ANALYSIS OF THE FUNCTIONAL PROPERTIES OF RICIN B CHAIN

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by

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ABBREVIATIONS.

RTB	-	Ricin B polypeptide
RTA	-	Ricin A polypeptide
RNA	-	Ribonucleic acid
mRNA	-	Messenger ribonculeic acid
rRNA	-	Ribosomal ribonucleic acid
RNase	-	Ribonuclease
DNA	-	Deoxyribonculeic acid
A	-	Adenine
G	-	Guanine
U	-	Uracil
C	-	Cytosine
GTPase	-	Guanidine triphosphatase
GTP	-	Guanidine triphosphate
E. coli	-	Escherichia coli
GlcNAc	-	N-acetyl glucosamine
Xyl	-	Xylose
Fuc	-	Fucose
Gal	-	Galactose
Man	-	Mannose
Gal NAc	-	N - acetyl galactosamine
Trp	-	Tryptophan
Asp	-	Aspartate
Val	-	Valine
Asn	-	Asparagine
Lys	-	Lysine
His	-	Histidine
Tyr	-	Tyrosine
Arg	-	Arginine
Cys	-	Cysteine
Co	-	Cobalt
K	-	Potassium
Con A	-	Concanavalin A
DMSO	-	Dimethyl sulphoxide
SPDP	-	N-Succinimidyl-3-(2-pyridyldithio) propionate
hr	-	Hour(s)
PBS	-	Phosphate buffered saline
SDS-PAGE	-	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TCA	-	Trichloroacetic acid
EGTA	-	Ethylene Glycol-bis(-Amino-ethyl ether) N, N, N', N'-tetraacetic acid
EDTA	-	Ethylene diaminetetraacetic acid

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SUMMARY.

Ricin is isolated from the plant Ricinus communis, and is an extremely toxic protein. The protein consists of two functionally distinct polypeptides, ricin A chain which will inhibit protein synthesis in eucaryotic cells, and the ricin B chain which acts as a lectin binding to galactose-containing cell surface macromolecules. Ricin has been extensively studied in attempt to determine both the biochemical and physical properties of the protein.

Ricin has been attributed with anti-tumorogenic properties, and has recently been used in the synthesis of immunotoxins. Studies on the interactions of immunotoxin molecules have indicated that the ricin B polypeptide may not function simply as a lectin. When cells were treated with a weakly toxic dose of an antibody - ricin A conjugate, the cytotoxicity was less than that of an antibody - ricin conjugate. The cytotoxicity of the antibody - ricin A conjugate can be greatly enhanced by the addition to the culture system of either free ricin B polypeptide or an antibody - ricin B complex. Thus it would appear that the ricin B chain was responsible for promoting access of the ricin A chain into the cytosol. It is this putative transport function of the ricin B chain that this project sought to address.

Before the principle experimental investigation could be undertaken a method for the purification of the commercially produced ricin B chain had to be developed, for it was found to contain ricin holotoxin. This was achieved by chromatofocusing for the isoelectric point of ricin is 7.1 and ricin B chain is 4.8, and this difference allowed separation. Affinity chromatography using Sepharose 4B pretreated with 1.0M propionic acid, as the column matrix, was utilised to separate the polybuffer (chromatofocusing buffer) from the purified ricin B chain. This also had the added advantage of confirming that the ricin B polypeptide retained

its ability to function as a lectin. The purity of the ricin B chain was ascertained by silver staining of SDS-PAGE gels and cytotoxicity assays.

To study the 'hypothetical' transport function of the ricin B polypeptide, a complex was synthesised between bovine pancreatic RNase A and ricin B chain. Bovine pancreatic RNase A was chosen for a number of reasons, the main one being the possibility of determining the internalisation of the RNase A by the ricin B chain due to the enzymatic digestion of cellular RNA which could be measured by a decrease in incorporation of [^{35}S]-methionine or [^{3}H]-thymidine by the cell cultures. The heterobifunctional cross-linking reagent, SPDP, was chosen to link the RNase A molecule to the ricin B polypeptide, so that a disulphide bridge was formed between the two proteins. This duplicates the disulphide linkage normally found between the ricin A and ricin B chains. The formation of the RNase A - ricin B complex was determined by silver staining of SDS-PAGE gels. The putative complex had a Mr of 45,000, indicating a complex containing equimolar amounts of RNase A and ricin B chain. Immunoblotting experiments confirmed that the 45kD species visualised on SDS-PAGE contained antigenic determinants derived from both RNase A and ricin B chain molecules. Two dimensional gel electrophoresis of iodinated RNase A - ricin B complex confirmed the formation of the complex as well as showing that the complex was basic having an isoelectric point in the range pH 8.0-8.5.

The RNase A - ricin B complex had to retain the functional activity of both the proteins, so that its effect on Daudi cells in culture could be examined. The enzymatic activity of the RNase A present in the RNase A - ricin B complex was assessed using activity gels. Areas of enzymatic digestion of the yeast RNA present in the activity gel were visualised as clear plaques. Iodination of RNase A then linkage of this to the ricin B polypeptide showed that the ricin B chain was binding to

galactose-containing molecules on the cell surface. Unfortunately, when the effects of the RNase A - ricin B complex were examined on cells in culture, no effect on DNA or protein synthesis due to the enzymatic action of RNase A was observed.

The results therefore confirmed earlier work which showed that insulin - ricin B conjugate, bound to galactose containing receptors present on the cell surface. The question of the internalisation of the chimeric protein was not answered, although it is known that hybrid proteins formed between abrin A -ricin B or gelonin - ricin B are toxic to cells which could reflect a synergistic mode of action of the toxic molecule and the lectin molecule from plants.

INTRODUCTION

1. INTRODUCTION.

1.1 GENERAL INTRODUCTION.

There is a strong historical relationship between the seeds of Ricinus communis (castor bean) and medicine. Castor beans have been utilised in the treatment of a wide variety of diseases as described in classical Greek medicine, folk medicine, and in the Susruta Ayurveta, a Sanskrit work from the sixth century B.C. Paul Ehrlich (1891, 1892) using the plant toxin, ricin, isolated from castor beans, carried out extensive studies which resulted in the discovery of some of the fundamental principles of immunology. He showed that immunisation resulted in the formation of serum proteins capable of specifically precipitating and neutralising the toxins, that immuno-specificity was associated with the antiserum, and that immunity could be transferred to a foetus via the mother's blood during pregnancy. Furthermore, it was demonstrated that after the birth specific immunity could be passively transferred to the neonate via the breast milk.

The severe toxic effects of ricin are historically well-documented. A recent, well-publicised example was provided by the murder of the Bulgarian radio reporter Georgi Markov by the injection of ricin via a modified umbrella tip. The debilitating effects of ricin are dependent upon the means of administration, with parenteral injection producing the most serious symptoms (Olsnes and Pihl, 1976; Olsnes and Sandvig, 1983; Rauber and Heard, 1985). The symptoms are nausea, vomiting, pain in the stomach, colic, diarrhoea, hemorrhage from the anus, anuria, cramps, dilation of the pupillae, fever, thirst, burning in the throat, headache and shock symptoms. Death occurs in exhaustion or cramp. The autopsy findings are usually multiple ulcera in the stomach and the small intestine, and numerous

hemorrhages around these ulcera (Abdulkadir-Lutfi 1935; Dirheimer,et al., 1966; Guggisberg, 1968). Although the toxin is lethal to all individual cell types, the precise organ which sustains the damage leading to death is unknown.

1.2. PLANT TOXINS.

Abrin, modeccin, viscumin, volkensin and ricin are isolated from plants. Abrin is isolated from the seeds of Abrus precatorius (Olsnes and Pihl, 1976). Modeccin and volkensin are found in highest concentration in the roots of Adenia digitata and Adenia volkensii (Refsnes, et al., 1977; Stirpe et al., 1978; Barbieri, et al., 1984) respectively, whilst viscumin is found in all parts of the Viscum album (Olsnes and Sandvig, 1985). These toxins, like ricin, are all glycoproteins consisting of two polypeptide chains linked by a disulphide bond (Table 1). Each peptide chain mediates a distinct biological activity. The A chain possesses an enzymatic activity and the B chain has lectin properties binding to carbohydrates with terminal galactose residues (Olsnes and Sandvig, 1985).

1.3. RICIN.

Structurally, ricin consists of two functionally distinct N-glycosylated polypeptide chains (ricin A and ricin B) linked by a single disulphide bond. The ricin A subunit acts by enzymatically inactivating the 60S ribosomal subunits, thereby irreversibly inhibiting eucaryotic cellular protein synthesis (Sperti, et al., 1973; Olsnes, et al., 1974; Hedblom, et al., 1976). The other subunit, ricin B chain, possesses two galactose binding sites (Baenziger and Fiete, 1979) and is therefore thought to function principally as a lectin, binding to the galactose or N-acetylgalactosamine residues present on the cell surface

TABLE 1.

Biochemical properties of plant toxins and their subunits.

Toxin	Mr	Plant
Ricin	62,000	Ricinus Communis.
A2	32,000	"
A1	30,625	"
B	31,432	"
Abrin	65,000	Abrus Precatorius.
A	30,000	"
B	35,000	"
Modeccin	63,000	Adenia digitata.
A	28,000	"
B	38,000	"
Viscumin	57,000	Viscum album.
A	29,000	"
B	33,000	"
Volkensin	62,000	Adenia volkensii.
Subunits	36,000	"
	29,000	"

macromolecules. The carbohydrate chains present on the subunits may also provide a means by which ricin can bind to cells since animal cells have receptors for carbohydrates i.e. mannose - receptors present on reticuloendothelial cells (Thorpe, et al., 1985; Skilleter, et al., 1985; Skilleter and Foxwell, 1986).

The precise sequence of events involved in ricin intoxication of the cell have not yet been elucidated, but it is known that one molecule of ricin is sufficient to kill the cell (Eiklid, et al., 1980). This illustrated the enzymatic nature of ricin action. Studies have centred on the galactose - receptor mediated endocytotic pathway which, for experimental purposes, has been divided into four separate steps; binding to the cell surface, endocytotic uptake, entry into the cytosol, and inactivation of the intracellular target, the ribosomes (Olsnes, et al., 1985).

1.3.1. BINDING TO SURFACE MACROMOLECULES.

Ricin binds to oligosaccharides possessing terminal galactose residues which are attached to a variety of glycoproteins and glycolipids present on the cell surface. Ricin binding, as well as its toxic effect to intact cells, can be inhibited by lactose, galactose and glycoproteins with terminal galactose residues, presumably because of their competition with cellular receptors for ricin. The affinity of binding of ricin to its receptor varies with the cell type but falls within the range Ka = 10^7 - 10^{10} M^{-1} (Olsnes and Pihl, 1982; Olsnes and Sandvig, 1983). Binding is reversible at $0^{o}C$, but at higher temperatures endocytosis of the toxin causes irreversible binding of some of the toxin molecules (Sandvig and Olsnes, 1979). The number of receptors also differs between cell types and it is unclear whether all receptors promote the transfer of toxin to the cytosol (Olsnes and Sandvig, 1985).

1.3.2. ENDOCYTOSIS AND DIACYTOSIS.

Ricin bound to cell-surface receptors is internalised by endocytosis via the coated pit/coated vesicle pathway (Gonatas, et al., 1980; van Deurs, et al., 1985). Coated pits with bound ricin mediate its internalisation into coated vesicles. Shortly after entering the cell the coated vesicles lose their clathrin coats and the toxin becomes associated with the endosomes (smooth-surfaced vesicles and tubular structures). Following transit in the vacuolar portion of the endosomal system, ricin may recycle back to the cell surface via the tubulo-vesicular portion of the endosomal system and / or trans Golgi reticulum, pass to the cytosol, or be transferred to lysosomes (Olsnes and Sandvig, 1985). Diacytosis of ricin (Sandvig, et al., 1978; Sandvig and Olsnes, 1979) follows completely different kinetics from those of endocytosis, for there is an abrupt increase in the release rate at around 20^{o}C and the rate of release approaches its maximum at about 30^{o}C. The trans Golgi reticulum is thought to be involved given that ricin is found to accumulate there and movement to and from this organelle is inhibited at 20^{o}C (Sandvig and Olsnes, 1979). However, exocytosis of the toxin occurs by two processes, one of which is rapid, the other much slower, therefore indicating the existence of two possible routes (Sandvig and Olsnes, 1979). Very little ricin appears to accumulate in the lysosomes and it is degraded very slowly (Sandvig, et al., 1978a).

Endocytotic uptake of ricin has been followed by linking it to horse-radish peroxidase (Gonatas, et al., 1975, 1977), colloidal gold, (van Deurs, et al., 1985, 1986) and ferritin (Nicolson, 1974; Nicolson, et al., 1975, 1977). The complexes were bound to the cell surface at 4^{o}C, as this temperature arrests endocytosis; the temperature was then increased to 37^{o}C, and at various time intervals the cells were fixed, sectioned and examined by electron microscopy.

Nevertheless, there are problems associated with these studies, for the initial exposure of the cells to a temperature of 4°C may have a det rimental physiological effect. The properties of the complexes themselves may influence the intracellular routing. It has been suggested that it is not the molecules themselves, but the valency of the ligand or ligand-conjugate which determines the routing and sorting events at the level of the endosomal system (van Deurs, et al., 1986). This would account for differences in the results of Nicolson, et al., (1974, 1975, 1978), Gonatas, et al., (1975, 1977), and van Deurs, et al., (1985, 1986).

1.3.3. CYTOSOLIC ENTRY.

The subcellular site of cleavage of the disulphide bond and transfer of the enzymatically active A chain into the cytosol has not been definitively located. Endocytosed ricin appears to be able to enter the cytosol for many hours after uptake (Sandvig and Olsnes, 1982b). Transfer could occur from the endosome, phagolysosome or the Golgi complex. Evidence in favour of the Golgi apparatus is provided by experiments in which the temperature was lowered to 20°C. This blocked the transport of ricin from the endosome to the Golgi complex and inhibited its toxic effect (van Deurs, et al., 1987; Sandvig, et al., 1987a). Morphological changes induced in the Golgi by addition of a number of inhibitors of glycoprotein synthesis such as cycloheximide, tunicamycin, and swainsonine as well as compounds such as ammonium chloride, chloroquine and methylamine sensitise cells to ricin (Sandvig, et al., 1987a). It should be noted that a neutral or slightly alkaline pH is required for ricin toxicity (Sandvig and Olsnes, 1982b). Thus, ammonium chloride, chloroquine or methlyamine could exert their effect by their ability to increase the pH in acidic vesicles, or they could protect ricin from lysosomal degradation (Ray

and Wu, 1981; Sandvig and Olsnes, 1982b; Mekada, et al., 1981). The calcium ion has also been found to be required for transfer of ricin to the cytoplasm for the removal of calcium ions with EGTA , replacement with Co^{2+} or the presence of calcium transport inhibitors prevent the expression of a toxic effect whilst not effecting binding or endocytic uptake (Sandvig and Olsnes, 1982a).

The free A chain enzymatically inactivates the 60S ribosomal subunit without the requirement of a cofactor such that protein synthesis is abolished (Olsnes and Pihl, 1982). Experimental investigations have led to the suggestions that ricin A chain has a ribonuclease activity (Obrig, et al., 1985) or that it acts in a hydrolytic fashion cleaving the N-glycosidic bond of adenine residue 4324 in 28S ribosomal RNA (Endo, et al., 1987; Endo and Tsurugi, 1987).

There are indications that ricin can enter the cell by an alternative pathway distinct from the receptor mediated endocytic pathway (Moya, et al., 1985). Thus, when the coated pit/coated vesicle pathway was blocked by a method involving hypotonic shock and subsequent K^{+}-depletion or by acidification of the cytosol (Sandvig, et al., 1987b), the overall uptake of ricin was only reduced slightly.

Evidence is increasing that the mannose containing oligosaccharides on the A and B chains of ricin are recognised by the reticuloendothelial system in the liver (non-parenchymal cells; mainly Kupffer and sinusoidal endothelial cells) and spleen, macrophages, and fibroblasts (Youle, et al., 1979, 1981; Skilleter, et al. 1981; Simmons, et al., 1986). It was first shown using monophosphopentamannose ricin, that it could be efficiently internalised via the mannose receptor and exert a toxic effect (Youle, et al., 1979, 1981). Subsequent studies examined ricin uptake mediated exclusively via the mannose - terminating oligosaccharide

side chains and mannose receptor. Endocytosis of ricin mediated by mannose binding-receptors appears to differ from that seen after binding to galactose, a much more extensively studied system. Simmons, et al., (1986) showed that ammonium chloride, which potentiated the toxicity of ricin when endocytosed via the binding of the B chain, protected the macrophages when ricin was bound via the mannose receptor, thus suggesting the requirement for translocation through an acidified compartment for exertion of the toxic effect.

In conclusion, there appears to be a variety of mechanisms by which ricin can be internalised by the cell, but the intracellular release of the A chain, its enzymatic activity and its interplay with the B chains are vital factors which still need to be determined.

1.4. BIOSYNTHESIS OF RICIN.

Ricin is synthesised by endosperm cells, and stored in protein bodies for the utilisation of the developing castor bean seedling on germination (Tulley and Beevers, 1976; Youle and Huang, 1976). The castor bean genome apparently contains six ricin like genes (Halling, et al., 1985). It has been suggested that all members of this multigene family may all be transcribed and translated, with the products being several ricin-like proteins (Halling, et al., 1985). This is consistent with previous data reported by Cawley, et al., (1978) who found they could isolate three forms of ricin. Transcription and translation processes have been studied to elucidate the synthetic pathway of the ricin glycoprotein.

The nucleotide sequence of cloned DNA complementary to ricin precursor mRNA has been determined (Butterworth and Lord, 1983; Lamb, et al., 1985). The data indicated that the ricin precursor contains 567 amino acids corresponding to; a 24 amino acid N-terminal leader sequence (a putative signal peptide), 267 amino acids comprising the A chain, a 12 amino acid linker sequence, and 262 amino acids comprising the B chain. The sequencing data confirmed the amino acid analysis of

ricin (Yoshitake, et al., 1978), but there was a major difference at amino acid residue 26 of the A chain. Sequencing data predicted an asparagine residue as a potential site for asparagine linked glycosylation whilst the data of Yoshitake, et al., (1978) indicated that an aspartate residue was present. This anomaly has been explained by the isolation of the variant or 'heavy' form of ricin A chain which contains two N-linked oligosaccharide chains (Foxwell, et al., 1985). Halling, et al., (1985) have also sequenced DNA encoding a ricin gene and they found ten nucleotide differences between the cDNA sequence reported by Lamb, et al., (1985) as well as suggesting that the leader sequence may consist of thirty-five amino acids. Furthermore, it has been proposed that many of these differences arise from real variations between different genes in the family (Halling, et al., 1985).

The ricin precursor polypeptide is synthesised on membrane bound polysomes and segregated into the lumen of the endoplasmic reticulum (Lord, 1985). Formation of the disulphide bond linking the individual subunits of the mature heterodimer is thought to occur during synthesis or segregation, (Bergman and Kuehl, 1979; Roden, et al., 1982). The disulphide linkage is formed between the cysteine residue located at the ninth position from the C-terminus of the ricin A chain (cys-259) with the cysteine residue at the fourth position from the N-terminus of ricin B chain, i.e. cys-4 (Funatsu, et al., 1979). The precursor form is extensively modified in the lumen of the endoplasmic reticulum. The N-terminal leader sequence is removed, and oligosaccharide chains are added (Lord, 1985). Tunicamycin treatment of the endosperm tissue inhibited glycosylation of the precursor. However, this did not effect the intracellular transport of the toxin from the endoplasmic reticulum to the protein bodies (Lord, 1985).

Studies of the intracellular transport mechanism have shown that a dense vesicle fraction is involved, and that the oligosaccharide moieties of the precursors undergo further modification (Lord, 1985). This was ascertained using the enzyme endoglucosaminidase H, which deglycosylates the precursor isolated from the endoplasmic reticulum, but has no effect on the "more mature" form from the dense vesicle fraction. Fucosylation of the oligosaccharide chains is one of the modification steps and, by analogy with animal and viral glycoproteins, this would indicate that the precursor passed via the Golgi apparatus en route to the dense vesicle fraction (Munro, et al., 1975).

The twelve amino acid linker sequence present in the precursor is cleaved by an endoproteinase whilst in the process of transfer to or while present in the protein bodies (Harley and Lord, 1985).

It has been hypothesised that the gene for ricin was developed by fusion of a gene for ribosome-inactivating proteins with a lectin gene. Ribosome-inactivating proteins, are structurally and functionally closely related to the ricin A chain (Xuejun and Jiahuai, 1986; Ready, et al., 1984; Jimenez and Vazquez, 1985). They are present in the majority of plants which have been studied for this type of protein including, for example, pokeweed, wheat germ, Croton tiglium, Jatropha curcas and Gelonium multiflorum, whilst lectins are extremely common in plants (Brown and Hunt, 1978).

1.5. RICIN A CHAIN.

The ricin A chain has been found to inhibit protein synthesis by inactivating the 60S ribosomal subunit, (Sperti, et al., 1973; Benson, et al., 1975). This was demonstrated by separate treatment of the 40S and 60S ribosomal subunits with ricin A chain. Treated and untreated subunits were mixed in different combinations and their effect on polyphenylalanine synthesis determined. Highly purified ricin A chain inactivates salt-washed ribosomes in simple buffer solutions at a rate of about 1500 ribosomes per minute per ricin A chain. The Km with respect to ribosomes is about 2×10^{-7} M, which ensures that ricin A chain acts in the cytosol at close to its maximal rate, (Olsnes, et al., 1975). Bacterial ribosomes, and mitochondrial ribsomes are resistant to ricin A chain (Olsnes, et al., 1973; Greco, et al., 1974).

Carrasco, et al., (1975) reported that ricin inhibits the enzymatic binding of aminoacyl tRNA to ribosomes, but other groups were unable to demonstrate this effect (Montanaro, et al., 1973; Nolan, et al., 1976). It has also been suggested that the ricin A chain acts at the elongation factor binding site(s) on the ribosome. Thus, in the presence of excess elongation factor 2 and GTP, under which conditions the elongation factor is bound, the ribosomes were partially protected against the action of the A chain (Fernandez-Puentes, et al., 1976). Conversely, A chain treated ribosomes had lost their ability to bind elongation factor 2 in a GTP dependent manner (Carrasco, et al., 1975; Nolan, et al., 1976). The A chain also inhibited the ribosome dependent elongation factor 2 linked hydrolysis of GTP (Montanaro, et al., 1973).

Benson, et al., (1975) released an 8S complex from the 60S ribosomal subunits by treatment with EDTA. The 8S complex consisted

of 5S RNA and a single ribosomal protein. The complex was shown to have some GTPase activity, (Grummt, et al., 1974). Inhibition of the GTPase activity occurs on treatment with ricin A chain.

Studies of polysome profiles have been carried out using cell free protein synthesising systems, cells in culture, and animals. Olsnes and Pihl, (1972) examined the effect of ricin on the polysome profile of a cell-free protein synthesising system. The polysome structure appeared to be preserved. Grollman, et al., (1974) showed that in Hela cells incubated with ricin, protein synthesis stopped after approximately one hour and, concomitantly, that there was disaggregation of the polysome structure with the appearance of monosomes instead. Polysome break down was also observed in rats during the course of ricin intoxication (Lin, et al., 1972). A similar result was obtained in ricin-intoxicated mouse myeloma cells, the polysomes again disaggregated, but it was felt that this alone was not great enough to account for the inhibition of protein synthesis (Onozaki, et al., 1975). Lin, et.al., (1972) had shown that there was an increase in RNAse activity in the supernatant fraction of rat liver cells on intoxication by the plant protein abrin, and assumed ricin would have a similar effect. The increase in the enzymatic activity of the ribonuclease coincided in time with the disaggregation of polyribosomes. Other groups were, however, unable to reproduce this effect (Grollman, et al., 1974; Onozaki, et al., 1975). However, treatment of 5S or 5.8S rRNA substrates with ricin A chains generated distinctive digestion patterns suggesting the existence of a ribonuclease activity associated with ricin A chain (Obrig, et al., 1985). However, Paleologue, et al. (1986) were unable to detect any ribonuclease activity associated with the ricin A chain and, indeed, their results suggested that it induced only a limited conformational change in the 60S subunit, which affected non-covalent associations between ribosomal proteins and rRNA.

