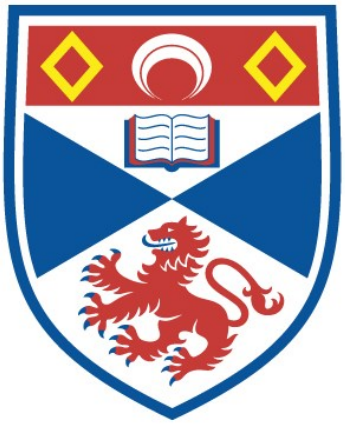
AN INVESTIGATION OF THE IGA1 PROTEASE OF UREAPLASMA UREALYTICUM

R. Katharine Spooner

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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An investigation of the IgA1 protease of *Ureaplasma urealyticum*

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Presented for the degree of Doctor of Philosophy at the University of St. Andrews,

August 1993.

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DECLARATION

I, Rachel Katharine Spooner, hereby certify that this thesis has been composed by myself, that it is a record of my own work, and that it has not been accepted in partial or complete fulfilment of any other degree or professional qualification.

Signed: Date: 13·8·93

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Dedicated to the memory of my mother, Frances Spooner

ABSTRACT

It was confirmed that *U. urealyticum* produces an IgA1 protease, which cleaves human IgA1 only into intact Fabα and Fcα fragments. By N-terminal amino acid sequencing of Fcα fragments, the site of digestion was identified as a Pro_{235}-Thr_{236} peptide bond within the α chain hinge-region of IgA1.

A number of assay systems were examined for their ability to detect and estimate IgA1 protease activity. A reliable and reproducible immunoblotting method was developed, in conjunction with a quantifiable assay utilising [^{125}I] IgA1. By these methods, IgA1 protease activity was identified in fourteen serotypes of *U. urealyticum*, all of which appeared to digest IgA1 at the same Pro_{235}-Thr_{236} peptide bond. The enzyme was active over a broad range of pH (pH 3-10) and was inhibited by the serine-protease inhibitors 3,4-DCI and DFP.

The IgA1 protease was not located in 'spent' ureaplasma cultivation medium but appeared to be cell-associated. The activity was solubilised by a number of non-ionic detergents which were required in purification buffers to maintain enzyme stability, further suggesting a membrane-bound location. Although the enzyme was not purified to homogeneity, a number of protocols were established which provide a basis for future work.

A genomic library of *U. urealyticum* DNA was produced and a variety of strategies adopted for identification of the *iga* gene. Radiolabelled DNA probes were generated from a plasmid containing the *iga* gene for *N. gonorrhoeae* (pIP503). By Southern blot hybridisation, no significant homology was identified between the heterologous probes and ureaplasma genomic DNA. Based on regions of high nucleotide conservation between the *iga* genes from *N. gonorrhoeae* and *H. influenzae,* degenerate PCR primers were designed. While amplification products did not appear to contain regions of the *iga,* such an approach may be adapted and extended for use in future studies.

ABBREVIATIONS

A	adenine
$A_{\#}$	absorbance at # nm
AA	acetic acid
ADCC	antibody-dependent cell-mediated cytotoxicity
AIDS	acquired immunodeficiency syndrome
amp	ampicillin
ATP	adenosine 5'-triphosphate
BCDS	bathocuprine disulphonate
BSA	bovine serum albumin
C	cytosine
CCU	colour-changing units
Cen A	endogluconase A
$C_{H}\#$	constant domain of an antibody heavy chain
CIAP	calf intestinal alkaline phophatase
CLD	chronic lung disease
ConA	concanavalin A
cpm	counts per minute
CRB	Cambridge Research Biochemicals
CSF	cerebral-spinal fluid
dATP	2'-deoxyadenosine 5'-tris(phosphate)
dCTP	2'-deoxycytidine 5'-tris(phosphate)
3,4-DCI	3,4-dichloroisocoumarin
DEE	diethyl ether
DFP	di-isopropylfluorophosphate
dGTP	2'-deoxyguanosine 5'-tris(phosphate)
DMAP	4-dimethylaminopyridine
DMF	dimethyl formamide
DMSO	dimethyl sulphoxide
DNA	2'-deoxyribonucleic acid
dNTP	2'-deoxynucleotide 5'-tris(phosphate)
DTT	dithiothreitol
dTTP	2'-deoxythymidine 5'-tris(phosphate)
E64	*L*-trans-epoxysuccinyl-leucylamide-(4-guanidino)-butane
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycol-bis(β-aminoethylether)*N,N,N',N'*-tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
Fab	antigen-binding fragment of antibody
Fc	crystalisable fragment of antibody
Fcα	crystalisable fragment of IgA antibody
FcαR	IgA Fc receptor
Fmoc	fluorenyl methoxycarbonyl
FPLC	fast protein liquid chromatography
FSD	full-scale deflection

G	guanine
HIV	human immunodeficiency virus
HLA B27	human lymphocyte antigen (major histocompatibility molecule) B27
HPLC	high performance liquid chromatography
IAA	iodoacetic acid
IEP	isoelectric point
Ig	immunoglobulin
IPTG	isopropyl-β-*D*-thiogalactopyranoside
kb	kilobases
kbp	kilobase pairs
LB	Luria broth
LPS	lipopolysaccharide
Mab	monoclonal antibody
MB	multiple-banding antigen
Mr	relative molecular mass
NaAc	sodium acetate
NAD^{+}/ NADH	nicotinamide adenosine dinucleotide (oxidised and reduced forms)
NAO	NADH oxidase
$NADP^{+}$/ NADPH	nicotinamide adenosine dinucleotide phosphate (oxidised and reduced forms)
NGU	non-gonococcal urethritis
NP40	nonidet P-40
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCMB	*p*-chloromercuribenzoate
PCR	polymerase chain reaction
PEG	polyethylene glycol
PFGE	pulse-field electrophoresis
pfu	plaque-forming units
pH	pondus hydrogen ($-\log_{10}[H^{+}]$)
PID	pelvic inflammatory disease
PMSF	phenylmethylsulphonylfluoride
PPLO	pleuropneumonia-like organisms
RFLP	restriction fragment length polymorphism
RIEP	rocket immunoelectrophoresis
RNA	ribonucleic acid
rpm	revolutions per minute
rRNA	ribosomal RNA
SAO	superoxide dismutase
SARA	sexually acquired reactive arthritis
SBTI	soya bean trypsin inhibitor

SDS	sodium dodecyl sulphate
SSC	salt-sodium citrate buffer
strep	streptomycin
T	thymine
TAA	tertiary amyl alcohol
TBE	Tris-borate-EDTA buffer
TBS	Tris-buffered saline
TCA	tricarboxylic acid or trichloroacetic acid
TE	Tris-EDTA buffer
TEMED	*N,N,N',N'*-tetramethylethylenediamine
tet	tetracycline
TFA	trifluoroacetic acid
TLCK	tosyl-lysine-chloromethyl ketone
TPCK	tosyl-phenylalanine-chloromethyl ketone
Tris	2-amino-2-(hydroxymethyl)propane-1,3-diol
tRNA	transfer RNA
TX100	Triton X-100
SC	secretory component
S-IgA	secretory IgA
UV	ultra-violet
V_H	variable region of an antibody heavy chain
V_L	variable region of an antibody light chain
v/v	volume per volume ration
w/v	weight per volume ratio
X-gal	5-bromo-4-chloro-3-indolyl-β-*D*-galactoside

GENETIC CODE

TTT phe F	TCT ser S	TAT tyr Y	TGT cys C
TTC phe F	TCC ser S	TAC tyr Y	TGC cys C
TTA leu L	TCA ser S	TAA OCH Z	TGA OPA Z
TTG leu L	TCG ser S	TAG AMB Z	TGG trp W
CTT leu L	CCT pro P	CAT his H	CGT arg R
CTC leu L	CCC pro P	CAC his H	CGC arg R
CTA leu L	CCA pro P	CAA gln Q	CGA arg R
CTG leu L	CCG pro P	CAG gln Q	CGG arg R
ATT ile I	ACT thr T	AAT asn N	AGT ser S
ATC ile I	ACC thr T	AAC asn N	AGC ser S
ATA ile I	ACA thr T	AAA lys K	AGA arg R
ATG met M	ACG thr T	AAG lys K	AGG arg R
GTT val V	GCT ala A	GAT asp D	GGT gly G
GTC val V	GCC ala A	GAC asp D	GGC gly G
GTA val V	GCA ala A	GAA glu E	GGA gly G
GTG val V	GCG ala A	GAG glu E	GGG gly G

ABBREVIATIONS FOR AMINO ACIDS

Alanine	ala	A
Arginine	arg	R
Asparagine	asn	N
Aspartic acid	asp	D
Cysteine	cys	C
Glutamine	gln	Q
Glutamic acid	glu	E
Glycine	gly	G
Histidine	his	H
Isoleucine	ile	I
Leucine	leu	L
Lysine	lys	K
Methionine	met	M
Phenylalanine	phe	F
Proline	pro	P
Serine	ser	S
Threonine	thr	T
Tryptophan	trp	W
Tyrosine	tyr	Y
Valine	val	V

UNITS

^{o}C	degrees Celsius (temperature)
g	gram (mass)
m	metre (length)
mol	mole (quantity)
s	second (time)

Ci	Curie (radioactivity of 3.7×10^{10} disintegrations.s^{-1})
Da	Dalton (relative molecular mass)
g	gravitational acceleration
h	hour (time)
l	litre (volume)
M	molar concentration (mol.l^{-1})
min	minute (time)
S	Svedberg (sedimentation)
U	unit of enzymatic activity

ORDER PREFIXES

c	centi	10^{-2}	k	kilo	10^{3}
m	milli	10^{-3}	M	mega	10^{6}
μ	micro	10^{-6}	G	giga	10^{9}
n	nano	10^{-9}	T	terra	10^{12}
p	pico	10^{-12}			

CONTENTS

LIST OF FIGURES

LIST OF TABLES

Chapter 1:
Introduction

SECTION A
PROPERTIES OF *U. UREALYTICUM*

1.1 TAXONOMY AND PHYLOGENY

1.1.1 The class *Mollicute*

Mollicutes are a group of free living prokaryotes that have been isolated from a wide range of hosts including humans, animals, birds, insects and plants. They possess a number of unusual properties, the most notable being a lack of cell wall which places them within the division of wall-less bacteria, *Tenericutes*. Their small genomes and unusual nutritional requirements justifies the creation of *Mollicute* as a distinct class within this division (Edward and Freundt, 1967) (Fig. 1.1). Further sub-divisions are based on phenotypic characteristics such as genome size, oxygen sensitivity and sterol requirements, producing a total of six genera; *Acholeplasma, Anaeroplasma, Asteroleplasma, Spiroplasma, Mycoplasma,* and *Ureaplasma* (Robinson and Freundt, 1987). By convention, the trivial name 'mollicute' is used to denote any member of this class and the names 'acholeplasma', 'anaeroplasma', 'asteroleplasma', 'spiroplasma', 'mycoplasma' and 'ureaplasma' denote members of the corresponding genera.

The evolution of mollicutes is unclear; as wall-less bacteria they may represent primitive forms of prokaryotes or, alternatively, they may have evolved later as degenerate forms of walled relatives (Razin, 1978). Genomic comparisons have given some insight into their phylogeny and also support the broad taxonomic groupings described above. Initial characterisation of 16S RNA revealed that mollicutes were related to gram-positive bacteria with low G+C compositions, such as the *Bacillus-Lactobacillus* cluster and *Clostridia* (Woese *et al.*, 1980). Sequence comparisons of 5S RNA (Rogers *et al.*, 1985) and 16S RNA from over forty species of mollicute (Weisburg *et al.*, 1989) confirmed and extended these findings and has led to the construction of a detailed phylogenetic tree (Maniloff, 1992) (Fig. 1.2). From these analyses, it has been proposed that asteroleplasmas

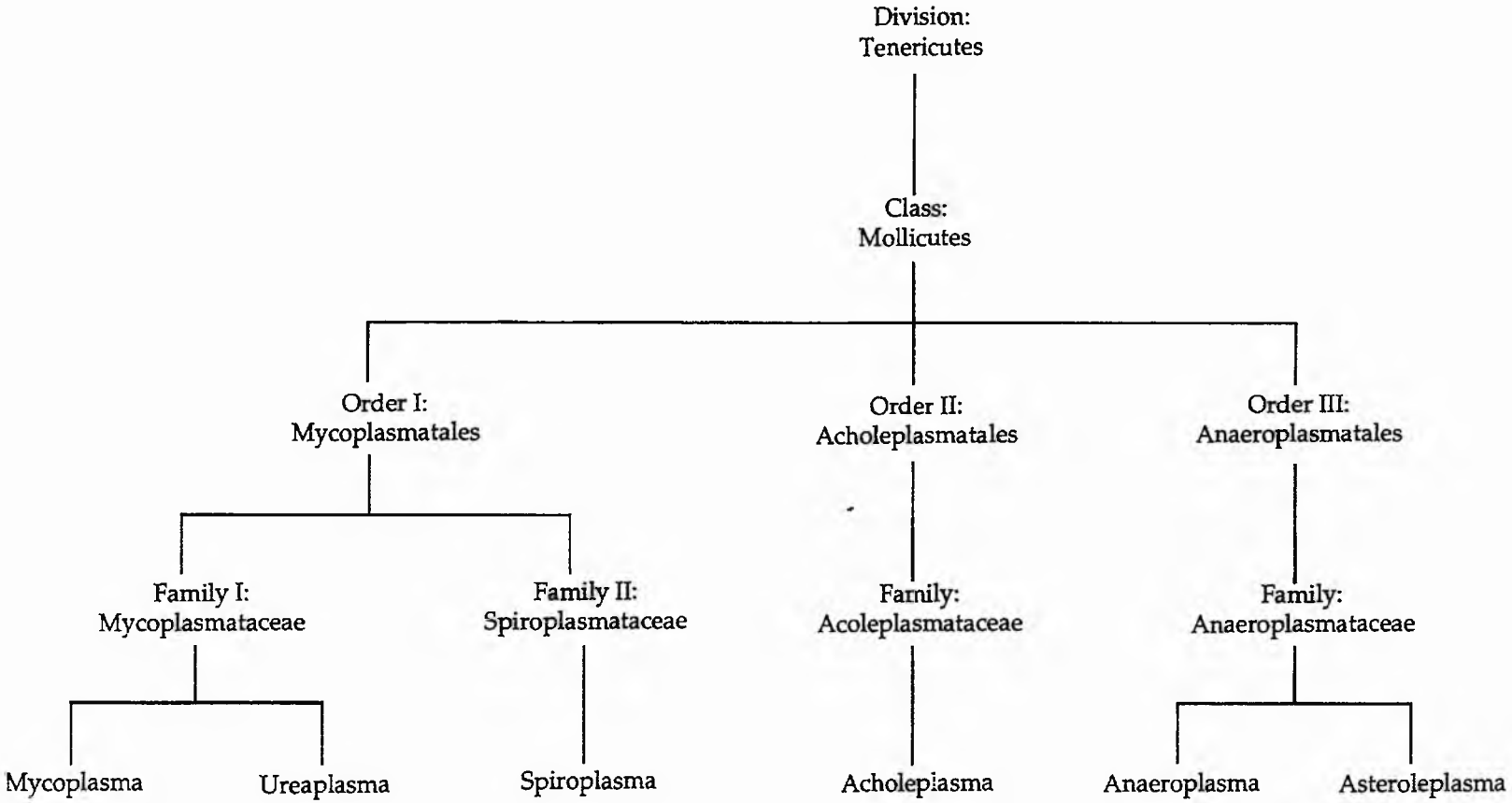


Fig. 1.1 The taxonomy of *Mollicutes*

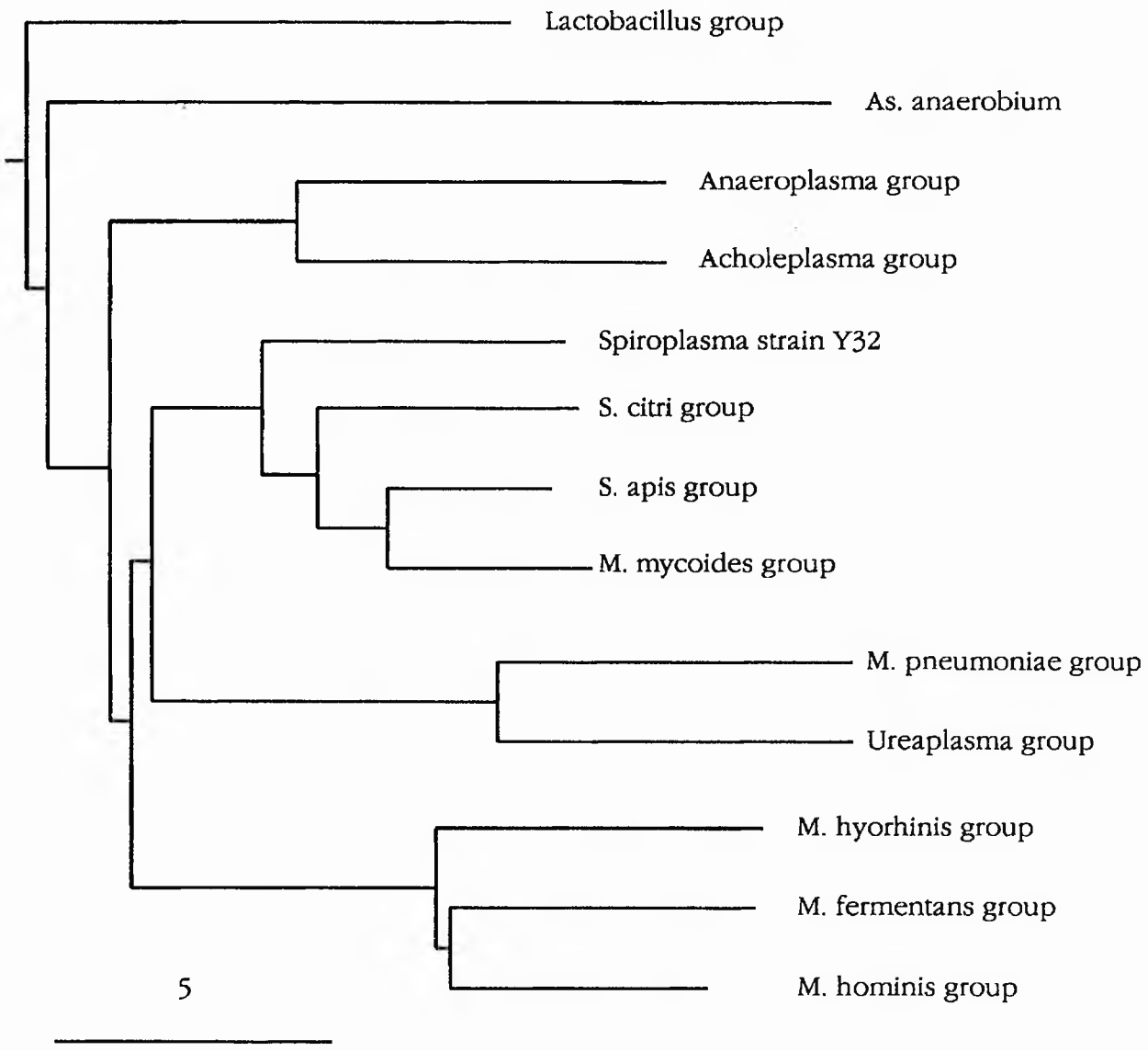


Fig. 1.2 Phylogenetic tree for *Mollicutes*

Taken from Maniloff (1992), the mollicute phylogenetic tree has been reconstructed from 16S RNA sequence comparisons. *E. coli* provided the root of the tree and closely related phylogenetic sublines have been grouped together. Branch lengths are proportional the number of base changes per 100 nucleotides, i.e. the evolutionary distance between groups. The scale to the left denotes the branch distance corresponding to five base changes per 100 nucleotides.

and acholeplasmas have evolved from the *Lactobacillus* group by a decrease in chromosome size to 1640-1700 kbp and that further divergence has produced the sterol-requiring anaeroplasmas (1700 kbp) and helical spiroplasmas (1350-1700 kbp). A number of independent and repeated genome reductions in the spiroplasma branch may have finally yielded mycoplasmas and ureaplasmas with genome sizes of 620-1160 kbp (Rogers *et al.*, 1985, Maniloff, 1992).

These genomes are the smallest recorded for any self-replicating prokaryote and mollicutes therefore represent the simplest free-living biological system known (Razin, 1992). Their limited coding capacity imposes restrictions on their biosynthetic capabilities so that they live as parasites both in their hosts, where they are frequently pathogenic, and in rich tissue culture systems, where they are unwelcome contaminants. It is for these reasons that mollicutes are of scientific, medical, veterinary and agricultural importance.

1.1.2 The genera *Mycoplasma* and *Ureaplasma*

Of all mollicutes, the genus *Mycoplasma* has aroused the greatest interest as within its eighty species are a number of human pathogens that have been associated with respiratory, arthritic and urogenital diseases (Krause and Taylor-Robinson, 1992). These include *Mycoplasma pneumoniae,* (*M. pneumoniae*), *Mycoplasma hominis* (*M. hominis*), and *Mycoplasma genitalium* (*M. genitalium*). Most recently *Mycoplasma fermentans* (strain *incognitus*), *Mycoplasma penetrans* (*M. penetrans*) and *Mycoplasma pirum*, have been implicated as cofactors in the progression of human immunodeficiency virus (HIV) infection to AIDS (Lo *et al.*, 1989, 1991, Lemaitre *et al.*, 1992, Chirgwin *et al.*, 1993).

Ureaplasmas have many features in common with mycoplasmas, but their unique ability among mollicutes to hydrolyse urea places them within a separate genus *Ureaplasma* (Shepard *et al.*, 1974). They are widely distributed in nature and colonise most animal species that have been examined to date. Serologic and genomic analysis of some isolates has currently divided the genus into five species; *Ureaplasma urealyticum* (*U. urealyticum*), isolated from humans, *Ureaplasma diversum* (*U. diversum*), isolated from cattle (Howard and Gourlay,

1982), *Ureaplasma gallorale*, isolated from chickens (Koshimizu, *et al.*, 1987), *Ureaplasma felinum*, and *Ureaplasma cati*, both isolated from cats (Harasawa *et al.*, 1990). Identification of new species awaits further characterisation of isolates and the development of improved culture conditions, which at present may not support the growth of more fastidious organisms.

The human isolate, *U. urealyticum*, has presently been divided into fourteen serotypes on the basis of a complement-dependent mycoplasmacidal test (Lin and Kass, 1980), a modified metabolic inhibition test and a colony indirect epifluorescent test (Robertson and Stemke, 1982). The fourteen serotypes can be further sub-divided into two seroclusters based on their sensitivity to manganese salts (Robertson and Chen, 1984), protein profiles by 1D and 2D polyacrylamide gel electrophoresis (Mouches *et al.*, 1981, Swensen *et al.*, 1983), hybridisation using rRNA probes (Razin and Yogev, 1986), restriction endonuclease patterns (Tully and Taylor-Robinson, 1986) and, most recently, by polymerase chain reaction (PCR), (Blanchard, 1990).

Since its isolation in from patients with non-gonococcal urethritis (Shepard, 1954), research on *U. urealyticum* has centred on elucidating the role of this mollicute in disease, particularly of the human urogenital tract. In order to achieve this goal, fundamental characterisation of the organism has been a requirement.

