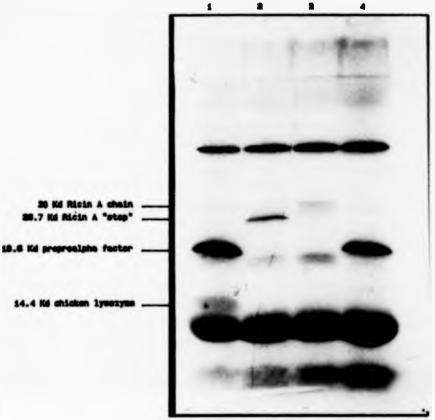
are not significantly inactivated by ricin A chain, the ricin A chain transcripts were translated in a rabbit reticulocyte lysate system as described in Section 2:25:4. The toxicity of each product was established by assessing the ability of the reticulocyte lysate to translate a second mRNA subsequent to the initial ricin A chain mRNA as described in Section 2:25:5.

5:7 Analysis of the toxicity of ricin A chain transcripts in a rabbit reticulocyte lysate translation system.

Transcripts of ricin A stop, and unmodified ricin A chain (see Section 2:25:2) were translated for 30 minutes in a rabbit reticulocyte lysate translation system as described in Section 2:25:4. A second transcript, for prepro-alpha factor, was then added to each translation reaction and incubated for a further 30 minutes. The products from each translation reaction were separated by SDS-PAGE under reducing conditions and then visualised by fluorography and autoradiography as shown in Figure 5:8.

Track 2 of Figure 5:8 shows the translation products from the addition of the truncated ricin A chain (A stop) transcripts, followed by a prepro-alpha factor transcripts. In this reaction, the reticulocyte lysate was able to support the translation of a limited amount of truncated ricin A stop product, but could not translate any of the second, prepro-alpha factor message. This result is identical to that shown in track 3 in which the reticulocyte lysate system supports the translation of a limited amount of the full length ricin A chain mRNA, but does not translate any of the second prepro-alpha factor mRNA. In contrast to these results, when chicken lysozyme mRNA is used first, the reticulocyte lysate system readily supports the translation of the second prepro-alpha factor mRNA (track 1). Track 4 shows the background detected with no first mRNA but followed by addition of prepro-alpha factor mRNA.

Figure 5: 8 Analysis of ricin A chain transcripts in a rabbit reticulocyte in vitro translation system.



Autoradiograph of rabbit reticulocyte lysate in vitro translation products analysed on 15% SDS-PAGE under reducing conditions.

The ribosome inactivating activity of the translated ricin A chain products was analysed by the ability of the translation system to support the translation of a second preproalpha factor message.

Track 1 contains the translation products from the addition of chicken lysozyme mRNA followed by preproalpha factor mRNA.

Track2 contains the translation products from the addition of the truncated ricin A chain message, followed by preproalpha factor mRNA.

Track 3 contains the products from the translation of complete ricin A chain message, followed by preproalpha factor mRNA.

Track 4 contains only preproalpha factor mRNA as background.

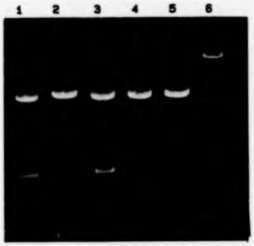
The results shown in Figure 5:8 clearly shows that the translation of A "stop" transcripts and unmodified ricin A chain transcripts results in the inactivation of the translation system, as judged by its inability to support the translation of a second mRNA. This effect was not observed when chicken lysozyme was used as the first mRNA (track 1). It may be assumed therefore, that the loss of translational activity observed in tracks 2 and 3 is the result of the translation of active ricin A chain products. Thus from these types of assay it may be concluded that the truncated ricin A chain product, devoid of 30 terminal amino acid residues has retained it ribosome inactivating activity. In contrast ribosome inactivating activity is abolished in ricin A chain after the removal of only a few amino acid residues from the N-terminus of ricin A chain (M. May, personal communication).

The data described so far in this chapter has demonstrated that by the use of site-directed mutagenesis it is possible to modify the ricin A chain encoding DNA sequence to produce a truncated protein which has retained its ribosome-inactivating activity. In order to study the effect, if any, of removing the 30 C-terminal amino acids on the ability of ricin A chain to enter mammalian cells, it was necessary to obtain reasonably large amounts of material. For this purpose the truncated ricin A chain clone was ligated into the *E. coli* expression vector pDS5/3.

5:8 Analysis of pDS5/3 A stop by limited restriction endonuclease mapping.

pDS5/3 A stop was created by ligating the 893 base pair BamHI fragment from pUC8RA stop into BamHI restricted pDS5/3 as described in Section 2:26:1. The new vector, pDS5/3 A stop (Figure 2:5) was analysed by limited restriction endonuclease mapping, as shown in Figure 5:9. Table 5:3 shows the expected fragment sizes from each digest.

Figure 5: 9 Restriction endonuclease mapping of pDS5/3 A "stop"



pDS5/3 A "stop" was digested with the following restriction enzymes.

Track 1 Xho I /Bgl II

Track 2 Xho I / Cla I

Track 3 Bam HI

Track 4 Bam HI /Bgl II

Track 5 Bam HI / Cla I

Track 6 lambda DNA . Eco RI / Hind III

Table 5:3

TRACK NO.	RESTRICTION ENZYMES	FRAGMENT SIZES
1	XhoI BglII	709
2	XhoI ClaI	459
3	BamHI	893
4	BamHI BglII	630, 263
5	BamHI ClaI	380, 513
6	λ EcoRI HindIII Markers	3530, 2027, 1904, 1584 1530, 983, 831, 564

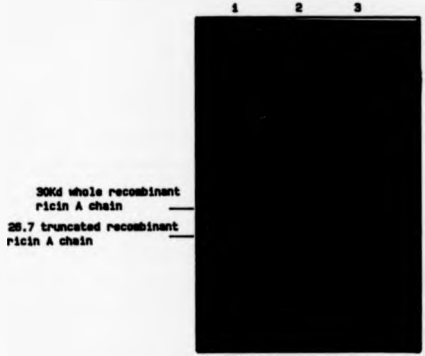
Figure 5:9, track 1, XhoI/BglII digest demonstrates that the BamHI fragment derived from pUC8RA stop has been ligated into pDS5/3 in the correct orientation. Tracks 3-5 shows the fragment sizes from the various digests coincides with the expected sizes.

The vector containing the correctly orientated fragment (pDS5/3 A stop) was transformed into BMH 71.18 E. coli cells as described in Section 2:15:1.

5:8 Western blot analysis of transformed E. coli cells upon induction.

BMH 71.18 E. coli cells were transformed with either pDS5/3 A stop or pDS5/3 A chain (O'Hare et al., 1987) and induced with 1 mM IPTG, as described in Section 2:26:2. Whole cell extracts from these cultures were analysed by SDS-PAGE under reducing conditions and Western blotting as shown in Figure 5:10. Track 3 of this figure contains a whole ricin control, which indicates the mobility of the native glycosylated ricin

Figure 5: 10 Expression of ricin A "stop" in E.coli. : Analysis of whole cell products by western blotting.