Experiments by Endo and colleagues (Endo, et al., 1987) have shown that in 28S rRNA isolated from ricin-treated ribosomes, the adenine is missing from position 4324 although the phosphoribose backbone remains intact. The loss of the base renders the phosphodiester bond between guanine residue 4323 and adenine residue 4324 nuclease-resistant, whereas the equivalent linkages on both sides of the adenine become hypersensitive to cleavage by alkali and by aniline at low pH. Ricin removes approximately one adenine residue per ribosome, indicating that the enzymatic cleavage of the N-glycosidic bond of adenine 4324 by hydrolysis is the sole site of its action. It has been found that the 60S subunits are extremely sensitive; the 28S rRNA is also modified although at a reduced rate. However, denatured 28S rRNA is resistant to the putative N-glycanase activity of the ricin A chain, thus indicating that the A chain recognises the three-dimensional structure around adenine 4324. Additional evidence supporting this conclusion comes from the finding that the target sequence AGUACGAGAGGAAC has been conserved between eucaryotes and procaryotes, yet procaryotic ribosomes are resistant to the enzymatic activity of the ricin A chain, suggesting that primary sequence alone is not sufficient for ricin A chain activity (Olsnes, et al., 1973; Greco, et al., 1974).

Modification of arginine residues in the N-terminal part of the molecule strongly reduced the inhibitory activity of ricin A chain in a cell free protein synthesising system (Watanabe and Funatsu, 1986). It is therefore postulated that these residues may be involved in the enzymatic reaction. By analogy with other enzymes, which act on negatively charged substrates, arginine residues have been implicated in their active sites. Thus, in the case of ricin A chain, it is possible that this site interacts with the RNA in the 60S subunit.

The A chain of ricin is not enzymatically active, unless it is released from the B chain. It is therefore very likely to undergo a conformational change which activates the enzymatic function. This may also expose some of the continuous sequences of hydrophobic amino acids which occur along ricin A chain, (Funatsu, et al., 1978), and these hydrophobic region(s) could be crucially involved in transmembrane interactions leading to the release of ricin A chain into the cytosol. Treatment of ricin with 2-mercaptoethanol strongly increased its binding to liposomes (Beugnier, et al., 1982). Ischida, et al., (1983) studied the interaction of ricin and its subunits with Newcastle disease virus containing a photoreactive glycolipid. The photolabelling experiment showed that the ricin A chain would spontaneously insert into the viral membrane. Utsumi, et al., (1984) using liposomes prepared from dipalmitoylphosphatidylcholine showed that ricin A chain penetrated into the lipid bilayer and destabilised their organisation.

The X-ray crystallographic structure of ricin A chain at 2.8-Å resolution has been determined (Montfort, et al., 1987). It has been found to be a globular protein consisting of three domains. The putative active site cleft is created at the interface between all three domains. Ricin A chain is heterogeneous containing two components of molecular weight 30,000 and 32,000 kilodaltons, a difference due only to a difference in carbohydrate units. The lighter component has been designated the A_1 chain and the heavier component the A_2 chain (Foxwell, et al., 1985). Characterisation of the A_1 carbohydrate side chain has shown it to contain $(GlcNAc)_2(Xyl)(Fuc)(Man)_{4-6}$ (Olsnes and Pihl, 1976; Foxwell, et al., 1985). It therefore lacks the trimannosidic core for which concanavalin A has high affinity, so it has been termed A chain con A(-). The A_2 chain contains an extra simple mannose-rich oligosaccharide unit, which enables concanavalin A to bind, and is therefore termed con A(+), although there does appear to be some heterogenity within this

carbohydrate unit. The carbohydrate chains can be removed by ∝-mannosidase digestion and deglycosylated ricin A chain retains the ability to inactivate ribosomes in a cell-free protein synthesising system (Foxwell, et al., 1987). Ricin formed from deglycosylated ricin A chain was toxic to AKR-A cells and CBA mice. E. coli cells transformed with a plasmid which contains DNA encoding ricin A chain, express ricin A chain which is not glycosylated (O'Hare, et al., 1987). The purified, recombinant ricin A is a potent inhibitor of eucaryotic ribosomes, both in the rabbit reticulocyte lysate system and in cultured cells. The removal of the carbohydrate chains is therefore important when ricin A chain is being used therapeutically, for their presence renders ricin more susceptible to clearance by the reticuloendothelial system, as shown by the intoxication of mannose receptor bearing cells, macrophages, by highly purified ricin A chain (Simmons, et al., 1986).

1.6. RICIN B CHAIN.

Ricin B chain, molecular weight 34,700 daltons, (including the oligosaccharide side chains) possesses two sugar binding domains, which bind two galactose residues in a non-co-operative manner (Zentz, et al., 1978; Houston and Dooley, 1982). Tyrosine-248 has been implicated in the 'strong' galactose binding site (Mise, et al., 1986; Wawrzynczak, et al., 1987) and tryptophan-37 is believed to be important in the weak binding site (Hatakeyma, et al., 1986; Rutenber, et al., 1987). The mode of galactose binding is nearly identical at the two sites, the lactose lying in a shallow pocket (see figure 1a). One side of the pocket being formed by the aromatic ring of a side group (of Trp-37 in domain 1 and Tyr-248 in domain 2), whilst the other side is formed by a slight kink in the chain, involving a

highly-conserved Asp-Val-Arg tripeptide sequence. In domain 1, the galactose forms hydrogen bonds to Asn-46 and to Lys-40. These bonds involve hydroxyls 3 and 4 of the sugar and help determine the epimeric specificity of the binding site. Hydrogen bonding between Asn-46 and Asp-22, maintains the position of Asn-46 which is the key residue (see Figure 1b). In domain 2, Asn-255 is crucial to epimeric specificity, binding to hydroxyl 4 of galactose. The position of Asn-255 is stabilised by a hydrogen bond with Asp 234. A water molecule, bound to Asn-255, forms a hydrogen bond to hydroxyl 3 of the galactose and the water is in a position analogous to Lys-40 of domain 1, Figure 1c (Rutenber, et al., 1987; Montfort, et al., 1987).

Study of the amino acid sequence of the B chain indicated that it could be divided into two homologous domains (Villafranca and Robertus, 1981). This was confirmed by X-ray crystallography, which showed that the B chain did indeed fold into two separate globular domains with identical folding topologies, but that the lactose sugars were bound at non-homologous sites in the two domains. This result can be explained by an analysis of the primary and tertiary structure of the B chain which reveals that each of the homologous domains can be divided into four peptides, termed λ, α, β, and γ.

The individual units have undergone considerable divergence, but the similarities have been found to be statistically significant. The λ peptide of domain 1 is homologous to the λ peptide of domain 2, but these are not related to the other peptides α, β and γ, which are all homologous with each other. It has therefore been hypothesised that the ancestor of the modern B chain was a galactose binding peptide which resembled the 1 subdomain which is the only unit which retains all the key structural and functional features. Gene duplication and fusion then produced the $\alpha\beta\gamma$-molecule similar to the modern B chain domain. The β subdomains could then become specialised

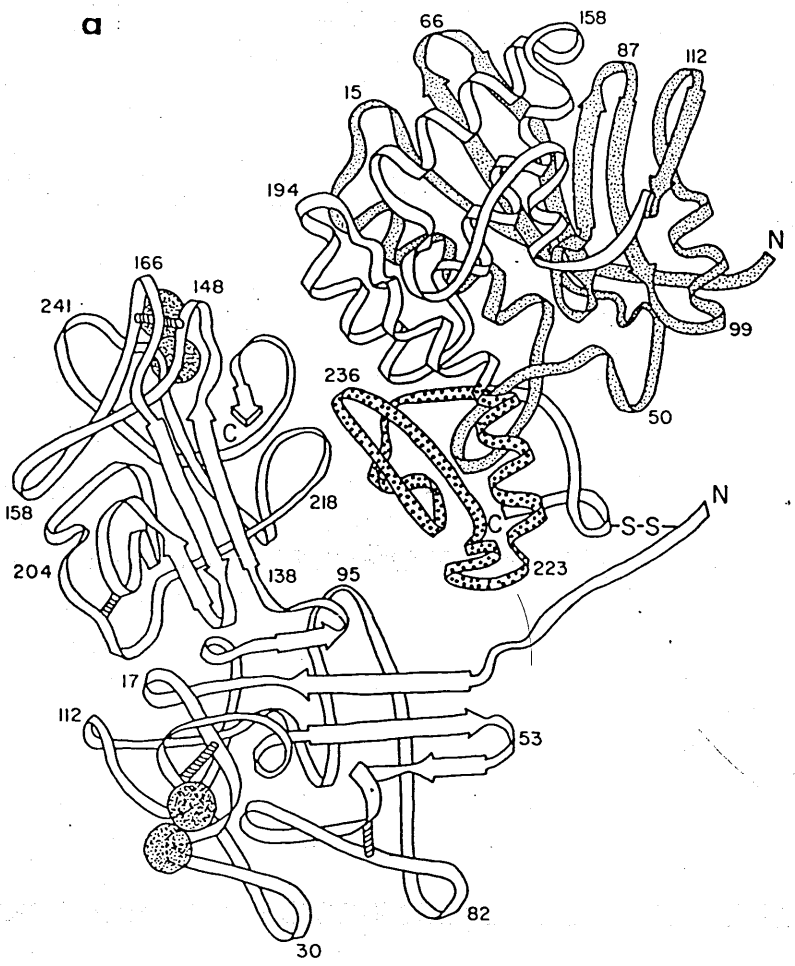
Figure 1. The Three Dimensional Structure of Ricin at 2.8Å.

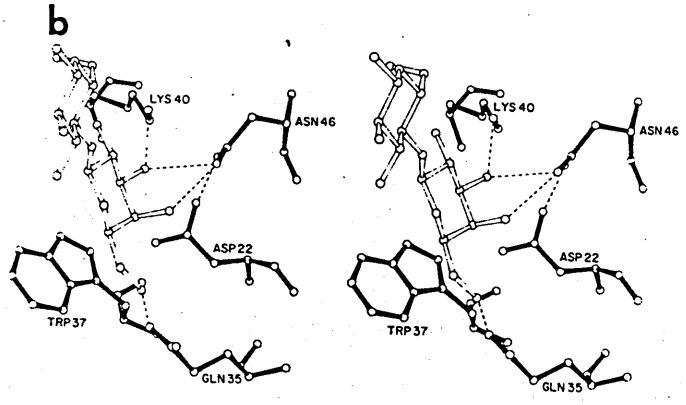
a. Ribbon representation of the ricin backbone. The A chain is in the upper right of the figure and the B chain at lower left. The two lactose moieties bound to the B chain are each represented as pairs of discs. The disulphide bond linking the chains is indicated in the lower right of the molecule.

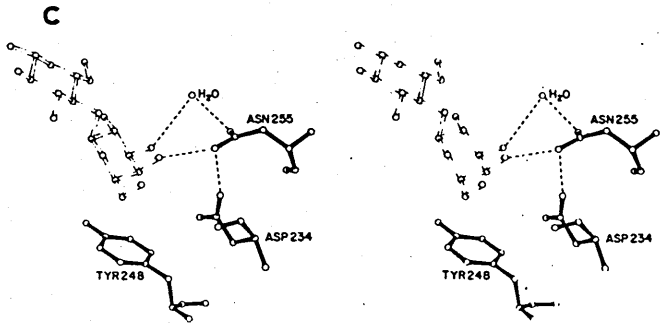
b. Stereographic view of the lactose binding site in domain 1. The lactose molecule has 'open bonds' whereas the protein residues are black.

c. Stereographic view of the lactose binding site in domain 2.

(From Montfort, et al., 1987)


a
66
158
87
112
15
194
N
166
148
241
99
236
50
C
C
S-S
N
158
218
223
204
138
95
17
112
53
82
30


b
LYS 40
ASN 46
ASP 22
TRP 37
GLN 35
LYS 40
ASN 46
ASP 22
TRP 37
GLN 35


c
H₂O
ASN 255
ASP 234
TYR 248
H₂O
ASN 255
ASP 234
TYR 248

for a structural role, whilst the α and γ would still bind the sugars. Fusion of the peptide would then further stabilise the structure. Another duplication would then give rise to the $(\lambda\alpha\beta\gamma)_2$ configuration. Thus, duplication probably blocked galactose access to two of the subdomains, causing the present day B chain to bind only two saccharide units.

Studies of the toxicity of immunotoxins, hybrid molecules consisting of an antibody component of defined fine specificity coupled to a potent cytotoxic agent, have led to the finding that ricin-containing immunotoxins have greater toxicity than immunotoxins containing only the ricin A chain. This has led to the hypothesis that ricin B chain may facilitate the translocation of ricin A chain into the cytosol, in addition to its receptor binding function. It has been demonstrated that the addition of free ricin B chain or B chain coupled to an antibody will potentiate the cytotoxicity of ricin A chain immunotoxins (Youle and Neville, 1982; McIntosh, et al., 1983; Vitetta, et al., 1983, 1984). This synergy apparently occurs even with ricin B chain antibody complexes in which the B chain has been chemically-modified to attenuate its capacity both to bind to cells and to form a disulphide bond with the A chain (Cushley, personal communication). However data does indicate that lectin activity may be essential for the A chain enhancing function of the ricin B chain. Youle and Colombatti, (1987) using a cell line which secreted a monoclonal antibody which bound specifically to the ricin B chain, showed that this antibody was capable of blocking ricin toxicity within the cell. This indicates that ricin galactose-binding sites are required intracellularly for toxicity. It also showed that these receptors exist in the intracellular compartment containing secretory

proteins (putatively the Golgi apparatus which contains more ricin binding sites than any other cellular compartment (Yokoyama, et al., 1980). This may now be settled using site directed mutagenesis of the ricin B chain which has been successfully cloned and expressed in COS-M6 cells by Chang, et al., (1987).

Ricin B chain has two oligosaccharide chains consisting of mannose and N-acetyl glucosamine (one of saccharide composition $(GlcNAc)_2$ $(Man)_6$, and the other $(GlcNAc)_2$ $(Man)_7$) attached to asparagine residues at position 93 and 133 of the peptide chain (Funatsu, et al., 1979). The influence of these carbohydrate chains on the intoxication procedure has been studied. Pure ricin B chain has been enzymatically deglycosylated using ∝-mannosidase or endoglycosidase H (Foxwell, et al., 1985, 1987), and intact ricin was treated with sodium metaperiodate at low pH to oxidise the carbohydrate chains (Simmons, et al., 1986). Periodate treated ricin and intact ricin were compared in toxicity assays on L929 cells, and it was found that the toxicity of periodate treated ricin was two orders of magnitude lower than that of intact ricin (Simmons, et al., 1986). This effect could not be explained by a decrease in the ability of the B chain to bind to its receptors, or to be a change in the binding affinity or to an alteration in the enzymatic activity of the ricin A chain. It therefore appeared to be attributable to the removal of the oligosaccharide side-chains (Simmons, et al.,1986). The effect was confirmed by the enzymatic deglycosylation of ricin B chain by ∝ − mannosidase (Foxwell, et al., 1985, 1987). Recombination of ∝-mannosidase-treated B chain with ∝ − mannosidase-treated A chain led to reduced cytoxicity in vitro. However deglycosylation of ricin B chain using endoglycosidase H (Foxwell, et al., 1985, 1987) which is only able to cleave one of the oligosaccharide side chains due to the protection of the other by the

polypeptide structure, when recombined with deglycosylated ricin A chain was exceedingly toxic *in vitro*. This suggests that the terminal mannose residues are required to maintain the conformation of the B chain or of intact toxin, and their removal may reduce the ability of the B chain to assist the A-chain across the cell membrane to the cytosol, or could render it more susceptible to cleavage by cellular thiols or proteolytic enzymes (Foxwell, *et al.*, 1987). The final destination of the ricin B chain within the cell is still undetermined.

There are many continuous sequences of hydrophobic amino acids along the ricin B chain, especially at the C-terminus (Funatsu, *et al.*, 1979). It has been shown that ricin B chain will insert into the membrane of Newcastle disease virus (Ischida *et al.*, 1983). Experiments which investigated the association of ricin with the lipid bilayer of GM_1-liposomes at acidic pH demonstrated that for a hydrophobic interaction to occur, the binding must be mediated by binding of ricin to GM_1-liposomes containing galactose moieties (Utsumi, *et al.*, 1987). However, the membrane interaction and its function in the release of ricin A chain into the cytosol have still to be clarified.

Ricin B chain has been used in the synthesis of a number of chimeric molecules, since it was hypothesised that the B chain may be able to mediate the translocation of attached molecules to an intracellular site appropriate to expression of their biological activity. Insulin, abrin A chain and gelonin have all been linked to ricin B chain. The insulin - ricin B conjugate was shown to bind to receptors for ricin, and that cellular responses due to insulin were observed (Roth, *et al.*, 1981; Hofmann, *et al.*,1983). The location of insulin or ricin B chain within the cytoplasm was not ascertained.

The conjugates of abrin A - ricin B chain (Olsnes, et al., 1974b) and gelonin - ricin B chain (Goldmacher, et al., 1987), were toxic to cells in culture. Gelonin, a ribosome-inactivating protein isolated from Gelonium multiflorum, and abrin A chain both inactivate the 60S ribosomal subunit, and it could therefore be argued that it is an inherent property of these toxic molecules isolated from plants which allows interaction with the ricin B chain such that it facilitates their entry into the cytosol.

1.7. ROLE OF THE DISULPHIDE BOND.

The disulphide bond that links the A and B moieties of ricin is also a property common to most toxins, e.g. diphtheria toxin, shigella toxin, cholera toxin, E. coli heat-liable toxin, abrin, modeccin and viscumin. It has been suggested that the disulphide bond does not simply prevent dissociation of the A and B chains, for there are sufficient weak interactions present to keep them together, mediated mainly by the side-chains of prolines and phenylalanines (Montfort, et al., 1987), but that it is necessary for toxicity. Lewis and Youle (1986) studied the toxicity of reduced ricin and found that the reduced subunits associated only by non-covalent bonds and were more toxic to the cell than disulphide bonded subunits. This was in contradiction to the findings of Lappi, et al., (1978) which stated that associated reduced ricin was less toxic than native ricin. This difference can be explained in terms of the concentrations of associated and native ricin used for at 1-100ng per ml the equilibrium constants would favour complete dissociation of the subunits rendering them non-toxic.

It has also been proposed that the disulphide bond may play a role in toxin entry. This has been tested experimentally by the synthesis of immunotoxins held together by covalent linkages other

than a disulphide bridge (Jansen, et al., 1982; Masuho, et al., 1982). When ricin A chain was linked via a thioether bond to an antibody against trinitrophenol, no toxic effect was expressed towards TNP-derivatised target cells, whereas a similar hybrid containing a disulphide bridge was highly toxic (Jansen, et al., 1982). Similar experiments were performed using hybrids consisting of ricin A chain linked to either $F(ab)'_2$ or F(ab) fragments of IgG antibodies specific to L1210 cells (Masuho, et al., 1982). These workers found that when the link was not a disulphide bond the conjugates were 100 times less toxic than when a disulphide bond was employed. Wright and Robertus, (1987) alkylated the cysteine residues which link the A and B chains of ricin with iodoacetamide. It was found that the alkylated ricin would still bind to HeLa cells and alkylated A chain was toxic to ribosomes in an in vitro protein synthesis assay. However, the alkylated molecule showed little or no cytotoxicity towards cells in culture suggesting that a functional interdomain disulphide bridge is essential probably for transmembrane transport (Wright and Robertus, 1987).

It is not known when reduction of the disulphide bond occurs, but if reducing agents are not present in the system, then even high concentrations of toxin will not inactivate ribosomes. Reduction could occur at the cell surface due to transient interactions with sulphydryl groups on the membrane during the entry. However, the enzyme glutathione protein disulphide oxidoreductase, present in the vesicular compartments, can efficiently reduce the interchain disulphide bond (Barbieri, et al., 1982). Reduction of intracellular glutathione concentrations with diethyl maleate decreased the rate of inhibition of protein synthesis by ricin, whereas ribosome inactivation by reduced associated ricin was accelerated. This indicates that the reduction of the disulphide bond is probably a rate

limiting step in the intoxication process (Lewis and Youle, 1986).

Inhibition of protein synthesis does not appear to require free thiol groups, for the inhibiting effects of reduced toxins or isolated ricin A chain was only moderately lowered after treatment with N-ethylmaleimide which binds irreversibly to thiol groups (Olsnes, et al., 1975).

1.8. DETERMINATION OF THE EXISTENCE OF A 'TRANSPORT' FUNCTION OF RICIN B CHAIN.

Observations from the interactions of immunotoxin molecules have indicated that the ricin B polypeptide has functions other than simple ligand binding. Thus in a system where target cells were treated with a weakly-toxic dose of an antibody-ricin A chain conjugate, the cytotoxicity expressed by that immunotoxin can be greatly enhanced by addition to the culture system of either free ricin B polypeptide or an antibody - ricin B complex (Vitetta, et al., 1983, 1984). This synergy appeared to occur even when the ricin B chain had been chemically modified so that it was no longer capable of binding to cells or forming a disulphide bond with the ricin A polypeptide (Cushley, personal communication). The conclusion drawn from this data was that the ricin B polypeptide was responsible for promoting the access of the ricin A chain into the cytosol and hence potentiates the cytotoxic effect.

The 'hypothetical' transport function of the ricin B polypeptide could therefore be used to target reagents (chemicals, enzymes, or antibodies) which do not possess the ability to reach the cytosol themselves, a means of gaining access to it. The main objectives of this work was to synthesise a complex between ricin B polypeptide and ribonuclease A; establish that the two polypeptides of the complex retained their biological function; purification of the complex and

development of a cell assay system so that the enzymatic effects of the ribonuclease A could be observed *in vitro* and therefore provide evidence that the ricin B polypeptide was capable of internalising molecules.

MATERIALS AND METHODS

2. MATERIALS AND METHODS.

2.1. MATERIALS.

2.1.1. Proteins and Enzymes.

Ricin A chain, ricin B chain and ribonuclease A (from bovine pancreas) were supplied by Sigma Chemical Co. Ltd., Dorset, U.K. Ricin B chain was also obtained from Xoma Corporation, San Fransisco, U.S.A, and E.Y. Laboratories, San Mateo, U.S.A.

2.1.2. Chemicals.

N-succinimidyl-3-(2-dithiopyridyl) propionate (SPDP), 4-chloro-1-napthol, periodic acid, tris (hydroxymethyl) aminomethane (Trizma base), dimethyl sulphoxide were obtained from Sigma Chemical Co. Ltd., Dorest, U.K.

Routine reagents, chemicals and solvents were supplied by BDH Chemicals Ltd., Dorset, U.K., and were of the highest grade available.

Ribonucleic acid (from yeast) was obtained from Sigma Chemical Co. Ltd., Dorset, U.K. Freunds complete and incomplete adjuvant were from Difco Laboratories, Detroit, Michigan.

2.1.3. Chromatographic Materials and Equipment.

PD-10 prepacked disposable columns, Sephacryl S-300, Sepharose 4B, Polybuffer exchanger PBE 94 and Polybuffer solution PBE 74 were obtained from Pharmacia, Uppsala, Sweden. The gel filtration (Superose 12) and the ion exchange column (Mono-Q) for the FPLC System were purchased from the same source.

2.1.4. Electrophoretic Materials.

Acrylamide, N, N, N' N' tetramethylene diamine (TEMED), N'N'-methylene bis acrylamide, ammonium persulphate, urea, nonidet P40 (NP-40) and sodium dodecyl sulphate (SDS) were supplied by BDH Chemicals Ltd., Poole, Dorset, U.K. 2-mercaptoethanol was obtained from Koch-Light Laboratories, England, U.K. Low molecular weight marker proteins and 'Pharmalyte' pH 5-8 and pH 3-10 (carrier ampholytes) were supplied by Pharmacia Fine Chemicals, Hounslow, Middlesex, U.K.

2.1.5. Stains.

Coomassie blue (G) and napthol blue-black were obtained from Sigma Chemical Co. Ltd., Dorset, U.K. Silver nitrate was supplied by Johnson Matthey Chemicals, U.K.

2.1.6. Radiochemicals.

L-[^{35}S]-Methionine, specific activity 1200Ci/mmole, [^{125}I]-iodine (100 mCi/ml) carrier free were obtained from Amersham International plc, Amersham, England.

2.1.7. Photographic Materials.

Photographic films, X-ray films (Kodak X-Omat S), DX-80 developer and FX-40 X-ray liquid fixer were obtained from Kodak Ltd., Hemel Hampstead, U.K.

2.1.8. Serological Reagents.

Goat anti-rabbit IgG (H+L) horse radish peroxidase was supplied by Miles Laboratories Ltd., Slough, U.K.

Normal goat serum and normal rabbit serum were obtained from Scottish Antibody Production Unit, Wishaw, Scotland, U.K.

Rabbit anti-*Ricinus communis* lectin was purchased from Sigma Chemical Co. Ltd., Dorset, U.K.

2.1.9. Cell Culture Materials.

RPMI-1640, foetal calf serum (FCS) and L-glutamine were obtained from Gibco Ltd., Paisley, U.K. Penicillin and streptomycin were supplied by Glaxo Pharmaceutical Ltd., London.

2.1.10. Disposable Plasticware.

Tissue culture flasks were supplied by Nunc, Trident House, Paisley, U.K. Tissue culture plates (96 wells and 24 wells) were supplied by Costar, Northumbria Biologicals Ltd., Northumbria, U.K. Sterile universals were obtained from Sterilin Ltd., Feltham, England, U.K.