1.2 CHARACTERISATION OF *UREAPLASMA UREALYTICUM*

1.2.1 Growth

As a consequence of their lack of cell wall and limited coding potential, ureaplasmas require an osmotically balanced cultivation medium, rich in a supply of metabolic intermediates. Many of the complex media described for ureaplasma growth are based on that devised by Edward (1947). PPLO broth provides the base and consists of beef heart infusion, bactotryptose and sodium chloride. It is supplemented with yeast extract (containing nucleic acids, their derivatives and precursors) and with horse serum which provides essential cholesterol, urea, and

other unidentified components beneficial to ureaplasma growth (Shepard and Masover, 1979). Additional urea may be included to give an optimum concentration of 0.01 M (Shepard and Lunceford, 1967). The hydrolysis of urea to ammonia and carbon dioxide by a urease enzyme seems to be an absolute requirement for growth in ureaplasmas, as indicated by the use of urea-free media and specific urease inhibitors (Kenny and Cartwright, 1977, Kenny, 1983, Smith *et al.*, 1993).

It has been proposed that *U. urealyticum* has the unique ability among mollicutes to synthesise unsaturated and saturated fatty acids from acetate (Romano *et al.*, 1976), but other workers have disputed this finding (Pollack *et al.*, 1984). Fatty acids provided in the serum may therefore also be necessary for growth. Very little is known about the nutritional requirements of ureaplasmas for amino acids, vitamins and cofactors. Thiols can improve the growth of *U. urealyticum*, but they may be serve to prevent inhibition of the urease enzyme rather than being incorporated for metabolic purposes. Supplements of L-histidine, arginine, methionine and cysteine can stimulate growth and calcium may also be a requirement (Shepard and Masover, 1979).

Inclusion of an indicator in the cultivation medium allows ureaplasma growth to be monitored, since the liberation of ammonia from urea hydrolysis results in a detectable pH rise. Utilising this property, cell numbers are estimated by serially diluting a suspension of the organism to extinction in liquid medium. After a suitable incubation period (at least 24 h after the last colour change was seen), the reciprocal of the highest dilution in which growth occurs (indicated by the colour change) is the estimated population size, expressed in colour-changing units ($CCU.ml^{-1}$). By this method, it been established that the optimum pH for growth is 6.0-6.5 and that ureaplasmas follow the lag, exponential and stationary phases typical for bacterial growth. Beyond pH 8, however, there is rapid cell death so that characteristically the $CCU.ml^{-1}$ never rises above 10^8 (Shepard and Masover, 1979). This may be due to inactivation of the urease enzyme at pH 8, but buffering of the media fails to alleviate this effect (Razin *et al.*, 1977a, Thirkell, personal communication). Growth is also not improved by replenishing urea,

demonstrating that substrate is not limiting (Razin *et al.*, 1977a). It is possible that growth is inhibited by an unidentified toxic product of metabolism (Furness, 1973).

An important consequence of this inhibitory effect is the very low yield of ureaplasma cells obtained from growth in artificial media (O' Brien and Barile, 1983). Furthermore, the organisms are frequently harvested in association with non-specific adherents, probably serum components, which interfere with subsequent analysis of cell surface antigens (Masover and Hayflick, 1973). It may be possible to overcome this growth medium contamination by replacing the horse serum with PPLO serum, as recently described by Horowitz and Gal (1991).

1.2.2 Morphology, size and ultrastructure

Ureaplasmas were first identified on solid media as exceedingly small colonies (7-15 μm) and it is for this reason that they were originally named 'tiny form PPLO' or 'T mycoplasmas' by Shepard (1954). They grow with an irregular border and only under certain conditions have the classical 'fried egg colony' appearance of other mycoplasmas (Razin *et al.*, 1977b). In liquid culture, ureaplasmas generally exist as round or ovoid elements but show varied morphology, depending on medium composition, their stage of growth and the methods used for sample preparation (Reviewed by Shepard and Masover, 1979). It has been important to establish the minimum size of these simple life forms, but there are errors inherent in the methods used to determine this. For example, passage through defined filters may only indicate that the wall-less organism can squeeze through a pore smaller than its own diameter. Likewise, measurements from electron micrographs vary according to the type of fixation and the plane of section taken. There is general agreement, however, that ureaplasmas and mycoplasmas are no smaller than 300 nm, which represent the smallest living units reported to date that are capable of self-reproduction (Razin, 1978).

The means by which ureaplasmas reproduce is unclear, but identification of surface outgrowths suggests that it may be by budding (Razin *et al.*, 1977b, Myles *et al.*, 1991). In other mycoplasmas, there is evidence for binary fission.

Electron micrographs following the stages of reproduction in *M. hominis* showed that rapidly growing cocci temporarily became filamentous (Bredt *et al.*, 1973). This has been interpreted as binary fission where cytoplasmic division has lagged behind genome replication, thus producing multi-nucleate filaments. Filamentous forms have been observed in ureaplasma cultures by some workers (Rottem *et al.*, 1971, Black *et al.*, 1972a) but not others (Whitescarver and Furness, 1975, Razin *et al.*, 1977b). Since the absence of filaments may have been due to the poor growth of these organisms in the available media, binary fission has not been discounted as a form of replication in *U. urealyticum*.

There is more agreement on the ultrastructure of ureaplasmas; electron micrographs show them to be bound by a typical trilaminar membrane (7.5-10 nm) with an extra membranous electron-dense layer present in some preparations (Black *et al.*, 1972a, Whitescarver and Furness, 1975). Staining by osmium-ruthenium red and ConA/ion dextran stain shows that this 'capsule' contains mucopolysaccharide, and it may influence ureaplasma aggregation and adherence (Robertson and Smook, 1976). The precise means by which ureaplasmas adhere to cells is unknown, but small hair-like projections seen by freeze-fracture analysis may be involved (Black *et al.*, 1972a). Specialised tip structures that mediate adhesion in *M. pneumoniae* and *M. genitalium* (Razin and Jacobs, 1992) are not obvious in ureaplasma preparations.

Within the cells, the cytoplasm is quite uniform and contains ribosomes, vacuoles, and a double stranded circular DNA molecule. This has a low but constant G+C content of 28% (Bak and Black, 1968) and, as with cellular dimensions, estimates of its size vary depending on the methods used. Early studies using renaturation kinetics suggested a genome size of 620 kbp-720 kbp for *U. urealyticum* (Black *et al.*, 1972) but later work using pulse-field electrophoresis (PFGE) gave larger estimates of around 900 kbp (Neimark and Lange, 1990, Pyle *et al.*, 1988). These differences may be due to high A+T levels in ureaplasma DNA producing anomalous results. A refinement of PFGE, contour-clamped homogenous field agarose gel electrophoresis has divided *U. urealyticum* genome sizes into two groups; 760 kbp for the small serocluster

and 840-1140 kbp for the large serocluster (Robertson *et al.*, 1990). These values seem plausible, since by summing a series of overlapping cosmid clones that map the whole genome, a method that is not affected by the G+C content of the DNA, the genome size for *M. pneumoniae* has been estimated as 835-849 kbp (Wenzel and Herrmann, 1989). Since 29 restriction sites have now been mapped in the *U. urealyticum* genome (Cocks *et al.*, 1989), such an approach may be possible in this organism.

Assuming that the minimum genome size for ureaplasmas is 750 kbp then, along with mycoplasmas, these are the smallest genomes recorded for any self-reproducing prokaryote. It has been predicted that genomes of this size (600-800 kbp) contain no more than 500 genes which should encode all the compounds necessary for growth and replication (Morowitz, 1967). These include enzymes for translation, transcription and protein synthesis, messenger, transfer and ribosomal RNA, structural proteins for ribosomes and cell membranes, and enzymes for the biosynthesis of cellular components and fuelling reactions. With such a small genome, it is likely that ureaplasmas have retained only the essential genetic information necessary to perform all these functions. This is indicated by their lack of a cell wall, simple ultrastructure and extensive nutritional requirements. If all the essential processes can be delineated in these simple free living organisms, then progress will be made in understanding the fundamental workings of a cell (Razin, 1992).

Information on the cellular reactions operating in *U. urealyticum* is scarce however, and a list of all the various protein functions is far from exhaustive. Limited progress has been made in examining each of the predicted functions described above and, for many, the economy in genetic material is apparent.

1.2.3 Transcription, translation and protein synthesis

Since the only sequence data available for *U. urealyticum* is from the urease gene (Blanchard, 1990), knowledge of ureaplasma genetics is limited and very fragmented. In the urease operon, a putative promoter upstream from the initiation site contains a -35 region and a Pribnow box. Ribosome binding sites are

located upstream from the g subunit in the urease operon (Blanchard, 1990) and ribosomes appear have a typical 30S:50S prokaryotic structure (Ohse and Göbel, 1987). The basic mechanism of protein synthesis is retained in ureaplasmas but there are considerable savings in genetic information. Compared with the seven rRNA operons in *E. coli*, only two copies are found in *U. urealyticum*. rRNA genes are organised in the order 5'-16S-23S-5S-3' (Ohse and Göbel, 1987). There is also a saving in the number of tRNA species. For mollicutes in general, up to thirty different tRNA genes encoding 29 tRNA species have been identified, which is less than half of the 78 tRNA genes found in *E. coli* (Razin, 1992). tRNAs appear to be highly conserved and have G+C values closer to prokaryotic tRNA. In many mycoplasmas, including *U. urealyticum*, the codon UGA codes for tryptophan rather than a stop signal (Blanchard, 1990). As with Gram-positive bacteria, mycoplasmas carry only one copy of the *tuf* gene encoding the elongation factor Ef-Tu (Yogev *et al.*, 1990). Gram-negative bacteria carry two copies of this gene.

Within the DNA replication complex of *U. urealyticum*, a single copy of the DNA polymerase gene, coding for an enzyme lacking 3'-5' exonuclease activity (Maurel *et al.*, 1989) and a *dnaA* gene have been found (Miyata *et al.*, 1992). A number of DNAases and RNAases have also been identified, which may have a role in DNA repair and restriction of foreign DNA (Romano and La Licata, 1978, O'Brien *et al.*, 1983).

1.2.4 Membrane structure

In the absence of a cell wall, structural stability is maintained by the plasma membrane, which, like typical biological membranes, is composed mainly of proteins (50-60%), lipids (30-40%) and a small amount of carbohydrate (Shepard and Masover, 1979). The lipid fraction contains predominantly cholesterol and its esters, free fatty acids, glycolipids, phosphatidic acid and phosphatidyl ethanolamine (Rottem *et al.*, 1971, Romano *et al.*, 1972). Cholesterol is an integral part of the membrane and the acidic lipids may serve to neutralise the copious quantities of ammonia produced by urea hydrolysis. Purified membranes from several strains of *U. urealyticum* have been found to contain

mannose, glucose and galactose (Whitescarver *et al.*, 1975). It is thought that these are complexed as membrane-associated lipoglycan, which may constitute the capsular material described earlier (Smith, 1985).

Over 30 membrane proteins of *U. urealyticum* have been identified by various methods including [^{125}I] Bolton and Hunter surface-labelling and phase-separation of hydrophobic proteins using Triton X-114 detergent, followed by SDS-polyacrylamide gel electrophoresis (Thirkell *et al.*, 1989a). A number of these are antigenic, as demonstrated by Western Blotting with polyclonal anti-sera raised against *U. urealyticum* and three in particular have been characterised using monoclonal antibodies. These are a 17 kDa antigen (17K), a serogroup specific 16 kDa antigen (16K) and a serotype 8 specific 96 kDa antigen (96K), (Precious *et al.*, 1987, Thirkell *et al.*, 1989a). The 16K and 17K are located internal to the membrane and may perform a structural role (Myles *et al.*, 1991) and the 96K is surface exposed having at least four different epitopes, as detected by monoclonal antibodies (Thirkell *et al.*, 1990). Binding by these antibodies suppresses growth and metabolism of *U. urealyticum* for six hours, suggesting an important metabolic function for the 96K antigen (Precious *et al.*, 1987). In addition to other membrane proteins (~25%), the 96K can be labelled with ^{3}H palmitic acid, which suggests a degree of acylation (Thirkell *et al.*, 1991). Acylation may serve to anchor proteins into the lipid bilayer (Schmidt *et al.*, 1979) and may also be necessary for maintaining cellular integrity (Dahl *et al.*, 1983).

The functions of the other membrane proteins are unknown but it is presumed that some may act as adhesins (Manchee and Taylor-Robinson, 1969) and others may be involved in transport mechanisms. Since ureaplasmas require a number of preformed metabolites, efficient transport mechanisms are vital. A transport protein that has been characterised in some detail is the ureaplasma adenosine triphosphatase (F_0F_1 ATPase), first identified by Masover *et al.*, (1977a). This integral membrane protein translocates protons into the cell under the influence of an electrochemical potential, resulting in concomitant production of ATP.

1.2.5 Biosynthesis and fuelling reactions

It is likely that an F_0F_1 ATPase is the primary source of ATP production for ureaplasmas as a large number of enzymes involved in the conventional energy generating pathways of glycolysis, tricarboxylic acid cycle (TCA cycle) and the electron transport chain are missing in this organism (Fig. 1.3). Low cell yields and enzyme instability may have prevented the identification of these metabolic pathways, or they may indeed be absent. The use of radiolabelled precursors has helped to resolve this problem and to answer the fundamental question of how ureaplasmas obtain energy and convert medium constituents into cell mass.

Unlike fermentative mycoplasmas, glucose is not an energy source for ureaplasmas (Pollack, 1986) due to the lack of hexokinase and phosphoglucose isomerase early in the glycolytic pathway. Other carbon-containing compounds may enter glycolysis at the glyceraldehyde 3 phosphate locus from either lipids (via glycerol-3-phosphatase) or from nucleic acids via the non-oxidative enzymes of the pentose phosphate shunt. Here pentose phosphates from RNA and DNA degradation are converted to fructose-6-phosphate and glyceraldehyde-3-phosphate by the action of transketolase and transaldolase enzymes. These reactions appear reversible, so that nucleosides and nucleotides can be synthesised from glycolytic intermediates by pentose phosphates entering purine and pyrimidine salvage pathways. Ureaplasmas therefore have the potential to interchange carbon reversibly between lipids and nucleotides (Cocks *et al.*, 1985). The contribution of this pathway to energy generation is at present unclear as there are no glycolytic enzymes beyond 1,3-bisphosphoglycerate to generate ATP from the common intermediate, glyceraldehyde-3-phosphate.

Localisation of enzymes at later stages of glycolysis suggest that pyruvate may be a metabolically useful intermediate. However, pyruvate is not converted into lactate or ethanol nor does it enter the TCA cycle via acetyl CoA since enzymes for these pathways are lacking in ureaplasmas. Davis *et al.*, (1990) proposed that pyruvate can enter into a regenerative cycle involving pyruvate dehydrogenase, malate synthase, malate dehydrogenase and pyruvate carboxylase

Fig. 1.3 Carbohydrate catabolism in *U. urealyticum*

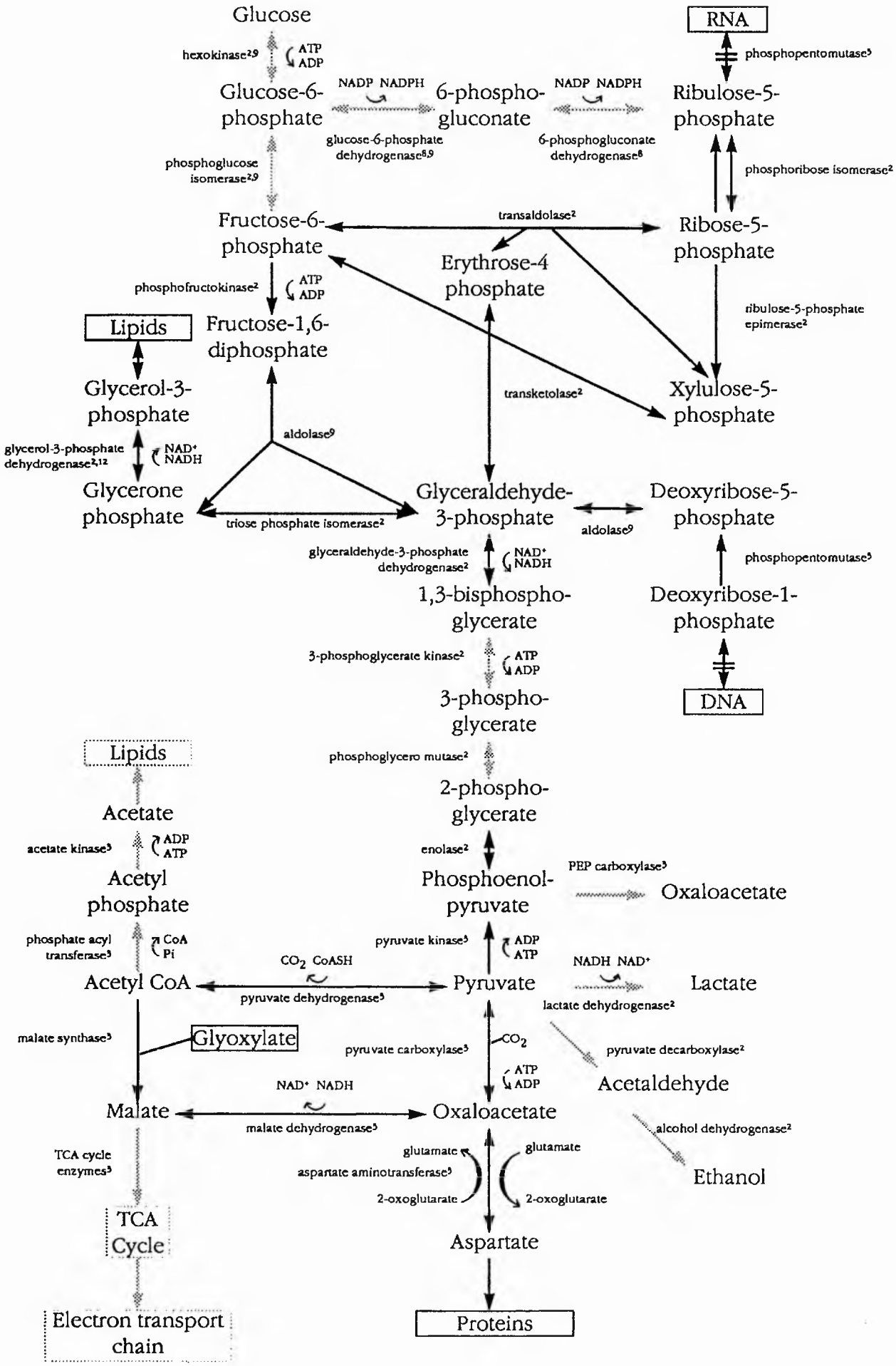
(modified from Miles,[1992] and Pollack, [1992]). Bold arrows indicate reactions reported in *U. urealyticum*, shaded arrows represent those not yet identified in the organism. Numbers in superscript refer to the following publications:

2 Cocks *et al.*, 1985

3 Davis *et al.*, 1990

8 O'Brien *et al.*, 1983

9 Pollack, 1986


Glucose
hexokinase[2,9]
ATP
ADP
Glucose-6-phosphate
NADP NADPH
glucose-6-phosphate dehydrogenase[8,9]
6-phospho-gluconate
NADP NADPH
6-phosphogluconate dehydrogenase[8]
RNA
phosphopentomutase[5]
Ribulose-5-phosphate
phosphoglucose isomerase[2,9]
phosphoribose isomerase[2]
Fructose-6-phosphate
transaldolase[2]
Ribose-5-phosphate
Erythrose-4 phosphate
phosphofructokinase[2]
ATP
ADP
Lipids
Fructose-1,6-diphosphate
ribulose-5-phosphate epimerase[2]
Glycerol-3-phosphate
transketolase[2]
Xylulose-5-phosphate
aldolase[9]
glycerol-3-phosphate dehydrogenase[2,12]
NAD+
NADH
Glycerone phosphate
triose phosphate isomerase[2]
Glyceraldehyde-3-phosphate
aldolase[9]
Deoxyribose-5-phosphate
glyceraldehyde-3-phosphate dehydrogenase[2]
NAD+
NADH
phosphopentomutase[5]
1,3-bisphospho-glycerate
Deoxyribose-1-phosphate
3-phosphoglycerate kinase[2]
ATP
ADP
DNA
3-phospho-glycerate
Lipids
phosphoglycero mutase[2]
2-phospho-glycerate
Acetate
acetate kinase[3]
ADP
ATP
enolase[2]
PEP carboxylase[3]
Acetyl phosphate
Phosphoenol-pyruvate
Oxaloacetate
phosphate acyl transferase[3]
CoA
Pi
pyruvate kinase[3]
ADP
ATP
CO2 CoASH
NADH NAD+
Acetyl CoA
pyruvate dehydrogenase[3]
Pyruvate
Lactate
lactate dehydrogenase[2]
malate synthase[3]
Glyoxylate
pyruvate carboxylase[3]
CO2
pyruvate decarboxylase[2]
ATP
ADP
Acetaldehyde
NAD+ NADH
Malate
malate dehydrogenase[3]
Oxaloacetate
alcohol dehydrogenase[2]
TCA cycle enzymes[3]
glutamate
aspartate aminotransferase[3]
2-oxoglutarate
glutamate
2-oxoglutarate
Ethanol
TCA Cycle
Aspartate
Electron transport chain
Proteins

(Fig. 1.3) which results in the overall production of ATP and carbon dioxide from one molecule of glyoxylate. The source of carbon (glyoxylate) for this cycle is unknown, since a number of enzymes from the glyoxylate cycle are missing in ureaplasmas, as are glycine oxidase and glycine dehydrogenase (Alozie *et al.*, 1992). The cycle may be biosynthetically useful, since pyruvate may be converted to aspartate via aspartate amino transferase and it also may serve to regenerate NAD^+ necessary for carbohydrate metabolism.

In other mollicutes, it has been proposed that the oxidation of NADH is catalysed by a copper or iron containing oxygen-dependant NADH oxidase (NAO) (Pollack, 1992). In this reaction, electrons pass directly from NADH to oxygen, resulting in the formation of a superoxide radical (O_2^-). This is subsequently converted to H_2O_2 and finally H_2O and O_2 via superoxide dismutase (SOD) and catalase enzymes, respectively. Known as flavin-terminated respiration, NADH oxidation may be responsible for the conversion of reducing equivalents to ATP, analogous to electron-transport chain reactions in *E. coli*. Although SOD and catalase enzymes have been identified in *U. urealyticum* (Meier and Habermehl, 1990), NAO appears to be absent (Pollack, 1986), which suggests that carbohydrate metabolism results in the production of ATP from substrate level phosphorylation alone.

It is unlikely, however, that ATP production from substrate level phosphorylation in the reactions described so far will be sufficient to sustain life, so other energy-generating systems must operate in *U. urealyticum*. It has been proposed by Romano *et al.*, (1986) that urea hydrolysis within the ureaplasmas generates a transmembrane potential which drives protons into the cell via an ATPase, thus generating ATP via a chemiosmotic process. Smith *et al.* (1993), have shown that an ammonium ion potential is indeed generated by urea hydrolysis and that this can result in *de novo* ATP synthesis. Furthermore, inhibition of the urease by fluorofamide abolishes the chemical potential and ATP synthesis is reduced by 95%.

Not only does this proposed mechanism provide an energy source for ureaplasmas but it also answers the question of why these organisms have an

absolute requirement for urea. It had been shown previously that the carbon component did not enter biosynthetic pathways but was metabolised completely to carbon dioxide (Ford *et al.*, 1970). Urea nitrogen, however, may enter into a metabolically useful pathway by the carbamoyl synthetase-catalysed conversion of ammonia to carbamoyl phosphate (Fig. 1.4). This in turn combines with ornithine in the urea cycle to form citrulline. Detection of three enzymes (argininosuccinate synthetase, argininosuccinase, arginine deaminase), which would complete the cycle of citrulline to aspartate, would confirm urea as a potential source of nitrogen for biosynthetic reactions, but to date these enzymes have not been identified (Smith *et al.*, 1992). The role of urea in this pathway seems secondary to its role in energy generating mechanisms.