Whole cell extracts from induced cultures of E.coli cells transformed with either pCRR/2 A chain or pCRR/2 A 'stop' were analysed by western blotting. Filters were probed with polyclonal anti-ricin A chain antibodies.

Track 1 contains whole cell extract from pCRR/2 A "stop" transformed and induced E.coli cells. A band of the correct mobility for the truncated ricin A chain and weakly reactive with the antibody probe is indicated on the figure.

Track 2 contains whole cell extract from pCRR/2 A chain transformed and induced E.coli cells. A 30Kd band, reactive with the antibody probe has been indicated on the figure.

Track 3 contains a purified native ricin control.

subunits. Track 2 of this figure reveals a protein band, reactive with anti-ricin A chain antibodies, and with an electrophoretic mobility slightly faster than native ricin A chain obtained from a whole cell extract of pDS5/3 A-transformed E. coli. This protein band is known to be full length recombinant ricin A chain. Track 1 of this figure contains a whole cell extract from pDS5/3 A stop-transformed E. coli cells. In this track, two bands which react with anti-ricin A chain antibodies are observed. Both protein bands have an increased electrophoretic mobility compared to either native or recombinant ricin A chain, the upper band coinciding closely with the electrophoretic mobility of the truncated product observed in the in vitro transcription/translation analysis (see Figure 5:8). The nature of the lower band in track 1 is unclear. Possibly this band represents some breakdown product of the truncated ricin A chain, or premature translation termination or downstream translation initiation, or some irrelevant E. coli protein which cross reacts with the anti-ricin A chain antibodies. In this particular example the first possibility is more likely since pDS5/3 vector alone produces no reactive bands corresponding to the positions of the bands in either track 1 or 2 (data not shown). No early initiation or termination of translation has ever been observed when expressing full length recombinant ricin A chain and it would seem unlikely here, discounting the other possibilities.

In both tracks 1 and 2 approximately equal protein loadings were applied, however it was consistently observed that the unmodified recombinant ricin A chain produced a much stronger reaction with the anti-ricin A chain antibodies than did the truncated form of the ricin A chain. It is possible that this reduced reactivity is the result of a reduced level of expression with pDS5/3 A stop compared to pDS5/3 A chain. However, for this reasoning to hold true, both expressed forms of the ricin A chain would have to appear equally reactive with the anti-ricin A chain antibodies. Another possible explanation for this observation could be

that the truncated form of ricin A chain is less antigenic than the full length polypeptide. The 30 amino acid region deleted from the C-terminus of the ricin A chain represents a significant hydrophobic domain which has been implicated in possible subunit interactions, (Montfort et al., 1987). Furthermore, if as has been suggested by Montfort et al. (1987), that this "hydrophobic disc-like structure" interacts by inserting between the two sugar-binding domains of ricin B chain, then it is likely to be exposed to the media in the isolated ricin A chain. These predictions therefore, go some way to reinforcing the suggestion that this region may possess important antigenic determinants, and thus explain, at least in part, the reduced reactivity observed in track 1, Figure 5:10, which shows the C-terminal truncation of ricin A chain known as A stop. Neither of the possibilities mentioned above have been vigorously examined. Another possibility, again not explored, is that the shorter A chain may be less stable than full length ricin A chain and be turned over more rapidly. The kinetics of turnover were not investigated.

5:9 Partial purification of ricin A stop by chromatofocusing.

In view of the success of chromatofocusing for the purification of recombinant ricin A chain from E. coli (O'Hare et al., 1987), this technique was employed here with the aim of obtaining milligram amounts of pure, enzymatically active, truncated ricin A chain.

Samples from the scaled-up expression system were prepared as described in Section 2:26:3 and applied to a chromatofocusing system with a pH range 9-6 as described in Section 2:26:4. The protein elution profile from this purification step is shown in Figure 5:11. As is shown in this figure, a significant proportion of the protein applied to this column either passed straight through without binding, (pI >9.6) or eluted in the final salt wash (pI <6.0). Samples across the elution profile were

Figure 5:11 Protein elution profile : Partial purification of recombinant truncated ricin A chain by chromatofocusing.

Figure 5:11 shows the protein elution profile from the partial purification of recombinant truncated ricin A chain by chromatofocusing using a 9-6 pH range system.

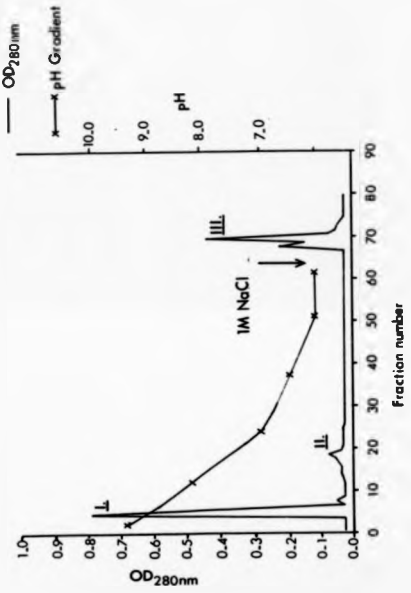
Protein eluting from the column was monitored by measuring the absorbance at 280 nm.

Peak I represents material passing straight through the column i.e. *E. coli* proteins with pIs greater than 9.6.

Peak II represents material eluting with a pI close to 7.5, and including the recombinant truncated ricin A chain.

Peak III represent material eluting in the final salt wash i.e. *E. coli* proteins with pIs less than 6.0.

Figure 5: ii Protein elution profile : Partial purification of recombinant truncated ricin A chain by chromatofocusing.



analysed by Western blotting, as shown in Figures 5:12 and 5:13. In Figure 5:12, tracks 1 and 12 contain native ricin controls electrophoresed under reducing conditions, as are all the other fractions. Tracks 2 and 3 represent material from fractions 5 and 7 which did not bind to the column. None of the proteins in either of these two fractions reacted with the anti-ricin A chain antibodies. Tracks 4-11 of this figure represent fractions 12-19 from the chromatofocusing protein elution profile inclusively. Across these fractions the pH falls from approximately 8.0 to 7.5. In fractions 18 and 19 (tracks 10 and 11) a protein band which both cross-reacts with anti-ricin A chain antibodies and has an electrophoretic mobility corresponding to the truncated ricin A chain (A stop) is apparent. In tracks 8 and 9 some material, with an electrophoretic mobility corresponding to native glycosylated ricin A chain, and cross-reacting with anti-ricin A chain antibodies, can be observed. It is thought to be unlikely however, that this material represents full length ricin A chain which may have been the result of some read through of the introduced stop codon. The electrophoretic mobility of this product on SDS-PAGE does not show the slight but characteristic increase associated with non-glycosylated recombinant A chain (O'Hare et al., 1987) and as seen in Figure 5:10, track 2).