2.1.11. Cell Line.

The human B lymphoma cell line Daudi (Klein, *et.al.*, 1968) was employed in cellular cytotoxicity assays. The line was routinely maintained as a suspension culture.

2.1.12. Rabbits.

Male New Zealand Whites.

2.1.13. Miscellaneous Materials.

Eppendorf tubes (1.5ml capacity) were obtained from Anderman and Co., Surrey, U.K. Dialysis tubing was supplied by Visking Tubing, Scientific Instruments Centre, London. Micropipettes and tips were obtained from Gilson Anachem Ltd., Luton, U.K. Multi-channel micropipettes were obtained from Flow Laboratories, Ayrshire, U.K.

2.2. PURIFICATION OF RICIN B CHAIN.

Due to the presence of ricin in the 'purified' ricin B chain supplied by E.Y. Laboratories and Sigma Chemical Co. a purification procedure had to be developed.

2.2.1. Sample Preparation.

METHOD A.

500ug ricin B chain in 0.1M Tris-HCl, pH 7.2 containing 0.1M lactose and 0.02% sodium azide was microdialysed against the following buffers overnight at $4^{\circ}C$:-

a) 0.1M Tris-HCl, pH 7.2

b) PBS, pH 7.2 (170mM NaCl, 3.4mM KCl, 10mM Na_2HPO_4 , 1.8mM KH_2PO_4).

The dialysed sample was then centrifuged in a fixed angle rotor at 10,000 x g for 15 min, before application to the Superose 12 column (gel filtration).

METHOD B.

Method A was followed, but after dialysis the sample volume was measured and solid ammonium sulphate was added to give a final concentration of 60% (w/v) in the solution. The sample was then centrifuged in a fixed angle rotor at 10,000 x g for 20 min. The supernatant was removed, and the precipitate resuspended in 250ul of 0.1M Tris-HCl, pH 7.2. The sample was then dialysed against 0.1M Tris-HCl, pH 7.2 to remove the ammonium sulphate. After dialysis the sample was again centrifuged at 10,000 x g for 15 min., before application to the Superose 12 column.

2.2.2. Gel Filtration (Superose 12).

There are a number of high performance techniques available with Pharmacia FPLC (fast protein liquid chromatography) system, including

chromatofocusing, reversed phase, affinity chromatography and ion exchange, but gel filtration was the technique selected. Thus, rapid results, high resolution, maintenance of biological activity and structural integrity of the applied sample could be obtained.

The Pharmacia Superose 12 column (1cm x 30cm packed with a cross-linked, agarose-based medium) was used to achieve separation of ricin from ricin B chain. The Superose 12 column was equilibrated with 0.1M Tris-HCl, pH 7.2 (0.22uM filtered).

The 200ul sample was loaded on to the equilibrated column and eluted with 0.1M Tris-HCl, pH 7.2 (flow rate 1ml/min). 0.5ml fractions were collected and their absorption profile at 280nm was recorded along with the corresponding fraction number by the chart recorder (1cm/ml). When the sample had eluted from the column, the equilibration buffer was replaced by 24% ethanol (0.22um filtered) under which the column material was stored. The fractions corresponding to the peaks were pooled, or individual fractions were analysed by SDS-PAGE (Section 2.5.). The polyacrylamide gels were silver stained (Section 2.7.), to determine the purity of the ricin B chain.

The Superose 12 column was also equilibrated with PBS, pH 7.2 so that this buffer could be used in conjunction with the column to purify the ricin B chain.

2.2.3. Chromatofocusing.

The isoelectric point of ricin (pI 7.1) and ricin B chain (pI 4.8) are quite different and, therefore, chromatofocusing can be utilised to separate these proteins.

Polybuffer exchanger (PBE 94), 10ml, was poured into a sintered glass funnel, and the gel was equilibrated with 0.025M histidine-HCl, pH 6.2 (150ml). The equilibrated gel was then packed in the column (10cm x 1cm).

Ricin B chain, 3mg, was dialysed against 1 litre of 0.025M histidine-HCl, pH 6.2 for 4hr. The sample was then applied to the column. The column was eluted using degassed polybuffer 74-HCl, pH 4.0 (1:8 dilution, polybuffer:distilled water), the flow rate was approximately 0.5ml/min. 1ml fractions were collected and the absorbance at 280nm of each was plotted on a chromatogram. The fractions containing the ricin B chain were pooled.

The ricin B chain polypeptide was separated from the polybuffer 74-HCl using a Sepharose 4B column (L. Roberts and G. Clements, Warwick Uni., personal communication). Sepharose 4B was pre-treated with 1M propionic acid for three weeks, this was then poured into a sintered glass funnel. The Sepharose 4B was equilibrated with PBS, pH 7.2 and then packed in a column (20cm x 1cm). The ricin B chain/polybuffer 74-HCl was then applied to the column. PBS, pH 7.2 was used to elute the polybuffer from the column, 1ml fractions were collected and the absorbance at 254nm of these were recorded to determine when the polybuffer had been completely eluted. PBS, pH 7.2 containing 0.1M galactose was used to elute the ricin B chain which had bound to the Sepharose 4B column. 1ml fractions were again collected and the absorbance at 280nm of each was recorded and plotted on a chromatogram.

12.5% SDS-PAGE electrophoresis (Section 2.5.), silver staining (Section 2.7.), and toxicity assays (Section 2.13.2) were used to determine the purity of the ricin B polypeptide.

2.3. PROTEIN CHEMICAL PROCEDURES.

2.3.1. Derivatisation of Ribonuclease A.

The heterobifunctional cross-linking agent, SPDP, was selected to derivatise ribonuclease A such that hybrid proteins linked by a disulphide bond could be synthesised.

An assay to determine the concentration of SPDP required for optimal derivatisation of ribonuclease A was performed. A stock solution of 2ug/ul SPDP in dimethyl sulphoxide (DMSO) was prepared, then the following increasing concentrations of SPDP in 20ul DMSO (0ug, 1ug, 2ug, 5ug and 10ug) were added to individual eppendorfs containing 1mg/ml ribonuclease A in PBS, pH 7.2. The samples were incubated for 1hr at room temperature, then micro-dialysed against PBS, pH 7.2 overnight at $4^{o}C$ to remove excess reagent and DMSO. A Carlsson Assay, (see section 2.3.2.) was carried out so that the substitution ratio of moles pyridyl dithiopropionate groups per mole ribonuclease A could be determined.

Routinely, to derivatise 10mg/ml ribnonuclease A in PBS, pH 7.2, 50ug SPDP in 25ul DMSO was added. The only alteration to the procedure described above was that microdialysis was replaced by gel filtration using a PD-10 Sephadex G25 column. The absorbance at 280nm of individual 1ml fractions was measured and protein-containing fractions pooled. The substitution ratio was determined using the Carlsson Assay (See Section 2.3.2.).

2.3.2. Carlsson Assay.

This spectrophotometric assay described by Carlsson, et al., (1978) was used to calculate the substitution ratio of pyridyl diothiopropionate groups per mole protein.

The absorbance at 280nm (A_{280}) and (A_{343}) of 1ml derivatised ribonuclease A was measured and recorded. The substituted ribonuclease A

was then placed in an eppendorf tube and 5ul 2-mercaptoethanol was added and mixed. The sample was then incubated at 37°C for 30 min. A second measurement of the absorbance at 343nm (A_{343}') of the sample was then made. The absorbance at 280nm of 1mg/ml ribonuclease A (unsubstituted) in PBS, pH 7.2 was measured and recorded.

The substitution ratio is calculated by dividing the moles of pyridine-2-thione released by the moles of protein present. The concentration of pyridine-2-thione released in micromolar terms, [P2T], was calculated from the difference in absorbance readings at A_{343} of the sample according to the equation;

$$[P2T] = (A_{343}' - A_{343}) \div 0.008.$$

The true absorbance value of the substituted protein at 280nm (A_{280}') can be calculated from the reading which was recorded and which includes absorption by the dithiopropionate groups, according to the equation;

$$A_{280}' = A_{280} - (5.1 \times 10^3 \times [P2T]).$$

(Note that in this instance the concentration of P2T is expressed in molar terms).

To determine the concentration of protein in mg/ml the A_{280}' value is divided by the A_{280} value of the unsubstituted ribonuclease A, and this can then be easily converted to moles protein present.

2.3.3. Synthesis of Ribonuclease A and Ricin B Chain Complex.

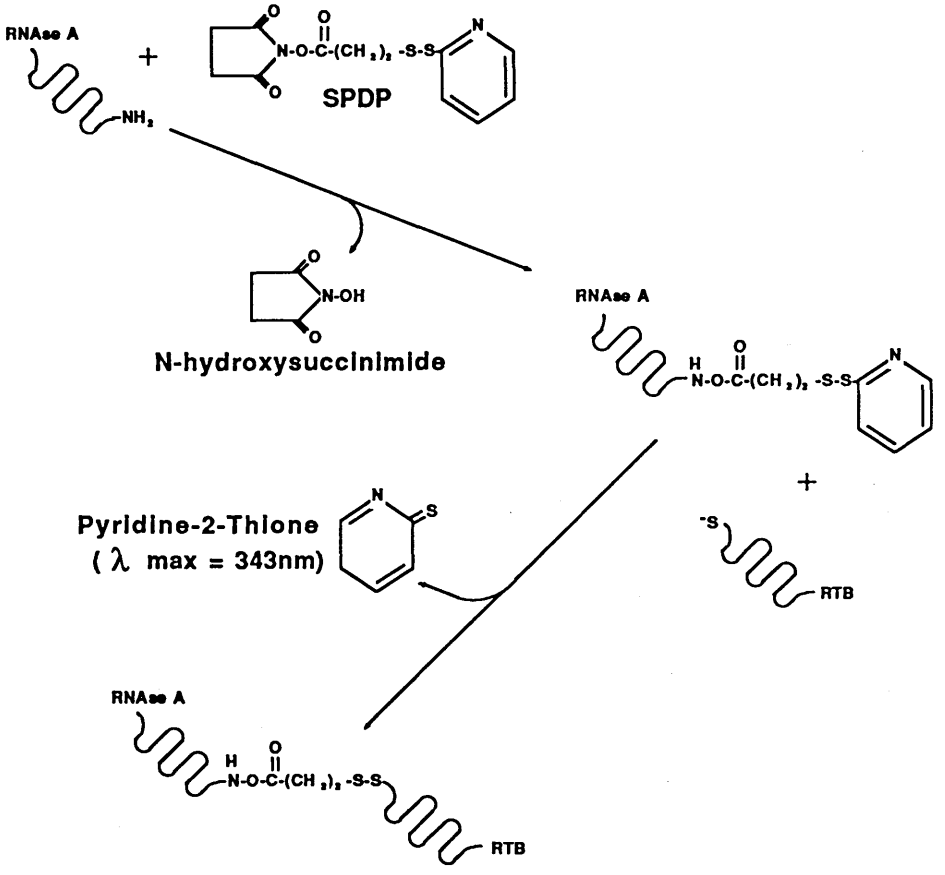
Five microlitres of 13M 2-mercaptoethanol was added to 500ul ricin B chain (2mg/ml), mixed, and left at room temperature for 30 min. The sample was then applied to a PD-10 Sephadex G25 column pre-equilibrated with PBS, pH 7.2. The ricin B chain was then eluted from the column using PBS, pH 7.2, 1ml fractions being collected. The absorbance at 280nm of the fractions were measured and a chromatogram plotted.

To the fraction(s) containing the freshly reduced ricin B chain an equal concentration of derivatised ribonuclease A, (See Section 2.3.1), was added, mixed and allowed to stand for 1hr at room temperature. Formation

Figure 2. Bovine Pancreatic Ribonuclease A : Ricin B Complex Synthesis.

The amino group of lysine residues present in RNase A react with the carboxyl group of the cross-linking reagent, SPDP, releasing N-hydroxysuccinimide. The derivatised RNase A then reacts with the reduced sulphydryl of the ricin B chain, such that pyridine-2-thione is released and the hybrid protein is formed.

BOVINE PANCREATIC RIBONUCLEASE A: RICIN B CHAIN COMPLEX SYNTHESIS



of the complex was then determined by SDS-PAGE (See Section 2.5.). The SDS-PAGE Gels were silver stained (See Section 2.7.) to allow visualisation of the complex. (See Figure 2)

2.3.4. Iodination of Proteins.

Proteins were iodinated by the 'Iodogen' method of Fracker and Speck, (1978). 1, 3, 4, 6 tetrachloro - 3 , 6 diphenylglycouril ('IODOGEN') was dissolved at 1mg/ml in chloroform and 1ml was evaporated on to the walls of a glass bijoux bottle.

Five hundred microlitres protein (0.5mg/ml) in PBS, pH 7.2 was added to the iodogen coated bijoux bottle and incubated on ice for 30 min. 100uCi of carrier-free [^{125}I] was then added, and following a 30 min. incubation on ice the reaction mixture was separated on a PD10 Sephadex G25 column equilibrated with PBS, pH 7.2. 1ml fractions were collected, 5ul of each being counted in an LKB Wallac '1275 Minigamma' counter. The radioiodinated protein peak was pooled and stored in a lead pot at -20^{o}C.

2.3.5 Synthesis of Iodinated Ribonuclease A and Ricin B Chain Complex.

The synthesis of iodinated ribonuclease A and ricin B chain complex was carried out exactly as described in Section 2.3.3. Formation of the complex was determined by autoradiography of 12.5% SDS-PAGE gels,(see Section 2.5. and 2.8.).

2.3.6. Amidination of Ribonuclease A.

The method that was followed for amidination of the ribonuclease A was described by Coggins, (1978).

A 20mM dimethylsuberimidate solution in 100mM buffer (1 vol 200mM triethanolamine - HCl, pH 8.5 and 1 vol 40mM sodium hydroxide) was prepared and used immediately. The ribonuclease (50uM in 100mM triethanolamine -

HCl, pH 8.5) was added to an equal volume of the dimethylsuberimidate solution and incubated at 20°C for 2 hr. To remove decompositon products, excess reagent and change the buffer present in the amidinated ribonuclease A, the sample was applied to a PD-10 Sephadex G25 column equilibrated with PBS, pH 7.2 and eluted with the same buffer. 1ml fractions were collected. The absorbance at 280nm of each of the fractions was measured in an L.K.B. spectrophotometer, and the absorbance versus fraction number was plotted on a chromatogram, so that the amidinated ribonuclease A fraction could be isolated and pooled. The polymerised ribonuclease A was then analysed by SDS-PAGE gel electrophoresis, (see section 2.5.) and by ribonuclease A activity gel, (see section 2.9.).

2.4. FPLC-MONO Q PURIFICATION OF THE RIBONUCLEASE A-RICIN B COMPLEX.

2.4.1. Sample Preparation.

The sample containing RNAse A-ricin B complex, RNAse A and ricin B polypeptide was centrifuged in a fixed angle rotor at 10,000 x g for 15 min. The supernatant was removed and this was then applied to the FPLC-Mono Q column.

2.4.2. Pre-equilibration Of The Column.

Buffer A:- 10mM NaCl in 20mM Tris-HCl, pH 7.0.

Buffer B:- 1M NaCl in 20mM Tris-HCl, pH 7.0.

The FPLC-Mono Q anion exchanger column was pre-equilibrated with the buffers according to the protocol from Pharmacia for use in conjunction with their columns. The buffer flow rate was set at 1.0ml/min and the chart recorder was set at 0.5cm/ml to record the equilibration profile. Firstly, 5.0ml buffer A was passed over the column, then in 0.1ml the buffer was changed to buffer B and 9.9ml of this was passed over the column. The buffer was again changed to buffer A in 0.1ml and 4.9ml of this buffer was passed over the column.

2.4.3. Purification Of The RNAse A - Ricin B Complex.

The sample (500ul) was applied to the column and the proteins which were bound, were eluted by increasing the salt concentration of the buffer. After the sample had been loaded on the column 2ml buffer A was used to elute the column, the gradient was subsequently formed in the following way:- a gradual mixing of buffers A and B so that after 18ml the salt concentration of the eluting buffer was 0.614M (equivalent to 60% (w/v)), at 27ml the salt concentration of the buffer was 1.0M (equivalent to 100%(w/v)), 6.0ml of this buffer i.e. B was used to elute the column 0.5ml fractions were collected and the absorbance at 280nm of each fraction was measured and the gradient profile was recorded by the chart recorder (chart speed 0.5 cm/ml). The fractions were analysed by silver staining SDS-PAGE gels (Section 2.5. and 2.7.).

This method was useful, as the sample volume was not restrictive, and multiple applications of the 500ul samples could be made to the column, as long as the protein concentration of the total sample was between 20-50mg. This method therefore enabled purification and concentration of the RNAseA - ricin B complex.

2.5. POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE).

Polyacrylamide gel electrophoresis was performed using the discontinuous tris-glycine buffering system (Laemmli, 1970).

2.5.1. Stock Solutions.

Solution A.
45% (w/v) Acrylamide,
1.2% (w/v) N, N' - methylenebisacrylamide.

The solution was deionised with Amberlite MB-2 ion exchange resin, filtered, and stored at $4^{o}C$ in a dark bottle.

Solution B.

1.5M Tris-HCl, pH 8.8
0.13% (v/v) TEMED.

The solution was filtered and stored at $4^{o}C$.

Solution C.

12% (w/v) sodium dodecyl sulphate (SDS).

The solution was filtered and stored at $4^{o}C$.

Solution D.

20% (w/v) Ammonium persulphate in H_2O.

This solution was prepared immediately prior to use.

Solution E.

0.65m Tris - HCl, pH 6.8.

The solution was filtered and stored at $4^{o}C$.

Electrophoresis Buffer.

0.25M Tris
0.132 M Glycine
0.2% (w/v) SDS

Non-Reducing Sample Buffer (x5).

0.625M Tris-HCl, pH 6.8
2% (w/v) SDS
10%(w/v) Glycerol
0.001% (w/v) Bromophenol blue

Reducing Sample Buffer (x5).

As for non-reducing loading buffer but with the addition of 5% (v/v) 2-mercaptoethanol.

2.5.2. Separating Gel Preparation.

12.5%, 10%, and 8.5% polyacrylamide separating gels were prepared from the stock solutions as follows:-

Acrylamide Gel	8.5%	10%	12.5%
Solution A	11.33ml	13.33ml	16.66ml
Solution B	15.00ml	15.00ml	15.00ml
Solution C	0.5 ml	0.5 ml	0.5ml
Distilled Water	32.66ml	30.67ml	27.4 ml
Solution D	0.5 ml	0.5 ml	0.5 ml

The solution was then poured to within 25% of the top of the front plate of the casting apparatus. 1ml of butan-2-ol was then used to overlay the acrylamide solution. The acrylamide solution was then allowed to polymerise.

2.5.3. Stacking Gel Preparation.

The stacking gel was prepared from the stock solutions as follows:-

2.64ml Solution A
2.40ml Solution E
0.20ml Solution C
18.67ml Distilled water
0.20ml Solution D
0.024ml TEMED

After polymerisation of the separating gel, the butan-2-ol layer was poured off, and the acrylamide surface rinsed with distilled water.

The stacking gel was then poured and allowed to polymerise around a 10 or 20 well teflon comb.

The final dimensions of the gel were 16.5cm x 14.5cm x 0.15cm., or, if "mini-gels" were employed, 8.0cm. x 7.0cm. x 0.05cm.

2.5.4. Electrophoresis Conditions.

Electrophoresis was performed for 3 - 4hr at room temperature at a constant current of 40mA per slab gel or overnight (at room temperature) at a constant current of 9mA per slab gel.

2.5.5. Molecular Weight Standards.

The low molecular weight standards (Pharmacia) comprise the following proteins:-

Protein	Mr	Source
Phosphorylase b	94,000	Rabbit muscle
Albumin	67,000	Bovine serum
Ovalbumin	43,000	Egg white
Carbonic Anhydrase	30,000	Bovine erythrocyte
Trypsin Inhibitor	20,100	Soybean
-Lactalbumin	14,400	Bovine milk

0.1 ml of non-reducing loading buffer was added to the lyophilised low molecular weight standards. The standards were then boiled at 100^{o}C for 3 minutes. Five microlitre samples of molecular weight standards were loaded into the appropriate slots of the gel.

2.5.6. Sample Preparation.

All samples contained between 1ug - 10ug of protein. To each protein sample, one-fifth volume of reducing or non-reducing sample buffer was added. The samples were then boiled at 100^{o}C for 3 minutes before loading

into the individual wells of the stacking gel.

2.6. COOMASSIE BLUE STAINING.

2.6.1. Coomassie Blue Stain.

0.25% (w/v) Coomassie blue G-250
45% (v/v) ethanol
10% (v/v) glacial acetic acid

2.6.2. 'Destain' for Coomassie Blue.

45% (v/v) ethanol
10% (v/v) glacial acetic acid

After electrophoresis the gels were stained with Coomassie blue for 1hr. The gels were then placed in 'destain' to remove the background colour and allow examination of the stained protein bands.

2.7. SILVER STAINING.

The silver stain method of Tsai and Frasch (1982) was also used to detect protein bands. Double-deionised water was used both in preparation of all glassware and throughout the experimental procedure.

2.7.1. Solutions for Silver Stain.

Fixing Solution.

40% (v/v) ethanol
10% (v/v) glacial acteic acid

Oxidising Solution.

0.7% (w/v) periodic acid in fixing solution.

Silver Stain.

28 ml 0.1N sodium hydroxide
2 ml concentrated ammonia
5 ml 20% (w/v) silver nitrate
115 ml water

Developer (200ml).

0.005% (v/v) citric acid
0.1 ml 37% w/v formaldehyde

Stabilising Solution.

3% (v/v) glacial acetic acid

2.7.2. Experimental Procedure.

After the completion of electrophoresis the gel was placed in the fixing solution overnight. The gel was transferred to 100ml oxidising solution for 5 min. with shaking, and then washed for 45 min. with at least 4 changes of water (500ml/wash). After the last wash, the silver stain (150ml) was added and the gel incubated at room temperature for 10 min. with shaking. The silver stain was removed and the gel washed for 30 min. with 4 changes of water (500ml/wash), followed by addition of the developer (200ml) until the protein banding pattern of required intensity was observed. The developed gel was stabilised by washing with water and then adding 200ml of stabilising solution. Before the gel could be dried, excess water had to be removed by placing the gel in 45% (v/v) ethanol; 10% (v/v) glacial acetic acid.

2.8. AUTORADIOGRAPHIC PROCEDURES.

2.8.1. Gel Preparation.

The gels were immersed in 45% (v/v) ethanol, 10% (v/v) glacial acetic acid for 15 -18hr and then dried under vacuum at 80^{o}C on to Whatman 3 MM filter paper. The gel and the X-ray film were then placed in a cassette containing a Dupont 'Cronex' intensifier screen, and placed at -70^{o}C for the required time.

2.8.2. Development of Autoradiograph.

After exposure the autoradiographic images were visualised by

immersing the film in DX-80 developer for 5 min., rinsing with water, then immersing in FX-40 fixer for 1 min. The film was then rinsed with water and allowed to air-dry.

2.9. ACTIVITY STAINING FOR RIBONUCLEASE A.

2.9.1. Solutions Required for Gel Activity Staining for Ribonuclease A.

Solution A:- 25% (v/v) isopropanol
0.01M Tris-HCl, pH 7.4

Solution B:- 0.01M Tris-HCl, pH 7.4

Solution C:- 0.1M Tris-HCl, pH 7.4

Toluidine Blue 'O' stain:- 0.1% (w/v) toluidine blue 'O'
0.01M Tris-HCl, pH 7.4

All solutions were filtered and stored at room temperature.

2.9.2. Ribonuclease A Activity Gel.

To detect ribonuclease A activity polyacrylamide gels containing yeast ribonucleic acid, an enzymatic substrate, were utilised. The method is that described by Blank et al., 1982.

To the 12.5% SDS-polyacrylamide gel mixture, as described for SDS-PAGE (Section 2.5.2) yeast ribonucleic acid was added to give a final concentration of 0.5 mg/ml. The gel mixture was then poured into the casting apparatus and allowed to polymerise around the teflon comb.

To each protein sample, one-fifth volume of non-reducing loading buffer was added. The sample was boiled for 3 min. at 100^{o}C, before being loaded. Electrophoresis at a constant current of 50mA for 3hr, was carried out at 4^{o}C using pre-cooled tris-glycine buffer (electrophoresis buffer).

The gel was then washed twice in 400ml of solution A for 30 min. at

room temperature, whilst shaking constantly. Isopropanol was included to facilitate the removal of SDS from the gel. The gel was then rinsed with 400ml of solution B (two changes) for 30 min. Enzymatic digestion of the yeast ribonucleic acid contained within the gel matrix occurred when the gel was incubated in 400ml solutin C at 37°C for 2hr. Again the gel was rinsed with 400ml solution B for 10 min. The undigested ribonucleic acid is stained with the toluidine blue '0' solution for 10 min. at room temperature. The gel was placed in 400ml solution B to destain. Solution B was replaced daily over a period of 2-3 days. Any clear "plaques" which were visualised were the result of ribonuclease A activity.