The urease enzyme is located in the cytosol (Masover *et al.*, 1977a, Myles *et al.*, 1991) and catalyses the conversion of urea to ammonia, carbon dioxide and water. A number of specific monoclonal antibodies have been raised against the enzyme which have been used in immuno-affinity chromatography for purification from cell extracts (Thirkell *et al.*, 1989b). The urease enzyme appears to exist as two nickel-requiring isoenzymes of approximate molecular weight 200 kDa. Denaturation produces three subunit polypeptides; 72 kDa, 14 kDa and 11 kDa, which probably form a hexamer in an $\alpha 2$, $\beta 2$, $\gamma 2$ configuration to give urease activity (Thirkell *et al.*, 1989b). Other workers have suggested that the enzyme is a dimer, consisting of two 75 kDa subunits (Saada *et al.*, 1990a). The urease gene has been cloned and sequenced and contains three open reading frames coding for deduced polypeptides of molecular weights 11.2 kDa, 13.6 kDa and 66.6 kDa (Blanchard, 1990). These are in close agreement with the subunit molecular weights reported by Thirkell *et al.*, (1989b) and support the proposal for a hexameric urease structure.

An understanding of the basic properties of *U. urealyticum*, and in particular the urease enzyme, have allowed a number of specific diagnostic techniques to be developed for identification of the organism in clinical samples.

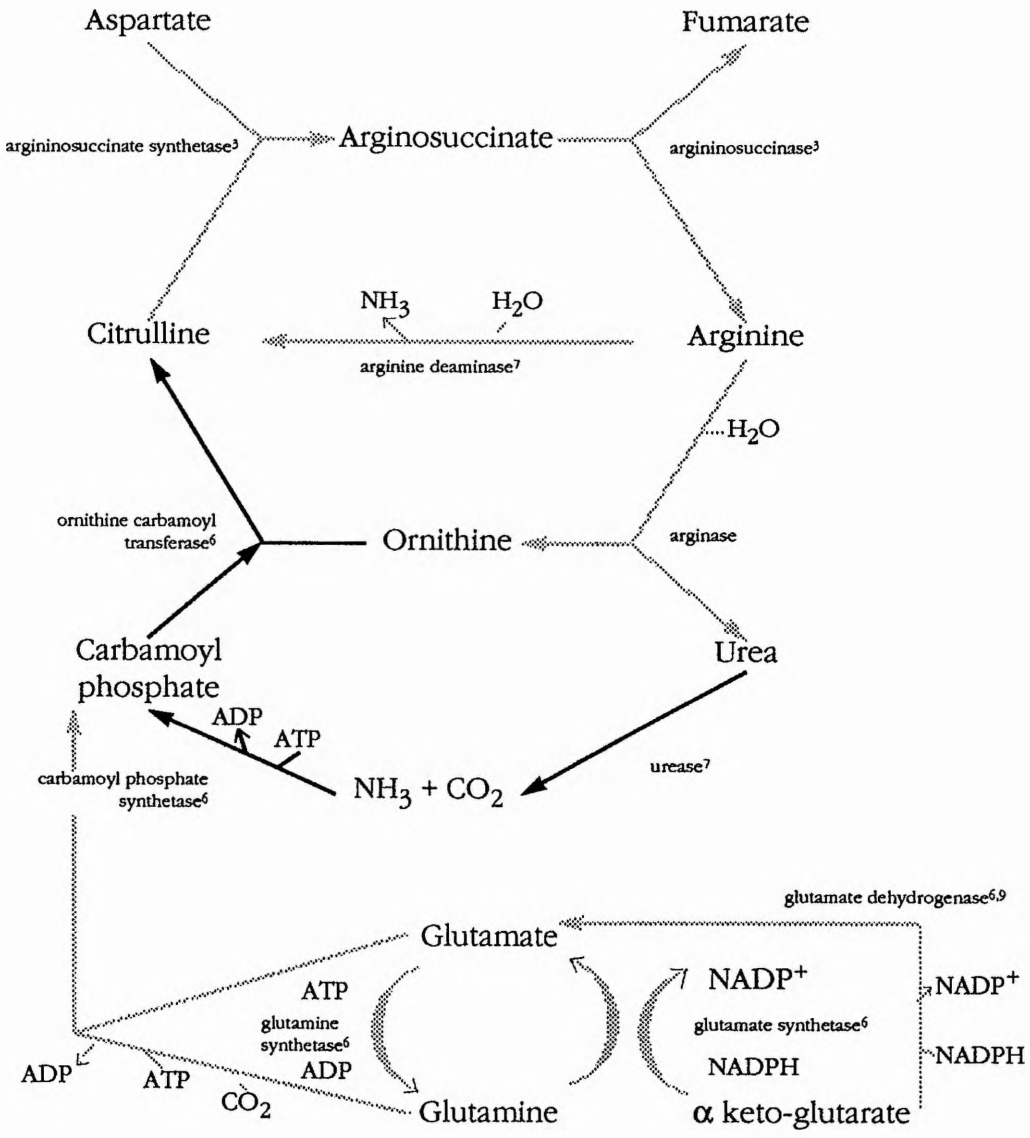


Fig. 1.4 The urea cycle in *U. urealyticum*

The cycle proposes an anabolic function for urea nitrogen, via the synthesis of carbamoyl phosphate to citrulline. In the absence of argininosuccinate synthase and argininosuccinate, the fate of citrulline is unclear. Other related enzymes that have not been detected in the organism are indicated by shaded arrows. Numbers in superscript refer to the following publications:

3 Davis *et al.*, 1990

6 Smith *et al.*, 1992

7 Shepard and Lunceford, 1979

9 Pollack, 1986

1.3 IDENTIFICATION OF *U. UREALYTICUM* IN CLINICAL SAMPLES

1.3.1 Cultivation

Early detection methods for *U. urealyticum* were based on the demonstration of urease activity in cultivation media (Shepard and Masover, 1979). Ureaplasmas grown on 'A7' differential agar (later improved to A8 by Shepard, 1983) appear as deep brown colonies due to the reaction of ammonia with manganese sulphate which produces deposits of manganese dioxide. For liquid cultures, 'U9' media, containing phenol red (Shepard and Lunceford, 1970) or bromothymol blue media (Robertson, 1978), give a visible colour change on ammonia production. Although cultivation methods are accepted as a sensitive means of ureaplasma identification, in theory detection requires the presence of only one cell, they are not selective for different ureaplasma serotypes and for diagnostic purposes they are time consuming. Furthermore, an understanding of the complex and varied growth requirements of ureaplasmas is often restricted to specialised workers in the field and in diagnostic laboratories ureaplasmas are generally not screened for routinely. It has therefore been necessary to devise rapid and accurate diagnostic techniques which do not require the growth of the organism and which may be used by non-specialists. These include methods that identify host antibody raised in response to ureaplasma infection (serodiagnosis), and those that directly detect ureaplasma proteins or DNA.

1.3.2 Serodiagnosis

There is increasing evidence that ureaplasma infections are associated with a good humoral immune response (Krause and Taylor-Robinson, 1992) and methods such as Metabolic Inhibition (Purcell *et al.*, 1966), ELISA (Brown *et al.*, 1983, Wiley and Quinn, 1984) and Immunoperoxidase assays (Quinn and Th'ng, 1990) have detected patient antibody levels that correlate well with ureaplasma cultivations data. For serodiagnosis, ELISA-based methods are particularly useful since large numbers of samples can be processed together, the results are easily quantifiable and serotype-specific antibody can be detected. The high background

levels sometimes reported for this technique may be eliminated in future by the use of species-specific and serotype-specific proteins, such as the 16K or 96K protein described in Section 1.2.4. The ability to clone such proteins and obtain them in large quantities may further reduce the cost and improve the sensitivity and selectivity of serodiagnostic techniques.

1.3.3 Protein detection

Alternatively, these species-specific and serotype-specific proteins may be used to generate monoclonal and polyclonal antisera for the detection of *U. urealyticum* in secretions, tissues and body fluids. Immunofluorescence using polyclonal antisera raised against whole organisms has detected ureaplasmas in clinical samples (Rudd *et al.*, 1989) and the value of monoclonal antibodies (Mabs) for this technique has recently been assessed (Myles, 1990). The species specific anti-16 and 17K Mabs and the serotype 8 specific anti-96K Mab detected as low as 10 ureaplasmas per ml of culture (10^1 ml^{-1}) but the anti-urease Mab was not effective in immunofluorescence, probably because the urease enzyme is not surface-expressed. However, this Mab has been utilised in a 'catch test' where it is immobilised onto a 96-well plate or a wooden support ('dip stick'). In this form the Mab may capture soluble urease enzyme present in a clinical swab or urine. Addition of a urease-specific colour developing reagent (Berthalot reagent) leads to an easily quantifiable test that by the 96-well plate method has detected as low as 10^2 ureaplasma cells ml^{-1} (Precious, *et al.*, 1987, Myles, 1990). Such preliminary experiments suggest that these two techniques may be of value in clinical diagnosis.

1.3.4 DNA detection

Since the identification of a number of ureaplasma genes, diagnostic tests based on the detection of ureaplasma DNA in clinical samples have been developed. Early work involved the use of DNA probes from ureaplasma rRNA. Although these hybridised to mycoplasma DNA, unique and identifiable patterns were produced with ureaplasma DNA (Razin *et al.*, 1984). It has also been shown

that rRNA probes can distinguish between the two seroclusters of *U. urealyticum* (Razin and Yogev, 1986). A probe generated from serotype 8 whole-chromosome DNA has been shown to hybridise with all other serotypes of the organism tested, but not with other mycoplasmas or bacteria from the urogenital tract (Roberts *et al.*, 1987). In clinical samples containing low ureaplasma cell numbers, however, the probe has shown limited sensitivity.

Diagnostic techniques based on DNA hybridisation have recently been superseded by the Polymerase Chain Reaction (PCR), which can amplify as low as 1 ng of DNA using specific primers and a heat stable *Taq* polymerase. Cloning and sequencing of the urease gene has enabled this technique to be developed for *U. urealyticum* and in a preliminary clinical trial, positive identification of the organism in urogenital swabs, amniotic fluids and endotracheal aspirates by PCR was in good agreement with results obtained from cultivation (Blanchard *et al.*, 1992) Other workers have used this technique to detect *U. urealyticum* in arthritic joints of a hypogammaglobulinemic patient (Lee *et al.*, 1992).

If PCR-based methods prove to be accurate and cheap diagnostic techniques, they may lead to a deeper understanding of the involvement of ureaplasmas from the many disease states from which they have been isolated. In this respect, establishing the pathogenic potential of *U. urealyticum* has been an area of great interest but of little conclusive evidence.

1.4 PATHOGENESIS OF *U. UREALYTICUM*

1.4.1 General principles

The isolation of *U. urealyticum* from men and women displaying a variety of genito-urinary infections has led to them being implicated as important human pathogens. The presence of the organism in the lower genito-urinary tract of over 50% of sexually active individuals who show no obvious signs of disease (Cassell and Waites, 1984), however, has led to speculation as to the validity of these assumptions. In elucidating the role of ureaplasmas in disease, it has therefore been important not to equate association with cause and a number of criteria have

been established which should be met before an etiologic role for *U. urealyticum* in any disease is proposed (Taylor-Robinson, 1983). These criteria apply to all mycoplasmas diseases and are described below:

a) Isolation

The organisms should be isolated more frequently and/or in larger numbers from patients with disease than from those without. It is critical that suitable isolation sites are chosen and that quantitative measurements are made.

b) Serology

Antibody responses should occur in patients with disease, and preferably follow the path of the disease. Detection of specific IgM or an increase in IgG titre of 4-fold or greater indicates an active infection.

c) Antibiotic treatment

Treatment with effective antibiotics should lead to the concomitant disappearance of organisms and symptoms. Interpretation of such studies is not always clear-cut as antibiotics are rarely selective for specific organisms.

d) Re-inoculation

Isolated organisms should be able to reinfect an animal host, produce disease similar to that seen in man and be subsequently be recovered. The disease process in an animal model, however, may never be identical as that seen in a human.

It is also critical to select appropriate human controls. The colonisation of individuals with ureaplasmas varies according to their age, sex, race, socio-economic status, smoking habits, the number of sexual partners and the type of contraceptives used (Cassell and Waites, 1984). Many of these characteristics are predictive for other venereally transmissible organisms such as *Chlamydiae* and group B *Streptococci*. The presence of these and other microorganisms should therefore be taken into account in any assessment of causality (Cassell *et al.*, 1991).

The majority of studies have associated ureaplasmas with genito-urinary tract infections of the male, reproductive tract disorders in the female and a variety of diseases in new born infants. In the following section the evidence for these

associations will be reviewed, highlighting where possible to what extent the above criteria and conditions have been satisfied.

1.4.2 Non-gonococcal urethritis

U. urealyticum may be responsible for some cases of acute urethral syndrome in women (Schiefer and Weidner, 1990) but the evidence for its association with Non-Gonococcal Urethritis (NGU, urethritis in the absence of *N. gonorrhoeae*) is largely confined to within the male (Krause and Taylor-Robinson, 1992).

NGU may be caused by a variety of microorganisms including *Chlamydia trachomatis (C. trachomatis), Haemophilus vaginalis, Trichomonas vaginalis*, herpes simplex virus, *M. hominis* and *U. urealyticum* (O'Leary, 1990). Although in a large number of cases (40-50%) *C. trachomatis* appears to be the primary pathogen, there is considerable dispute about the relative involvement of the other agents in the remainder (Taylor-Robinson, 1983).

Since their original identification in urethral discharge from a patient displaying NGU (Shepard, 1954), ureaplasmas have been isolated from 70-80% of all cases of NGU and there is substantial evidence to suggest that *U. urealyticum* may be the primary pathogen in some of these cases. In fact, of all of the ureaplasma-associated diseases, NGU is the only one in which all of the requirements for incriminating a mycoplasma as the causative agent of a disease, as outlined above, have been fulfilled. The subject has been reviewed by Taylor-Robinson (1985) and the following sections summarise his findings:

a) Isolation

U. urealyticum has been isolated more frequently (qualitative studies) and in larger numbers (quantitative studies) from cases of NGU than from healthy controls. In one patient with hypogammaglobulinemia, NGU occurred intermittently over a period of years, only at times when ureaplasmas were present in the urethra and in large numbers (10^6-10^8) (Webster, *et al.*, 1982, Taylor-Robinson *et al.*, 1985a). Moreover, no other microorganisms were co-isolated, suggesting that *U. urealyticum* was the primary pathogen in these instances. It has

been proposed that ureaplasmas only cause NGU in the absence of *C. trachomatis* but the precise interrelationship between microorganisms in mixed infections of the urogenital tract has not been established.

b) Serology

Early serological tests only detected an immune response to *U. urealyticum* in a small number of patients with NGU but in a later study using an ELISA with broad serotype cross-reactivity a change in antibody levels was detected in over 50% of NGU patients who were culture positive for ureaplasmas (Brown *et al.*, 1983). Of these, 80% displayed a change in IgM levels which suggested an active infection. A more recent report found an antibody response in 48% of patients with NGU, 24% of these were colonised with *C. trachomatis*, suggesting that *U. urealyticum* can induce a response in the presence of this organism (Deodhar and Gogate, 1990).

c) Antibiotic treatment

It was first demonstrated by Shepard (1974) that ureaplasma isolation followed a clinical course of infection by treating NGU patients with suboptimal levels of tetracyclines. Culture positive patients showed signs of recovery following initial antibiotic treatment and ureaplasma isolations fell to negligible levels. A subsequent relapse and return of symptoms was accompanied by an increase in ureaplasma colonisation. Although other workers have reported a similar effect with tetracycline, this antibiotic is not ideal for such investigations due to *Chlamydia*-sensitivity and the emergence of tetracycline-resistant strains of *U. urealyticum*. The use of selective antibiotics may therefore provide more valuable information.

By using aminocyditols (streptomycin and spectinomycin), which are active against ureaplasmas but inactive against *Chlamydia*, Bowie *et al.*, (1976) found that persistent urethritis was associated with persistence of *Chlamydia*. Furthermore, using an antibiotic which is active against *Chlamydia* but not ureaplasmas (sulphonamide) these workers found a significant association between persistent urethritis and the re-isolation of ureaplasmas after treatment. In trials using rifampicin, which is also effective against *Chlamydia* but not

ureaplasmas, 55 out of 68 men continued to be ureaplasma-positive after treatment. Of these, 44% did not recover from symptoms of NGU, whereas in the group that had become ureaplasma-negative, only 8% were not cured (Coufalik *et al.*, 1979). These findings emphasise the importance of both of these organisms in urethritis.

d) Re-inoculation

Intraurethral inoculation of two adult male volunteers with *U. urealyticum* that had been isolated from patients with NGU (2 strains of serotype 5) resulted in the onset of disease symptoms accompanied by ureaplasma colonisation, both of which receded after minocycline treatment (Taylor-Robinson *et al.*, 1977). In animal studies, a urethral polymorphonuclear leukocyte response has been produced in male goats after intraurethral inoculation of caprine ureaplasmas (Taylor-Robinson, 1979) and urethritis has also been observed in male chimpanzees following inoculation of unpassaged human ureaplasmas (Taylor-Robinson *et al.*, unpublished data cited by Krause and Taylor-Robinson, 1992).

These results provide convincing evidence for a role of ureaplasmas in the development of NGU. It is still a matter of controversy as to what proportion of cases can be attributed to *U. urealyticum,* but it has been predicted as 10-30% (Cassell and Waites, 1984). The question of why infection is only produced in a small proportion of individuals colonised with the organism will be addressed later in this section, since it is a question pertinent to all diseases with which ureaplasmas have been associated.

1.4.3 Complications of NGU

Once the association between ureaplasmas and NGU had been established, the involvement of ureaplasmas with diseases that can occur in conjunction with NGU, such as sexually acquired reactive arthritis (SARA), Reiter's syndrome (a triad consisting of urethritis, conjunctivitis and arthritis) and inflammation of the prostate (prostatitis), has been examined. Results are difficult to interpret in these cases since isolation rates or antibody titres should be elevated to levels higher than in patients with uncomplicated NGU before an association is

suggested. Thus, although ureaplasmas have been isolated from the urethra of patients with SARA (Taylor-Robinson, 1985) and Reiter's syndrome (Martin *et al.*, 1984) they were not at significantly higher levels than from control patients. In contrast, specific antibodies to *U. urealyticum* have been detected in a higher proportion of patients with Reiter's syndrome than in patients attending a venereal disease clinic for other reasons (Ford and Henderson, 1976), suggesting a role for the organism in this syndrome.

In the case of chronic prostatitis, which can occur in over a quarter of NGU patients, it has been shown that ureaplasmas colonise the prostate in a higher percentage of symptomatic men than in healthy controls (Weidner *et al.*, 1980). Brunner *et al.*, (1983a, b) reported that following prostatic massage, high titres of *U. urealyticum* were present in prostatic secretions and urine from 14% of men suffering from chronic prostatitis. After tetracycline treatment, over 80% of these men were free of both ureaplasmas and symptoms. As with other antibiotic studies, another tetracycline-sensitive organism may have been the causative agent and the role of ureaplasmas in this condition remains unclear.

1.4.4 Urinary tract involvement

The ability of ureaplasmas to ascend the urinary tract to the bladder has been demonstrated only infrequently (Birch *et al.*, 1981), but in some of these cases the organisms have been associated with the formation of a type of urinary tract stone.

Urinary stones composed of struvite and carbonate-apatite account for 20% of all urinary tract stones and are induced by the breakdown of urea by bacterial ureases, such those found in *Proteus* species. *U. urealyticum* inoculated into artificial urine has been shown to induce crystallisation of struvite and calcium phosphates, but at a slower rate than *Proteus mirabilis* (Takebe *et al.*, 1984). Such renal caliculi have also been produced experimentally by the inoculation of *U. urealyticum* into the bladder and renal pelvis of rats (Frielander and Braude, 1974). Furthermore, *U. urealyticum* has been isolated from stones recovered by surgery in 6 of 15 humans and in four of these no other urease-producing

organisms were isolated (Pettersson *et al.*, 1983). As with NGU, it now seems generally accepted that ureaplasmas can elicit the production of urinary stones, but less frequently than other urease-positive bacteria.

Beyond the bladder, ureaplasmas have produced kidney infections in animal models (Kreiger and Kenny, 1986) but have not yet been implicated in human pylonephritis.

1.4.5 Infertility

It has been suggested that NGU, and prostatitis in particular, may impair male fertility (Taylor-Robinson, 1985), but there is little evidence to demonstrate that infertility occurs subsequent to ureaplasma-induced disease. Numerous studies, however, have attempted to relate ureaplasma colonisation to infertility in men who are apparently otherwise healthy (reviewed by Taylor-Robinson, 1986a).

U. urealyticum has been isolated more frequently from the semen of infertile than fertile men (Friberg and Gnarpe, 1974, Cracea *et al.*, 1982, Busolo *et al.*, 1984a) and in some of these cases and others an associated decrease in sperm quality, numbers and motility has been reported (O'Leary and Frick, 1975, Toth *et al.*, 1978, Naessens *et al.*, 1986). These findings are not universal (Taylor-Robinson and Furr, 1973, Taylor-Robinson *et al.*, 1985) and it has been argued that ureaplasmas are unlikely to interfere with spermatogenesis since they have not been isolated from the vas deferens or testis (Taylor-Robinson, 1985).

Scanning electron microscopy (Fowkes *et al.*, 1975) and fluorescent-light microscopy (Swensen and O'Leary, 1980) have revealed that ureaplasmas can bind to the mid-piece of sperm and thereby decrease motility and encourage multi-sperm agglutination. Other workers have found no significant change in the morphology or motility of sperm after overnight incubation with ureaplasmas but have recorded a reduction in penetration of zona-free hamster cells when tested in a human-sperm-hamster egg fertilisation test (Busolo and Zanchetta, 1985). In an *in vitro* fertilisation study, it was found that fertilisation parameters were not affected by ureaplasma-infected serum but that there was a reduction in the pregnancy rate per embryo transfer in these circumstances (Montagut *et al.*, 1991).

Neither of these studies clarify the effect of ureaplasmas on sperm functions but they do suggest that ureaplasma colonisation of the female may influence conception. For this reason, it has been more instructive to consider the effect of ureaplasma colonisation within an infertile couple, rather than in the male alone.

Most studies in this area examine either ureaplasma isolation rates from infertile couples or the effect of antibiotic treatment on conception success. Gnarpe and Friberg, (1972) and Cassell *et al.*, (1983a), isolated *U. urealyticum* more frequently from infertile couples than from fertile couples but De Louvois *et al.*, (1974) and Gump *et al.*, (1984) could not substantiate these findings. Doxycycline treatment produced a significant increase in conception rates in couples where the male had become free of ureaplasma colonisation, compared to couples where the male remained positive (Toth *et al.*, 1983 and Busolo *et al.*, 1984b). In contrast, double blind-controlled trials found that while antibiotic treatment reduced ureaplasma colonisation, the rate of conception in these couples was no higher than in controls (Harrison *et al.*, 1975, Upadhyaya *et al.*, 1983).

After over fifty studies attempting to elucidate the role of *U. urealyticum* in infertility, it is still only possible to suggest an association of the organism with the condition, which may only be significant in individuals who are subfertile for other reasons (Cassell *et al.*, 1987).