Figure 5:13 shows Western blot analysis of the remaining fractions from the chromatofocusing profile (Figure 5:11). Tracks 2-8 of Figure 5:13 contains samples from fractions 20-26 inclusively. Fraction 20 (track 2), like fraction 19 (Figure 5:12, track 11) contain a protein band which reacts with the anti-ricin A chain antibodies and electrophoreses with the appropriate mobility for ricin A stop. No significant reactivity with antibodies can be detected in the other tracks, including material from the final salt wash i.e. fractions 68, 69 and 70 (tracks 9-11). The failure to detect any truncated ricin A chain material in the salt wash possibly indicates that none of this material (A stop) has been denatured during the

Figure 5: 12 Purification of recombinant A "stop" I.: Western blot analysis of chromatofocused fractions.

I. SDS-PAGE Analysis

II. Western blot Analysis

Figure 5: i2 Purification of recombinant A "stop" I.: Western blot analysis of chromatofocused fractions.

I. SDS-PAGE analysis of soluble E.coli protein fractions after chromatofocusing, electrophoresed under reducing conditions.

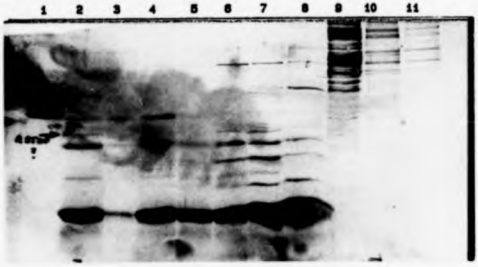
Tracks 1 and i2 contain samples of whole ricin electrophoresed under reducing conditions. Tracks 2 and 3 contain material from fractions 6 and 7 respectively, which did not bind to the chromatofocusing column. Tracks 4-11 contain samples from fractions i2-i8 inclusively.

II. Western blot analysis.

All the above fractions were transblotted onto nitrocellulose filters and probed with anti-ricin A chain antibodies. Track 11 (fraction i8) contains a protein band reactive with the antibody probe and with a corresponding electrophoretic mobility to that expected for the truncated A chain. It's position has been indicated by an arrow on the figure. Material from track i0 (fraction i8 was also weakly reactive with the antibody probe , although this band is not apparent after photography).

Figure 5: 13 Purification of recombinant A "stop" II. : Western blot analysis of chromatofocused samples.

I. SDS-PAGE Analysis



II. Western blot Analysis

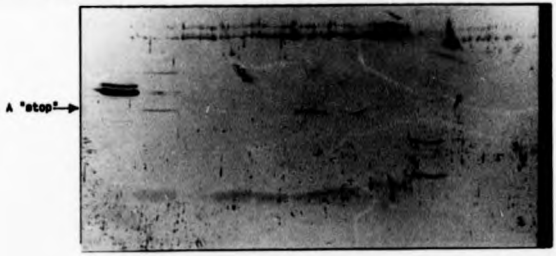


Figure E: 13 Purification of recombinant A 'stop' II. : Western blot analysis of chromatofocused samples.

I. SDS-PAGE analysis of chromatofocused fractions of soluble *E.coli* proteins under reducing conditions.

Tracks 2-8 contain samples from fractions 20-26 inclusive. Fractions 9-11 contain fractions 68, 69, and 70 from the final salt-wash. Track 1 is a whole ricin control electrophoresed under reducing conditions.

II. Western blot analysis.

As with figure E: 12, track 11 , a protein band reactive with the antibody probe , and with an electrophoretic mobility corresponding to that expected for ricin A 'stop' is evident in track 2 (fraction 20). No other reactive bands, of appropriate mobility can be detected in any other track. Some proteins, with mobilities quite different from that of ricin A 'stop', can be detected in the final salt-wash, indicating that some cross-reactivity occurs between the anti-ricin A chain antibodies and some *E.coli* proteins.

preparative and purification steps. It is interesting to note that some proteins, with mobilities quite different from either complete or truncated A chain cross-react with the polyclonal anti-ricin A chain antibodies, which may help explain the cross-reacting material observed with fractions 16 and 17 (Figure 5:12, tracks 8 and 9).

These results indicate that the truncated ricin A chain (A stop) has been partially purified to 7 or 8 other proteins by a single chromatofocusing step and that this material elutes with a pI of approximately 7.5 (fractions 19 and 20). The polyclonal anti-ricin A chain antibodies used as the probe in the Western blot analysis do show some limited cross-reactivity with a number of E. coli proteins, the most significant being fractions 16 and 17. The cross-reacting material in these tracks electrophoreses with a mobility close to, but slightly higher than that expected for recombinant whole ricin A chain. The partially purified truncated ricin A chain has apparently not become denatured during sample preparation or subsequent purification steps.

A particular problem encountered during this type of analysis was the apparently low level expression, high turnover, or low antigenicity of ricin A stop which required subsequently higher protein loadings onto gels. The effect of this was to raise the background levels of co-purifying proteins thereby hampering the interpretation of results. In an attempt to avoid the use of an antibody-based detection system for the validation of subsequent purification procedures, the RNA modification analysis described in Section 2:11 was employed.

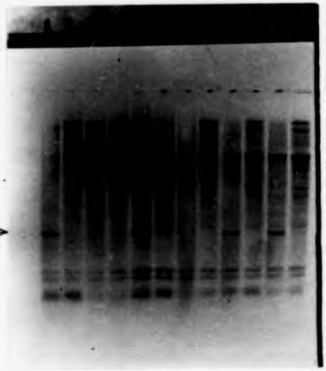
5:10 Determination of ricin A chain-specific activity in chromatofocused fractions.

Aliquots from fractions 19 and 20 were analysed for their ability to catalyze a ricin A chain specific modification of ribosomal RNA in rabbit reticulocyte lysate ribosomes (after Endo *et al.*, 1987).

Figure 5:4 is a 1.2% (w/v) agarose, formamide gel showing the results from the application of aliquots of fraction 19 and 20 directly onto rabbit reticulocyte ribosomes. Tracks 1-4 are controls, demonstrating that the diagnostic fragment indicated by the lower arrow is produced only after appropriate incubation with ricin A chain and aniline. In tracks 5 and 6, 20 µl of fraction 19 have been incubated with rabbit reticulocyte lysate plus and minus aniline treatment respectively. In track 5 the fragment, diagnostic of ricin A chain specific activity can be clearly seen (lower arrow). Equally when either 20 µl or 30 µl of fraction 20 (tracks 9 and 11 respectively) are incubated with reticulocyte lysate followed by aniline treatment the characteristic fragment is apparent.

This result clearly indicates the presence of an enzymically active form of ricin A chain present in these two fractions (i.e. fractions 19 and 20). This finding is in agreement with the result described in Section 5:7 where the *in vitro* translation product of pSP64ΔBam A stop was shown to be biologically active, as judged by the inability of the rabbit reticulocyte system to support the translation of a second prepro-alpha factor transcript. Assuming that all the ribosomal RNA modifying protein purified by chromatofocusing in fractions 19 and 20 is the truncated form of ricin A chain, then this result demonstrates the expression and purification of soluble, non-denatured ricin A chain which exhibits ribosomal RNA modification activity despite the absence of 30 amino acids from the C-terminus.

Figure 5: 14 RNA modification assay : Analysis of partially purified recombinant ricin A "stop".



Formamide gel of RNA samples extracted from reticulocyte lysate incubations with fractions of chromatofocused recombinant ricin A "stop".

Tracks 1 and 2 shows ricin A chain treated RNA incubated with and without aniline respectively.