2.10. IMMUNOBLOTTING.

Proteins transferred on to the nitrocellulose paper were detected using enzyme conjugated anti-sera as described by Towbin, et al., (1979).

2.10.1. Immunoblotting Stock Solutions.

Transfer Buffer.

0.025M Tris-HCl,pH 8.6
0.16M glycine
20%(v/v) methanol
0.02% (w/v) sodium dodecyl sulphate

Stain.

0.01% (w/v) napthol blue-black
10% (v/v) glacial acetic acid
50% (v/v) methanol

Destain.

10% (v/v) glacial acetic acid
50% (v/v) methanol

Substrate Stock Solution.

0.3% (w/v) 4-chloro-1-napthol in methanol

Stored in dark bottle at 4°C.

Working Solution.

3ml 4-chloro-1-napthol stock solution
5ml PBS, pH 7.2
0.01% (v/v) hydrogen peroxide

Blocking Buffer.

5% (v/v) normal goat serum in PBS, pH 7.2

Antibody.

Anti-ricin or Anti-RNase A, 1:200 dilution of antibody in blocking buffer.

Conjugate.

1:1000 dilution goat anti-rabbit IgG (H+L) horse radish peroxidase in blocking buffer.

2.10.2. Experimental Protocol.

Samples were applied as triplicate or quadruplicate sets to 12.5% SDS-PAGE polyacrylamide gels (See Section 2.5.). After electrophoresis the gel was sectioned. Sponge layers, filter papers and nitrocellulose papers were soaked in transfer buffer then layered within the blotting cassette(s), ensuring that no air bubbles were present between the gel and nitrocellulose paper which would inhibit transfer of protein band(s). The cassette was then closed and placed in the transfer tank containing 3 litres of transfer buffer. The negatively charged protein(s) are then transferred to the nitrocellulose paper by means of a constant current of 350mA for 2.5 - 3hr.

When the transfer was completed one section of nitrocellulose paper

was stained in napthol blue-black for 3 min. and then destained to determine whether the proteins had actually transferred.

The remaining sections of nitrocellulose were then incubated in 20ml blocking buffer for 1hr at room temperature, in order to block any remaining sites on the paper. The nitrocellulose was then washed with PBS, pH 7.2 (3 buffer changes) for 30 min. at room temperature. The appropriate antibody in blocking buffer was added and incubated overnight at $4^{o}C$.

The protocol for the PBS, pH 7.2 wash was repeated. The nitrocellulose strip was then incubated with goat anti-rabbit IgG (H+L) horse radish peroxidase in blocking buffer, for 1hr, at room temperature. The nitrocellulose was again washed with PBS, pH 7.2 (3 changes) for 30 min. at room temperature. The addition of the substrate for horse radish peroxidase, 4-chloro-1-napthol, present in the working solution, allowed visualisation of bands, on the nitrocellulose filter where protein / antibody / goat anti-rabbit IgG horse radish peroxidase interactions occurred. The nitrocellulose strips were developed at room temperature in the working solution until distinctly coloured bands were seen at which point the filters were rinsed in deionised water and air dried.

2.10.3. Preparation of Rabbit Anti-Ribonuclease Antiserum.

Primary immunisations were prepared by mixing 0.5ml PBS, pH 7.2 containing 100-200ug of purified protein with 0.5ml of Freunds complete adjuvant (Difco). An emulsion was formed by sonication for 10 seconds at probe setting 'low'. The emulsion was injected sub-cutaneously in 3 - 5 sites on the rabbit's back (0.1 - 0.2 ml per site). Four weeks after the initial challenge the rabbit received a booster dose, made using Freunds incomplete adjuvant (Difco). Immune serum, denoted RD7, was collected one week later by bleeding from the ear and again at intervals following subsequent booster injections.

The blood was centrifuged at 1000 x g for 15 min. and the serum was removed. This procedure was repeated twice, and the serum removed was pooled.

2.11. TWO-DIMENSIONAL GEL ELECTROPHORESIS.

In order to determine the net charge of the protein complexes, two dimensional gel electrophoresis was used.

2.11.1. Isoelectric Focusing in the First Dimension.

Gel Solution.

5.5% (w/v) Urea
1.33ml 28.3% (w/v) Acrylamide / 1.62%(v/v) bisacrylamide
2.00ml 10% nonidet P-40
1.95ml de-ionised water
0.40ml Pharmalyte ampholines pH 5-8
0.10ml Pharmalyte ampholines pH 3.5-10
0.10ml 10% (w/v) ammonium persulphate
0.005ml TEMED

Electrophoresis Solutions.

Upper Resevoir.

20mM sodium hydroxide

Lower Resevoir.

10mM phosphoric acid

Sample Buffer.

9.5M urea
2.0%(v/v) NP-40
1.6% (v/v) Pharmalyte ampholines pH 5-8
0.4% (v/v) Pharmalyte ampholines pH 3-10

Overlay Buffer.

50% (v/v) sample buffer in deionised water.

NEPHGE Buffer.

2.3% (w/v) SDS
10.0% (v/v) glycerol
0.1% (w/v) bromophenol blue

2.11.2. SDS-PAGE Gel Electrophoresis in the Second Dimension.

10% (w/v) acrylamide resolving gels and 5%(w/v) acrylamide stacking gels were employed. The procedures were as described in section 2.5.

2.11.3. Experimental procedure.

Gel tubes (12.0 x 0.2cm) with nescofilm enclosing the bottom end, were filled to within 1cm of the top with the first dimension gel solution. The acrylamide solution was then overlaid with deionised water and left to polymerise. The deionised water was removed, and replaced with 25ul sample buffer for 20 min. as this equilibrated the gels. The sample buffer was then replaced with a further 25ul of sample buffer and placed in the electrophoresis tank. Appropriate electrophoresis solutions were then placed in the upper and lower reservoirs and the gels pre-run at the following voltages, sequentially, 200 volts for 15 min., 300 volts for 30 min. and 400 volts for 30 min. After this treatment the solution was removed from the upper reservoirs and the liquid above the gels carefully aspirated. The sample and sample buffer were mixed at a ratio of 1:1 to give a total volume of 24ul. The sample was then loaded on to the tube gels and overlaid with 25ul of overlay buffer and electrophoresed for a total of 7000 volt hours.

The gels were then removed from the tubes and equilibrated for 1hr in NEPHGE buffer. A 10% SDS-PAGE separating gel was prepared, but the stacking gel was poured to within 1cm of the upper edge of the front plate and overlaid with 1ml of butan-2-ol and allowed to polymerise. The butan-2-ol was removed and the gel surface rinsed with deionised water. The equilibrated tube gel was gently inserted between the gel plates ensuring that there were no air-bubbles present between the tube gel and the stacking gel. Electrophoresis in the second dimension was carried out exactly as for SDS-PAGE (Section 2.5.4.). The second dimension gel was

then autoradiographed (Section 2.8.).

2.12. TISSUE CULTURE PROCEDURES.

2.12.1. Heat Inactivation of Foetal Calf Serum.

Foetal calf serum (FCS) was inactivated by heating at 56°C for 30 min. The serum was then stored at -20°C.

2.12.2. Cell Culture Medium.

The composition of RPMI-1640 is shown in Table 2. 10% (v/v) heat inactivated foetal calf serum was added, and this was termed complete RPMI-1640.

2.12.3. Routine Culture.

Daudi cells,as suspension cultures, were grown in complete medium at 37°C in a 95% air / 5% CO_2, humidified incubator. The cells were sub-cultured every three to four days.

2.12.4. Cell Counting and Determination of Cell Viability.

To ensure an even dispersal of cells throughout the medium, cells were gently pipetted a number of times. An aliquot of cells was then removed and counted in the Neubauer haemocytometer.

Cell viability was determined on the basis of the uptake of the dye trypan blue by dead cells. A known volume of cells was centrifuged at 500 x g for 5 min., the supernatant was removed and the cells were resuspended in the same volume of 0.2%(w/v) trypan blue in PBS, pH 7.2. The percentage cell viability was estimated by counting live and dead cells in the haemocytometer according to the following equation:-

$$\text{Viability} = \frac{\text{Number of live cells}}{\text{Total number of cells}} \times 100\%$$

TABLE 2

COMPOSITION OF RPMI-1640 MEDIUM

Amino Acids	mg/litre
L-Arginine (free base)	200.0
L-Asparagine	65.0
L-Aspartic Acid	20.0
L-Cysteine (2HCl)	65.0
L-Glutamic Acid	20.0
Glycine	10.0
L-Histidine (free base	15.0
L-Hydroxyproline	20.0
L-Isoleucine (allo free)	50.0
L-Leucine (methionine free)	50.0
L-Lysine HCl	40.0
L-Methionine	15.0
L-Phenylalanine	15.0
L-Proline (Hydroxy-L-Proline free)	20.0
L-Serine	30.0
L-Threonine (allo free)	20.0
L-Tryptophan	5.0
L-Tyrosine	28.94
L-Valine	20.0
Inorganic Salts	
$Ca(NO_3)_2\ 4H_2O$	100.0
KCl	400.0
$MgSO_4$	48.84
NaCl	6000.0
Na_2HPO_4 (anhydrous)	800.0
Vitamins	
Biotin	0.2
D-Calcium Pantothenate	0.25
Choline Chloride	3.0
Folic Acid	1.0
i-Inositol	35.0
Nicotinamide	1.0
Para-aminobenzoic acid	1.0
Pyrodoxine Hydrochloride	1.0
Riboflavin	0.2
Thiamine Hydrochloride	1.0
Vitamin B_{12}	0.005
Other Components	
Glucose	2000.0
Phenol red	5.0
Reduced glutathione	1.0

2000 mg/litre $NaHCO_3$ was added separately to dissolved medium.

The pH was adjusted to 7.0 with concentrated HCl. The media was sterilized by filtration and stored at $4^{\circ}C$.

2.13. CELLULAR CYTOTOXICITY ASSAYS.

2.13.1. [^{3}H]-Thymidine Labelling of cells.

Cells were counted and harvested from healthy log phase cultures, by centrifugation at 500 x g for 5 min. The supernatant was discarded and the cells resuspended in RPMI-1640 and again centrifuged at 500 x g for 5 min. This process was repeated once more before finally resuspending the cells at a concentration of 2.5 x 10^6 cells/ml in RPMI-1640. 1 x 10^5 cells were added to individual microwells of a ninety-six well plate.

1uM stock solutions of ricin, ricin B chain, RNAse A and RNAse A-ricin B were used to make 1:10 serial dilutions to give a final concentration of 10nM, 1nM, 0.1nM and 0.01nM in individual, cell-containing microwells. A set of control wells were also prepared. The final volume in each microwell was 100ul. The microwell tray was then placed for 1hr in a humidified CO_2 incubator at 37^{o}C.

After 1hr, the cells contained in the microwell tray were centrifuged at 500 x g for 10 min. the supernatant was removed and the cells were resuspended in RPMI-1640. The centrifugation and resuspension of the cells was repeated three times. After the final centrifugation the cells were resuspended in complete medium. The cells were then incubated at 37^{o}C for 24hr in a humidified CO_2 incubator. 10ul [^{3}H]-thymidine solution (1uCi) were added to each of the wells and then incubated at 37^{o}C for 4hr.

The cells were then harvested on to a glass fibre filter mat from the microwell tray using a Flow 'Titertek' Cell Harvester. After the filter mat had air dried, the individual discs were removed from the strip, placed in scintillation vials containing 3ml ecoscint and counted. The percentage incorporation of [^{3}H]-thymidine into the treated cells compared to the control were then calculated and the results shown graphically.

2.13.2. Cells labelled with [^{35}S]-Methionine.

Cells were counted, harvested and centrifuged at 500 x g for 5 min. The supernatant was removed and the cells were resuspended in RPMI-1640 minus L-methionine. The cells were again centrifuged and resuspended in RPMI-1640 minus L-methionine (repeated twice). The cells were finally resuspended at a concentration of 2.5 x 10^6 cells/ml. If the assay to be carried out was to determine the purity of the ricin B then the plate was laid out in the following way: to four separate wells in each of four rows of a twenty-four well costar plate, the following solutions were added (a) 60ul RPMI-1640 minus L-methionine, (b) 60ul 100nM - 0.1nM ricin solution, (c) 60ul 100nm - 0.1nM ricin B chain solution and (d) 60ul 100nM - 0.1nM purified ricin B chain, 540ul of cell suspension was added to each of the wells, gently mixed and incubated at 37^{o}C for 1hr in a humidified CO_2 incubator.

After the 1hr incubation 10ul FCS and 10ul of [^{35}S]-methionine (0.5uCi/ul), were added to each of the wells. The cells were again incubated at 37^{o}C, whilst at 3hr and 24hr time points, three 50ul samples were removed. To each of the samples 20ul ice-cold 60% TCA was added, mixed, and left on ice for 20 minutes. The total sample volume was then spotted on to 3MM Whatman filter discs and air-dried. The filter discs were then given two washes in ice-cold 10% TCA (150ml) over a 1hr period. The discs were then rinsed in absolute alcohol and allowed to dry.

Individual discs were then placed in scintillation vials containing 3ml ecoscint and counted for 1 min. in the scintillation counter. The resulting counts were used to calculate the percentage inhibition of protein synthesis by the toxins compared to the control.

If the toxicity of the RNAse A-ricin B complex was being assayed the protocol described was followed exactly but with the inclusion of this sample.

2.14. BINDING AND UPTAKE OF IODINATED RIBONUCLEASE A - RICIN B COMPLEX BY DAUDI CELLS.

2.14.1. Iodinated Samples.

Each sample contained 5×10^4 cpm per 50ul. Samples 'a', 'b', and 'c' contained only [^{125}I]-ribonuclease A, while samples 'd', 'e', and 'f' contained [^{125}I]-ribonuclease - ricin B chain complex. Samples 'a', 'b', 'd' and 'e' were prepared in RPMI-1640 and samples 'c' and 'f' were in RPMI-1640 containing 0.2M galactose.

2.14.2. Procedure.

Daudi cells from log phase cultures were counted, harvested, washed twice and resuspended at a concentration of 5.0×10^4 cells/ul in RPMI-1640.

To sterile, labelled eppendorf tubes containing the iodinated samples listed above, 2.5×10^6 cells / 50ul were added. The cells were incubated at 37°C for 2hr, then centrifuged at 500 x g for 5 min. and the supernatant discarded. Samples 'a', 'c', 'd' and 'f' were washed five times with RPMI-1640, whilst 'b' and 'e' were washed five times with RPMI-1640 containing 0.5M galactose. After the fifth wash, all cell samples were resuspended in 100ul RPMI-1640 and then transferred to counting tubes. The tubes were counted on an LKB Wallac '1275 Minigamma' counter. Results were calculated according to the following equation:-

$$\text{Uptake} = \frac{\text{Final cell-associated cpm.}}{\text{Input cpm.}} \times 100\%.$$

and presented as a bar chart.

RESULTS AND DISCUSSION

3. PURIFICATION OF THE RICIN B CHAIN.

3.1. Detection of Ricin in Commercially-purified Ricin B Chain.

SDS-PAGE gel electrophoresis followed by silver staining of the protein bands revealed that the ricin B chain obtained from the commercial suppliers, E.Y. Laboratories and Sigma Chemical Co. Ltd. contained ricin holotoxin (Figure 3). Silver staining was chosen to detect the protein bands since both ricin and ricin B are glycoproteins and the silver stain was able to detect both the carbohydrate and the protein, whilst coomassie blue stains glycoproteins very poorly (Tsai and Frasch, 1981).

Figure 3, track B shows ricin B chain obtained from the Xoma Corporation. The low molecular weight band of approximately 30 kilodaltons is ricin B chain whilst the higher molecular weight band corresponds to disulphide-bonded ricin B chain dimers of approximately 60 kilodaltons. Ricin in track D can be seen to exist as two molecular weight forms, 'heavy' (approx. 66 Kd) and 'light' (approx. 64 Kd). The 'heavy' form is due to the presence of two N-linked oligosaccharides on the ricin A polypeptide (therefore termed ricin A_2) of the ricin molecule, whilst the 'light' form has only one N-linked oligosaccharide on the ricin A polypeptide (termed ricin A_1) of the ricin holotoxin (Foxwell, et al., 1985). Track C shows the protein bands visualised by silver staining of electrophoretically-separated ricin B chain purified by E.Y. Laboratories and Sigma Chemical Co. The electrophoretic mobilities of the high molecular weight protein bands correspond precisely with those of 'heavy' and 'light' forms of ricin; dimers of the ricin B chain are also present.

Due to the presence of ricin in the ricin B chain preparations obtained from E.Y. Laboratories and Sigma Chemical Co., procedures for

Figure 3. Detection of Ricin Contamination in Commercially-produced Ricin B Chain.

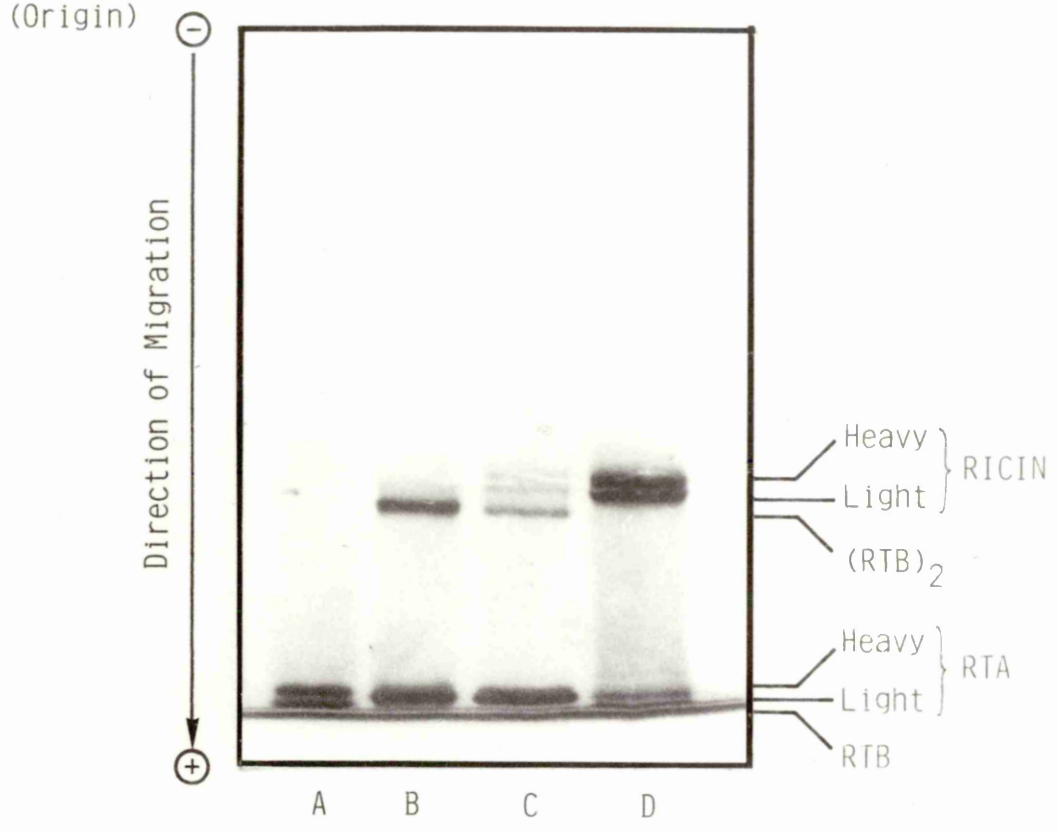
Samples of ricin A chain, ricin B chain (Xoma and E.Y. Laboratories) and ricin were analysed by SDS-PAGE on 8.5% (w/v) acrylamide gels. After electrophoresis protein bands were detected by silver staining.

Tracks:- A - ricin A polypeptide

B - ricin B polypeptide, Xoma

C - ricin B polypeptide, E.Y. Laboratories

D - ricin


(Origin)
Direction of Migration
Heavy
Light
RICIN
(RTB)2
Heavy
Light
RTA
RTB
A
B
C
D

purification of the B chain were developed.

3.2. FPLC - Gel Filtration.

As there is a two-fold difference in the molecular weights of ricin and ricin B chain, FPLC-gel filtration appeared to be a suitable system for rapid and reproducible purification of the ricin B chain.

Figure 4 shows the result of the initial experiment in which 200ul (1ug/ul) of protein which had been dialysed against 0.1M Tris-HCl, pH 7.2 (to remove sodium azide) was applied to the column. The chromatogram showed only one protein peak suggesting that there had been no separation of ricin B chain from ricin. This was confirmed by SDS-PAGE analysis (Figure 4, panel b). As these conditions did not result in the separation of ricin from ricin B polypeptide, the buffer was changed to PBS, pH 7.2. The resulting chromatogram and SDS-PAGE analysis (results not shown) of the fractions were indistinguishable from Figure 4. The explanation for this is probably the formation of aggregates of ricin and ricin B chain in solution. It can be argued that these aggregates are the result of either specific or non-specific interactions between the ricin B polypeptides and ricin holotoxin leading to the formation of complexed proteins. Thus, aggregation of ricin and the ricin B polypeptide could be a reflection of complexes which are naturally formed within the protein bodies in the endosperm tissue of Ricinus communis for the storage of ricin.

To disrupt these protein aggregates, ammonium sulphate was used to precipitate the proteins from solution. The precipitated proteins were then redissolved and dialysed before application to the FPLC-gel filtration column. The resulting chromatogram (Figure 5, panel a) showed that the sample had been separated into two protein peaks. This was consistent with the predicted behaviour of the sample during gel filtration since the column is unable to separate the three high

molecular weight input components (i.e. ricin 'heavy' and 'light' forms and ricin B dimers) due to the similarity of their molecular weights. When these protein peaks were analysed by SDS-PAGE (Figure 5, panel b) the data showed that the first protein peak (lane A) consisted mainly of ricin 'heavy' and 'light' forms, although there were traces of ricin B polypeptide present. Lane B showed that there was an extremely low level of ricin present in the second peak, and that it consisted mainly of ricin B chain.

Denaturing conditions are clearly required for the breakdown of the interactions occurring between ricin and ricin B polypeptides. The denaturing conditions of SDS-PAGE also leads to the separation of ricin and ricin B chain. This suggested, therefore, that it was not an inherent property of the ammonium sulphate which led to the separation of the two proteins by FPLC-gel filtration.

Although this combined method of precipitation and FPLC-gel filtration was leading to purification of the ricin B chain, there were a number of factors which detracted from this technique. Firstly, after ammonium sulphate precipitation, it proved extremely difficult to redissolve the protein precipitate even after dialysis to remove the ammonium sulphate, so there was a loss of protein at this initial stage. Secondly, the maximum sample volume which could be loaded on the FPLC-gel filtration column was 200ul containing a maximum protein load of 5-6mg. Therefore, in order to purify a large volume of the protein, multiple separations would be necessary, and the separated fractions would require to be pooled and concentrated, manoeuvres which would certainly incur further protein losses. Thirdly, since the ricin B polypeptide had not been purified to homogeneity, at least one more separation by FPLC-gel filtration would have been required. This would, again, have led to a loss of protein, and a decrease in total yield.

Figure 4. Purification of Ricin B Chain Using FPLC gel Filtration.

Panel a. The chromatogram resulting when 200ug unpurified ricin B polypeptide was applied to the FPLC-gel filtration column is shown. The column was equilibrated with 0.1M Tris-HCl, pH 7.2, and the sensitivity of the meter measuring the absorbance was set at 0.1.

Panel b. Samples from the three fractions forming the single protein peak, were analysed on 12.5% SDS-PAGE gels. The protein bands were silver stained. Lanes B, C and D show that all the fractions contain ricin, ricin B dimers and ricin B polypeptide.