1.4.6 Reproductive tract disease

If the adhesion of ureaplasmas to sperm does not affect fertilisation, it certainly provides a means of transfer for the organism from the male to the female genital tract. In this regard, there is some debate as to whether *U. urealyticum* can cause infection in female genital tract. *U. urealyticum* has been isolated from women suffering from vaginitis, cervicitis, endometritis, salpingitis, oophoritis and pelvicperitonitis (pelvic inflammatory disease, PID) but it has also been found in the genital tract of 40-60% of women who are apparently asymptomatic (Cassell *et al.*, 1987). Although evidence is contradictory, it is generally accepted that while ureaplasmas may be involved in PID (Sweet, 1986), they are not major pathogens

of the lower genital tract and that isolation from inflamed tissue generally represents secondary colonisation rather than infection (Cassell *et al.*, 1987). During pregnancy, however, up to 90% of women become colonised with *U. urealyticum* and there seems to be an increased susceptibility to ureaplasma-associated diseases (Cassell, 1986). This is a phenomenon that has been demonstrated for other infectious agents (Brabin, 1985), and may be due to changes in the local environment of the genital tract, brought about the altered metabolic, immunologic and hormonal status of the pregnant mother. In this regard, oestradiol treatment of female mice renders them more susceptible to ureaplasma colonisation (Furr and Taylor-Robinson, 1989).

In a proportion of pregnant women, ureaplasmas colonising the endocervix appear to ascend the genital tract to the uterus and placenta (Cassell *et al.*, 1991). Intact membranes do not prevent colonisation of the foetus (Cassell *et al.*, 1983b, Waites *et al.*, 1989) but colonisation is more common following membrane rupture and the onset of labour (Cassell *et al.*, 1991). In addition ureaplasmas are frequently transmitted to infants during their passage through the birth canal (Klein *et al.*, 1969). It is not always clear whether such colonisation has caused disease or whether it has followed disease resulting from another cause. In spite of this, there have been a plethora of reports implicating ureaplasmas with chorioamnionitis (the inflammation of foetal membranes, chorion and amnion), spontaneous abortion, still birth, prematurity, low birth weight, neonatal pneumonia and neonatal meningitis. In the following sections the evidence for the involvement of ureaplasmas with these adverse outcomes of pregnancy will be reviewed.

a) Chorioamnionitis (Histologic chorioamnionitis)

There are many reports where ureaplasmas have been isolated more frequently from placentas of patients with chorioamnionitis than from patients without but in three in particular the findings are significant since no other microorganisms were co-isolated and the studies were carefully controlled to take into account the duration of labour and membrane rupture (Kundsin *et al.*, 1984, Quinn *et al.*, 1985a, Hillier *et al.*, 1986). Quinn *et al.*, (1987) found that foetal

antibodies to *U. urealyticum* were elevated to four-times the maternal levels in cases of chorioamnionitis, suggesting an active infection. Further evidence has been provided by the examination of intra-amniotic fluid infection. Thus, Cassell *et al.*, (1983b) isolated *U. urealyticum* from the amniotic fluid of two women in early gestation, prior to the rupture of foetal membranes. In the absence of any other identifiable causes, there was an intense inflammatory reaction (chorioamnionitis) followed by premature labour and neonatal death. The organism was subsequently isolated in pure culture from a variety foetal organs and tissues. Similar results were found by Foulon *et al.*, (1986), which together with the work of Cassell and co-workers, implicates *U. urealyticum* as a cause of chorioamnionitis via an invasive process, and suggests that this condition may lead to an adverse pregnancy outcome. A strong association between chorioamnionitis caused by any agent and prematurity, congenital pneumonia, perinatal morbidity and mortality had been documented by other workers (Naeye, 1975, Russell, 1979).

b) Spontaneous abortion and still birth

Ureaplasma colonisation in the lower genital tract of habitual aborters does not differ from that in normal controls (Taylor-Robinson and McCormack, 1979) but the organism has been isolated from the endometrium of habitual aborters (Stray-Pedersen *et al.*, 1978) and from aborted placentas ((Embree *et al.*, 1980, Quinn *et al.*, 1983a, Kundsin *et al.*, 1984) more frequently than from the same site in control groups. At delivery, 49% of infants from women with a history of pregnancy wastage had a four-fold or greater elevation in specific antibody titre above the maternal levels (Quinn *et al.*, 1983a). A number of workers have also shown an increase in normal pregnancies following treatment with doxycycline or erythromycin, but the elimination of other microorganisms was not examined and the effects therefore may not be specific for *U. urealyticum* (Quinn *et al.*, 1983b, Kundsin and Driscoll, 1970, Toth *et al.*, 1983). Others have found no role for ureaplasmas in spontaneous abortion (Harrison, 1986, Romero *et al.*, 1989) and their involvement with this syndrome is only suggestive.

The evidence associating ureaplasmas with still birth is also contradictory. One study showed that only 8.3% of still-birth foetal-tissue was colonised with ureaplasmas (Madan *et al.*, 1988) but another isolated the organism in 78% of placentas from perinatal deaths compared to 32% from healthy births (Quinn *et al.*, 1985a). An elevated antibody response to *U. urealyticum* was found in 45% of the former cases and none of the latter. In a later study by the same workers, antibody was detected in 73% of still-born foetuses compared to 6.3% of healthy controls (Quinn, 1986).

c) Prematurity and Low birth weight

If the foetus survives *in utero*, there may still be a danger of preterm labour, low birth weight and neonatal disease. A strong association exists between maternal colonisation with ureaplasmas and prematurity, particularly since this commonly proceeds chorioamnionitis (Cassell *et al.*, 1986). It has been suggested that the cytotoxic effects of inflammation lead to prostaglandin release which stimulates labour (McGregor, 1988). In addition, the direct action of ureaplasma phospholipases on placental membrane phospholipids may release archidonic acid which in turn activates prostaglandin synthesis (De Silva and Quinn, 1991). Alternatively, digestion of membrane phospholipids could so weaken membrane structure that they rupture under the weight of the amniotic fluid pressure. Although all seem plausible, it has not been proved that any of these mechanisms occur *in vivo*.

The involvement of ureaplasmas with prematurity is frequently examined in association with the birth of infants weighing less than 2,500 g (low birth weight). Embree *et al.*, (1980) and Kundsin *et al.*, (1984) found that placental colonisation was inversely related both to gestational age and birth weight and Hillier *et al.*, (1986) confirmed that colonisation of this site was associated with preterm birth. Rudd *et al.*, (1984) and Izraeli *et al.*, (1991) reported that low birth weight and preterm infants were frequently colonised with *U. urealyticum* in the nose and throat and Rudd and colleagues found a specific antibody response to the organism in a number of cases. Serological evidence has also been provided by Kass *et al.*, (1986), who showed that a four-fold increase in type-specific

maternal antibody was linked to low birth weight. The same workers had previously shown, by a double blind placebo-controlled trial, that erythromycin treatment during the third trimester of pregnancy reduced low birth rates from 11% to 3% (Kass *et al.*, 1981). The presence of *Chlamydia*, but no other anaerobes, were controlled for in this study. In a review examining twelve other similar studies, Romero *et al.*, (1989) reported that there was no firm evidence to support an association of ureaplasmas with prematurity or low birth weight and its involvement with these syndromes therefore remains suggestive.

1.4.7 Infections of the new born

a) Respiratory tract disease

Low birth weight infants not only tend to show an increased incidence of ureaplasma colonisation but they are generally more susceptible to respiratory disease (Cassell *et al.*, 1988a). In addition, chorioamnionitis is almost always associated with congenital pneumonia (Naeye *et al.*, 1971). It is not surprising, therefore, that ureaplasmas have been associated with this syndrome but care must be taken to distinguish between respiratory distress caused by pulmonary immaturity and that caused directly by ureaplasma colonisation.

Early case studies reported that, in the absence of other microorganisms, ureaplasmas were isolated from the lungs of neonates with respiratory disease (Romano *et al.*, 1971, Dische *et al.*, 1979, Cassell *et al.*, 1983b). An elevated titre of IgM-specific antibody to *U. urealyticum* in new-born infants with similar respiratory distress suggested that ureaplasma colonisation produced an active infection (Quinn *et al.*, 1983c). Such a response was not found by other serological studies (Rudd *et al.*, 1986) and a number of prospective cultural studies have also failed to show an association (Taylor-Robinson *et al.*, 1984, Rudd *et al.*, 1984, Rudd and Carrington, 1985). A number of well documented case reports (discussed below), have provided definitive evidence that ureaplasmas are capable of producing clinical and histologic pneumonia.

It has been demonstrated that *U. urealyticum* induces ciliostasis and lung lesions in human foetal tracheal organ cultures (Dische *et al.*, 1979) and Quinn

et al., (1985b) reported that such effects may also occur *in vivo*. Scanning electron micrographs of lung sections from a case of fatal intrauterine pneumonia revealed that aggregates resembling ureaplasmas were closely associated with areas of the trachea completely devoid of cilia (Quinn *et al.*, 1985b). Immunofluorescence confirmed the identity of *U. urealyticum* (serotype 8) in lung lesions and an elevated antibody titre to this serotype was also identified in neonatal serum. In examinations of new born infants with pneumonia and persistent pulmonary hypertension, Waites *et al.*, (1989) found that *U. urealyticum* was the only organism to be isolated from multiple sites within the neonate, both before and after death, thus proving a causative role for ureaplasmas in pneumonia.

In addition to acute respiratory distress, *U. urealyticum* has been associated with chronic lung disease (CLD) of preterm infants, defined as the need for increased inspired oxygen at 28 days of age. Sanchez and Regan (1988) and Wang *et al.*, (1988) proposed that ureaplasmas contributed to the development of CLD and Cassell *et al.*, (1988a) identified a sub-population of pre-term infants which seemed particularly susceptible to ureaplasma associated CLD. Thus, very low birth weight infants (<1000 g) who were endotracheally colonised with *U. urealyticum* within 24 hours of birth were more likely to develop CLD and die than were uninfected infants of similar birth weight or infected infants who weighed greater than 1000 g. Age also seemed to be a critical determinant in the development and severity of disease and this finding has been reproduced in animal studies by Rudd *et al.*, (1989); when ureaplasma isolates from cases of CLD were inoculated into new born mice a histologic pneumonitis ensued, but only in mice younger than 14 days. Furthermore, exposure of new-born mice to 80% oxygen resulted in more severe lung lesions, organism persistence and death (Crouse *et al.*, 1990). Increased oxygen requirements of very low birth weight human neonates may predispose them also to ureaplasma respiratory infections or alternatively ureaplasma infection may potentiate oxygen-induced injury (Cassell *et al.*, 1988a). *U. urealyticum* is unlikely to be the primary cause of CLD but its contribution to the severity of this syndrome may be substantial.

b) Systemic infections

In a minority of new born infants, ureaplasmas are not confined to the respiratory tract but can migrate into blood or cerebro-spinal fluid. Waites *et al.*, (1989) and Brus *et al.*, (1991) have reported cases of neonatal pneumonia accompanied by septicaemia. In a prospective study of pre-term neonates with meningitis, Waites *et al.*, (1988) found that *U. urealyticum* and *M. hominis* were the most commonly isolated organisms from the cerebrospinal fluid (CSF). *U. urealyticum* has only once been identified as the cause of meningitis (Garland and Murton, 1987) and many workers have not been able to isolate the organism from the CSF of neonates with this disease (Taylor-Robinson and McCormack, 1979, Mardh, 1983, Likitnukul *et al.*, 1987). Others dispute any etiologic role for ureaplasmas in meningitis (Shaw *et al.*, 1989).

An unpublished study by Cox *et al.* (cited by Cassell *et al.*, 1991), reported that pure cultures of *U. urealyticum* isolated from the CSF of human infants induced meningitis and hydrocephalus in new born mice and beagles, indicating that this organism does have the potential to cause such diseases. Although the precise involvement of ureaplasmas in human CSF infections has not been established, clinicians have been advised to test for *U. urealyticum* and *M. hominis* in pre-term infants with symptoms of meningitis or progressive hydrocephalus (Waites *et al.*, 1990).

Following labour and delivery, *U. urealyticum* may also gain access to the blood of the mother and in some cases isolation has been associated with post-partum fever or septicaemia (Cassell and Cole, 1981). Such patients rarely have severe disease symptoms and frequently recover without antibiotic therapy (Risi and Sanders, 1989).

1.4.8 Extragenital infections

Ureaplasmas have been isolated in pure culture from septic arthritic joints in a number of hypergammaglobulinaemic individuals (Cole *et al.*, 1985). Since reinfection of chimpanzees with one of these isolates produces a severe arthritis (Barile *et al.*, 1990), it has been proposed that *U. urealyticum* is the principle

pathogenic agent in these cases. The organism does not appear to be responsible for disease in immunosuppressive patients and there is no evidence that ureaplasmas are associated with AIDS (Krause and Taylor-Robinson, 1992).

1.4.9 Conclusions

U. urealyticum can no longer be considered as a harmless commensal of the lower genito-urinary tract and there is much evidence to suggest that it causes a number of human diseases. Firm proof for most of these conditions has been difficult to obtain, mainly because ureaplasmas are found so frequently as apparently harmless members of the normal human flora. It has been proposed that the organism only elicits disease in a sub-population of individuals and it is for this reason that there has been difficulty in proving causal relationships by prospective studies. The predisposing factors that are operating in these sub-populations have not been delineated, but some host -related and pathogen-related properties have been suggested that may be relevant to the onset of disease in these individuals.

As far as the host is concerned, immunodeficient individuals are particularly susceptible to ureaplasma-associated diseases. This is emphasised by studies on hypogammaglobulinemic patients, which have provided the strongest evidence for the pathogenic capabilities of *U. urealyticum* (Webster *et al.*, 1982, Taylor-Robinson *et al.*, 1985b). Similarly, the gestational age-related susceptibilities of human neonates and mice may be due to a poorly developed immune system (Rudd *et al.*, 1989). It has been suggested that in these cases there is insufficient neutrophil and macrophage function, particularly since neonatal rabbit pulmonary macrophages have demonstrated a decreased rate of bacterial killing (Sherman *et al.*, 1982). In apparently immunologically competent hosts, there may be subtle differences in immune status that render some individuals more susceptible to ureaplasma infection than others.

Alternatively, the predisposing factors operating in these individuals may be pathogen-related. Taylor-Robinson (1986b) has proposed that ureaplasmas only cause disease when present in large numbers. In the case of the

hypogammaglobulinemic patient, it was clearly demonstrated that urethritis was related to the levels of ureaplasma colonisation. Such an association had been observed in other cases of NGU (Bowie *et al.*, 1976, Weidner *et al.*, 1980), but there are equally many reports which find no difference in ureaplasma numbers recovered from diseased and healthy men (Taylor-Robinson, 1985). It is a well-recognised phenomenon, however, that harmless members of the microbiological flora can cause disease if they are allowed to multiply excessively. For *Candida albicans* and *Clostridium difficile,* disease induced by increased levels of colonisation has been linked to excessive or prolonged use of broad-spectrum antibiotics (O'Leary, 1990). The conditions which might favour excessive ureaplasma growth have not yet been identified.

It has also been suggested that some serotypes of *U. urealyticum* are more pathogenic than others and that in healthy men the non-pathogenic serotypes prevail (Taylor-Robinson, 1983). There have been numerous attempts to determine whether certain serotypes are associated with disease states more than others, either by isolation studies or by comparing the pathogenic potential of different serotypes in animal and tissue culture systems.

Shepard (1974) reported that serotype 4 was recovered twice as frequently in men with NGU than from men who were symptom-free. Serotype 4 was also more effective than others in preventing sperm entry into hamster eggs in the *in vitro* fertilisation tests described earlier (Busolo and Zanchetta, 1984). When examining pregnancy complications, Naessens *et al.*, (1990) isolated serotype 4 more regularly from patients with a history of recurrent miscarriages than from normal pregnant women, and Quinn *et al.*, (1983a), found that serotypes 4, 7 and 8 were frequently associated with cases of spontaneous abortion. In trying to assess whether different serotypes produced different pathologies, Rudd *et al.*, (1989) demonstrated that lung lesions following intranasal inoculation were more severe with serotype 10 than serotype 1, but the experiments were performed at different times so could not be compared directly. Another murine study suggested that clinical isolates of *U. urealyticum* varied in their ability to persist in kidneys (Fodor, 1980), but the serotypes involved in this study were not identified. In

contrast, Zheng *et al.*, (1992) found that invasiveness was not restricted to a limited number of serotypes and that representatives of each gene cluster (serotypes, 1,3, 6, 8 and 10) were isolated from the CSF of thirteen infected infants.

There are numerous studies that have failed to relate any particular serotype of *U. urealyticum* to disease. These include examinations of female sterility (Cracea *et al.*, 1984), spontaneous abortion (Robertson *et al.*, 1986) and upper urinary tract disease (Hewish *et al.*, 1986). Taking all these reports into consideration, it has not yet been convincingly demonstrated that ureaplasma serotypes differ with respect to their pathogenicity.

There may well be differences in virulence between ureaplasma strains, but the properties that have been used to serotype the strains may not bear any relationship to the properties which determine their pathogenicity. If there are indeed differences between pathogenic and harmless strains of ureaplasmas, some other characteristics need to be identified which more reliably define these differences.

In summary, the colonisation of asymptomatic individuals with ureaplasmas does not rule out the possibility that these organisms can induce disease but suggests that this occurs only under certain circumstances. The precise circumstances have not been clearly elucidated but will depend on a number of interacting factors including the immune status of the host, the site and extent of ureaplasma colonisation, the presence of other microorganisms at this site and the pathogenicity of the ureaplasma strains involved.

It is now of value to examine the properties of *U. urealyticum* that could render it pathogenic as, once defined, these may serve as a more useful basis for classification than serotyping. Furthermore, an understanding of the pathogenic mechanisms operating in this organism may improve the therapy and prophylaxis of ureaplasma-induced disease.

1.5 VIRULENCE PROPERTIES OF *U. UREALYTICUM*

To establish that a particular property is important in virulence, it is necessary to demonstrate that selective attenuation of this property renders a virulent microorganism avirulent on subsequent reinfection of the host (Smith, 1984a). This is difficult to achieve for any microorganism since virulence is multifactorial and the pathogenic effect of a single factor can rarely be examined in isolation. For ureaplasmas there are additional problems. Avirulent and virulent strains have not yet been defined, genetic manipulation of the organism has not advanced to the stage where a particular property can be selectively attenuated and suitable animal models for pathogenicity studies have not been fully developed or evaluated. An understanding of the mechanisms of ureaplasma pathogenicity is therefore only in its infancy, but a number of biological properties have been identified which may prove to be important in determining the virulence of this organism.

These properties can be artificially divided into two categories, those that cause damage to the host and those which interfere with innate and acquired host defence mechanisms.

1.5.1 Damage to the host

A number of classical extracellular parasites, such as *Vibrio cholerae* and *Corynebacterium diphtheriae,* produce toxins whose apparent function is to cause damage to the host (Mims, 1987). Likewise, *Mycoplasma neurolyticum* and *Mycoplasma gallisepticum* release potent neurotropic toxins that have been associated with diseases of the brain and nervous system (Gabridge *et al.,* 1985). Other than an uncharacterised metabolite that inhibits their prolonged growth *in vitro* (Furness, 1973), no such toxin has been detected in ureaplasmas. It is more likely that ureaplasmas have a deleterious effect on their host simply as a result of their stringent metabolic requirements. Thus, metabolic enzymes may interact directly with host tissues or may exert an indirect effect by either depleting nutrients or releasing toxic waste products.

a) Ureaplasma toxins

In the absence of an electron transport chain, it is likely that a number of reactive oxygen species are produced during ureaplasma metabolism which may be cytotoxic to the host. In other mycoplasmas, hydrogen peroxide (H_2O_2) and superoxide anion-production has been well documented and for *M. pneumoniae*, high concentrations of H_2O_2 may be responsible for the lipid peroxidation of membranes observed *in vitro* (Cohen and Somerson, 1967). The ability of this mycoplasma to inhibit host cell catalase, possibly via the action of a superoxide radical, may further exacerbate the damage (Almagor *et al.*, 1984).

In ureaplasmas, H_2O_2 production and its impact on the pathogenesis of the organism has not been clearly established. Ureaplasma-induced cell lysis is inhibited by catalase, which suggests that H_2O_2 may be the haemolysin (Manchee and Taylor-Robinson, 1970). Although Bauman (1989) has failed to detect H_2O_2 production by *U. urealyticum*, Meier and Habermehl (1990) have reported both superoxide and H_2O_2 production in two isolates of this species. Furthermore, these workers identified superoxide dismutase (SOD) activity, which catalyses the recombination of superoxide anions to H_2O_2 and O_2. By this mechanism, ureaplasmas may protect themselves from the harmful effects of the superoxide anion and its reaction products.

By virtue of its urealytic activity, *U. urealyticum* has the potential to generate large quantities of ammonium ions from urea. This induces a detectable cytopathology in a variety of established cell lines (Cassell and Cole, 1981) and it has recently been reported that mice intravenously injected with ammonium ions (200 μM), died within five minutes (Ligon and Kenny, 1991). Furthermore, intravenous injection of ureaplasmas produced the same lethal response but concomitant intraperitoneal injection of fluorofamide, a potent urease inhibitor, provided protection. This not only demonstrated the harmful effects of high concentrations of ammonium ions *in vivo* but confirmed that, in a mouse model, *U. urealyticum* can induce the same effect via the action of its urease enzyme.

b) Interfering with host metabolism

The requirement of *U. urealyticum* for pre-formed purines and pyrimidines and the demonstration of RNA and DNA endonucleases activity in this organism suggests that ureaplasma infection may serve to reduce the pool of nucleotides available to the host. Although this has not been examined directly, mycoplasma infection of mammalian cells in tissue culture has been shown to alter host cell metabolism (Russell, 1966) and may induce chromosomal aberrations (Paton *et al.*, 1965). In the case of *M. pneumoniae,* damage to mycoplasma-infected hamster tracheal explants has been alleviated by incorporating adenine into the culture medium (Hu *et al.*, 1975).

Phospholipase enzymes may also cause damage to the host by degrading phospholipids in closely associated epithelial cell membranes. Phospholipase A and C have been located in the plasma membrane of three serotypes (3, 4, and 8) of *U. urealyticum* (De Silva and Quinn, 1991). The association of *U. urealyticum* with spontaneous abortion, stillbirth and prematurity has been linked to the impact of phospholipases on placental membranes, as discussed in Section 1.4.6. Likewise, the susceptibility of neonatal lungs to ureaplasma infections may be due to the combined effect of phospholipase activity and the depletion of sterols, resulting in decreased surfactant levels (Rudd *et al.*, 1989). Ureaplasma phospholipases require further characterisation before their role in ureaplasma metabolism and virulence is established.

When considering the above virulence properties, it seems likely that ureaplasma damage to the host occurs primarily as an undesirable side effect of their limited metabolic reactions. This is consistent with the proposal that it its not in the interests of a parasitic microorganism to cause damage to the host, since this may limit its chance of survival. In order to ensure colonisation and transmission, *U. urealyticum* must also be able to resist or modulate the innate and specific defence presented by the host.

1.5.2 Evading host defence mechanisms

a) Adhesion

U. urealyticum has a predilection for the urogenital tract and is transferred directly onto new mucosal surfaces by sexual contact. By this route, the organism avoids contact with the external environment and can gain access to the host via the mucosal epithelia rather than via the relatively impenetrable barrier of the skin. The urogenital tract, however, is maintained at a low pH and is continually flushed and cleansed by urine and mucocilliary action which serve to prevent the colonisation of invading microorganisms. The normally inhibitory acidic environment of the urogenital tract is favourable to the growth of ureaplasmas and the abundant supply of urea may in fact dictate the tissue specificity of this organism. To avoid expulsion, the organism may bind to mucosal epithelia, a property that has been demonstrated by a number of workers.