Tracks 3 and 4 shows buffer only treated RNA incubated with and without aniline respectively.

Tracks 5 and 6 shows RNA extracted from lysate samples incubated with 20ul of fraction 19 from the chromatofocusing step , treated with and without aniline respectively.

Tracks 7 and 8 are invalid as no RNA was recovered in track 7.

Tracks 9 and 10 show RNA extracted from lysate incubated with 20ul of fraction 20 from the chromatofocusing step with and without aniline respectively.

Tracks 11 and 12 show RNA extracted from lysate incubated with 30ul of fraction 20 incubated with and without aniline.

5:11 Evaluation of affinity chromatography using Blue Sepharose as a method for the purification of ricin A atep.

An aliquot (4-5 mls) of fraction 20 from the chromatofocusing step (see Section 5:9) was dialysed against 50 mM sodium phosphate buffer pH 7.5 at 4°C and then applied to a blue sepharose column as described in Section 2:26:6. Figure 5:15 shows the elution profile from this step. In theory the truncated ricin A chain should bind to the column matrix while the other proteins co-purified by the chromatofocusing procedure pass straight through the column.

At this stage, the use of Western blotting as an analytical technique was prohibited by the small amount of material obtained from the column. The validity of this chromatographic technique was analysed therefore by the much more sensitive RNA modification assay. Figure 5:16 shows the result of such an assay. As described before in Section 5:10, tracks 1-4 are controls which demonstrate that the production of the diagnostic rRNA fragment is a consequence of both appropriate ricin A chain and aniline incubations. In tracks 5 and 6 unbound material from fraction 8 was incubated with the reticulocyte lysate and subsequently with and without aniline respectively. The material from fraction 8 failed to produce the diagnostic rRNA fragment, a finding which is in contrast to the result when material from fraction 10, which also did not bind to the blue sepharose was tested. Without the subsequent aniline treatment (track 8) no fragment was produced. However with aniline treatment the diagnostic fragment is clearly visible indicating that ricin A chain specific activity is present in the material present in this fraction.

As expected all bound material, (eluting with 500 mM NaCl wash), fractions 28, 29 and 30 produced the diagnostic fragment when incubated with aniline (tracks 9, 11 and 14). No fragment was observed when the aniline incubation was not included (tracks 10, 12 and 13).

Figure 5:15 Protein elution profile : Partial purification of recombinant truncated ricin A chain by affinity chromatography.

Figure 5:15 shows the protein elution profile of the partial purification of recombinant, truncated ricin A chain by affinity chromatography using blue sepharose CL 6B.

Protein eluting from the column was monitored by measuring the absorbance at 280 nm.

Peak I represents material not binding to the column.

Peak II represents material which bound the column and was eluted with 0.5 M NaCl.

Material from each peak was evaluated for its RNA modification activity, see Figure 5:16.

Figure 6.15 Protein elution profile : Partial purification of recombinant, truncated ricin A chain by affinity chromatography using Blue Sepharose CL 6B.

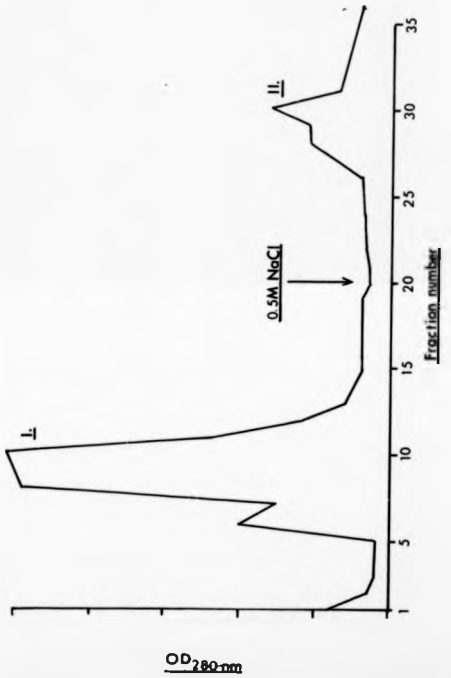


Figure 6: 18 RNA modification assay: Evaluation of blue sepharose affinity chromatography in the purification of recombinant truncated ricin A chain.

Formamide gel of RNA samples extracted from rabbit reticulocyte lysate incubations with fractions from the blue sepharose affinity chromatography step attempting to purify the recombinant truncated ricin A chain.

Tracks 1 and 2 are ricin A chain treated RNA samples incubated with and without aniline respectively.

Tracks 3 and 4 are buffer only treated RNA samples with and without aniline treatment respectively.

Tracks 5 and 6 show RNA extracted from lysate treated with fraction 8 (unbound material) with and without aniline treatment respectively.

Tracks 7 and 8 show RNA extracted from lysate samples incubated with fraction 10 (also unbound material) with and without aniline treatment respectively.

RNA extracted from lysate incubations with material bound to the blue sepharose column, fractions 28, 29 and 30, plus aniline treatment are shown in tracks 9, 11 and 14 respectively.

Tracks 10, 12 and 13 show RNA samples extracted from lysates treated with fractions 28, 29 and 30 without aniline treatment.

This result whilst demonstrating perhaps the unsuitability of blue sepharose for purification of A stop has demonstrated an application of the most recent method for detecting ricin A chain specific activity in a practical protein purification situation. This technique is particularly useful in these circumstances, as the small amounts of material available from this procedure cannot be analysed by Western blot analysis.

5:12 Discussion.

The means by which the A chain subunit of ricin gains entry into the cell cytoplasm during intoxication is poorly understood at both cellular and molecular levels. A body of evidence exists in the literature which suggests that the presence of ricin B chain may facilitate the entry of ricin A chain into cells (Youle and Neville, 1981; Houston, 1982; McIntosh et al., 1983; McIntosh et al., 1988). With the exception of Vitetta (1986), who was able to abolish the sugar-binding activity of ricin B chain by chemical means whilst retaining its putative A chain translocating activity, there is little direct evidence that ricin B chain is actively and solely involved in the translocation of ricin A chain into the cytosol. Furthermore, an analysis of the three dimensional structure of ricin (Montfort et al., 1987) has neither identified or even implicated any region of ricin B chain likely to have any potential for membrane interaction and translocation. Indeed the most likely candidate as a membrane translocating domain is found at the C-terminus of the A chain subunit. Montfort et al., (1987) have indicated that final 57 residues of the carboxyl-terminal of the ricin A chain forms a compact disc-like domain which protrudes so as to slide between the cleft formed between the two sugar-binding domains of ricin B chain. The final 27 amino acids of this region have been shown to be significantly hydrophobic, which may account for the relatively high association of ricin A chain with lipid vesicles

(Uchida et al., 1980). It is possible therefore, that this region of the ricin A chain subunit is involved in membrane interactions which may be important during the translocation process. This C-terminal domain of ricin A chain has also been implicated in strong interactions with domains within the ricin B chain. It is possible to speculate therefore, that the potentiation of the cytotoxicity, associated with ricin A chain containing immunotoxins, in the presence of B chain may be a result of this A chain C-terminal domain being protected (by the B chain), until it encounters the appropriate environment. Within this environment the C-terminal domain of the A chain becomes dissociated from the protective B chain and is able to perform its membrane interactions leading to translocation into the cell cytosol. Unfortunately there is no direct evidence to support the existence of such a mechanism, but as an approach to studying the possible role of the hydrophobic C-terminus of ricin A chain during translocation into the cytosol, a truncated form of the ricin A chain devoid of the 30 terminal amino acids was engineered. This was achieved by the introduction of a translation termination codon by site directed mutagenesis into the ricin A chain DNA sequence as described in Section 2:23.