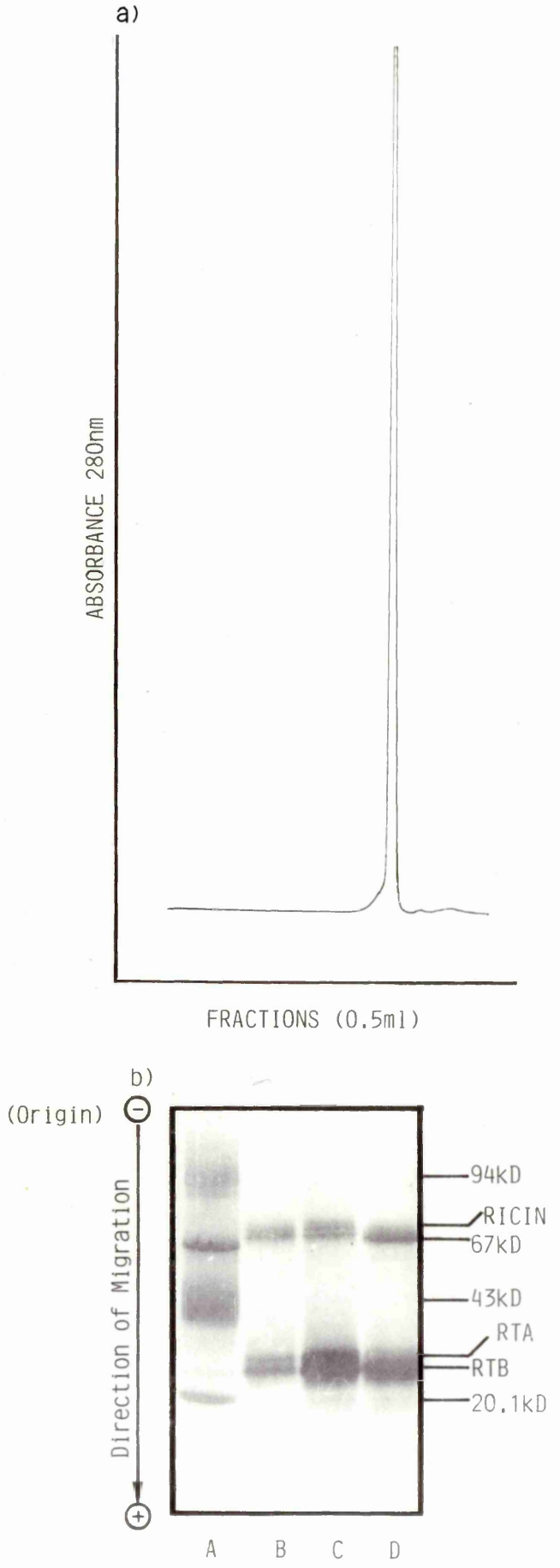
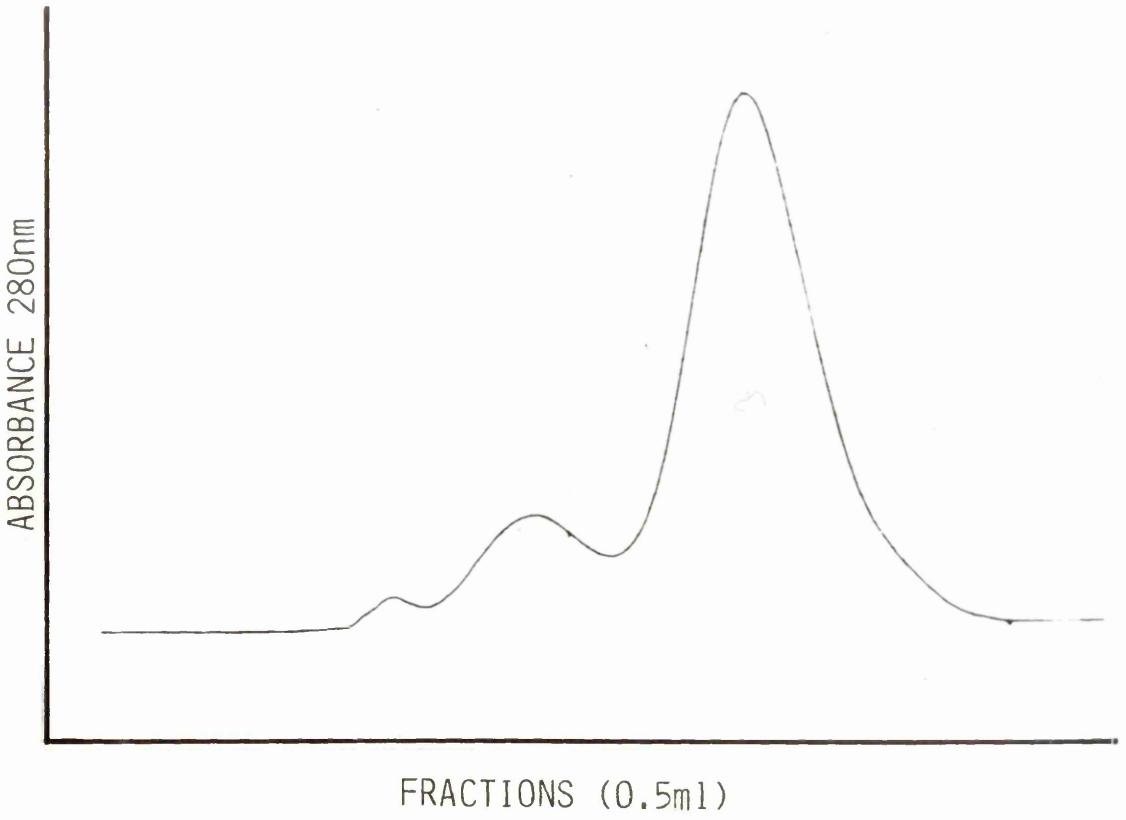

a)
ABSORBANCE 280nm
FRACTIONS (0.5ml)
b)
(Origin)
Direction of Migration
94kD
RICIN
67kD
43kD
RTA
RTB
20.1kD
A
B
C
D

Figure 5. Ammonium Sulphate Precipitation and FPLC Gel Filtration of Ricin B Chain.

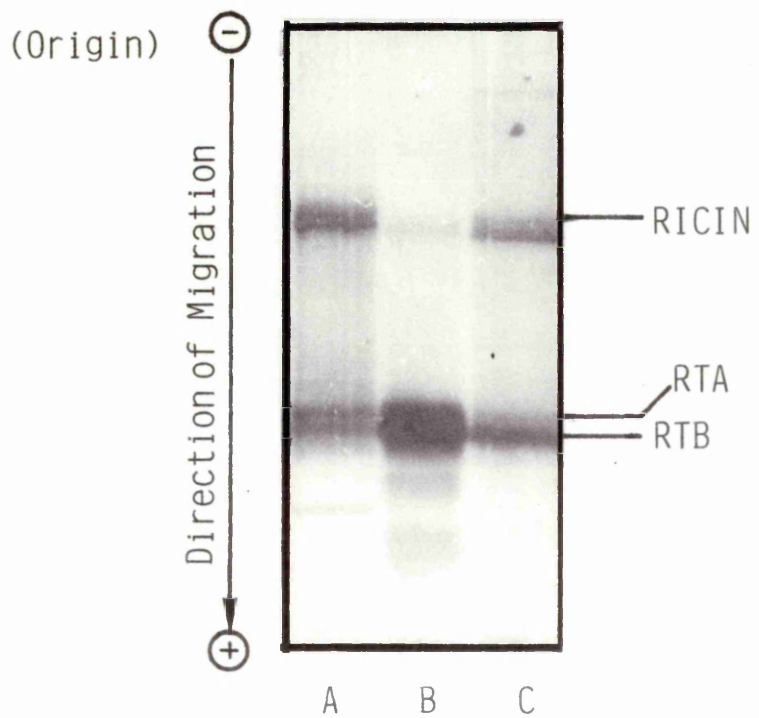
Panel a. The chromatogram showed that ammonium sulphate precipitation followed by gel filtration, resulted in separation of high molecular weight protein(s) from the lower molecular weight protein. The FPLC-Superose 12 column had been equilibrated with 0.1M Tris-HCl, pH 7.2 and the sensitivity of the meter measuring absorbance at 280nm was set at 0.1.

Panel b. The fractions forming the first protein peak were pooled as were the fractions forming the second protein peak. A sample was removed from each, and analysed by SDS-PAGE 12.5% (w/v) acrylamide. The protein bands were detected by silver staining, lane A-peak 1, lane B-peak 2 and lane C-unpurified ricin B chain.

a)


ABSORBANCE 280nm
FRACTIONS (0.5ml)

b)


(Origin)
Direction of Migration
RICIN
RTA
RTB
A
B
C

3.3. Chromatofocusing.

Figure 6, panel a, shows the development of the pH gradient in the column from pH 6.0 - 4.0 and Figure 6, panel b illustrates the A_{280} profile across the gradient. The first protein peak eluted at a pH of approximately 6.0. This peak was expected to contain ricin since the isoelectric point of the holotoxin is 7.1. The protein would carry a net positive charge at pH 6.0, would not adsorb to the column, and would therefore be predicted to elute in the void volume. There was a second major peak at approximately pH 4.8 and this would be predicted to contain only ricin B chain. Given that the isoelectric point of the ricin B chain is 4.8, it would bind to the anion exchanger, and only desorb from the column matrix once the pH of the eluting medium dropped below its pI. When the fractions from the protein peaks were analysed by SDS-PAGE (Figure 7) the data confirmed that ricin B chain had been separated from ricin; i.e. there was no minor contamination of either the ricin B fractions with ricin holotoxin, or vice versa.

It was interesting that the polybuffer 74, which is an amphoteric buffer designed specifically for chromatofocusing in the range pH 7-4 in conjunction with the polybuffer exchanger, separated the proteins without any prior denaturing treatment. Thus, any aggregates were probably dissociated because of the positively charged polybuffer exchanger which would strongly repel cations (ricin) from the matrix. This process could also have been aided by the ampholyte species present in the polybuffer 74.

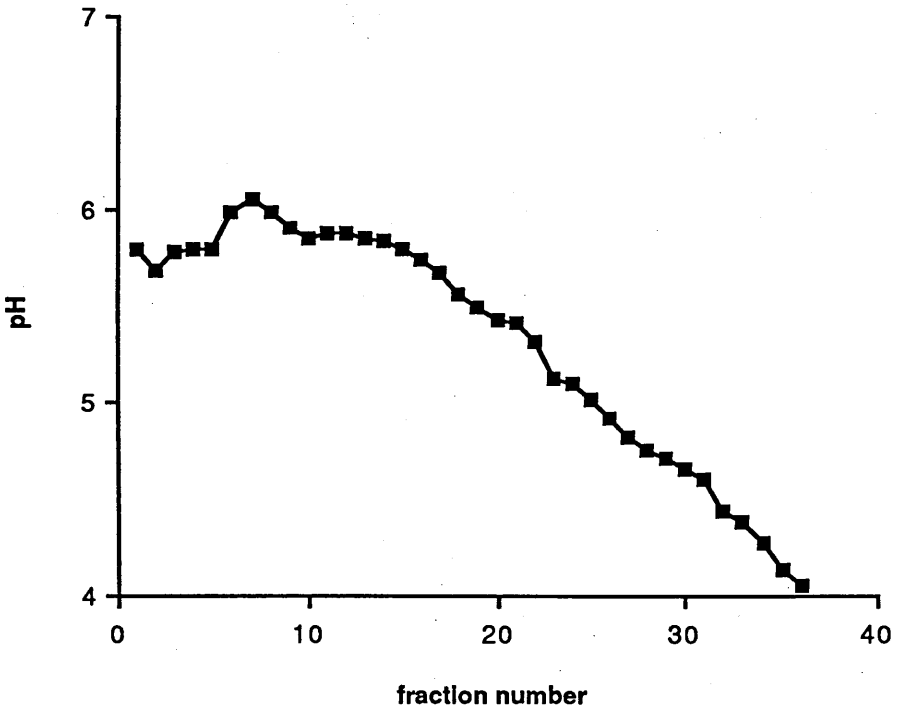
Before the 'purified' B chain could be used experimentally it had to be separated from the polybuffer. A gel matrix containing galactose binding sites, Sepharose 4B, was selected for this purpose. The ricin B polypeptide attached to the column matrix by means of its lectin binding sites, whilst the polybuffer passed through the column. The elution of

Figure 6. Chromatofocusing Profiles.

Panel a. The development of an approximately linear pH gradient for elution of the chromatofocusing column with polybuffer 94, pH 4.0, is shown.

Panel b. The absorbance at 280nm of each of the fractions is shown on the chromatogram. The first peak eluted at a pH of approximately 6 and the second peak at a pH of approximately 4.8.

a)


7
6
5
4
pH
0
10
20
30
40
fraction number

b)

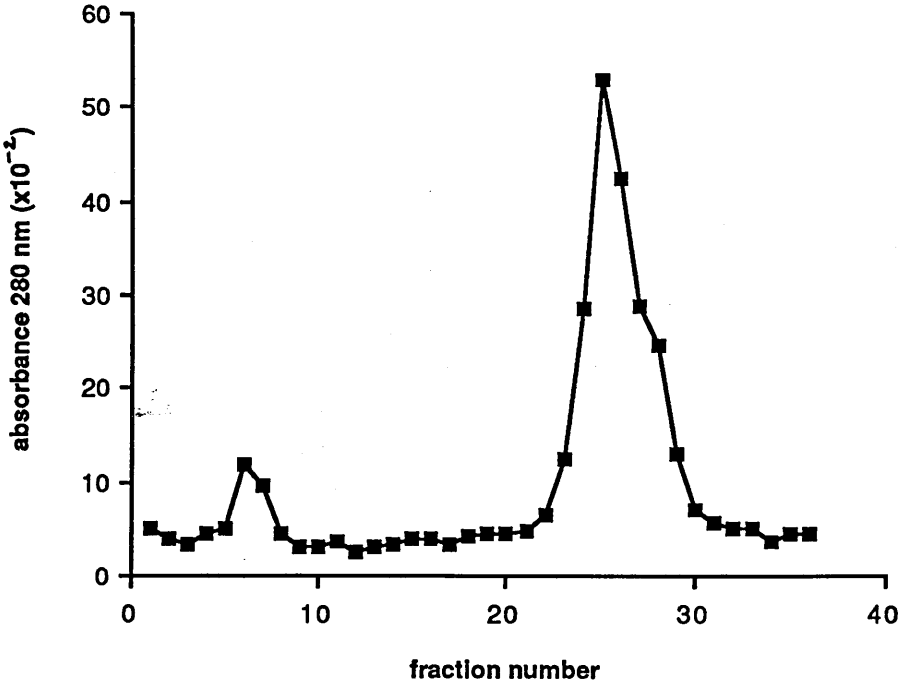
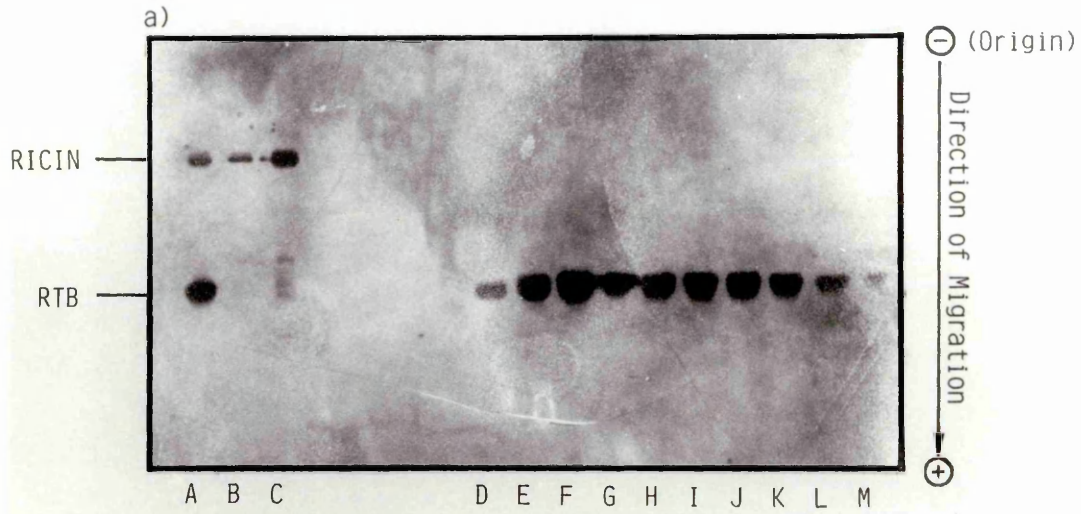
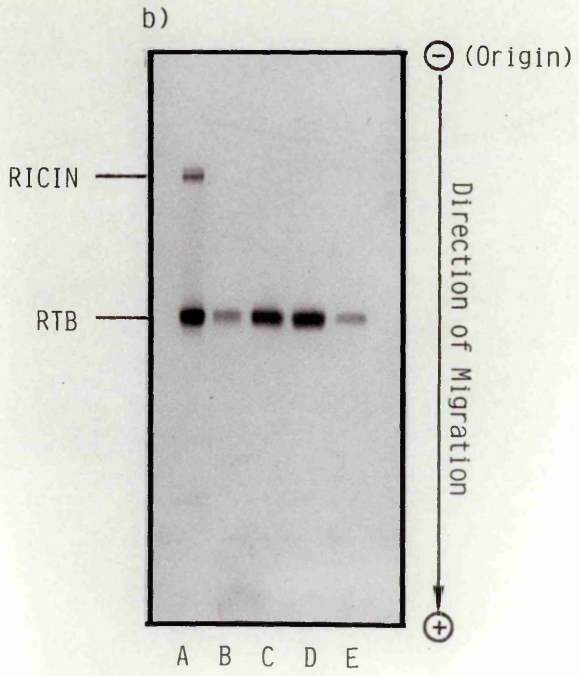

60
50
40
30
20
10
0
absorbance 280 nm (x10⁻²)
0
10
20
30
40
fraction number

Figure 7. SDS-PAGE Analysis of Protein Peaks.

Panel a. The fractions forming the protein peaks eluted from the chromatofocusing column were analysed by SDS-PAGE. The protein bands were detected by silver staining. Lanes B and C, samples from fractions constituting peak 1, lanes D to M, samples from fractions constituting peak 2.

Panel b. The fractions from the protein peak eluted from the Sepharose 4B column using PBS, pH 7.2 containing 0.1M galactose were analysed by SDS-PAGE 12.5% (w/v) acrylamide. This confirmed that the ricin B chain had been purified (lanes B,C and D) from ricin (lane A).


a)
RICIN
RTB
(Origin)
Direction of Migration
A B C D E F G H I J K L M


b)
RICIN
RTB
(Origin)
Direction of Migration
A B C D E

polybuffer from the column was determined by measuring the absorbance at 254nm since polybuffer absorbs light slightly at this wavelength. The ricin B chain was then eluted using PBS, pH 7.2 containing 0.1M galactose, as the ricin B polypeptide has a greater affinity for the free galactose than the binding sites of the column matrix. Analysis by SDS-PAGE of the fractions from the protein peak from the Sepharose 4B column (Figure 7, panel b) confirmed the purity of the ricin B polypeptide. The binding and desorption of the ricin B chain also confirmed that the polypeptide had retained its ability to function as a lectin.

The yield of purified ricin B polypeptide from the commercial preparations varied, but this was mainly due to the differences in the level of contamination of the commercial preparations with ricin.

3.4. Toxicity Assay.

Incorporation of [^{35}S]-methionine into the newly-synthesised proteins of cells in culture is a sensitive assay system for determining the toxic action of ricin. This assay was therefore used to ascertain the purity of the ricin B chain which had been separated from holotoxin contaminants by chromatofocusing.

Figure 8 shows the results of the assay after labelling cellular proteins for 3hr and 24hr with [^{35}S]-methionine, following a 1hr treatment of the cells with the different proteins. Ricin was extremely toxic to the cells, greatly reducing the level of protein synthesis. This was most pronounced in the data obtained from the 24hr labelling, since even the lowest concentration (0.1nM) was exerting a cytotoxic effect. The titration of toxicity for commercial, unpurified ricin B chain parallel those obtained for ricin after 3hr and 24hr labelling periods with [^{35}S]-methionine, whilst the ricin B chain purified by the techniques described above has little or no effect on protein synthesis

Figure 8. Toxicity of Purified Ricin B Chain to Daudi Cells.

Daudi cells were incubated at 37°C for 1hr with the various concentrations of ricin, unpurified ricin B chain and 'purified' ricin B chain indicated on the graphs. Foetal calf serum (10% v/v) and 10uCi [^{35}S] methionine were added to the cells and incubated at 37°C for 3hr or 24hr before being harvested, TCA precipitated and counted in the scintillation counter.

Panel a. 3hr labelling of the cells with [^{35}S]-methionine.

Panel b. 24hr labelling of the cells with [35S]-methionine.

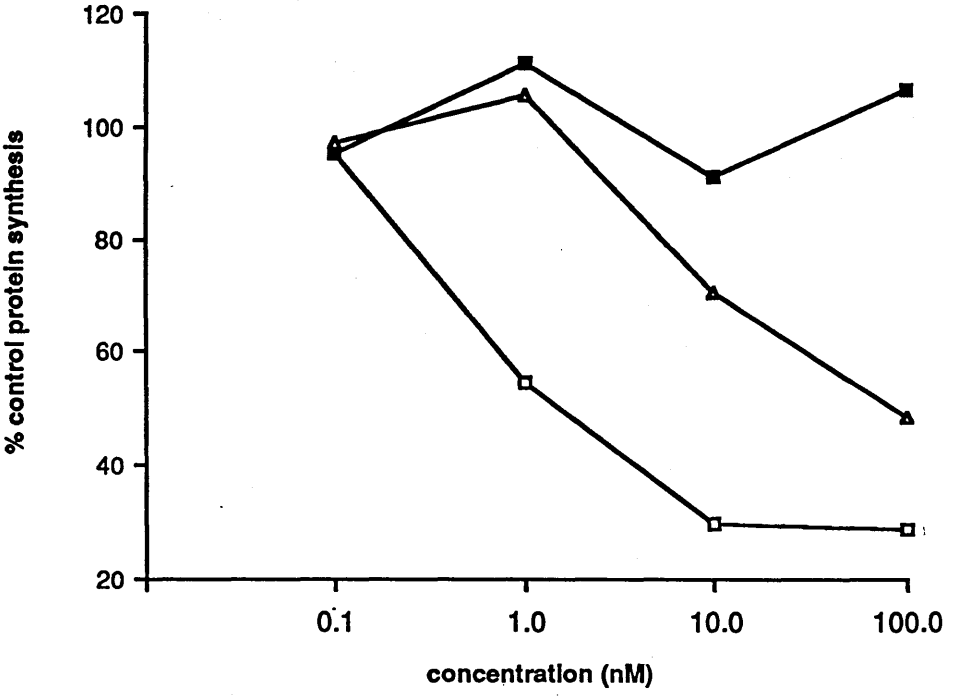
KEY:-

□ - Ricin

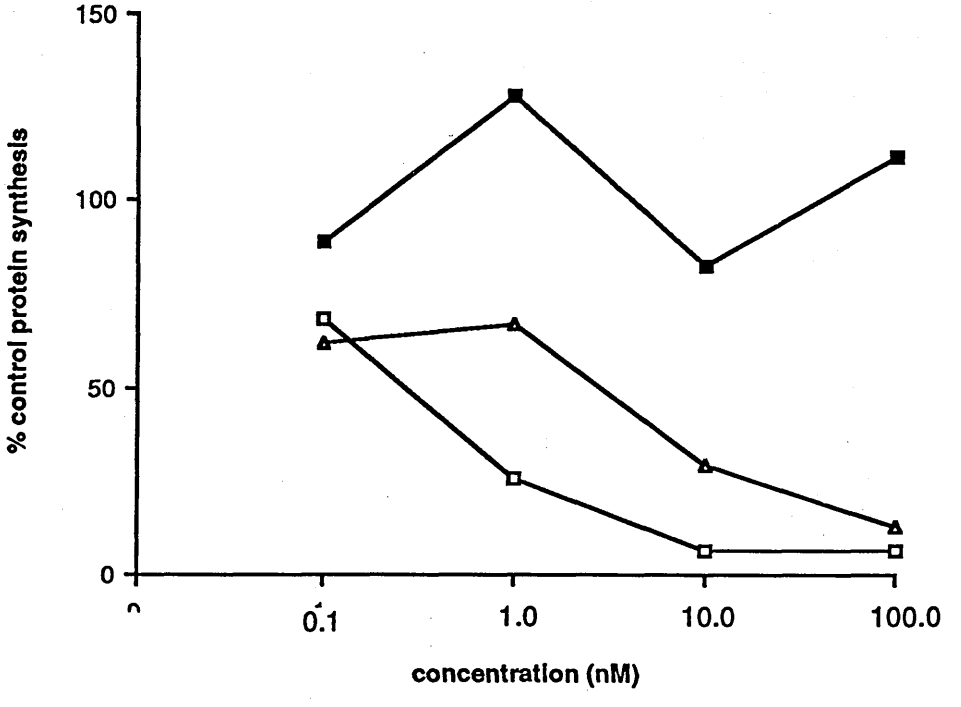
△ - Unpurified Ricin B chain

■ - Purified Ricin B chain

a)


120
100
80
60
40
20
% control protein synthesis
0.1
1.0
10.0
100.0
concentration (nM)

b)


150
100
50
0
% control protein synthesis
0.1
1.0
10.0
100.0
concentration (nM)

at these concentrations .

The variation (approximately plus or minus twenty per cent) is attributable to a number of factors including the stage of the cell cycle, environmental factors such as pH, temperature, and CO_2 content of the medium.

3.5. Conclusion.

Due to the yields, purity and functional activity of ricin B chain obtained after chromatofocusing and affinity chromatography, these techniques were utilised for the purification of ricin B chain from the commercial preparations.