In urethral scrapings and semen samples, a close association between *U. urealyticum* and host epithelial cells has been reported by Shepard (1957) and Busolo *et al.*, (1984a). *In vitro*, a number of ureaplasma serotypes adsorb a number of tissue-culture cell lines (Kotani and McGarrity, 1986) and a degree of binding to bovine fallopian tube mucosa cell cultures has recently been demonstrated (Saada *et al.*, 1990b). Using erythrocytes, which allow adhesion to be monitored easily but may not adequately represent mucosal epithelia, *U. urealyticum* colonies bind guinea pig red blood cells (Robertson and Sherburne, 1991). Saada *et al.*, (1991) have confirmed this property with human red blood cells and suggest that ureaplasma adhesins are protein in nature while erythrocyte binding sites appear to be sialyl and or sulphated compounds.

In this respect, adherence mechanisms in ureaplasmas may be analogous to those operating in *M. pneumoniae*. Here, a major adhesin (protein P1), present on a specialised attachment tip organelle, acts in concert with a number of other adhesins, accessory membrane proteins and cytoskeletal elements to bind to host surface glycoproteins (Razin and Jacobs, 1992). Adherence appears to be a prerequisite to infection in this organism, since reduced cytadsorbance has been

associated with loss of virulence (Razin, 1978). This may not be the case for ureaplasmas, as trypsin pretreatment of *U. urealyticum* improved their infectivity towards fibroblast cultures (Masover *et al.*, 1977b). However, the effect of trypsin on ureaplasma membranes was not examined in this study, which serves to emphasise that the precise adhesion mechanisms of ureaplasmas requires delineation.

Adherence not only prevents their removal but creates a close association between ureaplasmas and their hosts. While this may be nutritionally favourable for microorganism, the damage induced as result of its metabolic reactions (Section 1.5.1) may be enhanced. Thus, metabolic enzymes are held in close proximity to epithelial membranes and in the microenvironment created between host and parasite, the harmful effects of nutrient depletion and waste product accumulation may be amplified. The cytopathic effects of *M. pneumoniae,* for example, have been attributed to H_2O_2 production in conjunction with its adhesion properties. When the mycoplasma adheres to the host, high concentrations of H_2O_2 appear to act over short distances, causing cumulative damage to epithelial cell membranes (Almagor *et al.*, 1983).

For a number of pathogens, adherence is a preliminary to traversing epithelial membranes to the nutritionally rich environment of the host cell (Finlay and Falkow, 1989). Here they are removed from competing microorganisms and from specific host defence mechanisms. Electron micrographs have shown ureaplasmas within bovine fallopian tube cultures (Kahane and Adoni, 1990) and it has recently been proposed that *M. genitalium* and *M. penetrans* can enter human lung fibroblasts and various tissue culture cells, respectively (Baseman, 1992, Lo *et al.*, 1992). It is not clear whether ureaplasmas and mycoplasmas survive and replicate within these cells and, until this is demonstrated, they are not considered as intracellular pathogens. The organisms therefore remain vulnerable to phagocytosis and the induction of a specific immune responses. Alternative tactics may therefore operate to evade such host defences.

b) Resisting phagocytosis

Ureaplasmas possess an external capsule which is lipoglycan in nature, consisting of polysaccharides covalently attached lipids (Smith, 1985). There are serotype differences in capsule composition which may relate to antigenic variations observed between serotypes, but its function in the organism is presently unclear and can only be predicted from examining similar structures in other organisms.

Lipoglycans are found on the surface of some acholeplasmas, anaeroplasmas, and mycoplasmas and may provide a stabilising function in the absence of a cell wall or alternatively may be involved in adhesion mechanisms. They do not necessarily correlate with pathogenicity but are immunogenic, eliciting the production of mainly IgM antibodies (Smith, 1984b, 1987). Pathogens such as *Haemophilus influenzae* and *Neisseria meningitidis* have similar capsules which may serve to resist phagocyte mobilisation and stimulation (Finlay and Falkow, 1989). Although the capsule in ureaplasmas is different from the lipopolysaccharide found in piliated *E. coli* (LPS) it may provide a similar protective function, such as by preventing surface deposition of complement and thereby resisting phagocytosis. It has also been suggested that capsules may mask membrane proteins which may otherwise be antigenic (Joiner, 1988). In this respect, the pleomorphic nature of ureaplasmas allows them close contact with host membranes, which may also provide shielding of antigenic determinants from the immune system (Robertson, 1986). A number of other mechanisms may also operate in *U. urealyticum* to prevent the generation of a specific immune response, as discussed below.

c) Shielding antigenic determinants

For some microorganisms, virulence determinants are poor antigens, resulting in minimal induction of specific neutralising antibody. The epitopes responsible for adhesion on the P1 adhesin protein of *M. pneumoniae,* for example, have demonstrated poor antigenic properties (Razin and Jacobs, 1992). Information on the antigenicity of *U. urealyticum* functional proteins is limited, but a number may achieve the same effect by mimicking host structures. The

identification of epitopes on the urease enzyme that cross react with the HLA B27 antigen suggests one possible mechanism for avoiding an immune response. In this case, however, it has been suggested that the opposite occurs and that antibodies raised against *U. urealyticum* may cross react with the host and induce an auto-immune disease, such as Reiter's syndrome (Davis *et al.*, 1992). Similar molecular mimicry has also been reported in *M. pneumoniae* (Mims, 1987).

When proteins are antigenic, a secondary immune response may be avoided by continual alteration of antigenic epitopes, known as antigenic drift. In *U. urealyticum* (serotypes 3, 8 and 10), a number of predominant antigens have been detected that undergo a high rate of structural variation *in vitro* (Watson *et al.*, 1990a). These are among the major antigens recognised during human infections and invasive isolates demonstrate structural variability, suggesting antigenic drift is also operating *in vivo*. By using monoclonal antibodies, common epitopes have been identified between these antigens and each produces a characteristic multiple banding pattern on SDS-PAGE, hence the name 'multiple banding' antigen, 'MB' antigen.

A potential role for the MB antigen in ureaplasma virulence is evident when parallels are drawn with the V1 antigen of *M. pulmonis* (Watson *et al.*, 1990b). Also a 'multiple banding' protein, V1 is the major antigen recognised in the natural infection of mice with *M. pulmonis*. It undergoes a high rate of antigenic variation both *in vitro* and *in vivo* and in some cases structural alterations within V1 are the only differences between virulent and avirulent strains of *M. pulmonis*. The precise subunit composition of V1, the mechanism of antigenic variation and the relevance of the MB antigen in *U. urealyticum* awaits further investigation.

d) Interference with the immune response

If an immune response is elicited against *U. urealyticum,* the organism has the ability to cleave and inactivate the principle immunoglobulin at mucosal surfaces, IgA. This is mediated by an IgA1 protease, an enzyme that specifically cleaves human IgA1 to yield intact Fcα and Fabα fragments. IgA1 proteases have not been identified in any other species of *Mollicute,* but they are elaborated by a number of other pathogenic bacteria that colonise mucosal epithelia.

As the topic of this thesis, the properties of the IgA1 protease isolated from *U. urealyticum* will be discussed in a separate section, Section C. To introduce this area, Section B will concentrate on the function of IgA and the properties and significance of bacterial IgA1 proteases.

SECTION B
PROPERTIES OF IGA1 PROTEASES

1.6 THE IGA SYSTEM

The structure and function of IgA has recently been reviewed by Kilian *et al.*, (1988), Childers *et al.*, (1989) and Kerr (1990).

1.6.1 IgA structure

IgA has a typical immunoglobulin structure, consisting of two heavy chains (α) and two light chains (κ or λ), bound by disulphide bonds to create a 'Y-shaped' molecule. The heavy chains consists of two 'Fc' constant domains (C_H3 and C_H2) linked to third 'Fab' constant domain (C_H1) via a flexible hinge region. The heavy and light chain variable domains (V_H and V_L, respectively) together form the specific antigen-binding sites of IgA (Fig. 1.5). By varying the distances between these two sites, the hinge region controls the efficiency of antigen-binding and cross-linking reactions.

IgA is divided into two isotypes (or subclasses) known as IgA1 and IgA2. These differ in levels of α chain glycosylation and amino acid sequence, particularly in the hinge region which is extended in IgA1 (Fig. 1.5) but in IgA2 a stretch of thirteen amino acids is lacking. Genetic polymorphism is restricted to the IgA2 subclass, where the two allotypes are designated IgA2m(1) and IgA2m(2) (Fig. 1.2a and b). In IgA2m(2), the two light chains are disulphide-bound to each other and are only non-covalently associated with heavy chains. IgA2m(1) appears to be a hybrid molecule between IgA1 and IgA2m(2) as its C_H3 domain is identical to that found in IgA1 and, apart from four allotypic determinants, its C_H1 and C_H2 domains are identical to those found in IgA2m(2). It is thought that IgA isotypes and allotypes have evolved by a series homologous crossover and point mutational events (Tsuzukida *et al.*, 1979) and an examination of ancestral α chain genes suggests that IgA2 may have evolved prior to IgA1 (Kawamura *et al.*, 1990).

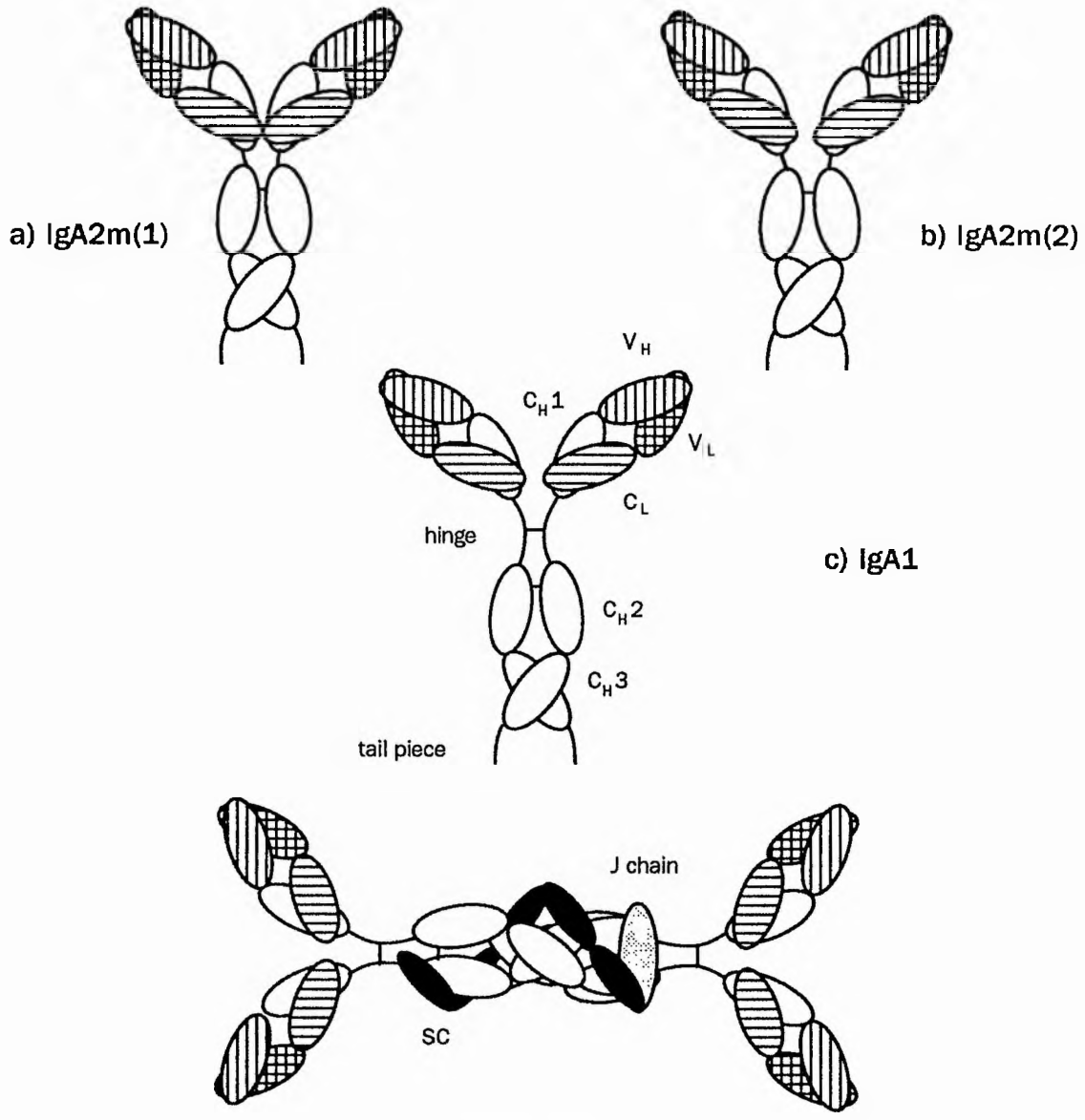


Fig 1.5 Theoretical structural models of monomeric IgA2m(1), IgA2m(2), IgA1 and secretory IgA1. (Modified from Kerr [1990]).

The models are based on the known structure of IgG. IgA consists of two heavy and two light chains associated by disulphide bonds to create a 'Y' shaped molecule. Each heavy chain is composed of three constant domains (C_H1, C_H2, C_H3) and one variable domain (V_H) which together with light chain variable domains (V_H) form the antigen binding site. In IgA2m(1), the terminal cysteine residues of each light chain constant domain (C_L) are disulphide-bound to each other rather than the C_H1 domain, as in IgA1 and IgA2m(2). An extended hinge region separates C_H1 and C_H2 domains in IgA1. In secretory IgA, two heavy chains are linked by the J chain and the dimer is enveloped by a heavily glycosylated secretory component.

These different isotypes of IgA are found in varying proportions in the numerous body fluids from which IgA has been isolated.

In serum, where IgA represents 20% of the total immunoglobulin present, IgA1 is the predominant isotype. It is produced in the bone marrow and exists mainly as monomers. In secretions, however, IgA constitutes the major immunoglobulin and 65%-90% of molecules are found in polymeric form, mainly dimeric. Up to 60% of secretory IgA can consist of IgA2, although the relative proportions of IgA isotypes appear to vary between secretory sites. In the distal gut, mammary, and salivary glands, the relative levels of IgA2 production are higher than in the spleen, peripheral lymph nodes and upper respiratory tract where IgA1-producing plasma cells predominate (Kett *et al.*, 1986, Brandtzaeg, 1992).

Following synthesis, IgA is released from plasma cells primarily as dimers, the Fc portion of α chains being linked by a cysteine-rich polypeptide, the J chain. To reach the mucosal surface, the dimers bind to a glycosylated immunoglobulin receptor on epithelial membranes. The receptor: IgA complex is internalised and transported through epithelial cells so that upon release IgA becomes enveloped by the now covalently associated 'secretory component' (SC).

The differences in cellular origin, subclass distribution and molecular form between secretory and serum IgA reflect the distinct functions that IgA is required to perform in the secretory and systemic immune systems.

1.6.2 IgA function

a) Secretory IgA

In the secretory compartment, the primary role of IgA is to prevent damage and systemic invasion by the large numbers of colonising microorganisms. For this function, S-IgA seems ideally suited. As a polymeric molecule, it has a high antigen binding potential and its association with the secretory component provides molecular stability and protection in the proteolytic-rich environment of mucosal epithelia. In addition, the highly glycosylated secretory component may confer particular properties beneficial to defence at secretory sites.

The means by which S-IgA provides protection are numerous but may include the prevention of epithelial attachment, antigen exclusion, neutralisation of harmful enzymes or toxins, microbial opsonisation for phagocyte uptake and cell-mediated killing. Since the levels of complement, phagocytic and cytotoxic cells are relatively low in the secretory mucosa, compared with the high microbial challenge, it has been proposed that S-IgA is not primarily involved with opsonisation and Fc-mediated cytotoxicity at this site (Childers *et al.*, 1989). It is more likely that S-IgA serves as an inhibitor of microbial adhesion, thereby allowing disposal of microorganisms by non-specific defence mechanisms such as cilliary-beating and mucous flow.

Microbial adhesion to mucosal epithelia may be via the specific recognition of host receptors by complementary adhesin molecules or via less-specific hydrophobic interactions occurring between microbial and host surfaces. Using host mucosal epithelial cells or salivary-coated hydroxyapatite (simulating dental enamel), it has been demonstrated that S-IgA can interfere with both of these processes (Kilian *et al.*, 1988). It is thought that the ability of S-IgA to interfere with hydrophobic interactions is mediated by the highly glycosylated secretory component, since serum IgA does not have such an effect (Hajishengallis *et al.*, 1992). This is supported by the fact that S-IgA Fabα fragments, while retaining their antigen binding potential, lose their ability to inhibit the binding of oral streptococci to saliva-coated hydroxyapatite (Reinholdt and Kilian, 1987). S-IgA may also act by increasing microbial agglutination, thereby reducing adhesion and encouraging disposal by innate defence mechanisms (Childers *et al.*, 1989).

b) Serum IgA

In the submucosa and systemic compartment, where, in contrast to the secretory compartment, microbial challenge is relatively light, it is likely that the role of IgA is to kill invading microorganisms via indirect Fc effector functions. For this, association with a secretory component and multivalency are not so critical. The interaction of IgA with humoral defence mechanisms, however, has been the subject of much controversy. For many years, it was assumed that IgA served only

to regulate the effects of IgG and IgM in complement activation and cell-mediated immunity, thereby reducing potentially harmful inflammatory side-effects of these reactions. It has recently become apparent that IgA may in fact perform a more active role in systemic host defence mechanisms than had previously been anticipated.

Although it is well documented that IgA can reduce IgM and IgG-mediated complement activation of the classical pathway, aggregates of IgA have also been shown to activate the alternative and, on occasion, the classical complement pathways (Kerr, 1990). These contradictions in function may be explained by the work of Jarvis and Griffiss, (1991) who demonstrated that the action of IgA1 was dependent upon the nature of the bound antigen. When specific for certain outer membrane proteins, IgA1-initiated complement-mediated lysis of *Neisseria meningitidis*, (*N. meningitidis*) but when bound to polysaccharide capsules, IgA1 appeared to block IgG-induced complement-mediated lysis. The likelihood and significance of these factors operating *in vivo* is not clear.

As far as IgA-dependent cell-mediated effector functions are concerned, receptors for IgA (FcαR) have been detected on polymorphonuclearleucocytes (PMN), monocytes, macrophages and lymphocytes. As mentioned above, early workers suggested that IgA inhibits the phagocytic, bactericidal and chemotactic effect of PMN at inflammatory sites (Kilian *et al.*, 1988) but more recent studies have demonstrated that IgA has opsoninic activity and can induce phagocytosis by neutrophils and macrophages (Kerr, 1990). Although the structure and function of the lymphocyte FcαR is not known, IgA-mediated ADCC (antibody-dependent cell-mediated cytotoxicity) by human T lymphocytes has been well documented (Childers *et al.*, 1989).

1.6.3 IgA degrading enzymes

A variety of human pathogens secrete proteases that digest IgA. A number have broad specificity, cleaving both IgA isotypes, IgG and other non-immunoglobulin proteins (Table 1.1C). The best characterised of these are the enzymes from *Serratia marcescens* (*S. marcescens*), *Candida albicans*

Table 1.1 IgA protease-producing bacteria

A. Classical IgA1 proteases

Disease	Species	% +ve	Reference
Meningitis	*S. pneumoniae*	>99	Male, 1979, Mulks *et al.*, 1980b
	N. meningitidis	>99	Plaut *et al.*, 1975, Mulks *et al.*, 1978
	H. influenzae	>95	Male, 1979, Kilian *et al.*, 1983a
Gonorrhoea	*N. gonorrhoeae*	>99	Plaut *et al.*, 1975, Mulks *et al.*, 1978
Urethritis	*U. urealyticum*	>99	Robertson *et al.*, 1984
Conjunctivitis	*H. aegyptius*	100	Kilian *et al.*, 1979, Kilian *et al.*, 1983a
Initial formation of dental plaque	*S. sanguis*	100	Plaut *et al.*, 1974b,
	S. oralis	100	Reinholdt *et al.*, 1990
	S. mitis biovar 1	>20	Reinholdt *et al.*, 1990
Destructive periodontal disease	*B. melaninogenicus*	~85	Kilian, 1981
	B. loeschii		Kilian *et al.*, 1983a
	B. oris		" "
	B. denticola		" "
	B. buccae		" "
	B. capillus		Frandsen *et al.*, 1987
	B. buccalis		" "
	B. oralis		" "
	B. veroralis		" "
	B. pentosaceus		" "
Miscellaneous	*H. parainfluenzae*		Male, 1979
	H. parahaemolyticus		Mulks *et al.*, 1980, Male, 1979
	G. haemolysans		Kilian *et al.*, 1983a
	Bifidobacterium (sp. undefined)		Fujiyama *et al.*, 1985
	Veillonella sp.		Frandsen *et al.*, 1986

B. IgA1/IgA2m(1) proteases

Disease	Species	% +ve	Reference
Inflammatory bowel disease	*Clostridium ramosum* *C. septicum* *C. tertium* *C. sporogenes*		Kobayashi *et al.*, 1987a Hashim and Hassan, 1991 " " " " " "

C. Broad specificity IgA proteases

Disease	Species	% +ve	Reference
Urinary tract infection	*P. mirabilis* *P. penneri* *P. vulgaris*	100 100 <10	Senior *et al.*, 1988 " " " "
Destructive periodontal disease	*B. gingivalis* *B. intermedius* *B. asaccharolyticus*	<50 100	Kilian *et al.*, 1983 Kilian, 1981 Kilian, 1981
Miscellaneous	*P. aeruginosa* *C. albicans* *S. marcescens*		Doring *et al.*, 1984 Rüchel *et al.*, 1982 Molla *et al.*, 1986, 1988

(*C. albicans*), *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Proteus mirabilis* (*P. mirabilis*). *Clostridium ramosum* (*C. ramosum*) is unique in releasing a protease that can digest only IgA2m(1) and IgA1 (Table 1.1B). Others are even more restricted in their choice of substrate and produce enzymes specific for IgA1 alone (Table 1.1A). These are known as 'classical' IgA proteases or IgA1 proteases. Since the enzyme elaborated by *U. urealyticum* falls into this category of IgA1 proteases, the remaining sections will be devoted to a discussion of the properties of these group of enzymes. Recent reviews on the properties of IgA1 proteases are provided by Mulks, (1985), and Kilian and Reinholdt (1986).

1.7 GENERAL PROPERTIES OF IGA1 PROTEASES

IgA1 proteases are characterised by their release of intact Fabα and Fcα fragments from IgA1. The identification of intact Fcα fragments in the faeces of patients with liver dysfunction (Mehta *et al.*, 1973) first alerted researchers to the existence of these enzymes since until then, enteric proteolytic activity had been confined to enzymes that extensively hydrolysed the Fcα portions of IgA, such as trypsin and chymotrypsin. Since then IgA1 proteases have been found in a small but clinically important group of pathogenic bacteria that all share predilection for human mucosal epithelia (Table 1.1A).

1.7.1 Protease-positive organisms

The most notable of these bacteria is the cause of gonorrhoea, *Neisseria gonorrhoeae (N. gonorrhoea)*, and the principle pathogenic agents in bacterial meningitis, *N. meningitidis*, *Haemophilus influenzae (H. influenzae)* and *Streptococcus pneumoniae (S. pneumoniae)*. These last three can all exist as harmless flora in the human upper respiratory tract but under some circumstances cause meningitis, pneumonia, septicaemia and middle-ear infections. IgA1 protease activity is not restricted to the pathogenic serotypes or biotypes of these species and over 95% of isolates elaborate the enzyme. This is also true for *N. gonorrhoeae* where ~100% of isolates have been found to be IgA1 protease-positive, irrespective of colonial type (piliated of non-piliated) or site of isolation.