Once the existence of the mutated ricin A chain sequence had been established by DNA sequence analysis (see Figure 5:4) the mutated clone was ligated into an *in vitro* transcription/translation vector (see Figure 5:6). The RNA transcripts of the mutated ricin A chain, catalysed by the activity of the bacteriophage SP6 RNA polymerase, were translated in both wheatgerm and rabbit reticulocyte lysate *in vitro* translation systems (see Figures 5:7 and 5:8 respectively). The first observation that can be made from the analysis of these *in vitro* translation results is that the introduced stop codon functions to produce a truncated protein whose mobility on SDS-PAGE under reducing conditions coincides with the expected mobility for ricin A chain after the removal of 30 amino acids (see Figure 5:7, track 2). The second observation, regarding the ability of the truncated ricin A chain to

inhibit protein synthesis can be made by considering the results of the *in vitro* translation of the protein in a rabbit reticulocyte lysate system. Because the ribosomes from this system are susceptible to inactivation by ricin A chain, the translation of an active form of ricin A chain will only be supported for a limited period, after which the level of translated product is sufficient to inactivate the translation system. Thus, a reticulocyte lysate system which translates an active form of ricin A chain will not support the translation of a second mRNA encoding a control protein. In Figure 5:8 track 2, the truncated form of the ricin A chain can be clearly visualized; electrophoresing on SDS-PAGE with an increased mobility compared with the non-mutated ricin A chain product visualized in track 3. In neither of these tracks is there any evidence of the protein product of the second prepro-alpha factor RNA transcript subsequently added to each reaction. In the positive control translation (Figure 5:8, track 1) the translation of an RNA transcript encoding for chicken lysozyme does not inhibit the translation of the second prepro-alpha factor transcript.

It is apparent from these results, that the removal of 30 amino acids has not affected the ribosome inhibiting activity of the A chain. In contrast the removal of just 12 amino acids from the N-terminus of the ricin A chain abolishes all ribosome inactivating activity. However no loss of activity was observed when only 9 amino acids were deleted (Mike May, personal communication). It has not been shown whether the loss of activity resulting from this 12 amino acid deletion of the N-terminus is a consequence of the loss of residues involved specifically with ribosome inactivation or, as seems more likely, the result of some other effect on protein structure, leading to misfolding and loss of enzymatic activity.

Having established the fidelity of the introduced stop codon, and the retained ribosome inactivating activity of the truncated ricin A chain, the next stage was to obtain enough of the purified protein in a soluble and active form to examine the effect, if any, of the 30 amino acid deletion

upon the ability of ricin A chain to become internalized into cells. The first step towards achieving this aim was to express, in a soluble and active form, the truncated ricin A chain in an E. coli expression system. The expression of the truncated ricin A chain, (A stop) in E. coli was carried out by following as closely as possible the methodology described by O'Hare et al. (1987) during the expression and purification of whole recombinant ricin A chain. An important feature of this expression system was the growth of the transformed and IPTG-induced E. coli at 30°C, which was essential for the production of biologically active expressed product (O'Hare et al., 1987). If grown at 37°C recombinant ricin A chain was found to form soluble aggregates which demonstrated greatly reduced biological activity. O'Hare et al. (1987) were able to estimate the expression level of recombinant A chain at between 2-3 mg/litre of culture under optimal conditions. The expression level of the truncated ricin A chain can not be so readily determined, as it is unclear as to whether the removal of the C-terminus has resulted in reduced antigenicity (see Section 5:8). If no difference in the antigenicity between whole and truncated ricin A chain is assumed, then the Western blot analysis of equal loadings of lysed whole cell cultures (see Figure 5:10) suggests that the expression level of the truncated form is reduced, possibly by as much as 75% compared to whole recombinant A chain. Because of this doubt as to the feasibility of the use of antibody detection as a measure of expression levels, a more suitable analysis might have been to determine the ribosome inactivating activity of the expressed products. Possibly the best method for achieving a measure of this activity would be to dilute the recombinant ricin A chains until the concentration which no longer produces the characteristic RNA cleavage product is reached, similar to the analysis described in Section 3:2:6. By assuming that the specific activities of native, whole recombinant and truncated recombinant ricin A chain are the same it would then be possible to estimate the concentration of the expressed products by

comparing the activities of dilutions of E. coli preparations to the known concentrations of native ricin A chain standards. Figure 3:14 shows that 30 pg is the smallest amount of native ricin A chain which produces the diagnostic rRNA fragment under the experimental conditions defined in Section 3:2:6. A further attraction of this type of analysis is that because such an assay detects a specfic ricin A chain modification it is not necessary to purify the recombinant proteins to homogeneity. Analysis of impure fractions in non-specific assay systems such as the cell-free assay described in Section 2:10 would not be able to differentiate between ricin A chain specific-inactivation or some non-specific inactivation of the rabbit reticulocyte translation system. A major drawback of this ricin A chain-specific RNA modification approach for the analysis of recombinant proteins is the susceptibility of the rRNA to enzymic cleavage of RNAases. If this problem can be overcome however, it represents a means of making a reasonably accurate assessment of expression levels, providing that RNA modifying activity of the recombinant protein is the same as the native ricin A chain standards. In this analysis of recombinant truncated ricin A chain this technique has only been successfully employed to determine that the expressed product had retained its biological activity after partial purification by chromatofocusing (see Figure 5:14). The ability of the recombinant protein to modify rRNA in a ricin A chain specific manner has been taken to indicate that the product has been expressed in a soluble form, however a rigorous evaluation of solubility has not been undertaken.

The expression of a eukaryotic gene in E. coli, yielding a soluble, active product is not a trivial event. Indeed, although expressed gene products can represent up to as much as 25% of the total cell protein, in the majority of cases these proteins are expressed in an insoluble form (Harris, 1983). The appearance of inclusion bodies concomitant with the accumulation of pro-insulin, insulin A chain or insulin B chain by Williams et al. (1982) was the first indication that the insoluble proteins might

accumulate in a discrete form. By isolating the inclusion bodies from cells expressing prochymosin, Marston et al. (1984) demonstrated that these inclusions were composed predominantly of the expressed product. In order to recover active, soluble protein from aggregated recombinant polypeptides it is necessary to first isolate the inclusion bodies. Then denaturants are used to unfold the proteins and finally appropriate conditions for the correct refolding of the polypeptides are introduced. The fact that many of these aggregated recombinant products form inclusion bodies is useful for purification, which is normally achieved by sedimentation using low speed centrifugation. Co-purifying E. coli proteins which sediment with these inclusion bodies can be preferentially solubilised using detergent solutions (Marston et al., 1984) which leave the recombinant material between 30% and 90% pure (Marston, 1982). Solubilization of the protein requires the disruption of non covalent, hydrogen bonds, ionic and/or hydrophobic interactions and unfolding of the polypeptides. A number of solubilization agents can be used for this procedure e.g. 8M guanidinium chloride, 6-8 M Urea, detergents and organic solvents. The choice of solubilization reagent will depend upon the nature of the protein, and on a number of other variables such as pH, temperature, time and ionic environment.