The purity of the ricin B polypeptide should be ascertained by SDS-PAGE separation followed by silver staining and assays to determine its effect on protein synthesis.

4.1. RICIN B CHAIN: POTENTIAL FUNCTIONAL ROLES.

The principal defined biological function of the ricin B polypeptide is binding to galactose-containing macromolecules expressed at the cell surface. However, it is thought that the ricin B polypeptide may also be responsible for facilitating entry of the ricin A chain into the cytosol (Youle and Neville 1981, 1982). This project was undertaken so that this potential property of the ricin B polypeptide could be studied in the context of its ability to effect the transport of other proteins across the membraneous elements of cells into the cytosol.

4.2. SELECTION OF THE PROTEIN TO FORM A COMPLEX.

Bovine pancreatic ribonuclease A (Hirs, et al., 1960 and Smyth, et al., 1963) was selected as the molecule to be linked to the ricin B polypeptide to form a hybrid complex. The reasons for this choice were:-

(a) RNase A is small, having a molecular weight of 13,680 daltons;

(b) the complete 124 amino acid sequence of the molecule is known;

(c) The protein is extremely stable;

(d) The protein is easily obtained;

(e) The biochemical properties of the RNase A have been determined;

(f) The enzymatic mechanism of action has been elucidated, and no cofactors are required. (RNase A catalyses the cleavage of the phosphodiester bond between the 3' and 5' positions of the ribose moieties in RNA with the formation of oligonucleotides terminating in 2',3' cyclic phosphate derivatives.);

(g) It had been postulated by others (Obrig, et.al., 1985) that the ricin A polypeptide had ribonuclease activity; and

(h) It was possible to assay the enzymatic activity of the RNase A, when present in a complex with the ricin B chain, both *in vivo* and *in vitro*.

4.3. CONJUGATE SYNTHESIS.

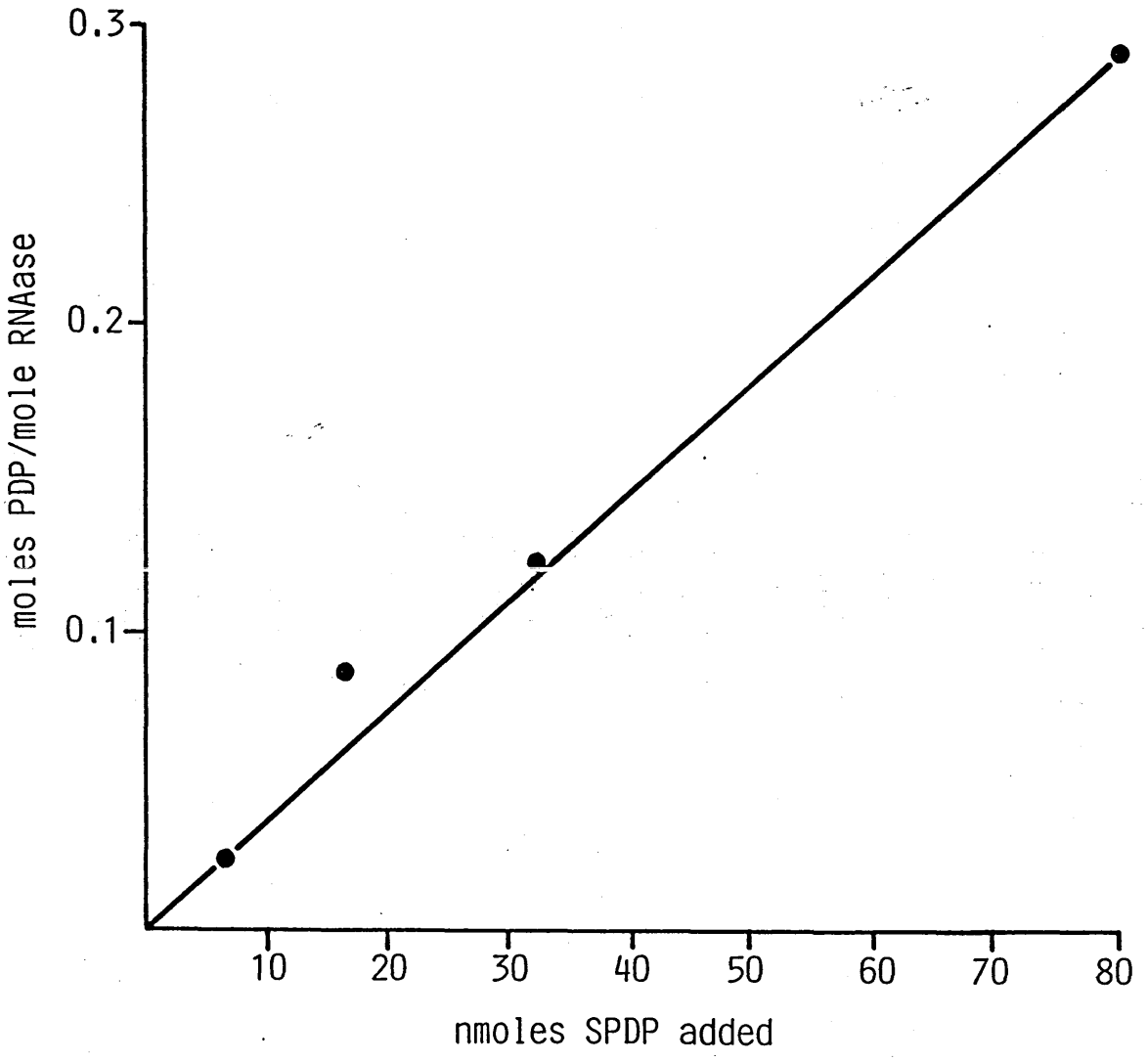
4.3.1. Derivatisation Of Bovine Pancreatic Ribonuclease A.

To enable the *in vitro* synthesis of a RNase A - ricin B chain complex, the heterobifunctional cross-linking reagent, SPDP, was used. To determine the efficiency of the reaction and the level of substitution of the RNase A which was required, substitution assays are shown in Figure 9. The results show that substitution ratios of 0.5 moles pyridyl dithiopropionate per mole RNase A were readily achieved using this procedure.

RNase A has a well-defined binding cleft for the substrate, in which are located residues His-12, His-119, Thr-45 and the side-chains of Lys-7, Lys-41 and Lys-66 (Pavlovsky et al., 1978; Wodak *et al.*, 1977; Borkakoti, 1983; Wlodawer *et al.*, 1983). The catalytic activity of the enzyme is dependent on the ionizations of the two histidine residues. Thr-45 is responsible for the specificity of the enzyme towards pyrimidines (i.e. uracil and cytosine), although this is not absolute since Ap diester bonds in a polynucleotide are also attacked (Beers, 1960). The lysine residues, particularly Lys-41, are an important part of the catalytic mechanism, for when they are acetylated there is a loss of enzymatic activity. The lysine side chains are very mobile in the free enzyme, but their mobilities are markedly reduced on the binding of the nucleotide substrate. Lys-41 interacts directly with the phosphate moiety and is thought to stabilise the pentavalent intermediate in the two-step reaction. A low substitution ratio was required when treating the RNase A with SPDP as this reagent is lysine-directed. The presence of phosphate (a competitive inhibitor of the natural substrate RNA ,

Figure 9. Derivatisation of Ribonuclease A by SPDP.

Seventy nanomoles of RNase A were treated with the indicated amounts of SPDP (dissolved in dimethyl sulphoxide) for 1hr at room temperature. The samples were then dialysed overnight, and substitution of PDP groups determined spectrophotometrically according to the method of Carlsson, et al., (1978).


0.3
0.2
0.1
moles PDP/mole RNAase
10
20
30
40
50
60
70
80
nmoles SPDP added

Blackburn and Stanford, 1982) in the buffer will protect Lys-41. Lys-41 is the only residue that the chemical shift alters on binding of phosphate (Brown and Bradbury, 1975; 1976). It was important to minimise any loss of enzymatic activity, for the assay systems which were to be used to determine if the complex had been internalised required a completely functional enzyme.

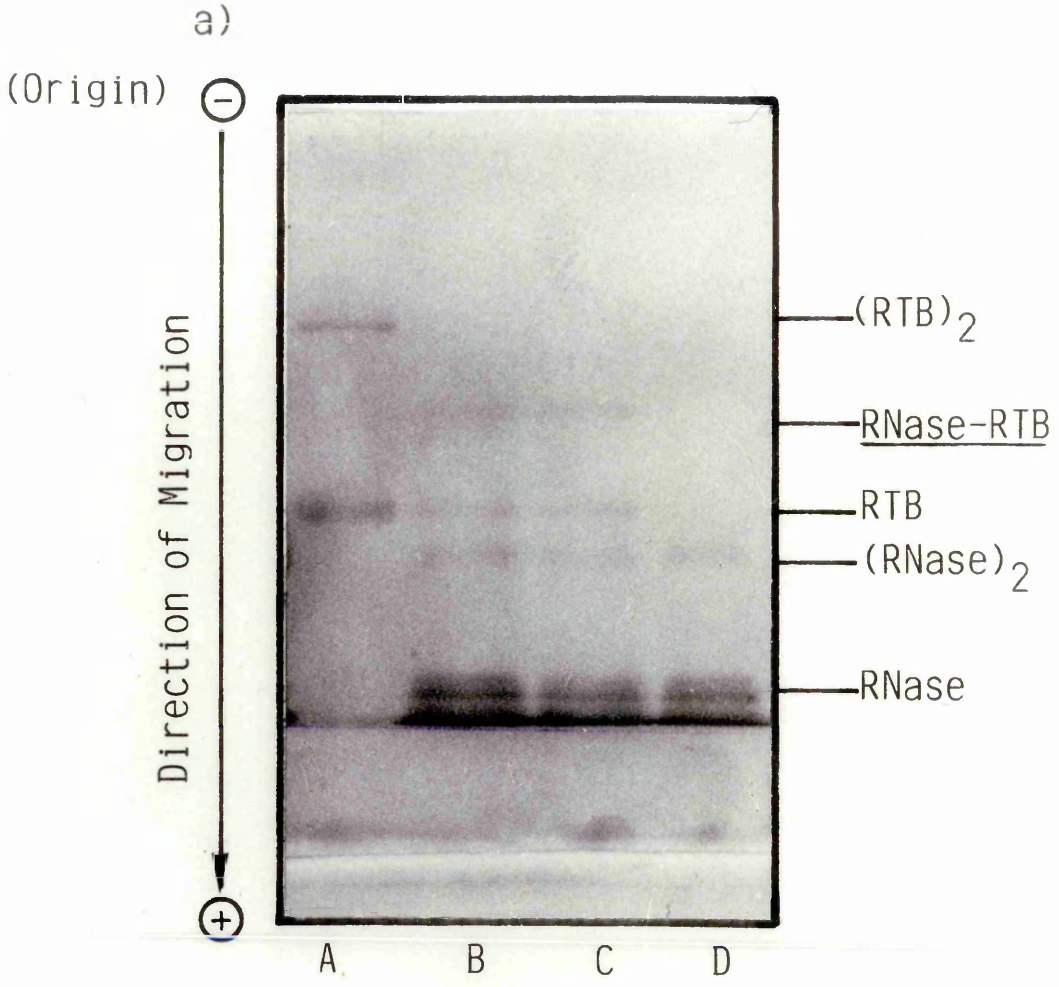
4.3.2. Synthesis Of The Hybrid Protein.

Derivatised RNase A (substitution ratio of 0.1 mole pyridyl dithiopropionate per mole RNase A (Figure 9) was reacted with freshly-reduced ricin B chain. The thiol groups of the reduced ricin B chain effect the elimination of the pyridine-2-thione group from the derivatised RNase, yielding a hybrid protein of RNase A - ricin B, linked by a disulphide bond, (Section 2.3.3., Figure 2). The formation of the complex was determined by separation of the reaction mixture by SDS-PAGE, followed by silver staining (Figure 10). Figure 10 'a' lane A showed the low molecular weight monomers of ricin B chain, approximately 30-33kD, and also indicated that ricin B chain dimers were present, approximately 60kD. Lane D revealed the presence of two high molecular weight bands of approximately 28kD (RNase A dimers) and 14kD (RNase A monomers). Lanes B and C indicated that a unique band of approximately 45kD was present which was in agreement with the values predicted for the apparent Mr of a covalent complex of ricin B (30-33kD) and RNase A (14kD). Also present in these tracks are the components which were used in the synthesis of the hybrid protein (i.e. RNase and ricin B chain).

On comparison of Figure 10 'a' with 10 'b' it can be seen that the level of derivatisation greatly influenced the formation of the complex. The derivatised RNase A used to synthesise the complex in Figure 10 'b' had a higher substitution ratio (0.5 mole pyridyl dithiopropionate) compared to 0.1 mole pyridyl dithiopropionate used normally for the

Figure 10. Ribonuclease A-Ricin B Complex Formation.

Panel a. The formation of a complex between RNase A and ricin B chain was determined initially by SDS-PAGE separation followed by silver staining of the protein bands: lane A - ricin B, lanes B and C - RNase A-ricin B complex and lane D - derivatised RNase A. Panel b. This showed that the level of derivatisation of the RNase A has a direct effect on the amount of RNase A-ricin B complex formed, lane 2. Lane 1 showed the separation of derivatised RNase A and lane 3 ricin B polypeptide.


a)
(Origin)
⊖
Direction of Migration
⊕
(RTB)2
RNase-RTB
RTB
(RNase)2
RNase
A
B
C
D
b)
(Origin)
⊖
Direction of Migration
⊕
(RTB)2
RNase-RTB
RTB
(RNase)2
RNase
1
2
3

synthesis of this complex. As it was necessary to maintain the enzymatic activity of the RNase A, then the simplest methods of improving the yield were by increasing the amount of both proteins used in its synthesis, or by increasing the pH of the disulphide exchange reaction.

It was important to form a disulphide-bonded complex between RNase and ricin B, since this imitates the natural bond which is formed between the ricin A polypeptide and ricin B polypeptide *in vivo*. The disulphide bond in the native toxin is thought to have a role in the internalisation of the toxin and is not simply present to link the two molecules (Section 1.7.).

4.4. BIOCHEMICAL ANALYSIS OF THE RNase - RICIN B CHAIN HYBRID PROTEIN.

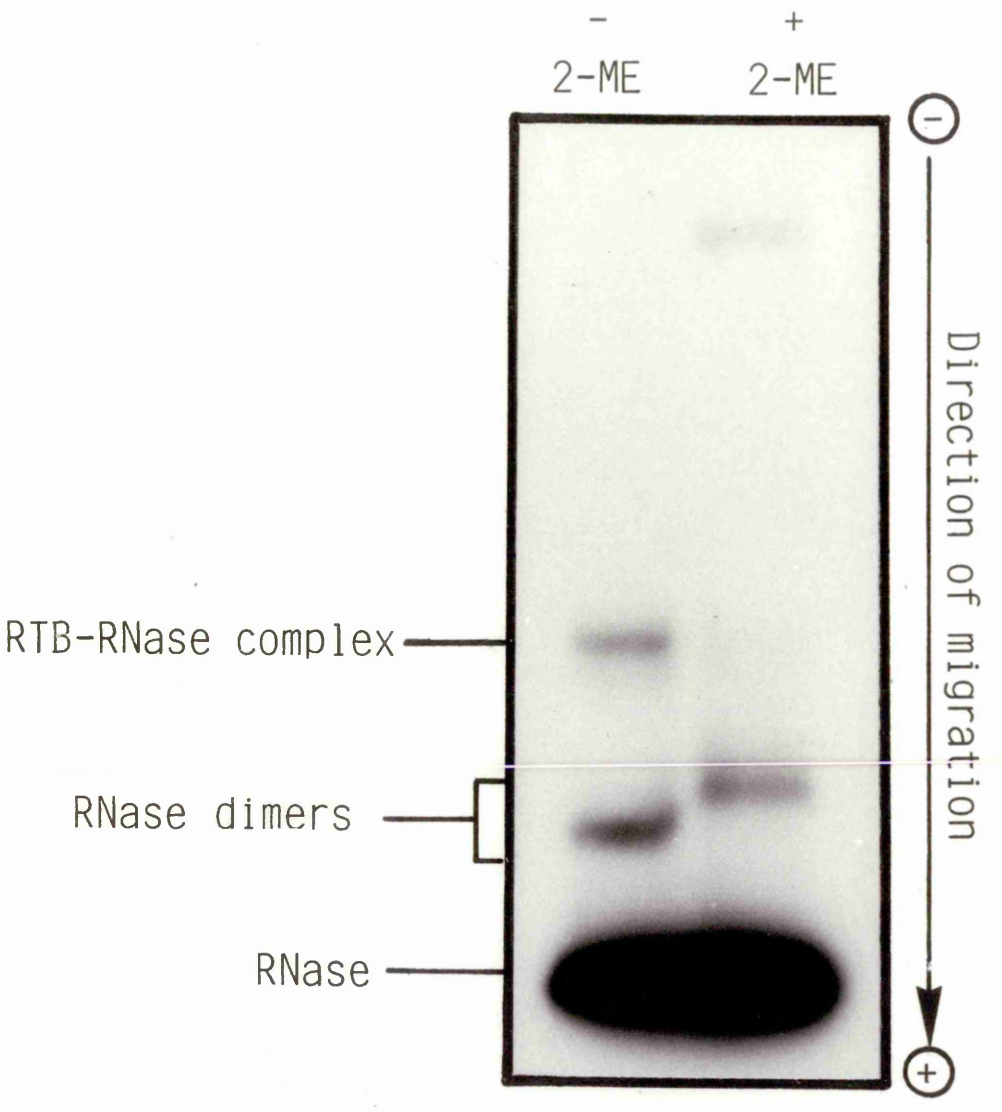
4.4.1. SDS-PAGE Analysis.

A radioiodinated complex was synthesised to facilitate analysis of a variety of biochemical and biological properties of the hybrid protein. In this procedure, RNase A was radioiodinated by the iodogen method prior to synthesis of the RNase A - ricin B chain hybrid. Ricin B chain was not iodinated because tyrosine residues have been implicated in the ligand binding sites of the ricin B chain, and it was important to maintain the functional integrity of this polypeptide (Mise, *et al.*, 1986; Wawrzynczak, *et al.*, 1987)

The autoradiograph (Figure 11) confirmed the results from the silver stained SDS-PAGE gels (see Figure 10). The lane labelled '+2Me' contained a sample of the synthesised iodinated complex which had been reduced using 2-mercaptoethanol. The reducing agent cleaved the disulphide bond which had been formed between the RNase A ricin B such that the complex was reduced to its component parts, such that no complex band was observed at approximately 45kD. However, the lane labelled '-2

Figure 11. Autoradiograph of Iodinated Complex.

The complex was synthesised between iodinated derivatised RNase A and ricin B chain. The track labelled '-2 Me' showed that a complex had been formed between iodinated derivatised RNase A and ricin B chain. Reduction (track '+2 Me') of the complex broke the disulphide bridge, so that the RNase A-ricin B complex band was no longer present.


-
2-ME
+
2-ME
RTB-RNase complex
RNase dimers
RNase
Direction of migration

Me' clearly showed that the complex had been synthesised. Again RNase A dimers were present in both tracks, but in the track labelled '+2-Me' the band has altered position. This was due to the reducing agent breaking the intrachain disulphide bonds (4 in each RNase A monomer) so that the molecule unfolds, leading to an alteration in the overall shape of the molecule and an apparently slower mobility on SDS-PAGE.

4.4.2. Two-dimensional Gel Electrophoretic Analysis.

The iodinated RNase A - ricin B complex was further analysed using two dimensional gel electrophoresis followed by autoradiography. The autoradiographs (Figure 12) again confirm that a complex has been formed between iodinated RNase A and ricin B chain, for when the complex was reduced using 2-mercaptoethanol the band disappeared (Figure 12, panel A). Panel B indicated that the RNase A, which has an isoelectric point of 9.4 and is therefore a 'basic' protein, has conferred a similar basic nature on the RNase A - ricin B chain complex. The isoelectric point of ricin B is 4.8, so the 'acidic' nature of this polypeptide could have influenced the acidic or 'basic' nature of the complex. The isoelectric point of the complex is estimated to be in the range pH 8.0 - 8.5.

4.4.3. Immunoblotting Analysis Of The Ribonuclease A - Ricin B Complex.

Immunoblots of the complex, RNase A and ricin B are shown in Figure 13. This was used to confirm that the putative complex, i.e. the 45kD band detected by SDS-PAGE analysis, was recognised by both the anti-ricin antibody and the anti-RNase antibody, thereby showing that antigenic determinants associated with the two proteins used in its synthesis were present. Simple protein staining (Figure 13, panel a) did not detect the 45kD species which was present. Staining of an identical blot with antibody RD7 (anti-RNase A) visualised the RNase species and the 45kD band (panel c). Development of a blot with an antiserum with specificity

Figure 12. Two-dimensional Gel Electrophoretic Analysis of Iodinated Complex.

Iodinated complex (panel b) and reduced iodinated complex (panel a) was analysed by two-dimensional gel electrophoresis. Isoelectric focusing was performed over the pH range 3.5 - 10, and a 10% (w/v) acrylamide gel was employed in the second (SDS-PAGE) dimension.

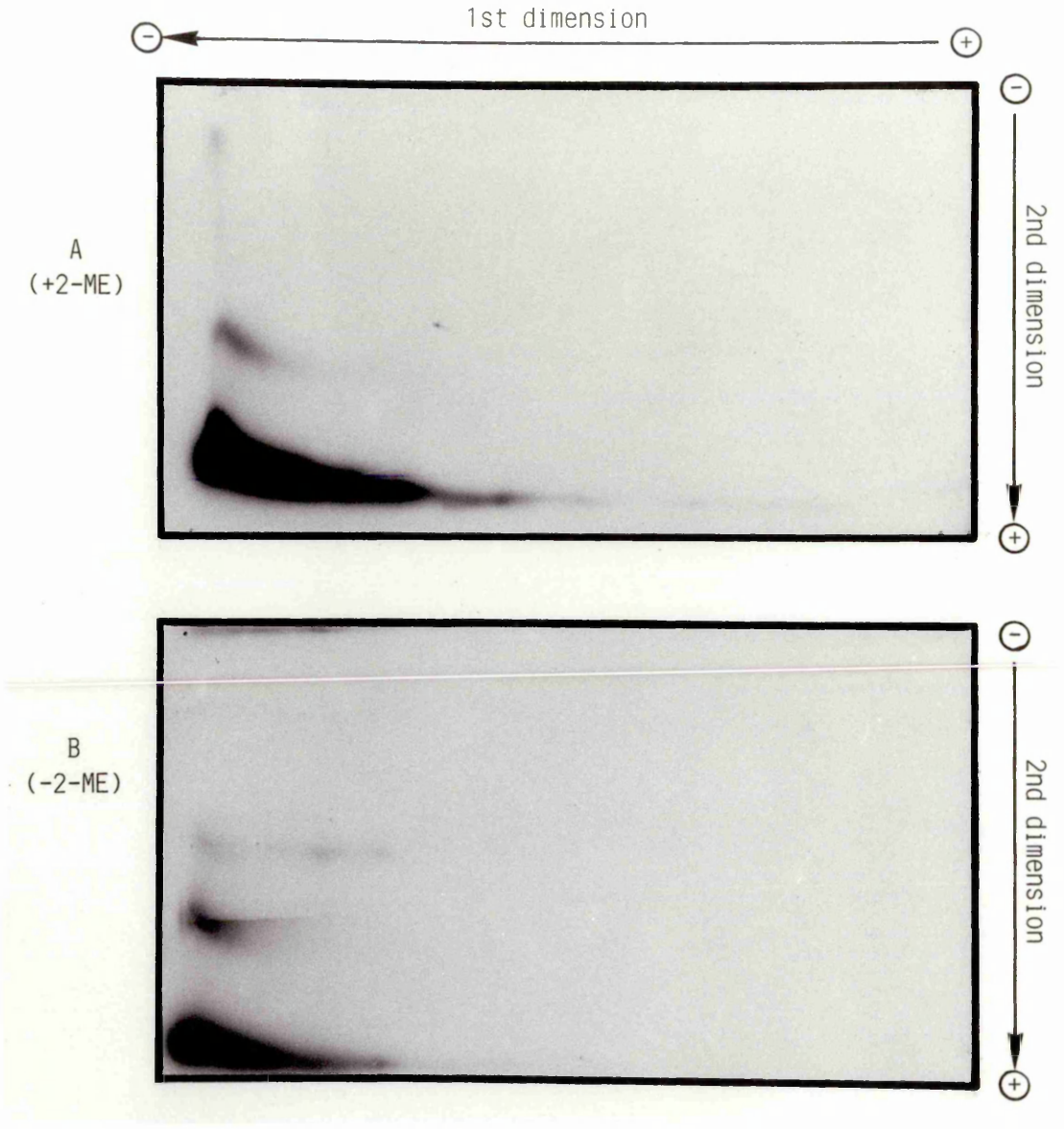

1st dimension
⊖
⊕
A
(+2-ME)
⊖
2nd dimension
⊕
B
(-2-ME)
⊖
2nd dimension
⊕

Figure 13. Immunoblots of Ribonuclease A - Ricin B Complex.