IgA1 proteases are expressed by members of the group 'viridans streptococci', which have been associated with dental caries and infective endocarditis. Of these, *Streptococcus sanguis, (S. sanguis)* and *Streptococcus oralis, (S. oralis,* formerly *S. mitior)* are dominant among bacteria that colonise a newly cleaned tooth surface and thereby may initiate the formation of dental plaque. Since the recent reclassification of oral streptococci (Kilian *et al.,* 1989), all isolates from these species have been found to be positive for IgA1 protease production (Reinholdt *et al.,* 1990). Also within the oral cavity, those bacteria implicated in destructive periodontal disease such as *Bacteroides (Prevotella)* and *Capnocytophaga* species elaborate an IgA1 protease. While some of these express 'classical' enzymes, digesting only IgA1, others have a broader specificity and digest both IgA2 and IgG. It is possible that in these cases an IgA1 protease is elaborated in addition to less specific immunoglobulin-digesting enzymes (Kilian, 1981).

A number of other, but less well characterised IgA1 proteases have been identified in *Gemella haemolysans, (G. haemolysans), Bifidobacterium* and *Veillonella species.*

1.7.2 Protease-negative organisms

There are many mucosal pathogens which have no apparent IgA1 protease activity and to date bacteria from over forty different genera have been examined for enzyme expression. These are detailed by Mulks, (1985) and Kilian and Reinholdt, (1986) and include strains of *Campylobacter, Corynebacterium, Enterobacter, Escherichia, Klebsiella, Legionella, Mycobacterium, Salmonella, Shigella, Staphylococcus, Vibrio* and *Yersinia. Herpes Simplex, Polio* or *Influenza* virus do not elaborate the enzyme nor do any of the class *Mollicute,* other than ureaplasmas.

1.7.3 Site of digestion within IgA1.

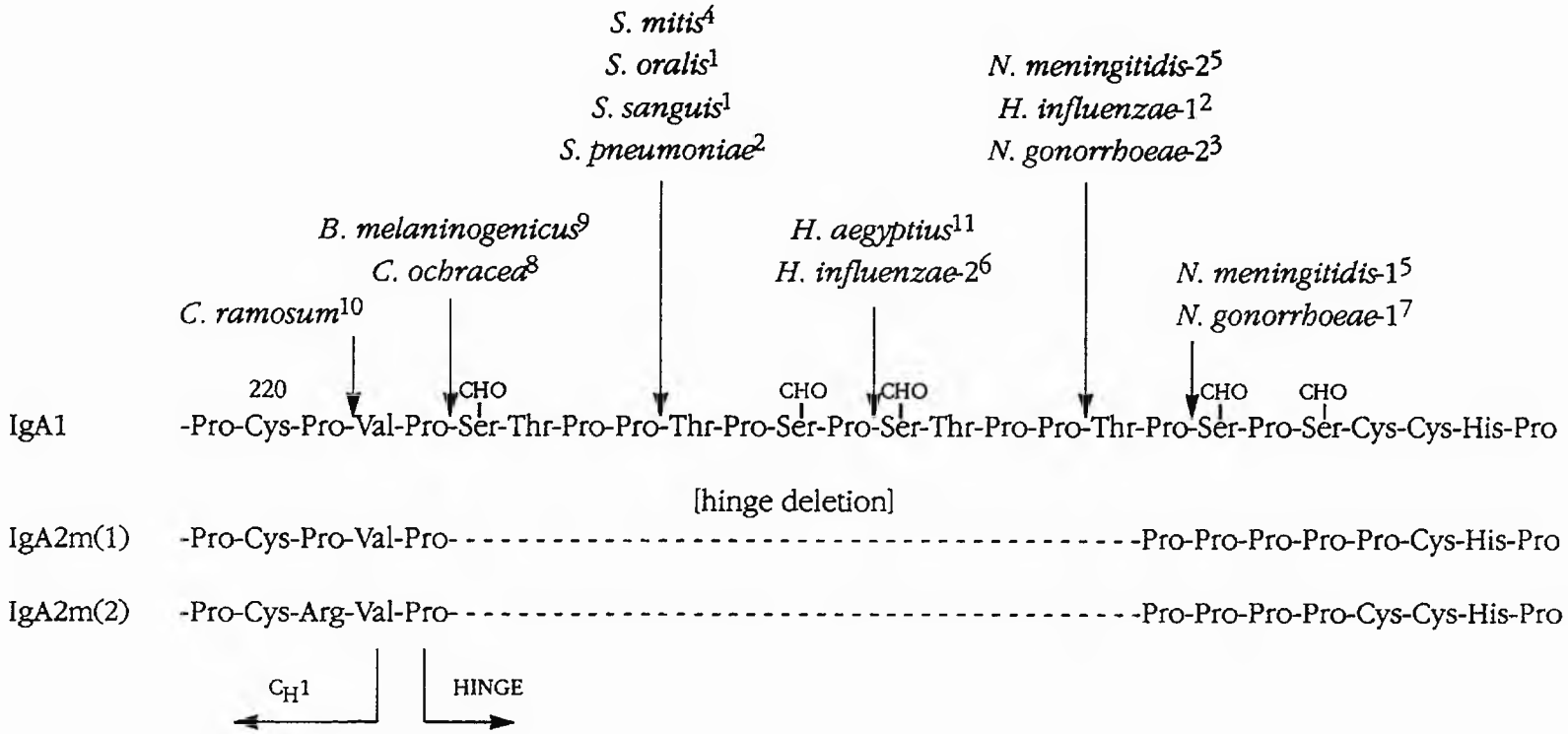
IgA1 proteases do not digest the light chain, secretory component or J chain of IgA1 (Kilian *et al.,* 1980) but cleave within the exposed hinge region of

the α chain (Fig. 1.6). The precise points of scission have been established by limited amino-terminal sequencing of Fcα fragments followed by comparison with the known sequence of IgA1 (Putnam *et al.*, 1979). IgA1 proteases do not share an identical substrate specificity but either cleave between Pro-Ser bonds (type 1 enzymes) or Pro-Thr bonds (type 2 enzymes) in a duplicated octapeptide present within the hinge region of IgA1 (see Fig. 1.6). IgA2 molecules are resistant to attack by classical IgA proteases since the thirteen amino acid region containing the duplicated octapeptide is lacking in this isotype.

1.7.4 Specificity types

The IgA1 proteases isolated from *Bacteroides melaninogenicus, (B. melaninogenicus), Capnocytophaga ochracea, (C. ochracea), S. sanguis* and *S. pneumoniae* only cleave a single bond in the IgA1 hinge region but two cleavage sites have been identified for the enzymes produced by *H. influenzae, N. gonorrhoeae* and *N. meningitidis*. For the neisserial species, a given isolate produces one but not both of the two enzyme phenotypes (type 1 or type 2). This is also true for the majority of *H. influenzae* strains, but a number have been identified that appear to digest IgA1 at both sites simultaneously (type 3). Inhibition studies using antibodies raised against type 1 and type 2 *Haemophilus* enzymes have suggested that these type 3 strains are expressing two different IgA1 proteases (Kilian *et al.*, 1983b). This was supported by Mortensen and Kilian, (1984b) who purified two proteins with IgA1 protease activity (74 kDa, 34 kDa) from one of these strains (HK 295). Hybridisation studies using a radiolabelled probe from the *Haemophilus* type 1 IgA protease gene identified only one homologous fragment in a type 3 strain (18 F), which suggested expression of a single enzyme with two distinct substrate specificities (Bricker *et al.*, 1983). Alternatively, the probe may have hybridised to only one of two separate genes specifying the IgA1 protease or a gene duplication event may have yielded two distinct but adjacent genes.

A single IgA1 protease gene (*iga* gene) is also found in *H. influenzae* strains expressing only one IgA1 proteases specificity type (Bricker *et al.*, 1983).



References:

1 = Plaut *et al.*, 1974b
2 = Kilian *et al.*, 1980
3 = Plaut *et al.*, 1975
4 = Reinholdt *et al.*, 1990.
5 = Mulks *et al.*, 1980a
6 = Mulks *et al.*, 1982
7 = Mulks and Knapp, 1987
8 = Frandsen *et al.*, 1987
9 = Mortensen & Kilian, 1984a
10 = Fujiyama *et al.*, 1986
11 = Kilian *et al.*, 1983a

Fig 1.6 Primary sequence of human IgA1 and IgA2 and the site of cleavage by microbial IgA1 proteases.

This suggests that conversion from one specificity type is not mediated through silent copies of the *iga* gene. By the construction and expression of hybrid type 1/type 2 genes, it has also been demonstrated that such a conversion does not occur via a simple inversion or deletion mechanism (Grundy *et al.*, 1990). It was established by these workers, however, that the cleavage site determinant for *H. influenzae* is located in a 370 base pair domain near the amino terminal coding region of the *iga* gene.

An examination of specificity type-distribution among IgA1 proteases from *Neisseria* and *Haemophilus* strains has revealed a degree of association between the site of IgA1 digestion and other phenotypic variables.

1.7.5 Association between specificity types and phenotypic variables

Among *N. meningitidis* strains, the specificity type may be linked to the outer membrane serotype and capsular serogroup of the organism. All serotype 2a, 2b and the majority of serotype 15: P1.16 strains produce a type 1 enzyme whereas all serotype 2c strains produced a type 2 protease. As far as capsular serogroups are concerned, all serogroup A isolates express type 1 enzymes but no clear association has been found between protease cleavage type and other serogroups (Mulks, 1985, Lomholt *et al.*, 1992). Analysis of IgA1 protease genes (*iga* genes) from representative isolates has failed to establish a correlation between restriction fragments length polymorphism (RFLP) patterns and IgA1 protease cleavage type or phenotypic characteristics but has served to demonstrate the high level of genetic polymorphism in this species (Lomholt *et al.*, 1992).

In *N. gonorrhoeae*, a more clearly defined phenotypic linkage between the specificity type and the nutritional auxotype, *dam* methylation and outer membrane protein I serovar-grouping of the strains has been demonstrated (Mulks and Knapp, 1987). The majority of type 1 IgA1 protease-producers require arginine, hypoxanthine and uracil (AHU) for growth, are *dam* methylase positive and belong to Protein IA-I or Protein IA-2 serovars. Isolates producing the type 2 enzyme are more diverse in auxotype and serovar grouping and can be *dam*+ or *dam*- (Mulks and Knapp, 1985). This pattern is reflected in gene organisation as

there are eight different RFLP types for gonococci type 2 proteases compared to only one gene type coding for the type 1 protease. The restriction site polymorphisms found in type 2 genes do not directly relate to the other phenotypic differences (Mulks and Knapp, 1987). Simpson *et al.,* (1988) have suggested that regions encoding these phenotypic traits may be closely linked on the gonococcal chromosome.

In contrast, it has been possible to relate the genotypic differences between encapsulated *H. influenzae* strains to capsular serotype and IgA1 protease specificity. Two independent studies have established that all serotype b and the majority of serotype a and d strains produce a type 1 enzyme while serotype c and most serotype e and f strains produce a type 2 enzyme (Mulks *et al.,* 1982, Kilian and Thomsen, 1983). Southern hybridisation analysis using an *iga* gene probe from a serotype d strain (type 1) with *Eco*RI-digested chromosomal DNA from five other *H. influenzae* serotypes revealed, with few exceptions, that each serotype produced a characteristic restriction enzyme pattern (Bricker *et al.,* 1983, 1985). In addition, serotype f strains gave two distinct patterns that correlated well with IgA1 protease specificity types.

Of perhaps more significance to the function of different IgA1 protease specificity types, these studies have revealed a potential correlation between the virulence of a particular bacterial isolate and the cleavage site specificity of its IgA1 protease. Thus, a type 1 IgA1 protease is expressed by all strains of the *H. influenzae* serotype primarily responsible for invasive disease (serotype b), in all but one of 58 *N. meningitidis* isolates obtained from meningococcal epidemics (Lomholt *et al.,* 1992), and in all *N. gonorrhoeae* strains from the AHU auxotype, which have been shown to be associated with disseminated gonorrhoea (Knapp and Holmes, 1975). While it seems unlikely that the function of cleaved IgA1 will differ according to such small differences in cleavage site, if other substrates important in a disease process are selectively digested by different IgA1 protease types, this correlation may prove to be significant.

1.7.6 Alternative substrates

In the search for an alternative substrate, IgA1 proteases have demonstrated an extremely narrow substrate specificity. For many years, the only known substrates for these enzymes were serum and secretory human IgA1 and serum IgA from the gorilla *(Gorilla gorilla)* and chimpanzee *(Pan troglodytes)*. All other classes of human and animal immunoglobulins (IgA2, IgG, IgM, IgD and IgE) and other substrates commonly digested by microbial proteases such as casein, gelatin, Azocoll and the oxidised β-chain of insulin are resistant to attack (A more detailed list of insusceptible substrates is provided by Kornfeld and Plaut, 1981). A systematic approach of designing peptides based on the hinge region of IgA1 failed to uncover a synthetic substrate for IgA1 proteases but a number have proved to be important inhibitors of the enzyme (Plaut *et al.*, 1982, Burton *et al.*, 1988, Wood *et al.*, 1989, Bachovchin *et al.*, 1990) (Table 1.2A).

It is only since the isolation of the *iga* gene from *N. gonorrhoeae* that this deadlock in finding alternative substrates for IgA1 proteases has been broken. By recombinant DNA technology, Pohlner *et al.*, (1987) have identified three sites in an IgA1 protease precursor molecule which are autoproteolytically cleaved during secretion of the enzyme (see Section 1.8.1). These are not identical to sites within the IgA1 hinge region, but contain a number of similar features (Table 1.2B). Furthermore, synthetic decapeptides based on sites B and C (but not site A) act as substrates for the *Neisserial* enzyme but the efficiency of digestion is only 1/10 of that observed with the native the IgA1 substrate (Wood and Burton, 1991).

Following the suggestion that IgA1 proteases may have a self-regulatory function, Shoberg and Mulks, (1991) have demonstrated that a number of proteins in the outer and cytoplasmic membranes of *N. gonorrhoeae* are digested by the gonococcal enzyme. The relative molecular masses of these proteins range from 21-78 kDa, but their precise cleavage sites and functions in the bacterial cell have not yet been established.

More recently, a substrate that may have applications for the assay of IgA1 proteases has been identified. Endogluconase A (Cen A) is isolated from the

Table 1.2 Inhibitors and substrates of IgA1 proteases

A. Synthetic substrate

Synthetic peptide	Sub	I_{50}	Reference
Based on IgA1 hinge region:			
P-V-P-S-T-P-P-T-P-S-P-S-T-P-P-T-P-S-P-S-C-C-H-P	-	8 mM	Plaut *et al.*, 1982
T-P-P-T-P-S-P-S-T-P-P-T-P-S-P-S	-	20 mM	
T-P-P-T-P-S-P-S	-	0.50 mM	Burton *et al.*, 1988
Ac-T-P-P-T-P-S-P-S	-	0.51 mM	
T-P-P-T-P-S-P-S-NH_2	-	0.18 mM	
(Ac-T-P-P-C-P-S-P-S-NH_2)$_2$	-	0.17 mM	
Ac-T-P-P-T-P-S-P-C-NH_2	-	0.49 mM	
Ac-T-P-P-T-P-C-P-S-NH_2	-	0.05 mM	
Ac-C-P-P-T-P-S-P-S-NH_2	-	0.02 mM	
Ac-S-T-P-P-	-	9.70 µM	Wood *et al.*, 1989
T-P-P-T	-	419 µM	
P-P-T-P	-	40.5 µM	
P-T-P-S-NH_2	-	0.5 µM	
T-P-S-P	-	33.1 µM	
Ac-A-P-boroP-OH	-	4 nM	Bachovchin *et al.*, 1990
Boc-A-P-boroP-OH	-	35 nM	
MeOSuc-A-A-P-boroP-OH	-	28 nM	
CenA-S-T-P-P-T-P-S-P-S-T-P-P-T-P-S-P-CenA	+		Miller *et al.*, 1992
Based on IgA1 protease precursor sites:			Wood and Burton, 1991.
Ac-V-V-A-P-P-S-P-Q-A-N-NH_2	+		
Ac-L-P-R-P-P-A-P-V-F-S-NH_2	+		

B. Natural substrates

IgA1 hinge region:		Reference
Type 1 cleavage	P-P-T-P-P-T-P-S | P-S-C-C-H -P-R	Mulks, 1985
Type 2 cleavage	T-P-S-P-S-T-P-P | T-P-S-P-S-C-C-	
IgA1 protease precursors:		
N. gonorrhoeae:		
Site A cleavage site	N-G-R-P-V-K-P-A | P-S-P-A-A-N-T	Pohlner *et al.*, 1987
Site B cleavage site	P-Q-N-I-V-V-A-P | P-S-P-Q-A-N-Q	
Site C cleavage site	R-R-I-A-L-P-R-P | P-A-P-V-F-S-L	
H. influenzae:		
Serotype D cleavage	V-E-T-A-P-V-P-P | P-A-P-A-T-P-S-	Pohlner *et al.*, 1991
Serotype B cleavage	V-D-E-A-P-V-P-P | P-A-P-A-T-P-S-	

bacterium *Cellulomonas fimi* and consists of two functional subunits, an N-terminal cellulose binding domain and a C-terminal catalytic domain, which are linked by a short proline/threonine polypeptide resembling the hinge region of IgA1. Although Cen A is not digested by IgA1 proteases, if the linker is replaced by the hinge region of IgA, the hybrid molecule acts as substrate for the neisserial enzyme (Miller *et al.*, 1992). The type 2 enzyme cleaves the hinge region at exactly the same site as within the native IgA1 molecule, but the type 1 enzyme cleaves the Pro-Ser bond in both octapeptide chains. The rate of digestion, however, is much slower than that found with the IgA1 heavy chain.

Although the precise determinants of IgA1 proteases substrate specificity have not yet been determined, the findings of both Wood and Burton (1991) and Miller *et al.*, (1992) have indicated that IgA1 proteases are not entirely sequence-specific, but show some conformational dependence. In the Cen A construct, the conformation of the first octapeptide Pro-Ser bond appears to resemble the second octapeptide bond more closely than in the native substrate, since both are digested by the type 1 enzyme. Likewise, peptides representing the gonococcal autocatalytic sites probably assume a more favourable conformation in free solution than those based on the hinge region of IgA1. In this respect, it would be of interest to examine the efficiency of digestion of a Cen A construct where the linker is replaced with a sequence corresponding to one of these autoproteolytic sites. In addition, sequencing of the cleavage site within the neisserial membrane proteins may give further insight into the specific substrate requirements of IgA1 proteases.

As far as developing an assay is concerned, the separation of the two functional Cen A domains by enzyme cleavage within the hybrid linker molecule may provide a cheap and rapid means of quantifying IgA1 protease activity. The recent expression of recombinant IgA1 in mammalian cell culture (J. Woof, personal communication) may have similar applications.

1.7.7 Enzyme assays

Until such systems are developed, assaying for IgA1 protease activity necessitates the use of human IgA1. Although, IgA1 paraproteins can be purified in large quantities from the serum of patients with plasma cell neoplasms their high relative molecular mass has impeded enzyme kinetic studies where known molarities of substrate are required. The insolubility of IgA1 at high protein concentrations and its instability at extremes of pH also pose a number of technical problems (Plaut *et al.*, 1978a). In addition, each assay involves the incubation of enzyme preparations with IgA1 followed by the separation of Fabα/Fcα fragments from intact IgA1 and a final step to detect hydrolysis products, all of which may be time consuming. Despite these problems, a number of assay systems have been described which have enabled the qualitative and quantitative measurement of IgA1 protease activity.

Using IgA1 and an appropriate antiserum, this has been achieved by immuno-electrophoresis (Plaut *et al.*, 1974a), radioimmunodiffusion (Labib *et al.*, 1978), rocket immunoelectrophoresis (Lassiter *et al.*, 1989), SDS-PAGE followed by immunoblotting (Proctor and Manning, 1990), and by ELISA (Reinholdt and Kilian, 1983). Alternatively, radiolabelled IgA1 has been used in combination with SDS-PAGE (Blake and Swanson, 1978), cellulose acetate electrophoresis (Plaut *et al.*, 1978b) and with IgA Fcα receptor-bearing haemolytic streptococci in an immuneprecipitation-based technique (Lindahl *et al.*, 1981) to quantitatively measure IgA1 protease activity. Two assays that detect IgA1 protease-hydrolysis products more directly by HPLC (Mortensen and Kilian, 1984b) and continuous spectrophotometry (Bleeg *et al.*, 1985) have also been described.

These various systems have been used successfully for the purification and basic characterisation of IgA1 proteases.

1.8 CHARACTERISATION OF IGA1 PROTEASES

1.8.1 Production of an extracellular enzyme

All of the IgA1 proteases identified to date appear to be released extracellularly during growth of the bacteria (Kilian and Reinholdt, 1986). For the type 2 enzyme from *N. gonorrhoeae*, it has been reported that over 96% of total IgA1 protease activity is present in the extracellular fraction but less than 2.5% is in periplasmic and cytoplasmic fractions (Simpson *et al.*, 1988). By using the marker enzymes, β-lactamase, glucose-6-dehydrogenase and succinate dehydrogenase (for the periplasmic, cytoplasmic and membranous fractions, respectively) it has been confirmed that the extracellular activity detected was not simply a result of cell lysis. Thus, during the stationary phase of growth, periplasmic leakage of β lactamase was detected but at a fraction the rate of IgA1 protease release. This suggested that a selective transport mechanism operated for neisserial IgA1 proteases which was later described in detail by Pohlner *et al.*, (1987).

Following the isolation and expression of the gene encoding the gonococcal type 2 enzyme in *E. coli*, these workers established that the IgA1 protease was initially expressed as a 169 kDa precursor which was extensively processed during the course of secretion from the cell (Halter *et al.*, 1984). Sequence analysis and expression of fusion proteins revealed that the precursor could be divided into three regions; a terminal leader peptide showing typical characteristics of a prokaryotic signal sequence, a central region of 106 kDa, coding for the IgA1 protease and a carboxy terminal helper sequence (β domain), containing three sites that resembled the IgA1 protease target sequence. From this, an elegant mechanism for IgA1 protease transport in *Neisseria* species has been proposed, which is described pictorially in Fig. 1.7.

In brief, an amino terminal leader peptide directs the passage of the IgA1 protease precursor across the bacterial inner membrane where the leader sequence is removed by a specific signal peptidase. Within the periplasm, the hydrophobic β domain forms a pore in the outer membrane which allows the remainder of the protease to be transported through to the exterior of the cell.

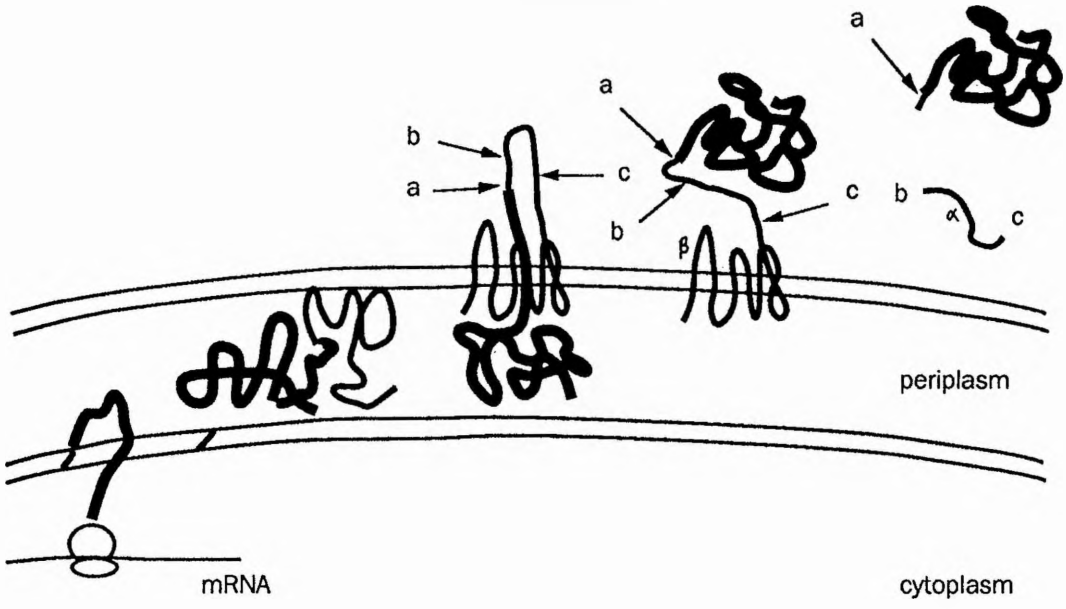


Fig. 1.7 Model for the extracellular secretion of IgA1 protease from *N. gonorrhoeae*.