Having disrupted the aggregated polypeptides, conditions must be adjusted for refolding. In some cases removal of the denaturant has been achieved by dialysis, successfully generating a soluble and active protein e.g. with bovine growth hormone (George et al., 1985) and urokinase (Winkler et al., 1985). Correct refolding can also be achieved by appropriately altering the pH of the environment, a variable which may be critical for correct disulphide bond formation, since thiol-disulphide exchange proceeds more rapidly at alkaline pH (Freedman and Hillson, 1980). In some cases, the correct formation of disulphide bonds may be critical during refolding, although this would not be necessary with ricin A chain

as no intra-chain disulphide bonds are formed.

In view of the apparent consistency with which recombinant proteins are expressed in an insoluble or aggregated form it is perhaps fortunate that a soluble truncated ricin A chain can be produced *de novo* without recourse to the denaturation or refolding procedures described earlier. Besides growing the transformed *E. coli* cells at 30°C and fortuitously having a low level expression system, the method by which the cell lysate was obtained may well have contributed to the extraction of a soluble, active product. The use of chromatofocusing to purify the truncated ricin A chain has indicated that this polypeptide has a pI of around 7.5-8.0, similar to the value for native ricin A chain. Furthermore, as no evidence of the truncated protein was apparent in any other fractions from the chromatofocusing column, including the final salt wash, it is probable that only non-denatured material was applied to the column from the cell lysate preparation. This is assuming that the pI of any denatured material will differ from non-denatured material, as experienced with the purification of native ricin B chain (see Section 3:3:2:3:6).

The data presented in the chapter has demonstrated that it is possible, by the use of site directed mutagenesis, to introduce a premature stop codon into the DNA sequence encoding for ricin A chain and successfully produce a truncated form of the A chain devoid of 30 amino acid residues from its C-terminus. Furthermore it has been demonstrated that both the *in vitro* translation product and the recombinant protein expressed in *E. coli*. have retained their ability to inactivate eukaryotic ribosomes (Figure 5:8 and 5:14 respectively). The deleted region is a hydrophobic stretch of amino acids which forms part of a disc-like domain that is believed to insert between the two sugar-binding domains of the B chain when the holotoxin is formed (Montfort *et al*., 1987). It has been hypothesised that this region interacts with lipid membranes and may be important during the events leading to the translocation of the A chain

into the cytosol (Uchida et al., 1980).

It is interesting to note that when the amino acid sequences of ricin A chain (Yoshitake et al., 1978) and the toxic plant protein trichosanthin (Gu Ziwei et al., 1984; Wang Yu et al., 1985) are compared, not only do they show a striking homology along much of their length, but trichosanthin terminates some 29 amino acid residues before ricin A chain and therefore lacks the ricin-like C-terminal hydrophobic domain. Thus trichosanthin is very similar to the truncated version of ricin A chain (A stop), created by the introduction of a premature stop codon into the ricin A chain DNA sequence. Possibly a more detailed analysis of the toxic nature of trichosanthin, which is extracted from the root tuber of Trichosanthes kirilowii maxim. may give some indication as to the significance of the C-terminal domain in ricin A chain. The proposed analysis of the function of this C-terminal region following on from the results described in this chapter, and possible improvements to the experimental strategy are considered later in the general discussion in Section 6:1.

6:1 General Discussion.

Despite the considerable interest generated by the potential of plant toxins for development as specifically targeted anticancer drugs, much of their basic biochemistry and mode of action remains poorly understood. The process of cellular intoxication by ricin and other type II RIPs may be considered in three stages, binding, internalization and ribosome inactivation.

I. Binding of the toxin to the cell surface.

With ricin, cell-surface binding is mediated through interactions between sugar-binding domains of the ricin B chain and galactosyl residues on the cell surface, probably in the form of terminal galactosyl residues on glycoprotein and glycolipid oligosaccharide side chains (Rosen and Hughes, 1977). Ricin may also bind to cells by a different mechanism; through its mannose rich oligosaccharide side chains. Cells of the reticuloendothelial system which bear mannose receptors on their cell surface can mediate the uptake of both whole ricin and ricin A chain (Simmons et al., 1986). It is likely therefore, that ricin may become internalized into cells via a number of different routes, which may be determined by the particular receptor(s) or ligand that the toxin binds or is bound to.

II. Internalization.

It is apparent that at least some cell surface bound ricin molecules are internalized via the coated pit-endosomal pathway (Van Deurs et al., 1985). This pathway however, may not necessarily represent the route taken by those ricin molecules whose A chains ultimately become translocated into

the cell cytosol, where they bring about the cessation of protein synthesis. A more detailed consideration of internalization is given in Chapter 1.

III. Inhibition of protein synthesis.

The inhibition of protein synthesis in the intoxicated cell is the result of the enzymic inactivation of 60S subunits of the ribosomes by the A chain polypeptide. Again a more detailed analysis of this activity is described earlier in Chapter 1. However, before the ricin A chain is able to inactivate the ribosomes, it must first gain entry to the cytosol. Very little is known about the nature of this translocation event or the respective roles, if any, of the ricin subunits in this process. The overall aim of the experiments detailed in this thesis has been to (a) examine the possible role of ricin B chain during this translocation process and (b) to examine the significance of deleting a hydrophobic C-terminal domain from the ricin A chain which has been implicated in both ricin subunit interactions (Montfort et al., 1987) and interactions with lipid vesicles (Utsumi et al., 1984).

As a prerequisite to studying the role of ricin B chain during the intoxication process it was necessary to obtain a source of ricin B chain available in milligram amounts, in a non-denatured form and free from contaminating ricin A chain. These requirements were met by purifying the reduced ricin subunits by the procedure outlined in Sections 2:2:3 to 2:2:4 and then assessing the purity of the subunits by increasingly stringent assay procedures. This analysis ranged from straightforward visual examination of silver stained SDS-PAGE for evidence of any contaminating subunit (see Section 3:2:1) through to the analysis of ribosomal RNA for a ricin A chain specific modification. The use of this RNA modification assay, developed from the work of Endo et al. (1987), represents the most

stringent analysis of ricin B chain purity possible at present (see Section 3:2:5). The application of this type of assay has not only indicated that some contaminating ricin A chain is present in the highly purified ricin B chain preparations at higher concentrations (see Figure 3:13), but has been extended to quantify the extent of contamination, see Section 3:2:6. In the example quoted in Sections 3:2:5 and 3:2:6, which represents purified ricin B chain used in later experiments, the contaminating ricin A chain represented only 0.003% of the total protein. This high degree of purity of ricin B chain was obtained after a single chromatofocusing step, which helped to minimise the loss of material experienced when subsequent purification steps were included.