Immunoblots of the RNase A-ricin B complex were used to determine that the complex did possess antigenic determinants which were recognised by anti-ricin antibody (panel 'b') and anti-RNase A antibodies (panel 'c'). The immunoblot (panel 'a') was stained with napthol blue-black to ensure that the proteins had transferred. The control using normal rabbit serum in place of the antibodies was completely clear (result not shown).

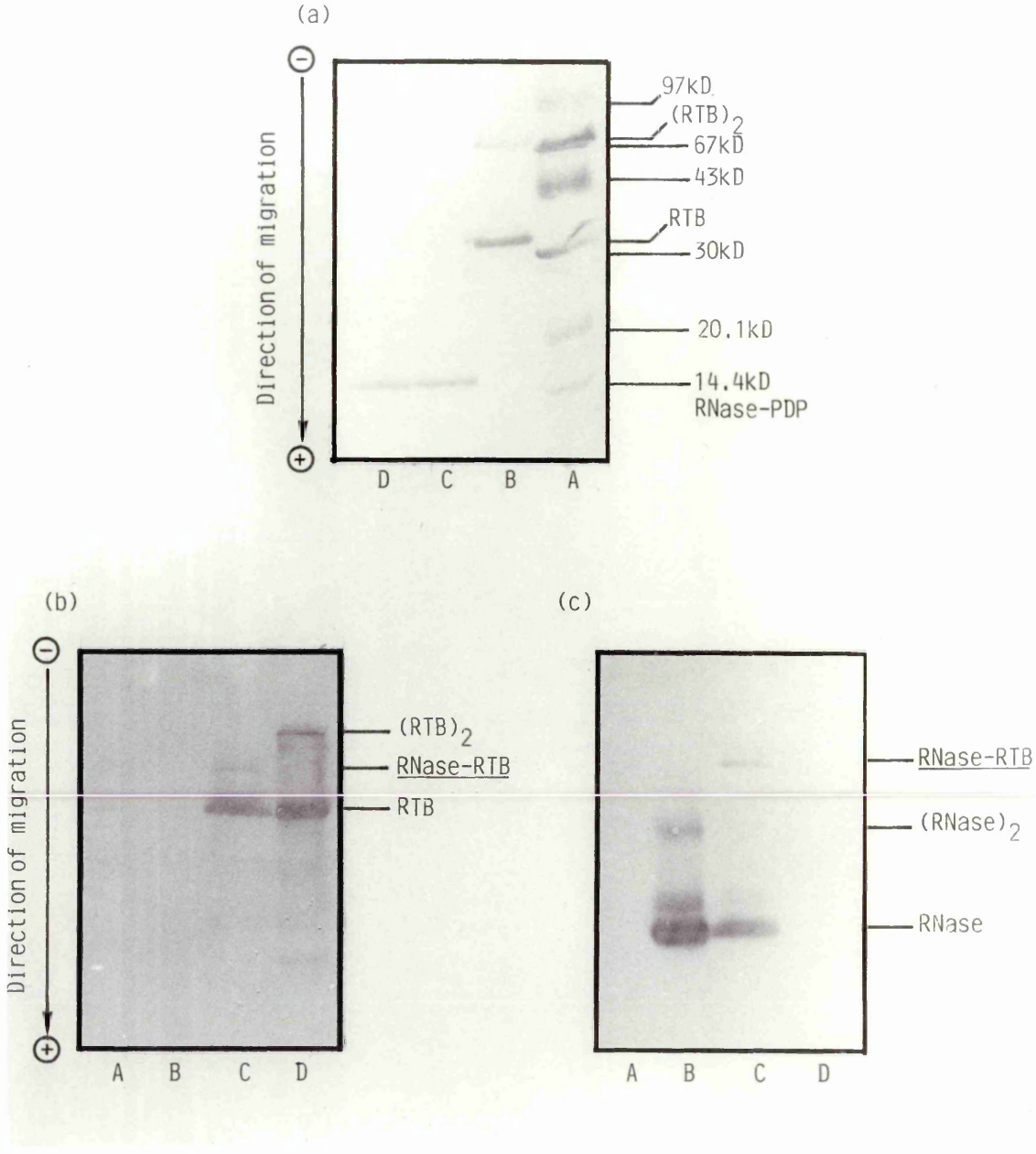
KEY:-

Lane A - low molecular weight markers

B - derivatised RNase A

C - RNase A-ricin B complex

D - ricin B


(a)
Direction of migration
97kD
(RTB)2
67kD
43kD
RTB
30kD
20.1kD
14.4kD
RNase-PDP
D C B A
(b)
Direction of migration
(RTB)2
RNase-RTB
RTB
A B C D
(c)
RNase-RTB
(RNase)2
RNase
A B C D

for ricin components (panel b) stained only ricin B chain monomers and dimers, and again recognised the 45kD species. A non-immune serum (not shown) failed to bind to any components on the blot. The fact that the 45kD band is recognised by an anti-RNase A reagent and by a ricin-specific antiserum provides strong evidence to support the hypothesis that the 45kD band possesses both RNase A and ricin B chain components, and is, therefore, a covalent complex of RNase A and ricin B chain.

4.5. ASSAY OF BIOLOGICAL ACTIVITY OF THE RNase A - RICIN B CHAIN COMPLEX.

4.5.1 Polymerised Ribonuclease A.

The protein modification agent dimethyl suberimidate was used to prepare cross-linked RNase A. Figure 14 'a' shows the SDS-PAGE profile of the resulting cross-linked RNase A preparation; dimers and trimers were easily detected by coomassie blue staining of the separated protein bands. Dimethyl suberimidate like SPDP is a lysine directed reagent, so it was useful to compare the effects of SPDP and dimethyl suberimidate on the enzymatic activity of the RNase A.

4.5.2. Ribonuclease Activity Gels.

To determine whether the dimethyl suberimidate-treated RNase A and the SPDP cross-linked RNase-A ricin B complex still retained enzymatic activity, they were subjected to SDS-PAGE in gels containing yeast RNA (Section 2.9.). After electrophoresis the SDS was removed from the gels to allow renaturation of the denatured proteins. The gels were then incubated at 37^{o}C to allow digestion of the yeast RNA by the RNase A. Figures 14 'b' and 15 clearly showed the areas of enzymatic digestion which were visualised as clear 'plaques' against the background of the toluidine blue 'O' stain. Figure 14 'b', lane A showed that RNase A monomers, dimers and trimers retained enzymatic activity, as did the

Figure 14. Polymerised Ribonuclease A.

Panel a. Treatment of RNase A with dimethyl suberimidate resulted in the formation of dimers and trimers, which were detected by coomassie blue staining of the protein bands after SDS-PAGE (12.5% (w/v) acrylamide).

Panel b. Samples of polymerised RNase A were subjected to SDS-PAGE on 12.5% (w/v) acrylamide gels containing 0.5mg/ml yeast RNA. After electrophoresis, the gels were soaked in isopropanol to remove SDS and then stained with toluidine blue. Upon destaining, RNase A activity was visualised as clear 'plaques' on a blue background. Sample loadings were 2ug polymerised RNase A in lane A and 1ug RNase A in lane B.

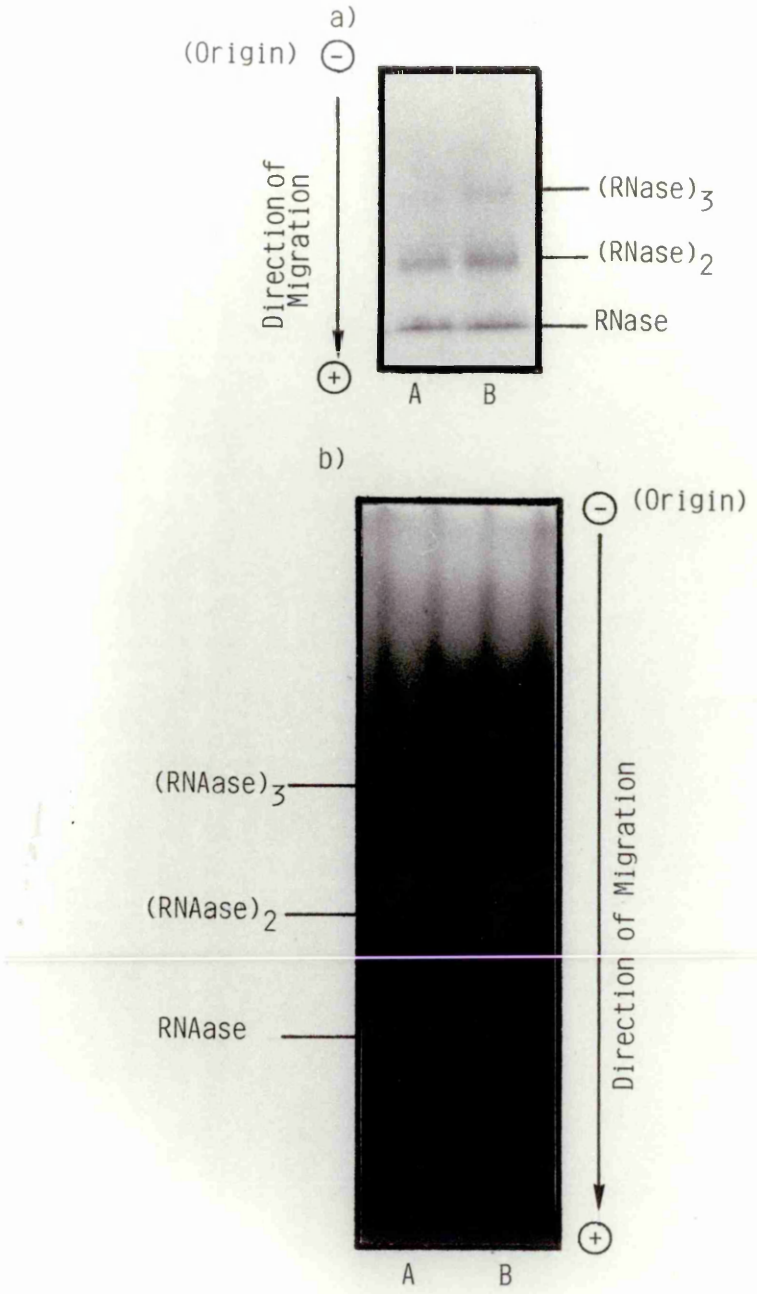
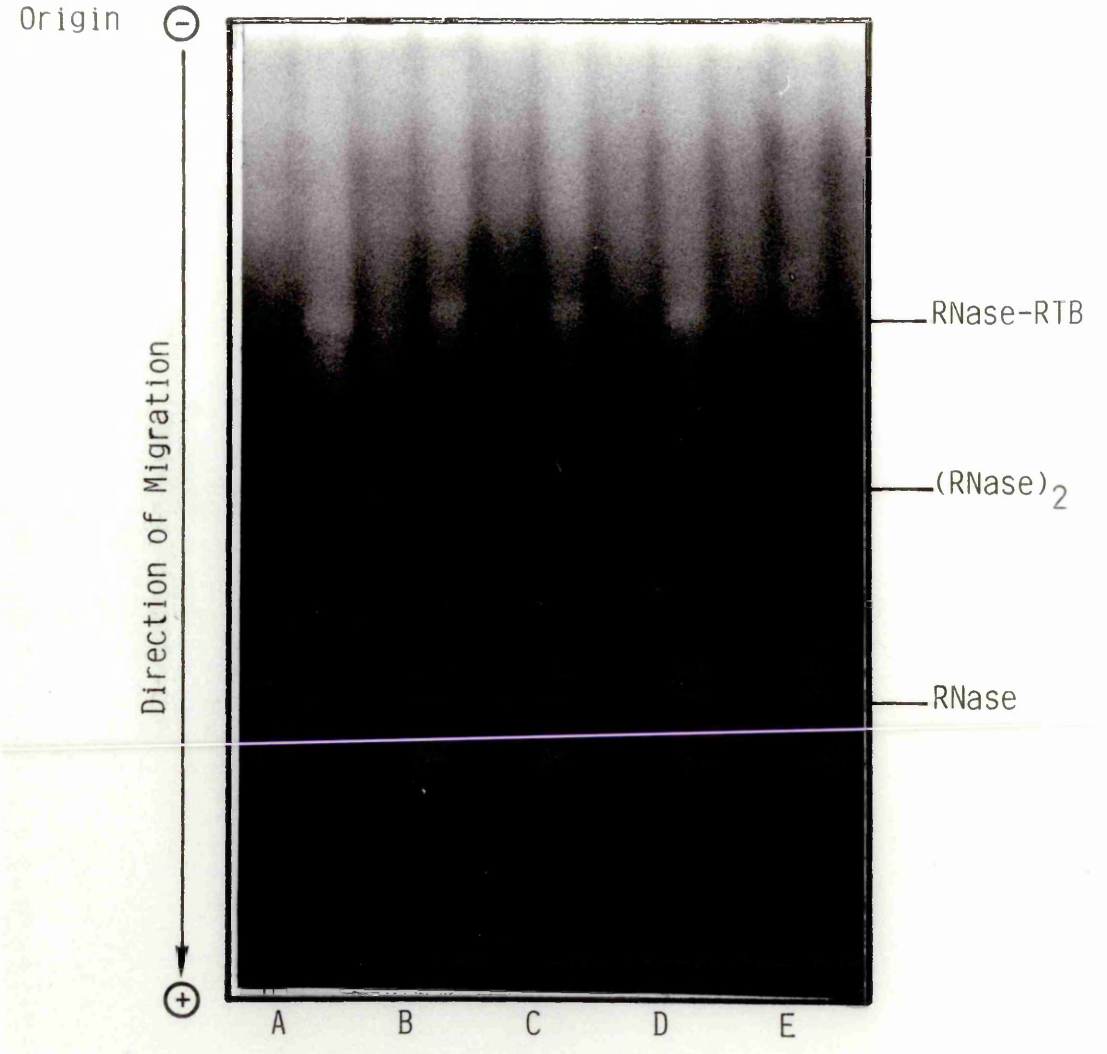

a)
(Origin)
Direction of Migration
(RNase)3
(RNase)2
RNase
A
B
b)
(Origin)
(RNAase)3
(RNAase)2
RNAase
Direction of Migration
A
B

Figure 15. Activity Gel of RNase A - Ricin B Complex.

Samples of RNase A - ricin B complex were subjected to SDS-PAGE on 12.5% (w/v) acrylamide gels containing yeast RNA. After electrophoresis, the SDS was removed by soaking the gels in isopropanol and then the gel was incubated at 37°C for 2hr. The areas of enzymatic activity were detected by staining with toluidine blue.

Lanes A to D Sample loadings were 10, 8, 4 and 2ug of RNase A - ricin B complex, respectively.

Lane E Sample loading was 2ug RNase A (derivatised).


Origin
Direction of Migration
RNase-RTB
(RNase)2
RNase
A
B
C
D
E

monomers and dimers of the untreated RNase A in lane B. Figure 15 showed that RNase A present in the complex was enzymatically active (lanes A to D), as was the derivatised RNase A used in its synthesis (lane E).

It could be argued that the enzymatic activity observed in Figure 15 lane E, putatively due to the derivatised RNase A (the highest mobility band), was infact due to underivatised RNase A. This could also apply to the activity of the RNase A dimers, for in the commercially produced RNase A dimers were sometimes present at an extremely low level in the preparation. However, the presence of RNase A trimers shown in Figure 14 'b' and the RNase A-RTB complex (Figure 15) argues against this. These results therefore suggest that a low level of substitution of the lysine residues does not abrogate the enzymatic activity of RNase, although a more significant effect may have been observed if the lysine residue(s) at the active site were involved in the cross-linking. The zymogram also showed that RNase A did not require to be released from the ricin B chain to be enzymatically active. Thus, the presence of the ricin B polypeptide was not causing alterations to the overall three-dimensional conformation of the RNase A, which might have resulted in inhibition of expression of its enzymatic activities.

Results (not shown) of a titration experiment to determine the lowest concentration of dimethyl suberimidate-treated RNase in which enzymatic activity could be detected, suggested a threshold of 100ng. This was in contrast to the findings of Blank et al., (1981) , who were able to detect activity in as little as 10pg of bovine RNase A. However, there are a number of factors which could have caused this loss of sensitivity, such as the degree of derivatisation of the RNase A, the incomplete removal of SDS from the gel or contaminants in the SDS which can inhibit renaturation of the enzyme (Blank et al., 1981). The contaminants in the commercial SDS which may cause this are thought to be long chain alkyl sulphates, such as hexa- and / or tetra- decyl sulphate

(Lacks, et al., 1979) and other impurities, as yet not fully defined. Other factors may also affect the rate and efficiency of the renaturation of the enzyme and enzyme complexes.

There are two flaws in this technique; namely, the variability of the commercially produced SDS which affects the enzymatic activity detected, and the uneven background staining of the yeast RNA by the toluidine blue 'O'. The upper section of the gels in Figure 14 'b' and Figure 15 were very weakly stained, due to the slow migration of the yeast RNA during electrophoresis, whilst the lower areas of the gels were stained very darkly. Therefore, this meant that it was difficult to unequivocally detect high molecular weight proteins, such as RNase A trimers and RNase A - ricin B complex, by activity staining. The uneveness of the staining could be overcome by carrying out the electrophoretic separation as for SDS-PAGE (Section 2.5.), then after the removal of the SDS incubation of the gel at 37^{o}C in tris buffer containing yeast RNA, so that the yeast RNA would intercalate evenly into the gel, and the digested yeast RNA would be seen as clear plaques against an extremely even background. This technique proved not to be applicable in this particular situation (data not shown).

4.6. PURIFICATION OF THE RNase A - RICIN B CHAIN COMPLEX.

Anion exchange chromatography, using the FPLC mono-Q column, was chosen as a potential system for the purification of the RNase A - ricin B chain complex. From knowledge of the pI values of the components of the reaction mixture, it was predicted that the ricin B chain polypeptide would bind to the Mono-Q column given its negative charge under the conditions of column elution; the RNase A - ricin B chain complex, and free RNase A were expected to elute in the void volume. Figure 16, panel A, illustrates the elution profile of the reaction mixture; three protein peaks are evident.

Aliquots of individual column fractions 1 to 16 were analysed by immunoblotting (Figure 16, panels b and c). The immunoblotting analysis showed partial purification of the RNase A - ricin B chain complex, since fractions 5, 6 and 7 apparently contained only free RNase and the hybrid. Further enrichment for the hybrid, by affinity chromatography on propionic acid-treated Sepharose-4B, resulted in significant loss of protein, possibly due to aggregation of the material eluted from the mono-Q column.

Figure 16. Purification of RNase A - Ricin B Complex Using Anion Exchange Chromatography.

Panel a. Chromatogram of the purification of RNase A - ricin B complex from the proteins used for its synthesis. The column was equilibrated with 20mM tris-HCl, pH 7.0 containing 10mM sodium chloride and was eluted with a gradient of sodium chloride (final concentration 1M) - gradient profile recorded . The sensitivity of the meter for measuring the absorbance of the individual fractions was set at 0.2.

One hundred microlitre samples were taken from the individual fractions, and acetone precipitated (1 vol:5 vol). The samples were then loaded on 12.5% (w/v) acrylamide gels and then immunoblotted.

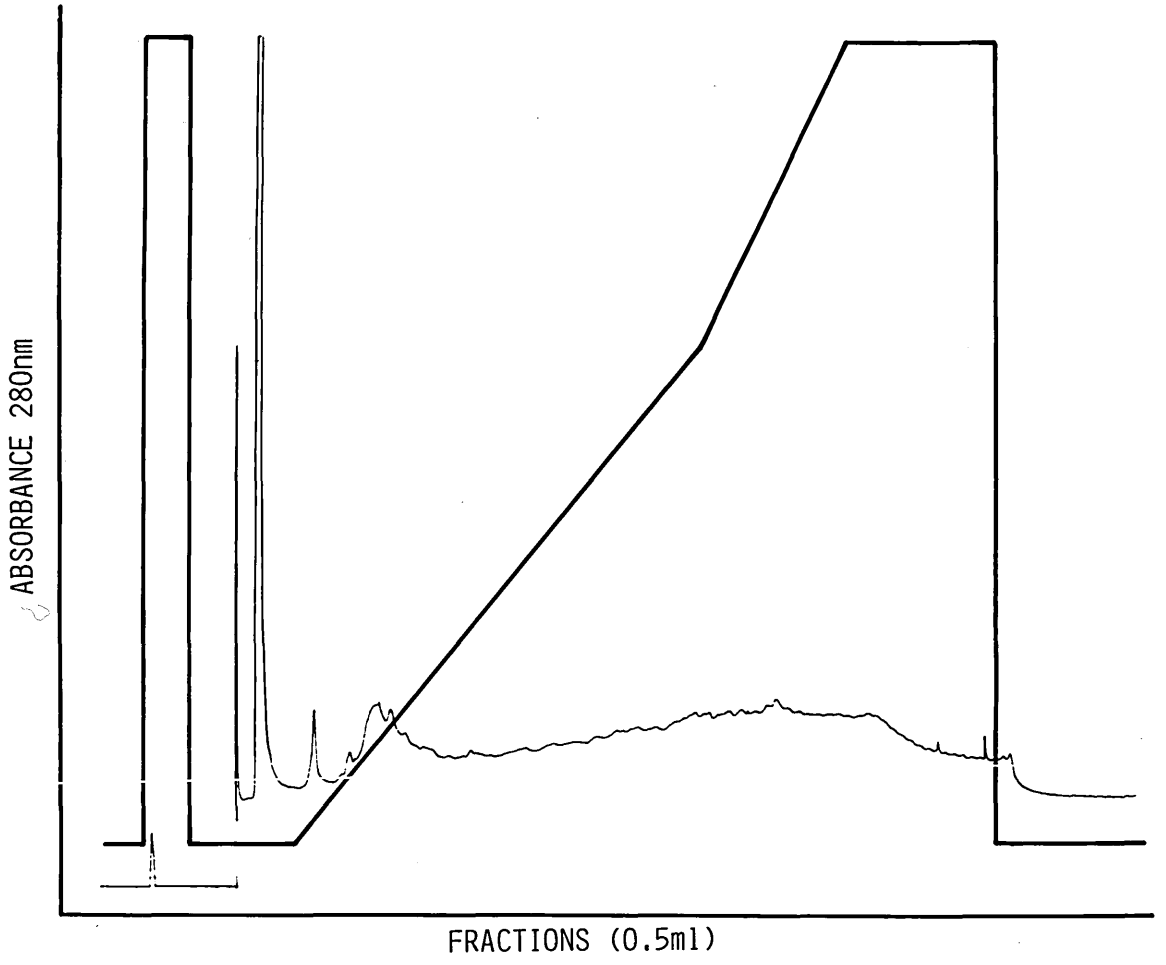
Lanes 1 to 16 individual samples corresponding to fractions from the chromatogram.

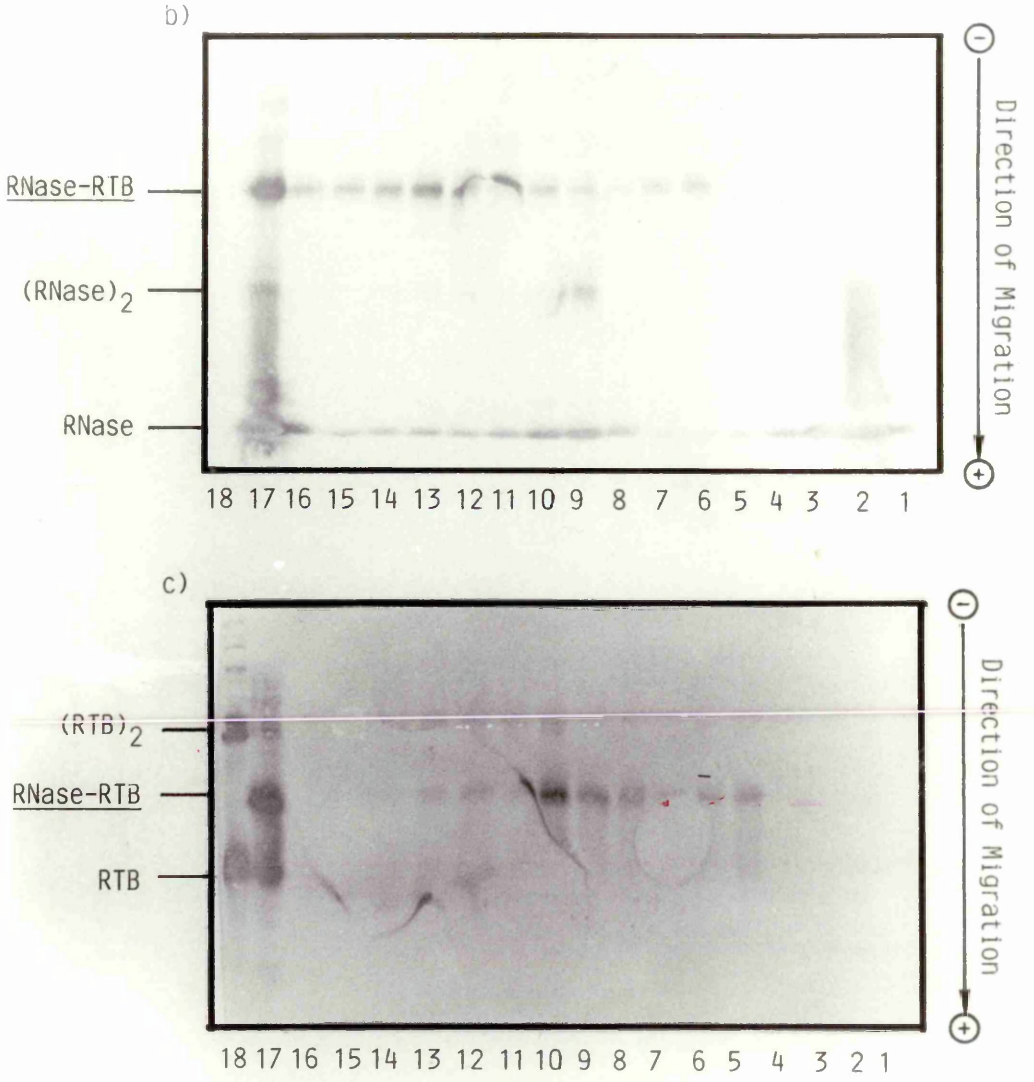
Lane 17 to RNase A - ricin B complex.