The model was first deduced from the *N. gonorrhoeae iga* gene sequence by Pohlner *et al.*, (1987). An amino terminal leader domain directs the passage of the proform IgA1 protease into the periplasmic space, where it is cleaved by a sequence-specific signal peptidase. In a second step, the hydrophobic β domain associates with the outer membrane to form a pore for the secretion of the molecule. Exterior to the cell, the protease achieves an active conformation and is released from the β domain by autoproteolysis (site 'b' or 'c'). Further cleavage at site 'a' results in the production of the α protein and a mature 106 kDa enzyme (thick line).

Here the protease is presumed to achieve an active conformation and by digestion of the internal site 'c' the extracellular enzyme (proform C) is released from the cell. Proform C is then further processed by enzymatic digestion at cleavage sites 'b' to yield proform B and the α protein. Final cleavage at site 'a' produces the mature 106 kDa IgA1 protease. The double band frequently seen in gonococcal IgA1 protease preparations examined by SDS-PAGE (Halter *et al.*, 1989), is presumed to represent the mature IgA1 protease and the extracellular proform B.

The IgA1 protease from *H. influenzae* is also synthesised as a larger precursor molecule that requires a C-terminal portion for full function and secretion of the enzyme (Bricker *et al.*, 1983; Grundy *et al.*, 1987). Although regions corresponding to a signal peptide and four potential autocatalytic cleavage sites have been identified in the deduced amino acid sequence from an *H. influenzae* serotype b *iga* gene, recent evidence suggests that only one of these sites is cleaved by the *H. influenzae* IgA1 protease. This is supported by the fact that immunoblotting using specific anti-IgA1 protease antisera detects only one molecular form of the enzyme is spent culture supernatants (Pohlner *et al.*, 1991). These workers also identified the IgA1 protease in *H. influenzae* cell lysates. To date, no other reports of a cell-associated IgA1 protease activity have been documented.

The deduced amino acid sequence of the *S. sanguis iga* gene has shown little structural similarity with the IgA1 protease from *N. gonorrhoeae* or *H. influenzae* (Gilbert *et al.*, 1991). Although a putative precursor enzyme (200 kDa) has been identified in the periplasm of *E. coli*-expressing cells an amino-terminal leader sequence and helper regions have not been identified (Gilbert *et al.*, 1988). One potential autoproteolytic site that resembles the sequence cleaved in the IgA1 hinge region by *S. sanguis* has been identified, but as it is not cleaved by the active enzyme accumulating in the periplasm it is likely that either this does not represent the processing site or that conditions in the *E. coli* periplasm are unfavourable for processing. It is therefore probable that

secretion in wild-type *S. sanguis* requires additional *iga* sequences or accessory gene products that have not yet been identified.

1.8.2 Enzyme purification

As a consequence of their extracellular production, bacterial spent medium has generally been used as the source of IgA1 proteases for the purposes of enzyme purification and characterisation. In some instances, extracellular IgA1 proteases are harvested from bacterial colonies grown on nutrient agar covered by dialysis membranes (Higerd *et al.*, 1977).

A number of protocols have been described for enzyme purification which differ according to the bacterial species and type of IgA1 protease involved. Early methods invariably included an initial ammonium sulphate 'salting-out' step followed by a series of chromatographic separations including anion exchange chromatography, isoelectric focusing and gel filtration. (*N. gonorrhoeae*; Blake and Swanson, 1978, Halter *et al.*, 1984, Simpson *et al.*, 1988, *S. sanguis*; Plaut *et al.*, 1974b, Labib *et al.*, 1978). More recently high performance liquid chromatography (HPLC) has been used for the purification of the enzymes from *N. gonorrhoeae* (Blake and Eastby, 1991) and *B. melaninogenicus* (Mortensen and Kilian, 1984a). These have been reported to give IgA1 proteases with a higher degree of purity and larger yields than have been obtained by more conventional means (Mortensen and Kilian, 1984a). Despite such refinements, no IgA1 protease has been isolated in large enough quantities for structural studies but the highly active preparations have been useful for biochemical, and immunochemical studies.

1.8 3 Biochemical characteristics

The biochemical properties of the bacterial IgA1 proteases characterised to date are summarised in Table 1.3. All the purified IgA1 proteases have an apparent relative molecular mass (Mr) of between 80 kDa and 100 kDa, apart from those isolated from *B. melaninogenicus* and *C. ochracea* which have a lower apparent Mr of 62 kDa and 48 kDa respectively. The type 2 gonococcal enzymes appear to

Table 1.3 Biochemical characteristics of bacterial IgA1 proteases

Species	Mr (kDa)	K_m (M)	pI	pH	Reference
N. gonorrhoeae					
Type 1	112		8-8.2		Simpson *et al.*, 1988
Type 2	114/109 110/105		8-8.2 8.6	 6-9	Simpson *et al.*, 1988 Halter *et al.*, 1984
N. meningitidis				7-8.5	Reinholdt and Kilian, 1983
H. influenzae					
HK 295	74/34	$8x10^{-5}$			Bleeg *et al.*, 1985 Mortensen and Kilian, 1984a
HK 393	105/80 85		6.6		Bleeg *et al.*, 1985 Reinholdt and Kilian, 1983
HK 50	86			6-9	Mortensen and Kilian, 1984a
S. sanguis	 100 100	$5.5x10^{-6}$	5.45	 6-7 8	Plaut *et al.*, 1978b Plaut *et al.*, 1978a Labib *et al.*, 1978 Reinholdt *et al.*, 1990
S. oralis	100	$3.4x10^{-6}$			Reinholdt *et al.*, 1990
B. melaninogenicus	62	$3.4x10^{-6}$	5	4.5-7.5	Kilian and Reinholdt, 1986
C. ochracea	48	$9.1x10^{-6}$			" "

have a slightly higher Mr than the type 1 enzyme (Simpson *et al.*, 1988) which has been linked to different cleavage site positions within the precursor molecule (Halter *et al.*, 1989). The enzymes appear to be active over a broad pH range (6-9; 4.5-7.5 for *B. melaninogenicus)* but show substantial variations in their isoelectric points (IEP). Those isolated from *N. gonorrhoeae* and *H. influenzae* have an IEP of 8.6 and 6.6 respectively, whereas those from *S. sanguis* and *B. melaninogenicus* have respective IEPs of 5.45 and 5. Such differences explain the variations observed between purification protocols.

1.8.4 Enzyme classification

The classification of IgA1 proteases has been based primarily on their sensitivity to diagnostic inhibitors from the serine, cysteine, metallo and aspartyl protease class. The inhibition of bacterial IgA1 proteases is presented in Table 1.4.

IgA1 proteases from *S. sanguis, S. pneumoniae* and *C. ochracea* all appear to be metallo enzymes as they are inhibited by low concentrations (5-10 mM) of ethylenediaminetetraacetic acid (EDTA) and other metal chelating agents. For *S. sanguis,* activity is recovered by dialysis with either calcium, magnesium, manganese or zinc ions (Labib *et al.*, 1978, Plaut *et al.*, 1974b). Sequencing of the *iga* gene from *S. sanguis* has identified a putative zinc-binding motif which is characteristic for metallo proteases from gram-negative and gram-positive bacteria (Gilbert *et al.*, 1991). Furthermore, site-directed mutagenesis of amino acid residues in this region has resulted in a catalytically inactive enzyme, further supporting the proposal that the streptococcal IgA1 protease is a metallo enzyme.

In *Bacteroides* species, the IgA1 proteases are also sensitive to the metallo protease inhibitor bathocuprine disulphonate (BCDS) (Frandsen *et al.*, 1987), but are clearly inhibited by compounds that interact with sulfhydryl groups, such as iodoacetamide and p-chloromercuribenzoate (PCMB) (Mortensen and Kilian, 1984a). These enzymes have therefore been defined as metal-ion dependent cysteine proteases (Frandsen *et al.*, 1987).

Table 1.4 Inhibitors of bacterial IgA1 proteases

Species	Inhibitor type			I_{50}	Reference
N. gonorrhoeae	Metallo	EDTA	10 mM	+	Plaut *et al.*, 1975
			125 mM	+/?	Kilian *et al.*, 1983a,
		BCDS	30 mM	+	Simpson *et al.*, 1988
		phenanthroline	35 mM	+	" "
		PCMB	90 mM	+	" "
	Serine	PMSF	2 mM	-	Blake and Swanson,
		TLCK/TPCK	1 mM	-	1978
		DFP	6×10^{-4} M	+	Bachovchin *et al.*, 1990
N. meningitidis	Metallo	EDTA	50 mM	+	Plaut *et al.*, 1975
			125 mM	+/?	Kilian *et al.*, 1983a
H. influenzae	Metallo	EDTA	125 mM	+/?	Kilian, 1981, Kilian *et*
		BCDS	10 mM	+	*al.*, 1983a
			20 mM	+	Bleeg *et al.*, 1985
	Serine	DFP	6×10^{-4} M	+	Bachovchin *et al.*, 1990
S. sanguis	Metallo	EDTA	5 mM	++	Plaut *et al.*, 1978a
		phenanthroline	500 µM	+	Labib *et al.*, 1978
	Cysteine	Iodoacetamide	10 mM	-	" "
	Cysteine	PCMB	10 mM	-	" "
	Serine	PMSF	10 mM	-	" "
		DFP	6×10^{-4} M	-	Bachovchin *et al.*, 1990
S. mitior/ S. pneumoniae	Metallo	EDTA	5 mM	+	Kilian *et al.*, 1983a.
Bacteroides sp.	Metallo	EDTA	125 mM	+/?	Kilian *et al.*, 1983a
		BCDS	10 mM	+	" "
	Serine	SBTI	1 $mg.ml^{-1}$	-	Mortensen and Kilian,
		PMSF	1 $mg.ml^{-1}$	-	1984a
	Aspartyl	Pepstatin	1 $\mu g.ml^{-1}$	-	" "
	Cysteine	$HgCl_2$	10 µM	++	" "
		PCMB	10 µM	++	" "
		DTNB	10 µM	+	" "
Capnocytophaga sp.	Metallo	EDTA	6 mM	+	Kilian, 1981
		BCDS	10 mM	+	Kilian *et al.*, 1983a
	Cysteine	Iodoacetamide	1 mM	-	Kilian, 1981

Classification of the IgA1 proteases from *N. gonorrhoeae* and *H. influenzae* has been more controversial. Early reports classified these enzymes as metallo proteases (Plaut *et al.*, 1975, Blake and Swanson, 1978) although 5-fold higher concentrations of EDTA were required for enzyme inhibition compared with streptococcal IgA1 proteases (Kornfeld and Plaut, 1981). Kilian *et al.*, (1980) reported that the IgA1 proteases from *H. influenzae* and *N. meningitidis* were completely insensitive to EDTA but later reported that all IgA1 proteases were inhibited by the metallo enzyme inhibitor BCDS (Kilian *et al.*, 1983a). The type 1 enzymes from *N. gonorrhoeae* are more sensitive to low concentrations of cysteine and metallo protease inhibitors than their type 2 counterparts (Simpson *et al.*, 1988). More recently, the inhibition of *N. gonorrhoeae* and *H. influenzae* type 1 and type 2 IgA1 proteases by diisopropyl fluorophosphate (DFP) and peptidyl boronic acids, which act as serine-protease transition state analogues, has suggested that these enzymes are serine proteases (Bachovchin *et al.*, 1990). These workers identified a possible consensus sequence for a chymotrypsin-like serine protease (GDS*GGPL) in the *iga* genes isolated from these species. Site-directed mutagenesis of the serine residue (S*) to a threonine residue in the *iga* gene from *H. influenzae* has resulted in enzyme inactivation and lends further support to the proposal that these IgA1 proteases are members of the serine protease class (Poulsen *et al.*, 1992).

1.9 THE DIVERSITY OF IGA1 PROTEASES

Although IgA1 proteases share a common function, the differences in substrate specificity and biochemical properties, both within and between bacterial species, suggests that these enzymes are highly diverse. Such diversity is further emphasised by complementary immunochemical and genetic comparisons of IgA1 proteases and their respective *iga* genes.

1.9.1 Immunochemical analysis

The preparation of neutralising antibodies to partially or totally purified preparations of IgA1 proteases has been reported by a number of workers. IgA1

proteases from *N. gonorrhoeae, N. meningitidis, H. influenzae* and *S. pneumoniae* all appear to be strongly immunogenic (Kilian *et al.*, 1983a, Stafford and Plaut, 1982, Blake and Eastby, 1991) but there has been some difficulty in raising neutralising antisera to the enzymes from *S. sanguis, S. oralis, Bacteroides* or *Capnocytophaga* species (Kilian and Reinholdt, 1986, Frandsen *et al.*, 1987). In these cases, a highly purified and concentrated preparation has been required for the inoculum (Frandsen *et al.*, 1987) or neutralising antiserum has been obtained from patients with an active bacterial infection (Reinholdt *et al.*, 1990).

1.9.2 Genetic analysis

Genomic libraries of chromosomal DNA from *N. gonorrhoeae, H. influenzae* and *S. sanguis* have been used to express the genes encoding the IgA1 protease enzymes (*iga* genes) (respectively; Koomey *et al.*, 1982, Bricker *et al.*, 1983, Gilbert *et al.*, 1988). Positively-expressing clones were identified by the digestion of radiolabelled IgA1 bound to either a nitrocellulose (Halter *et al.*, 1984) or Sephadex-containing agarose (Gilbert and Plaut, 1983) overlay. The region of the gene encoding the active enzyme was determined by deletion analysis (Halter *et al.*, 1984) or transposon mutagenesis followed by gene expression in mini or maxi cells (Bricker *et al.*, 1983, Grundy *et al.*, 1987), Gilbert *et al.*, 1988). The complete sequence of the *iga* genes from *N. gonorrhoeae* (type 2, Pohlner *et al.*, 1987), *H. influenzae* (type 1; Poulsen *et al.*, 1989, 1992) and *S. sanguis* (Gilbert *et al.*, 1991) have been published.

The relationship between different *iga* genes has been established by physical mapping, hybridisation studies or direct sequence comparison.

1.9.3 Intra-species variability

a) *H. influenzae*

H. influenzae demonstrates considerable polymorphism with respect to IgA1 protease specificity type, antibody inhibition type and *iga* gene structure. Using a battery of antisera raised against nine prototype *H. influenzae* IgA1

proteases, a minimum of fifteen inhibition types have been identified which show an association with capsular serotype and substrate specificity (Kilian *et al.*, 1983b), (see Section 1.8.3). All serotype b strains belong to inhibition type 1, which on further examination using additional neutralising anti-sera and inhibition titrations, have been subdivided into four inhibition groups (I-IV) (Poulsen *et al.*, 1992). Restriction enzyme analysis of *H. influenzae iga* genes has revealed characteristic restriction enzyme patterns (RFL pattern) which correlate well with each of the eighteen inhibition types (Poulsen *et al.*, 1988).

To examine in more detail the molecular basis for the serological variation within *H. influenzae* IgA1 proteases, *iga* genes encoding four serologically distinct enzymes have been cloned and sequenced (Poulsen *et al.*, 1989, 1992). A comparison of the deduced amino acid sequences has revealed long stretches of conservation divided by short regions of high variability. Between strains, these are organised in a mosaic-like structure with different combinations of identity within the variable regions. This may be explained by horizontal genetic exchange occurring between strains of this species via transformation and subsequent homologous recombination events.

b) *N. gonorrhoeae*

A similar mechanism of inter-strain transformation has been proposed for *N. gonorrhoeae,* as nucleotide sequence comparisons of three type 2 and one type 1 *iga* genes have revealed a mosaic-like organisation of variable regions (Halter *et al.*, 1989). Since restriction enzyme analysis has shown a high level of nucleotide conservation between type 1 genes, extensive polymorphism may be confined to type 2 *iga* genes, where eight different RFL groups have been identified (Mulks *et al.*, 1987) (see Section 1.8.3). Genetic differences have been linked to alterations in precursor molecule autocatalytic sites which lead to variations in the size of mature gonococcal IgA1 proteases (Halter *et al.*, 1989).

To examine if such polymorphisms have serological implications, inhibition studies using a single neutralising antisera have been performed by these workers. Although gonococcal strains revealed considerable antigenic diversity, the association of inhibition types with genetic differences has not been

as well defined as for *H. influenzae* (Halter *et al.*, 1989). However, using monoclonal antisera, differential inhibition can been demonstrated between type 1 and type 2 enzymes (Blake and Eastby, 1991)

c) *N. meningitidis*

Neutralising antisera raised against three selected strains of *N. meningitidis* has divided IgA1 proteases from this species into five different inhibition types (Lomholt *et al.*, 1992). These can be grouped according to IgA1 specificity type (a, b and c are type 1 enzymes, d and e type 2), but there is no correlation with the capsular serogroups or protein serotype of the strains. Examination of the *iga* gene region by restriction endonuclease analysis has revealed a high degree of genetic polymorphism which, unlike *H. influenzae*, could not be related to the inhibition or protease cleavage types. Extensive *iga* sequence comparisons have not been performed but it has been suggested that in *N. meningitidis* frequent inter strain recombination occurs, similar to that proposed for *N. gonorrhoeae* and *H. influenzae* (Lomholt *et al.*, 1992).

d) Other species

S. sanguis has been divided into two or three inhibition types, which correlate directly with other biological and serological properties of the isolates (biovar 1, 2 and 3 are found in type 1 and biovar 4 in type 2) but the genetic basis for this has not been examined (Kilian and Reinholdt, 1986, Reinholdt *et al.*, 1990). Two different inhibition groups have been identified within *S. mitis* biovar 1 (Reinholdt *et al.*, 1990, Kilian and Reinholdt, 1986) but all strains of *S. oralis* belong to one inhibition type only. There is little antigenic heterogeneity within *Bacteroides* and *Capnocytophaga* species (Frandsen *et al.*, 1987).

From this preliminary evidence it appears that the antigenic and genetic variability within the neisserial or *H. influenzae* IgA1 proteases is greater than within other species, although such detailed examinations have not been performed for these species. If the variation observed is due to immunologic selection pressure (Kilian and Poulsen, 1992), this suggests that the oral IgA1 protease-producers (*Streptococcus*, *Bacteroides* and *Capnocytophaga* species) are not exposed to significant levels of neutralising antisera *in vivo*. The difficulty in

obtaining neutralising antisera to these IgA1 proteases supports such a theory and may be explained by hidden active-site epitopes in the enzymes.

1.9.4 Intra-genus variability

Within the genus *Haemophilus*, IgA1 proteases of *H. aegyptius* isolates are inhibited by neutralising antisera from *H. influenzae* inhibition groups 1 and 2 (Kilian and Thomsen, 1983). Although it has been documented that these two species are genetically similar (Leidy *et al.*, 1965) homology between the *iga* genes has yet not been examined.

Hybridisation studies have also indicated a high level of sequence homology between *iga* genes within the two neisserial species (Koomey and Falkow, 1984) but, as expected, restriction enzyme analysis has revealed a degree of restriction site polymorphism, particularly at 5' terminal regions of the gene (Rahr *et al.*, 1985). Koomey and Falkow, (1984) suggested that *iga* genes encoding neisserial enzymes of the same specificity type shared a greater degree of homology than those coding enzymes of the different specificity type. This is supported by immunochemical studies where antisera raised against *N. gonorrhoeae* type 2 IgA protease inhibited the meningococcal type 2 but not type 1 enzymes (Stafford and Plaut, 1982). Other workers have raised antisera that cross-react with all type 1 and type 2 neisserial strains in a Western blot (Blake and Eastby, 1991).

For the genus *Streptococcus*, cross-neutralising tests using antisera towards IgA1 proteases from *S. pneumoniae, S. sanguis* and *S. mitior/S. oralis* (Kilian *et al.*, 1983a, Reinholdt *et al.*, 1990) has suggested that they are all mutually exclusive. This is supported by genetic hybridisation data which found no homology between the *iga* gene from *S. pneumoniae* and *S. sanguis*. Since these IgA1 proteases share an identical substrate specificity and enzyme mechanism, this is perhaps a surprising result. The overall level of DNA relatedness between these species, however, is only 20-30%; there is more similarity between the genomes of *S. oralis, S. mitis* and *S. pneumoniae* and an analysis of their *iga* genes may show higher levels of conservation (Reinholdt *et al.*, 1990).

An extensive study of antigenic relationships between *Bacteroides* species demonstrated considerable interrelationships; antisera raised against IgA1 proteases from *B. buccae*, *B. capillus* and *B. pentosaceus* caused complete mutual enzyme inhibition, from *B. veroralis* and *B. oralis* showed partial inhibition while enzymes from *B. oris*, *B. buccalis* and *denticola* were antigenically distinct (Frandsen *et al.*, 1987). For *Capnocytophaga species*, anti-IgA1 protease antisera from *C. ochracea* and *C. sputgena* produced reciprocal enzyme inhibition while the IgA1 protease from *C. gingivalis* was partially inhibited by antisera from *C. sputgena*.

1.9.5 Cross-genera interrelationships

The inhibition of IgA1 protease activity in *B. oralis* by antisera raised against three species of *Capnocytophaga* is the only evidence of shared active site-related antigens between genera (Frandsen *et al.*, 1987). Although inhibition tests using neutralising antisera raised against the IgA1 protease from *N. gonorrhoeae* and *H. influenzae* suggest that the enzymes are mutually exclusive (Stafford and Plaut, 1982, Kilian *et al.*, 1983a), a gonococcal-specific monoclonal antibody has been produced which cross reacts with the IgA1 protease from *H. influenzae* by Western blotting (Pohlner *et al.*, 1991). This relationship is supported by sequence analysis, where *H. influenzae* and *N. gonorrhoeae iga* genes show over 60% homology (Poulsen *et al.*, 1989).

1.9.6 Evolution of IgA1 proteases

The high degree of sequence homology between IgA1 proteases from the two distantly related genera *Haemophilus* and *Neisseria*, suggests that the *iga* genes have evolved from a common source by divergent evolution (Kilian and Reinholdt, 1986). On the other hand, the clear diversity found among IgA1 proteases has led Kilian and Poulsen (1992) to propose more recently that the enzymes from *H. influenzae*, *N. gonorrhoeae* and *S. pneumoniae* have evolved from three independent lines. Whatever their evolutionary path, the fact that IgA1

proteases show great variation in biochemical, antigenic and genetic characteristics while maintaining a common function attests to their biological significance.

1.10 THE BIOLOGICAL SIGNIFICANCE OF IGA1 PROTEASES

Since all IgA1 protease-producing bacteria colonise mucosal epithelia, where IgA is the major immunoglobulin, it has naturally been suggested that the role of the enzyme is to interfere with the specific host defences provided by IgA. The evidence implicating IgA1 proteases as important bacterial virulence factors will be discussed in the following section.