A further consequence of applying the RNA modification assay to analyse the purity of ricin B chain was that it enabled a distinction to be made between ricin A chain-specific ribosome inactivation and inactivation of rabbit reticulocyte lysate translation systems caused by some other factor(s) resulting from incubation with purified ricin B chain. Prior to publication of the work Endo *et al*. (1987) it was not possible to make such a distinction and some authors assumed that inhibition of cell free translation systems with ricin B chain preparations was the result of contaminating ricin A chain (Fulton *et al*., 1986).

A body of evidence exists in the literature indicating that the addition of free ricin B chain can lead to an enhancement of ricin A chain immunotoxin-mediated cytotoxicity (McIntosh *et al*., 1983) and that a similar "potentiation" of cytotoxicity can be observed if the ricin B chain is applied in the form of a suitably targeted immunotoxin (Vitetta *et al*., 1983). To examine the apparent "potentiation" of ricin A chain mediated cytotoxicity by ricin B chain, the potential of the latter as a carrier of polypeptides other than ricin A chain into cells was analysed. It was felt that if ricin B chain was solely responsible for directly or indirectly facilitating the cytoplasmic entry of ricin A chain, then it might be

expected that the B chain would act in the same manner when coupled with a similar A chain polypeptide. If successful, then the ability of B chain to help transport less related polypeptides into the cytoplasm could then be explored. In these experiments, native ricin A chain was replaced with the type I ribosome inactivating protein (RIP) gelonin. Gelonin was selected as the ricin A chain analogue for a number of reasons; firstly it is a glycoprotein of similar molecular weight to ricin A chain and secondly it s ribosome inactivating activity is similar to that of ricin A chain in a cell-free assay (Stirpe and Barbieri, 1986). In addition, it has subsequently been shown to modify rRNA in an identical manner to ricin A chain (Endo et al., 1988) and finally, it was relatively easy to obtain.

In order to link ricin B chain to gelonin via a reducible linkage, it was necessary to introduce a thiol group into the gelonin molecule. This was achieved by reacting the protein with 2-iminothiolane as described in Section 2:12:3. However, despite demonstrating that some conjugate, composed of one ricin B chain molecule disulphide bond linked to one gelonin molecule, had been synthesised (Figure 4:5), no cytotoxicity was associated with this construct. This result is perhaps surprising as cytotoxic activity has been associated with gelonin immunotoxins (Lambert et al., 1985; Thorpe et al., 1981). At the very least the association with ricin B chain would provide the gelonin with a cell binding moiety. It is perhaps interesting to note at this point that free gelonin was less cytotoxic than purified ricin A chain. It is unclear as to whether this higher cytotoxicity, associated with ricin A chain, is a result of slight B chain contamination or a consequence of some innate ability for promoting its own entry into the cytosol (Figure 4:3 and Figure 3:9). In contrast to the findings here, Goldmacher et al. (1987) have produced a cytotoxic gelonin-ricin B chain conjugate. However, it is interesting to note that in studies by McIntosh et al. (1988) neither the addition of ricin- or abrin B chains could potentiate the cytotoxic activity of a gelonin

containing immunotoxin.

Whilst considering the data presented in this thesis relating to the construction of a ricin B chain-gelonin hybrid toxin, it is worth recalling the experiences of other workers who observed that ricin B chain achieved poor association with abrin A chain (Olsnes et al., 1974) and diphtheria A fragment (Sundan et al., 1982). These results would suggest that any form of subunit reassociation, let alone correct reassociation, may require more than the formation of a single disulphide bond between the two subunits. The poor reassociation of ricin B chain with heterologous "A chains" observed both in this study and by other authors (see above) suggest that ricin B chain is perhaps unsuitable for use as a carrier molecule. A more suitable choice of carrier molecule might, in retrospect, be abrin B chain which, as well as possessing identical functions to ricin B chain (i.e. sugar-binding and A chain potentiation activities), more readily reassociates with alternative A chains (Olsnes et al., 1974). It should be added however that McIntosh et al. (1988) were unable to potentiate the cytotoxic activity of a gelonin containing immunotoxin with abrin B chain, as well as failing with ricin B chain.

Despite reports of cytotoxic activity associated with ricin B chain conjugates containing gelonin (Goldmacher et al., 1987), barley RIP (Ovadia et al. (1988) and abrin A chain (Olsnes et al., 1974), a true potentiation effect has only been demonstrated when ricin B chain has been added to systems containing either abrin A chain or ricin A chain containing immunotoxins (McIntosh et al., 1988). Recent amino-acid sequence analysis of abrin A chain has shown it to have considerable homology with the amino acid sequence of ricin A chain (Funatsu et al., 1988), including the C-terminal region which is absent from the sequence of the toxic plant protein trichosanthin (Xuejun and Jiahuai, 1986). It is possible to speculate therefore that ricin B chain may only associate correctly with closely related proteins and, in particular, may require an equivalent C-

terminal disc-like domain, as is present on ricin A chain, to form such an association. Following this argument through it would seem that correct subunit association is somehow necessary for cytotoxicity. Until more sequences of type I RIPS are published, it is only possible to speculate as to the significance of hypothetical domains within type I RIPs such as gelonin, in hybrid toxin formation and translocation events.

It may be concluded from the data presented in Chapter 4 that ricin B chain does not act to promote the entry of gelonin into cells. However, ricin B chain can apparently promote ricin A chain cytotoxicity (McIntosh et al., 1983, Vitetta et al., 1983) which might suggest a specific interaction between the two ricin subunits. In an attempt to analyse this possibility and to analyse the possibility that ricin A chain itself might have a role to play in promoting its own membrane translocation, a hydrophobic region of 30 amino acids was deleted from the C-terminus of ricin A chain.

The successful introduction of a functional stop codon into the DNA sequence encoding for ricin A chain has been shown by *in vitro* translation analysis of transcripts from the mutated sequence (see Figure 5:7). That toxicity had been retained in the truncated form of the A chain was also shown by *in vitro* analysis as described in Section 5:7. Following on from these observations, the truncated ricin A chain has been expressed in *E. coli* and partially purified in a soluble, active form (see Sections 5:8, 5:9 and 5:10). A further purification step involving the use of blue sepharose, see Section 5:11, was unsuccessful in isolating the truncated ricin A chain. However, the evaluation of this method demonstrates a further application of the RNA modification assay (see Section 2:11), by which the presence of the biologically active ricin A chain product can be detected at concentrations beyond the resolution of more conventional methods such as Western blotting.

The effect of removing 30 amino acids from the C-terminus of ricin A chain, on the ability of this truncated A chain to gain entry into the cell cytosol might be analysed by a number of ways. One such way would be to directly reassociate the truncated ricin A chain via a disulphide linkage to purified native B chain thereby reconstituting a modified ricin holotoxin. Such a procedure would require both relatively large amounts of completely purified truncated ricin A chain and the introduction of a thiol group into the truncated protein by chemical reagents, (see Section 2:12). The introduction of the thiol group is necessary to replace the thiol group normally provided by the cysteine residue at amino acid residue 259 in the ricin A chain sequence (Lamb et al., 1985). This residue is one of the 30 deleted from the A chain C-terminus as a result of the introduced stop codon. The large amount of starting material required to counter the anticipated losses during both the purification and derivatisation procedures would involve large-scale cultures of expressing E. coli, and possibly the use of a small chemostat. These proposals become less practical when the stringent safety regulations associated with Category III laboratory containment conditions are taken into account. Furthermore, the optimisation of growth conditions in chemostat cultures and considerations of plasmid stability upon continuous culture are also of paramount importance. The impracticalities of such a major endeavour under Catergory III containment therefore precluded attempts to scale up the production of truncated A chain.