Lane 18 to Ricin B polypeptide.

Panel b. Anti-RNase A antibody used in the detection of proteins on the immunoblot.

Panel c. Anti-ricin antibody used in the detection of proteins on the immunoblot.


ABSORBANCE 280nm
FRACTIONS (0.5ml)


b)
RNase-RTB
(RNase)2
RNase
18 17 16 15 14 13 12 11 10 9 8 7 6 5 4 3 2 1
Direction of Migration
c)
(RTB)2
RNase-RTB
RTB
18 17 16 15 14 13 12 11 10 9 8 7 6 5 4 3 2 1
Direction of Migration

4.7. ANALYSIS OF THE CELLULAR PROPERTIES OF THE RNase A - RICIN B CHAIN COMPLEX.

4.7.1. Cellular Cytotoxicity Assay.

The toxic action of ricin on cells in culture was readily determined by observing either its direct effect on protein synthesis by following the decrease in incorporation of radioactively-labelled methionine into newly-synthesised proteins, or by monitoring the decrease in the incorporation of radioactively-labelled thymidine into DNA.

Initially, the 'indirect' or 'long-term' effect of the RNase A - ricin B complex on the DNA synthesis of cells in culture was investigated. The cells were pulsed for six hours with [^{3}H]-thymidine, twenty-four hours after the treatment of the cells with the putatively toxic proteins. Figure 17 showed that derivatised RNase A, ricin B polypeptide and RNase A - ricin B complex had no effect on the DNA synthesis of cultured cells, whilst ricin caused complete inhibition of DNA synthesis at a concentration of 10nM.

The RNase A - ricin B complex may only be exerting an effect on protein synthesis. To test this hypothesis, the cells were subjected to pulse with [^{35}S]-methionine for 3 hours and 24 hours after exposure of the cells to toxin treatment. Figure 18 panels a and b, again showed that ricin was toxic to the Daudi cells, whilst RNase A, ricin B and RNase A - ricin B had no detectable effect.

The lack of a cytotoxic effect of the RNase A - ricin B complex on DNA or protein synthesis could have resulted from a number of different factors including:-

(a) the ricin B polypeptide was unable to bind to the receptors present on the cell surface, due to a conformational change on linkage to the RNase A;

(b) the inability of the ricin B chain to effect the

Figure 17. Toxicity of RNase A - Ricin B Complex to Daudi cells: Effect on DNA synthesis.

The Daudi cells were incubated for 1hr at 37°C with the various concentrations of the protein solutions in incomplete medium indicated on the graph. After 1hr the cells were washed then resuspended in complete medium and incubated for 24hr. 1uCi [^{3}H]-thymidine was then added to each of the wells and the cells incubated at 37°C for a further 4hr. The cells were then harvested and the incorporation of radiolabel was determined by liquid scintillation spectrophotometry.

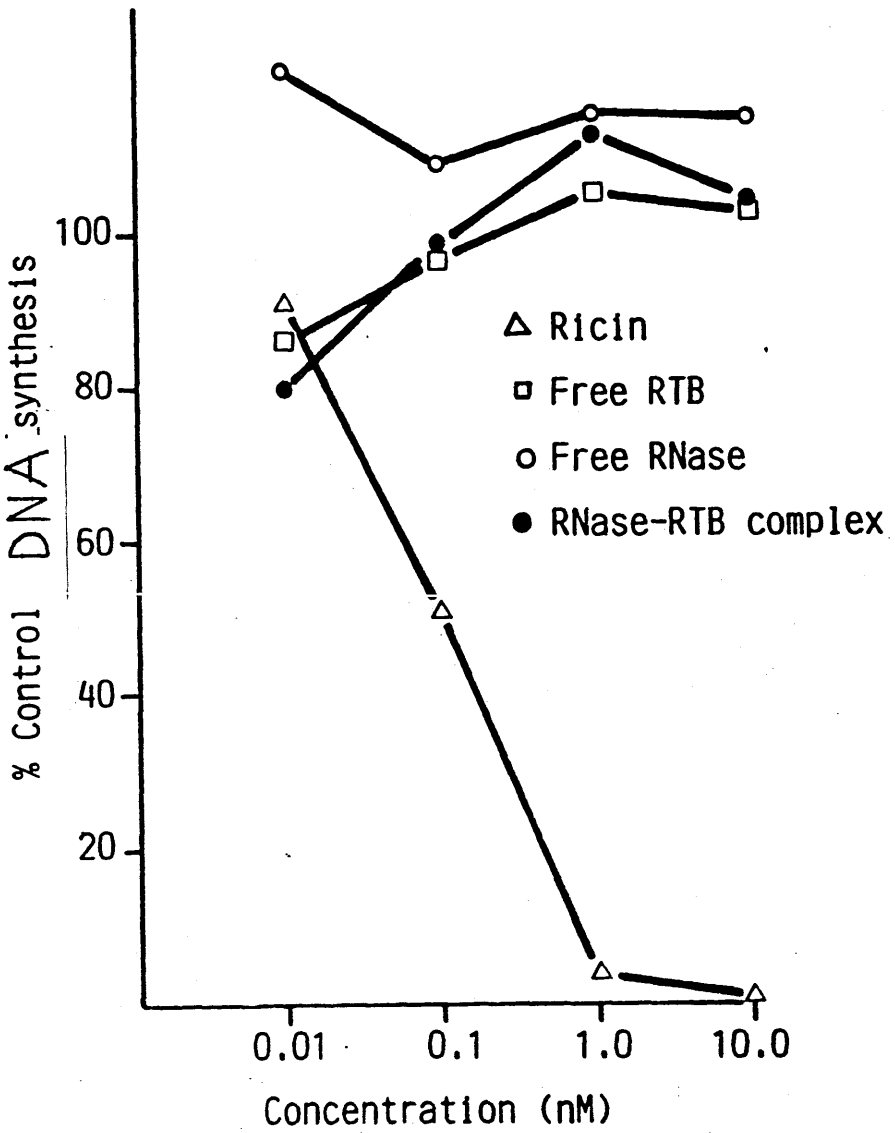

% Control DNA synthesis
100
80
60
40
20
0.01
0.1
1.0
10.0
Concentration (nM)
Ricin
Free RTB
Free RNase
RNase-RTB complex

Figure 18. Toxicity of RNase A - Ricin B Complex to Daudi cells: Effect on Protein Synthesis.

The Daudi cells were incubated for 1hr at 37°C with the concentrations of the proteins indicated on the graph. FCS (final concentration 10%) and 10ul [^{35}S]-methionine (5uCi) were added to each well and incubated for 3hr or 24hr, at 37°C. Samples were removed at the two time points, subjected to TCA precipitation and the incorporation of [^{35}S]-methionine into the proteins was determined by counting in a scintillation counter.

Panel a. 3hr incubation.

Panel b. 24hr incubation.

KEY;-

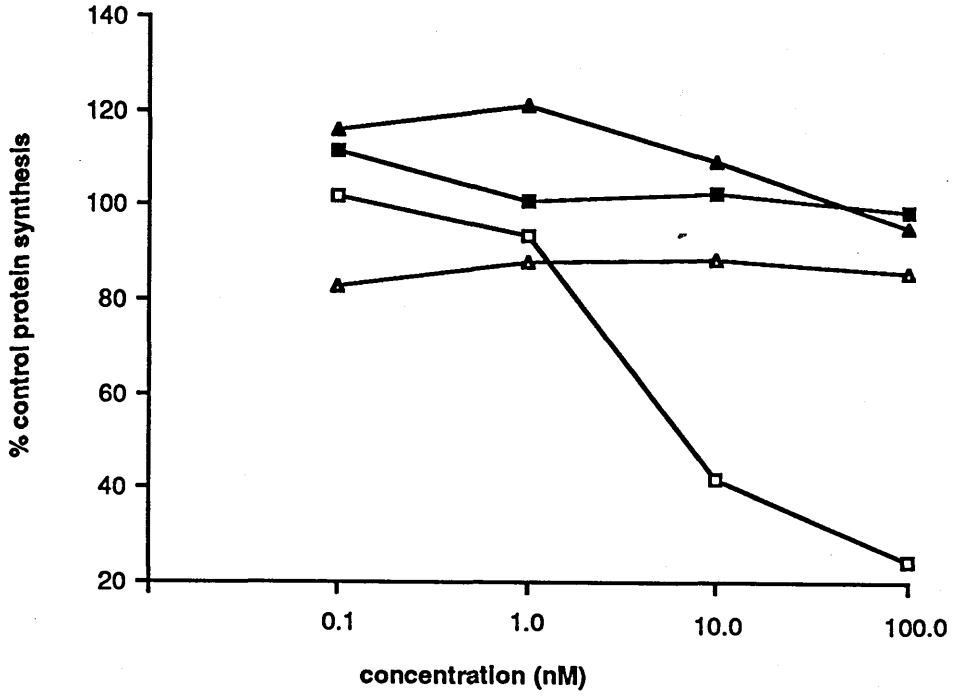
□ - ricin

▲ - purified ricin B chain

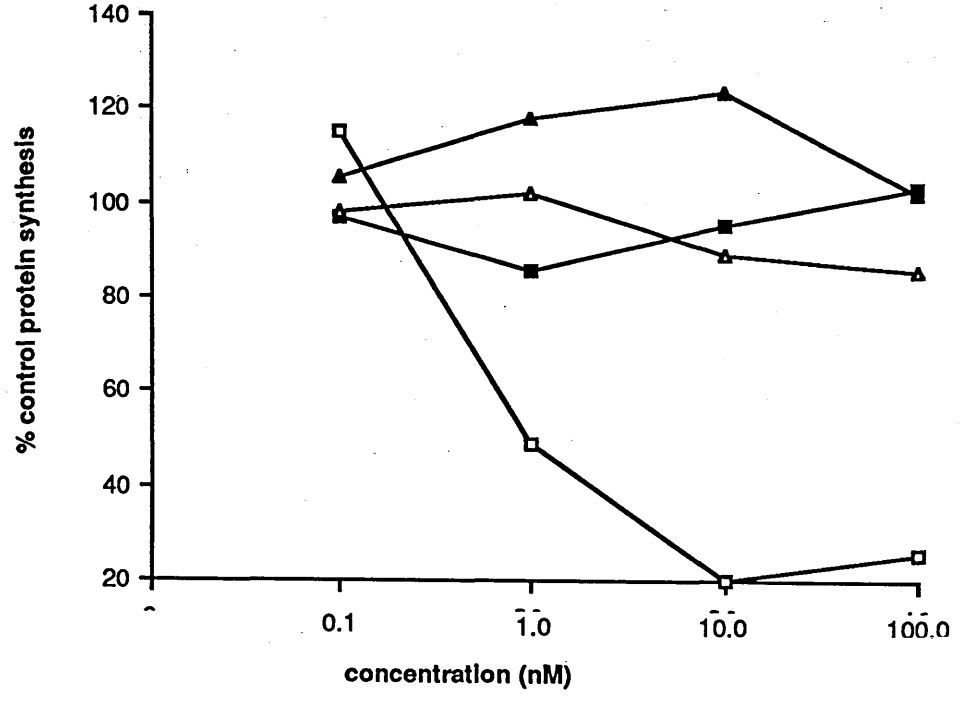
■ - RNase A -ricin B complex

△ - RNase A

a)


140
120
100
80
60
40
20
% control protein synthesis
0.1
1.0
10.0
100.0
concentration (nM)

b)


140
120
100
80
60
40
20
% control protein synthesis
0.1
1.0
10.0
100.0
concentration (nM)

internalisation of the RNase A - ricin B complex;

(c) the ricin B polypeptide was unable to transport the RNase A into the cytosol, after the complex had been internalised;

(d) any toxic effect which was expressed by the complex was transient, so was undetectable in the assay system used;

(e) the concentration of RNase A which was internalised was too low to illicit a measurable response;

(f) the RNase A was inhibited by the ribonuclease inhibitors which are present within the cell, and which appear to be specific for neutral RNases of the pancreatic type since they do not inhibit acid lysosomal RNase (Shortman, 1962). The ratio of the inhibitor to neutral RNase activity tends to increase in tissues characterised by increased rates of RNA synthesis and accumulation. Conversely, tissue in which protein synthesis decreases and catabolic activity increases usually express lower levels of the inhibitor and elevated neutral RNase activity (Liu, et al., 1975; Kyner, et al., 1979; Greif and Eich, 1972; Murthy and McKenzie, 1974; Brewer, et al., 1969; Liu and Matrisian, 1977; Karplus and Weaver, 1976). This arguement could therefore apply to the individual cell;

(g) bovine pancreatic RNase is an endonuclease , and its known that mRNA being translated by the ribosomes form stable complexes, i.e. polysomes (Strickberger, 1976), so it was perhaps difficult for the RNase A to gain access to the mRNA;

(h) magnesium is required for translation, the local concentration of Mg^{++} present in the polysomes may be too high and inhibit RNase A (inhibited by Mg^{++} at a concentration of 0.05mM). Phosphate acts as a competitive inhibitor (Blackburn and Stanford, 1982) and heavy metal ions also act as inhibitors, the concentration of these ions in the cytosol may inhibit the enzymatic activity of the RNase A; and

(i) Proteases within the cell may recognise the 'complex' as being

'foreign' and therefore degrade it. This could occur in the cytoplasm or, if the intracellular routing so directed the complex, to the lysosomal compartment.

4.7.2. Binding And Internalisation Of Radioiodinated RNase A - Ricin B Chain Complex

The RNase A - ricin B complex had no effect on DNA or protein synthesis and this could have resulted very simply from the inability of the ricin B polypeptide present in the complex to bind to receptors on the cell surface, so that the complex was not internalised.

Iodinated, derivatised RNase A was used to form a complex with the ricin B chain, and this iodinated complex was then used to study binding and internalisation. The formation of iodinated RNase A - ricin B complex was determined by autoradiography of SDS-PAGE gels as described in Section 2.5. and 2.8. Figure 19 panel a - bar 2 showed that the galactose washes did not remove any complex from the cell surface, suggesting that all the complex that had been bound was internalised. There is, however, a slight possibility that the galactose was for some reason unable to effect the removal of the iodinated complex from the receptor i.e. alteration of the binding affinity. The presence of galactose clearly acted as an inhibitor, Figure 19 panel a - bar 3, the iodinated RNase A - ricin B complex binding to the galactose, rather than the receptors on the cell surface, so that the percentage counts were similar to those obtained for the control percentage counts of the iodinated RNase A - PDP (Figure 19 panel b). This therefore provided evidence that the ricin B polypeptide present in the complex retained the one proven biological function associated with it that of lectin binding.

In this experiment it was assumed that the internalised iodinated complex would not have an effect on the cells which could be detected.

Figure 19. Binding/Internalisation of Iodinated Proteins.

a. Binding and internalisation of the RNase A - ricin b complex was studied by synthesising an iodinated RNase A -ricin B complex. Daudi cells were incubated at 37°C for 2hr with the iodinated complex, in the presence or absence of 0.1M galactose (a competitive inhibitor). The cells were then washed in PBS, pH 7.2 or PBS, pH 7.2 containing 0.5M galactose. The samples were then counted in a LKB Wallac '1275 Minigamma' counter.

b. Binding and internalisation of iodinated RNase A. The samples were treated exactly as described in 'a'.

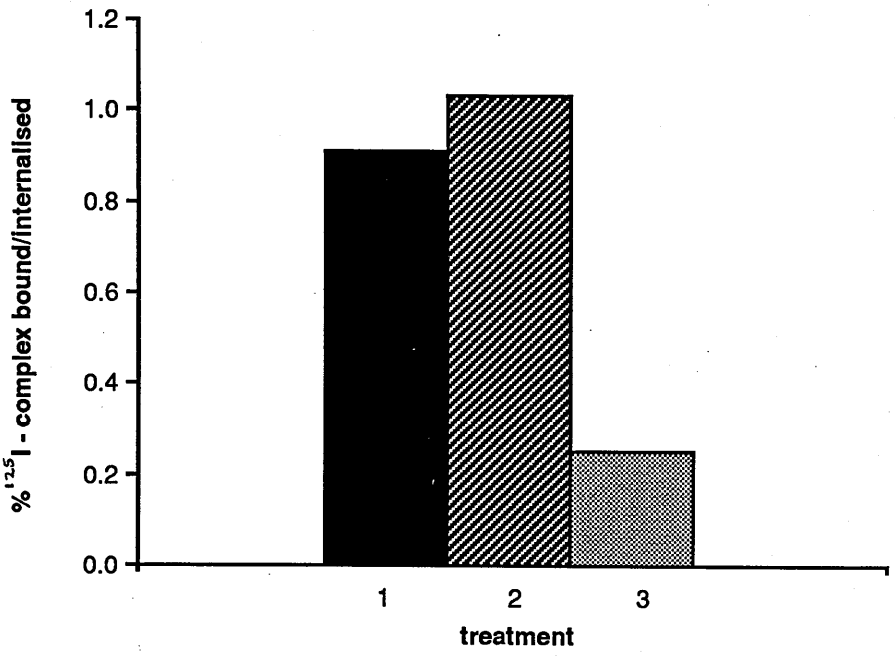
KEY:-

1 - Control

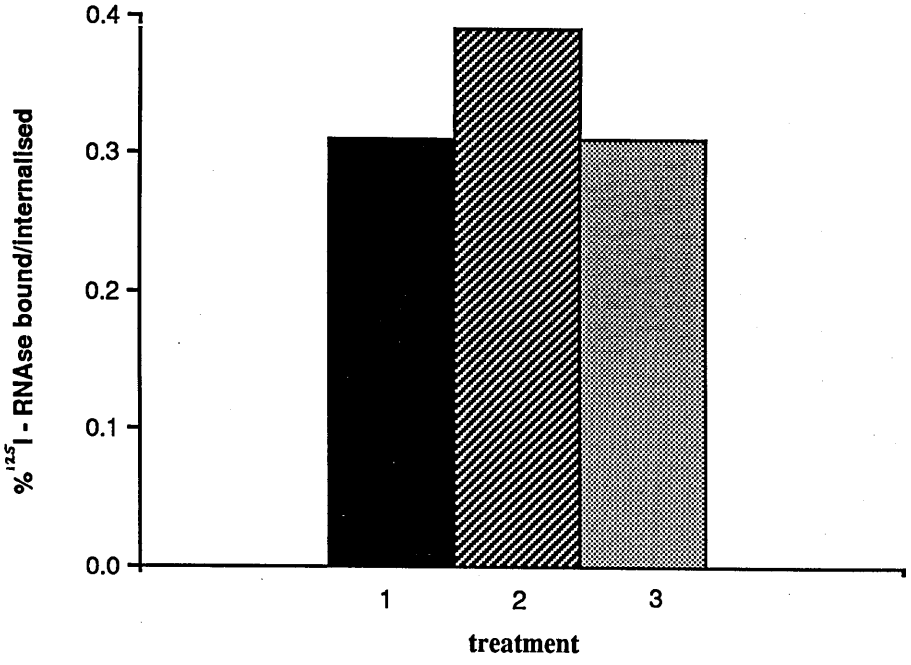
2 - Cells washed with PBS, pH 7.2 containing 0.5M galactose.

3 - Cells incubated with PBS, pH 7.2 containing 0.1M galactose.

a)


1.2
1.0
0.8
0.6
0.4
0.2
0.0
% 125I - complex bound/internallsed
1
2
3
treatment

b)


0.4
0.3
0.2
0.1
0.0
% 125I - RNAse bound/internallsed
1
2
3
treatment

4.8. CONCLUSION.

The results from the SDS-PAGE analyses, autoradiography and immunoblottting clearly showed that a disulphide-bonded complex could be formed between the ricin B polypeptide and RNase A, via derivatisation of the latter with the cross-linking reagent SPDP. The nuclease activity of the derivatised RNase A and the RNase A in the complex was assayed by applying samples to activity gels (zymograms). The results indicated that the chemical modification of the RNase A by amino-group directed derivatisation did not significantly reduce its nuclease activity. The zymogram also showed that the disulphide linkage between the RNase A and the ricin B polypeptide did not have to be cleaved for the RNase A to be functionally active, unlike the *in vivo* relationship between ricin A and B chains. For the RNase A-ricin B complex to gain efficient access to the cytosol, the ricin B polypeptide has to retain the ability to bind to galactose-containing molecules present on the cell surface. This was established by studying the binding of iodinated RNase A-ricin B complex to the lymphoblastoid cell-line, Daudi. The results indicated that the iodinated complex had indeed bound to the cell surface, but failed to determine whether internalisation had subsequently occurred. Unfortunately, the DNA and protein synthesis assays, which were used to demonstrate that the RNase A had indeed reached its intracellular location and was having a measurable effect on the Daudi cells, may well have been complicated by a wide range of interfering factors as discussed in the Results Section 4.7.1. Thus, it is perhaps not a total surprise that the complex appeared to have no biological activity even if it had been successfully internalised.

A number of other molecules have been linked to ricin B chain, including the toxin gelonin (Goldmacher, *et al.*, 1987), the toxic A chain moiety of abrin (Olsnes, *et al.* 1974b), insulin (Roth, *et al.*, 1981; Hofmann, *et al.*, 1983), and antibody molecules (Vitetta, *et al.*, 1983,

1984). These molecules were attached to the ricin B polypeptide for a variety of reasons. Abrin A - ricin B (both 'acidic' peptides) was synthesised so that the toxicity of this hybrid molecule and the effects of charge could be studied. Antibody-ricin B complexes were prepared to be used in conjunction with antibody - ricin A chain immunotoxins, due to the finding that the ricin B molecule could potentiate the cytotoxic effect of the ricin A chain immunotoxin. The non-specific binding of the ricin B polypeptide had to be nullified and this provided a method of targetting the ricin B chain to a specific cell type. Gelonin-ricin B complex was utilised in a comparative study on the toxicity of this complex, ricin, diptheria toxin and antibody conjugates (ricin A or gelonin) to somatic cell mutants of the human B cell-line Namalwa. The protein hormone insulin was linked to the ricin B polypeptide via a disulphide bond. The binding of this complex to cells which were deficient in insulin receptors was studied, and biochemical responses which could be attributed to insulin stimulation were measured (Roth, et al., 1980; Hofmann, et al., 1983).

Ricin B chain is capable of transporting either abrin A or gelonin both isolated from plants into the cytosol where they exert their toxic effect, the inhibition of protein synthesis. When the ricin B chain is attached to a mammalian protein i.e. insulin or RNase A, the only conclusion that can be drawn is that these complexes can be bound to the plasma membrane via the ricin B polypeptide.

The experimental approach which would probably yield the greatest amount of information on the possibility of using ricin B chain as a vector for transporting proteins into the cell is labelling with either colloidal gold or hydrogen peroxidase. The RNase A -ricin B complex could be labelled, and then the conjugate could be used for internalisation studies with monolayer cultures to determine the

compartments involved in the internalisation of the labelled RNase A - ricin B complex. The experimental procedures utilised by van Deurs, et al., (1986, 1987) could be followed. The electron microscopic analysis of serial sections may well provide evidence for the internalisation of a labelled RNaseA - ricin B complex. As with all techniques, there are associated problems the ligand i.e. colloidal gold or hydrogen peroxidase can effect the binding and the internalisation patterns, incorrect fixation and poor sectioning can lead to the formation of artefacts.

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