One strategy to reduce the amount of material required would be to introduce a cysteine residue into the amino acid sequence of the truncated A chain. The introduction of a naturally occurring thiol group into this protein would, in theory, eliminate the requirement for a chemically introduced group and therefore the relatively large amount of purified material required for this procedure. Ideally the introduced residue should be as close to the C-terminus of the truncated A chain as possible.

Suitable residues for modification might be the glycine residue at position 237 or the arginine residue at position 235. A single base change in either of these codons, to read TGT instead of CGT (for arginine) and GGT (for glycine), would encode for a cysteine residue at these positions. The sulphydryl groups of either of these cysteine residues would, in theory, be able to pair with the sulphydryl group of the cysteine at residue 4 of the ricin B chain amino acid sequence to create a disulphide bond between the two subunits.

There is however some uncertainty as to the importance of the disulphide bond for subunit association and toxicity of ricin. Whilst some authors suggest it is of little significance, other than to hold the two subunits together at low concentrations (Lewis and Youle, 1986); other authors believe that the interchain disulphide linkage is essential for toxicity (Wright and Robertus, 1987). Whatever its significance, to carry out experiments with the recombinant truncated A chain would still require that the product be purified from other E. coli proteins, before attempting reassociation with purified native ricin B chain. Furthermore even if such a reassociation between the truncated ricin A chain and purified native B chain were possible, such a "forced" reassociation may result in the formation of a non-cytotoxic molecule.

One approach, which may avoid the possibility of an incorrectly manufactured subunit association would be to make a truncated ricin A chain containing immunotoxin. Such an immunotoxin if linked to an appropriate antibody could be directed to the cell surface of the target cell and its cytotoxic activity analysed both with and without the addition of free ricin B chain. The addition of ricin B chain to this system would allow reassociation, if it was to occur, in a non-forced manner. The problem with creating such an immunotoxin, even with the introduction of a cysteine residue into the truncated A chain, would be obtaining an adequate amount of purified material to couple with the antibody. For this particular

reason a method, by which partially purified truncated ricin A chain could be coupled to antibodies targeted to cell surface determinants, is particularly attractive.

Such an approach might be possible by the use of bi-specific antibodies. Bi-specific antibodies, as their name implies have dual specificities, each associated with one arm of the antibody structure. In this example, the specificities would be against a determinant unique to the truncated ricin A chain and against some determinant on the cell surface of the target cells. In theory therefore, such a bi-specific antibody could be reacted with a partially pure truncated ricin A chain preparation and then be applied to the target cells. This, again in theory, would result in only the truncated ricin A chain being directed to the cell surface where, as suggested above, its cytotoxicity both with and without the addition of purified native ricin B chain could be assessed. Predictably the best analysis would be to compare the results from experiments with truncated ricin A chain with those using whole recombinant ricin A chain under identical conditions. This approach to immunotoxin production has been successful in producing a cytotoxic product using a bi-specific antibody raised against both a cell surface determinant of guinea pig lymphoblastic leukemia L_1C cells and against a determinant of the type I ribosome inactivating protein, saporin (M. J. Glennie, abstract from the International Symposium on Immunotoxins, Durham, North Carolina, 1988). The apparent ease with which these bi-specific antibodies can be produced, together with the advantage of using only partially purified products, which are not required to carry their own thiol linkage group, makes this approach the most attractive considered so far.

An alternative to these <u>in vitro</u> studies, in which the deletion at the C-terminus of the A chain is analysed in terms of its overall effect on cytotoxicity, would be to examine the interaction of the expressed truncated protein with lipid vesicles. In similar studies carried out by

Utsumi et al. (1984) ricin, ricin A chain and ricin B chain were tested for their association with dipalmitoylphosphatidylcholine (DPPC) vesicles. From these experiments they were able to make a number of interesting observations. The first of these was that intact ricin demonstrated no association with the vesicles, although both free ricin A chain and free ricin B chain could evidently associate with the lipid bilayer. These results suggests that in the intact ricin molecule no hydrophobic domains are accessible for lipid interaction. Once reduced however, hydrophobic domains on both ricin A and B chains are exposed, and can interact with the lipid membrane. This observation in which hydrophobic domains are not exposed in the holotoxin until dissociation of the subunits agrees with the hypothetical model proposed earlier, whereby the B chain subunit acts to protect the hydrophobic C-terminus of the A chain. However, Utsumi et al. (1984) observed that both subunits interacted with the lipid vesicles and indeed that the B chain had a greater affinity for the DPPC vesicles than did the A chain, with respective Ka values of $14.5 \times 10^{8}\ M^{-1}$ and $2.30 \times 10^{-8}\ M^{-1}$. This result would at first suggest that ricin B chain interaction with the lipid membranes has a greater possible significance in membrane translocation than ricin A chain interactions. However, in an extension to these studies it was observed that ricin A chain was able to induce the complete release of a carboxyfluorescein dye from the vesicles. Neither B chain or intact ricin could induce this release, even at concentration five times greater than those required for the complete release by ricin A chain. The overall conclusion from these findings is that although both ricin A chain and ricin B chain can associate with the lipid vesicles, only the A chain (as judged by dye release experiments) can penetrate the lipid bilayer and cause perturbation of both leaflets. The strong interaction of ricin B chain with the lipid vesicles without the concomitant release of dye suggests that this polypeptide does not achieve complete penetration and therefore does not disrupt the bilayer.

Clearly this type of analysis would be appropriate to determine the extent of membrane interaction with the truncated form of the ricin A chain. Ideally in this type of study, a comparison with whole recombinant ricin A chain should be carried out in parallel with the recombinant truncated material. This type of approach, coupled perhaps, with the bi-spacific antibody-immunotoxin approach, (which examines both the interaction to the truncated A chain with whole cells and the interaction with ricin B chain), represents perhaps the best way forward in analysing the overall activity of truncated ricin A chain.

It is impossible, without attempting such experiments, to predict the effect that the removal of this 30 amino acid region from the C-terminus of the A chain will have on the events contributing to the entry of this polypeptide into the cytosol of cells, or on its interaction with ricin B chain. What this work has shown however, is that the ribosome-inactivating activity of the A chain is not affected by this deletion. Furthermore, the fact that this truncated protein can be expressed and purified in a soluble, active form may offer a hitherto unexplored approach in helping to understand the apparently intricate and subtle mechanisms of toxin internalization and membrane translocation.

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TITLE STUDIES ON THE ENTRY OF RICIN SUBUNITS INTO CELLS

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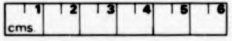

REDUCTION X 21

CAM. 1