1.10.1 Subclass specificity

The inability of IgA1 proteases to digest IgA2 was for many years a fundamental problem in justifying a role for these enzymes in bacterial pathogenicity, particularly since S-IgA may contain up to 50% IgA2 (Grey *et al.*, 1968, Delacroix *et al.*, 1982). A more recent analysis using specific anti-isotypic antibodies and refined immuno-histochemical techniques, however, has revealed that the proportion of IgA2-secreting immunocytes can vary considerably between different mucosal tissues (Kett *et al.*, 1986). Thus, in certain sites favoured by IgA1 protease-producing organisms, such as the upper respiratory tract or salivary glands, antibodies of the IgA1 isotype predominate. Even more significantly, perhaps, the major anti-bacterial in antibody in gingival fluid and secretion such as saliva has been found to be IgA1 (Brown *et al.*, 1991, Ahl and Reinholdt, 1991) which suggests that the physiological function as well as the relative distribution of IgA may vary between the different subclasses. In this regard, Mestecky *et al.*, (1990) have reported that salivary antibodies specific for proteins or glycoproteins are primarily of the IgA1 subclass but antibodies specific for lipopolysaccharide belong mainly to the IgA2 subclass. This may be of significance when considering the role of IgA1 proteases in enhancing bacterial adhesion (Section 11.1.5) since IgA1 antibodies are likely to predominate in the humoral response towards glycoprotein adhesin molecules.

1.10.2 Protease positive organisms

It is striking that all IgA1 protease-producing bacteria share predilection for human secretory sites and all, under some circumstances, can invade the mucosal barrier and cause systemic disease. In most cases, these bacteria do not appear to be opportunistic pathogens but produce infections in otherwise healthy hosts.

The clearest association between IgA1 protease activity and disease is in the case of bacterial meningitis, where the three major protagonists, *N. gonorrhoeae*, *H. influenzae* and *S. pneumoniae*, all elaborate the enzyme. Within the genera *Neisseria* and *Haemophilus*, IgA1 protease production is confined to pathogenic species and those species that exist as commensals within the human nasopharynx, such as *N. flava*, *N. sicca* and *H. parainfluenzae* do not elaborate enzyme activity (Mulks and Plaut, 1978). This pattern is not maintained within the genus *Streptococcus*, however, where the highly pathogenic Lancefield group A streptococci or group B β haemolytic species such as *S. pyogenes* or *S. agalactiae* are negative for IgA1 protease activity. Although *S. sanguis* and *S. oralis* are IgA1 protease-producers, the third major etiologic agent of dental caries, *S. mutans*, is devoid of enzyme activity.

Within each species, a direct correlation between IgA1 protease activity and pathogenicity has also not been established. In *N. gonorrhoeae*, both piliated infectious forms (colonial types 1 and 2) and non-piliated non-infectious forms (types 3 and 4) elaborate an IgA1 protease enzyme. Likewise, over 90% of *S. pneumoniae* and *H. influenzae* isolates are positive for the enzyme but only a proportion of these are associated with disease and many appear to be harmless members of the upper respiratory tract (Mulks, 1985). It is possible that the level of IgA1 protease activity varies between pathogenic and non-pathogenic isolates in these species, but semi-quantitative *in vitro* studies have suggested this is not the case (Kornfeld and Plaut, 1981). The relative levels of IgA1 protease activity *in vivo*, however, remain to be determined.

1.10.3 IgA1 protease activity *in vivo*

IgA1 protease activity *in vivo* was first suggested by the detection of intact Fcα fragments in the intestinal fluids of patients with liver dysfunction (Mehta, 1973). Since then, enzyme activity or specific IgA1 protease digestion products have been identified in vaginal secretions of women with gonococcal infection, (Blake *et al.*, 1979), in cerebrospinal fluid from patients with *H. influenzae* meningitis (Insel *et al.*, 1982), in nasopharyngeal secretions from children with respiratory disease (Sørensen and Kilian, 1984) and in incipient dental plaque from healthy individuals (Ahl and Reinholdt, 1991).

Indirect evidence of *in vivo* enzyme activity is provided by the presence of inhibitory anti-IgA1 protease antibodies in human secretory IgA and in serum (Kobayashi *et al.*, 1987b, Gilbert *et al.*, 1983). Enzyme-neutralising activity has been localised to the Fab fragments of S-IgA (Kobayashi *et al.*, 1987b) and to the IgG fraction of serum (Gilbert *et al.*, 1983), confirming that the inhibition is not due to competing IgA substrate or to plasma protease inhibitors such as α antitrypsin and α macroglobulin. Although antibody titres found in patients recovering from neisserial infections are generally higher than from uninfected individuals (Gilbert *et al.*, 1983, Mulks *et al.*, 1980c), there is no indication that anti-IgA1 protease activity protects against further infection (Mulks, 1985, Plaut, 1983). Furthermore, a recent study has reported that commensal carriage of meningococci is as efficient in antibody stimulation as active menigococcal disease (Brooks *et al.*, 1992). These workers suggest that IgA1 protease-production may not be a function of disease, but a more detailed examination of the IgG subclasses stimulated during disease and carriage states is required before the significance of this finding is known.

To directly assess the role of IgA1 proteases in disease necessitates the identification of a suitable host for *in vivo* pathogenicity studies. Since the enzymes are highly specific for human IgA1, traditional murine models are not appropriate for such investigations. A single report identifying an IgA1 protease in *H. pleuropneumoniae* specific for porcine IgA has not been substantiated by later

enzymatic or genetic studies (Mulks *et al.*, 1982). In addition, IgA1 proteases found in isolates of *S. sanguis* colonising *M. fascularis* and animal strains of *S. pneumoniae* are only specific for human IgA1 (Kilian and Holmgren, 1981, Proctor and Manning, 1990). It has therefore been necessary to confine such investigations to *in vitro* model systems.

1.10.4 IgA1 protease activity *in vitro*

a) Tissue culture models

Using human fallopian tube organ cultures, Cooper *et al.*, (1984) have reported that the rate of attachment, damage and invasion of *N. gonorrhoeae* IgA1 protease-negative mutants was indistinguishable from that produced by their wild-type isogenic counterparts. These workers obtained similar results using isogenic strains of *H. influenzae* in human nasopharyngeal organ cultures (Farley *et al.*, 1986) and concluded that bacterial IgA1 proteases were not responsible for the pathogenic effects observed *in vitro*. It is not possible to draw conclusions from such experiments concerning the role of the enzymes *in vivo*, since specific anti-bacterial S-IgA was not present in either of the model systems.

In assessing the function of IgA1 proteases in bacterial pathogenicity, *in vitro* studies have concentrated on the effect of enzyme activity on S-IgA functions.

b) IgA functional models

On of the best documented functions of S-IgA is the prevention of microbial attachment to cell surfaces (Section 1.6.2). There are numerous reports which suggest that IgA1 proteases can abrogate this function. The enzymes can act on free or antigen-bound IgA1 (Plaut *et al.*, 1977) and Mulks *et al.*, (1980c) have demonstrated that IgA1 protease-digestion of specific S-IgA counteracts its ability of to reduce the adherence of *N. gonorrhoeae* to human epithelial cells. Likewise, the capacity of adhesin-specific S-IgA to impede the adherence of oral streptococci to saliva-coated hydroxyapatite is neutralised by the action of IgA1 proteases (Reinholdt and Kilian, 1987, Hajishengallis *et al.*, 1992). It was originally proposed that this effect was due to a loss in binding capacity of IgA1 following enzyme treatment (Plaut *et al.*, 1977) but Mansa and Kilian (1986) demonstrated that the

resulting Fabα digestion fragments retained antigen-binding properties. Their ability to prevent bacterial adhesion, however, was severely compromised, which may have been due to a reduction in microbial agglutination resulting from the loss in antibody valency. Alternatively, removal of the relatively hydrophilic Fc/SC portion of S-IgA may have allowed adhesion via hydrophobic interactions, as described in Section 1.6.2. This is supported by the observation that in some circumstances association with Fabα fragments actually increases the adhesion of streptococci to hydroxyapatite beads (Reinholdt and Kilian, 1987 and Hajishengallis *et al.*, 1992). Thus, rather than prevent adhesion, Fabα fragments appear to mediate adhesion, presumably via their hydrophobic properties.

An additional consequence of retained Fabα fragment binding is that antigenic sites may be protected from recognition by intact functional antibody molecules or immunocompetent cells. A similar process of 'fabulation' has been described for a number of protozoa including *Tetrahymena pyriformis* (Eisen and Tallan, 1977). By elaborating an enzyme that selectively cleaves IgA1, IgA1 protease-producing bacteria may have evolved a more efficient means of escaping the immune system than by producing an enzyme that causes more extensive degradation of IgA.

The specific digestion of IgA1 may also interfere with any Fc-dependent immune-complex elimination. Although such Fc effector functions appear to predominate in the systemic compartment (Section 1.6.3), they may nevertheless play an important role in secretory immunity. Moreover, *Bacteroides* and *Capnocytophaga* species are exposed to gingival fluid, containing predominantly serum IgA and in an invasive infection, the action of bacterial IgA1 proteases on serum IgA may be significant.

Although knowledge of this area is sparse, it has been demonstrated that the inhibition of chemotaxis by serum IgA is reduced by the action of gonococcal IgA1 proteases (Van Epps *et al.*, 1980) and that released Fc fragments may also act as polyclonal B cell mitogens (Berman *et al.*, 1979). The increase in PMN and monocyte infiltration and polyclonal lymphocyte activation may serve to increase the inflammatory response in mucosal tissues and precipitate tissue damage. Such

effects may be significant in the case of destructive periodontitis, which is characterised by the inflammation of gingival mucosa leading to extensive periodontal breakdown (Kilian *et al.*, 1983a).

1.10.5 A hypothetical model for the role of IgA1 proteases in disease

On the basis of *in vivo* and *in vitro* observations, Kilian and Reinholdt, (1987) have described a hypothetical model for the development of invasive infection by bacteria elaborating an IgA1 protease (Fig. 1.8).

By this model, it is proposed that when a host is colonised by IgA1 protease-producing bacteria, antigen-specific antibodies (including anti-IgA1 protease antibodies) are elicited. Since the enzyme is neutralised and the bacteria are not coated by protective Fabα fragments, commensal carriage ensues. The specific immune response confers protection against subsequent attacks by the same bacteria. If, however, specific antibodies are already present in the host, from say a previous encounter with a similar but IgA1 protease-negative species, colonisation with an IgA1 protease-producer will result in digestion of these cross-reacting antibodies. The pathogen becomes coated with protective Fabα-fragments before an effective immune response to the IgA1 protease is elicited and the bacteria may become invasive. In addition, IgA1 digestion may extend to specific antibody directed against closely colonising but IgA1 protease-negative organisms, thereby promoting the establishment of other potential pathogens. In this regard, *S. gordonii* (IgA1 protease-negative) coated with IgA1 Fabα fragments has been identified following incubation of the bacteria with whole saliva (Ahl and Reinholdt, 1991).

Although there is no confirmation that these reactions occur *in vivo* the model provides an answer to some previously unexplained clinical observations, particularly with respect to infant meningitis (Kilian and Reinholdt, 1987). It also serves to reiterate that a disease process is a complex interrelationship between the host immune response and bacterial virulence properties. Thus conflicting evidence as to the role of IgA1 proteases in bacterial pathogenicity does not necessarily discount a function for the enzyme in this process but may

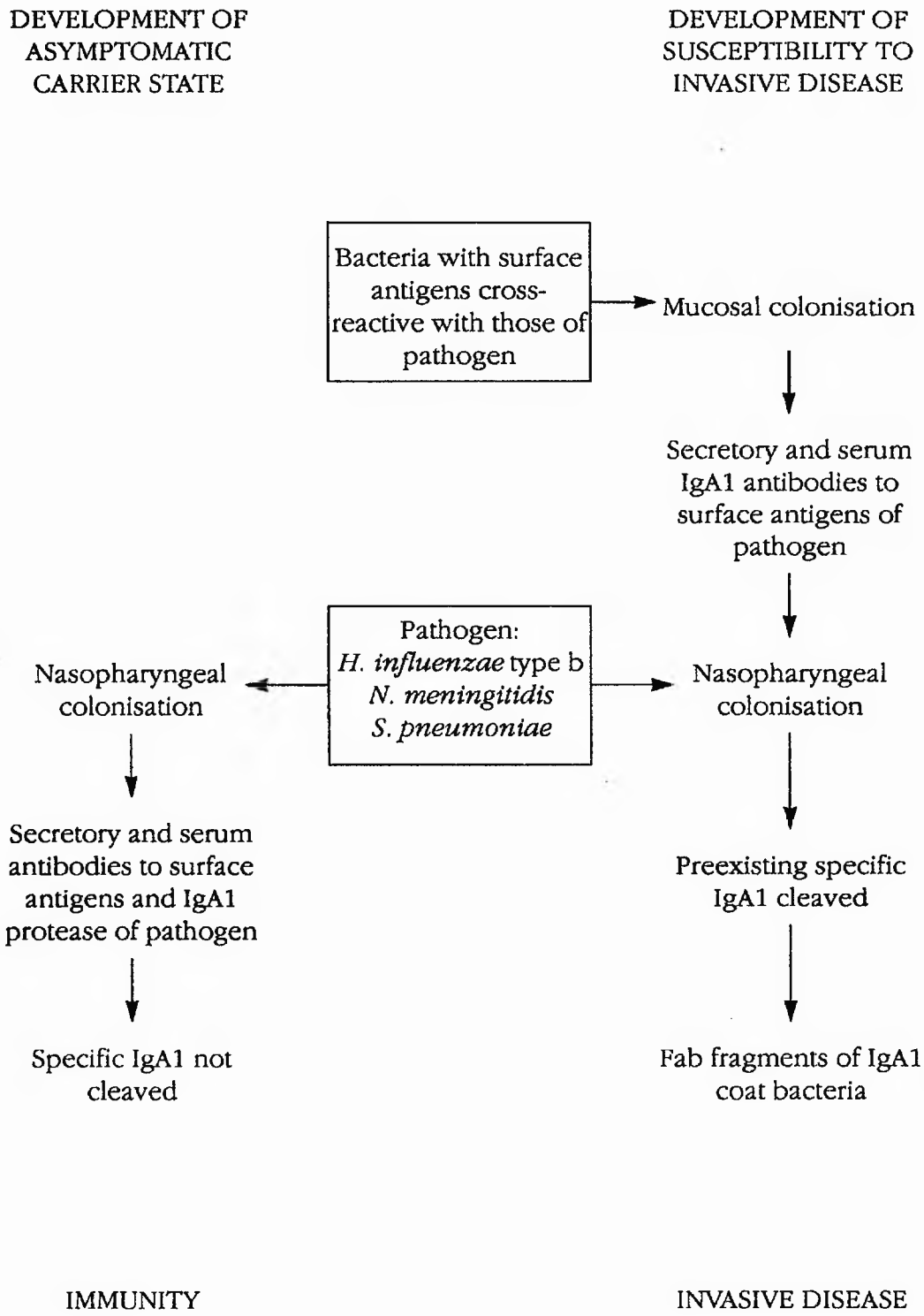


Fig.1.8 Hypothetical model of invasive infection due to IgA1 protease-producing bacteria. (Taken from Kilian and Reinholdt, 1987).

result from a lack of knowledge regarding the different factors operating in disease onset.

1.10.6 Alternative functions for IgA1 proteases

The identification of susceptible substrates other than IgA1 it has suggested that IgA1 proteases may perform functions additional to IgA1 digestion. Thus, the enzymes are likely to be responsible for autocatalytic processing and extracellular release (Pohlner *et al.*, 1987) as well as for the processing of a number of other bacterial components (Shoberg and Mulks, 1991). Since synthetic peptides based on the autocatalytic processing sites are more suitable enzyme substrates than those based on the IgA1 hinge region, the enzymes may primarily be required for the former function. The identification of IgA1-protease bacteria in animals whose S-IgA1 is resistant to IgA1 protease activity supports this suggestion. If it is indeed the case that the digestion of IgA1 is merely a fortuitous by-product of enzymes with alternative functions, it nevertheless remains likely that for colonisation of mucosal surfaces, IgA1 protease-expressing bacteria have a selective advantage over their IgA1 protease deficient counterparts.

SECTION C

THE IGA1 PROTEASE OF *U. UREALYTICUM*

1.11 GENERAL PROPERTIES

1.11.1 Isolation

Over fifty strains representing the genera *Mycoplasma*, *Ureaplasma*, *Acholeplasma* and *Spiroplasma* have been examined for IgA1 protease activity but, within the class *Mollicute*, enzyme expression appears to be limited exclusively to the genus *Ureaplasma*. IgA1 proteases have been identified in all 14 human serotypes of *U. urealyticum* and in over 50 clinical isolates obtained from a variety of mucosal sites (Kilian *et al.*, 1984, Robertson *et al.*, 1984, Stemke *et al.*, 1984, Kapatais-Zoumbos *et al.*, 1985). Ureaplasmas isolated from cases of NGU and from asymptomatic controls are positive for IgA1 protease activity (Robertson *et al.*, 1984).

For the bovine ureaplasma, *U. diversum*, two strains have been identified as IgA1 protease-negative, but the type strain from this species (A417) appears to extensively degrade both human and bovine IgA (Kilian and Freundt, 1984). This effect has not been reproduced by other workers and Kapatais-Zoumbos *et al.*, (1985) found that human, murine and porcine IgA was resistant to cleavage by strain A417. The digestion of bovine IgA was reported as inconclusive. IgA1 proteases expressed by ureaplasma isolates from non-human primates, feline, canine, ovine or avian hosts do not digest human IgA (Stemke *et al.*, 1984, Kapatais-Zoumbos *et al.*, 1985). Two strains of canine ureaplasmas (D1M-C and D11N-A), however, cleave canine IgA. The species specificity of *U. urealyticum* IgA1 proteases is confirmed by the inability of the human ureaplasma IgA1 protease to digest porcine, murine or canine IgA.

1.11.2 Assaying for the enzyme

To detect the presence of the IgA1 protease from *U. urealyticum*, crude cellular suspensions are incubated with IgA (18 h, 37 °C), and the supernatants

examined for digestion products by immunoelectrophoresis or SDS-PAGE. By immunoelectrophoresis (with anti-S-IgA antisera) of the digestion mixture, two separate precipitation lines are produced, differing in electrophoretic mobility from intact IgA1. Using specific antisera to different portions of the IgA molecule, these species have been identified as Fabα and Fcα fragments (Kilian *et al.*, 1984, Robertson *et al.*, 1984, Kapatais-Zoumbos *et al.*, 1985). SDS-PAGE analysis (reducing conditions) of the digestion products has estimated the relative molecular mass of the α chain fragments to be 29 kDa (Fabα) and 33.5 kDa (Fcα) (Kilian *et al.*, 1984) and as 28-29 kDa (Fabα) and 33 kDa (Fcα) (Kapatais-Zoumbos *et al.*, 1985). Using SDS-PAGE under non-reducing conditions, partial digestion products (110-115 kDa) and a single band representing co-migrating Fabα and Fcα fragments (50 kDa) have been identified (Robertson *et al.*, 1984). In this report, [^{125}I] IgA1 replaced unlabelled IgA1 and digestion products were identified by autoradiography rather than by staining with Coomassie blue.

1.11.3 Characterisation

Human serum IgA1 is a substrate for the ureaplasma IgA1 protease but human IgG, IgM, IgA2 and azocasein are resistant to digestion (Robertson *et al.*, 1984, Kilian *et al.*, 1984). In general, ureaplasma cell harvests have been used a source of IgA1 protease, but Kilian *et al.*, (1984) have detected activity in concentrated cell-free supernatants by immunoelectrophoresis. The IgA1 protease is not be inhibited by up to 25 mM EDTA (Robertson *et al.*, 1984).

These preliminary studies have established that the IgA1 protease of *U. urealyticum* specifically digests IgA1 into intact Fabα and Fcα fragments, is possibly released into the growth medium and does not appear to be a metallo enzyme. Identification of the enzyme in ureaplasmas isolated from asymptomatic controls as well as from NGU patients suggests that it does not correlate directly with virulence, but does not discount the IgA1 protease from being one of a number of virulence determinants for this organism. In this respect, serum IgA specific for *U. urealyticum* has been identified in patients with NGU (Brown *et al.*,

1983) but changes in S-IgA levels in response to ureaplasma infections have not been examined to date.

1.12 AIMS OF THE THESIS

The main aim of this thesis is to extend these preliminary studies and to further characterise the IgA1 protease from *U. urealyticum*. This would not only add to the limited knowledge available on the enzymatic functions operating in this simple life-form, but may, in the long-term, serve as a basis for understanding the role of the IgA1 protease in ureaplasma pathogenicity. Information on the ureaplasmal enzyme may also contribute to the field of bacterial IgA1 proteases in general. Thus, further comparisons between enzymes from different genera may provide a better understanding of their diversity, functions, and ultimate significance.

In order to achieve these goals, a primary aim should be to improve the assays previously developed for identifying IgA1 protease activity in *U. urealyticum*. Once this is achieved, it should be possible to characterise the enzyme in terms of its site of production, substrate specificity and enzyme classification. To further characterise the IgA1 protease, purification would be desirable, particularly if this enables the production of a specific anti-IgA1 protease antibody. Not only would this aid enzyme detection but it would allow comparative immunochemical studies between the ureaplasmal and the bacterial IgA1 proteases to be performed. Isolation and sequencing of the *iga* gene from *U. urealyticum* would serve to support and expand all of these findings, as has been possible for the IgA1 protease from *N. gonorrhoeae*.

In Chapter 1, progress made in developing an enzyme assay and characterising the IgA1 protease from *U. urealyticum* will be presented. Attempts to purify the enzyme and to identify the *iga* gene in *U. urealyticum* are covered in Chapters 2 and 3 respectively.

Chapter 2:
Characterisation of the IgA1 protease from *U. urealyticum*

SECTION A
MATERIALS AND METHODS

All chemicals and solvents described were AnalaR grade. Apart from gel electrophoresis buffers, which were freshly prepared, all other buffers were sterilised by autoclaving or, if stated, by filtration using 0.2 µm filters. H_2O refers to sterile double-deionised water obtained from a Milli Q water purification system (ELGA).

2.1 INITIAL DEMONSTRATION OF IGA1 PROTEASE ACTIVITY

2.1.1 Source and cultivation of bacteria

Ureaplasma urealyticum (*U. urealyticum*) strains of serotypes 1 (T7), 4 (11860), 6 (12253), and 8 (T 960) were gifts from D. Taylor-Robinson (Clinical Research Centre, Harrow, UK); serotype 7 strain (27819) was obtained from the American Type Culture Collection, and strains of serotypes 2 (T 23), 3 (DKF 3), 5 (NIH 5), 9 (9-Vancouver), 10 (10-Western), 11 (11-JsL 2), 12 (12-JsL 5), 13 (13-JsL 6), and 14 (14-JsL 11) were gifts from J. Robertson (University of Alberta, Edmonton, Canada). All strains were cultured and harvested as described previously (Thirkell *et al.*, 1989a). Briefly, ureaplasmas were grown in a sterile medium containing 70% (v/v) PPLO broth (Difco), 20% horse serum (NBL), 2.5% (w/v) fresh yeast extract, 0.1% (w/v) urea, 0.005% (w/v) phenol red and penicillin G (1000 I U.ml^{-1}) (Glaxo) at a starting pH of 6.0. Cultures were incubated without shaking at 37 °C until a pH of 7.6 was reached (at which stage cell numbers have been shown to be maximal, Myles, 1990), harvested by centrifugation (25,000 x *g*, 20 min, Beckman J2-21 centrifuge) and the pellets washed three times in Dulbecco's phosphate buffered saline 'A' (PBS), (Dulbecco and Vogt, 1954). The washed pellets were resuspended in PBS (5 ml per 10 l culture) to give a final protein concentration of approximately 5 mg.ml^{-1}, estimated by the method of Lowry *et al.*, (1951). These preparations were used fresh or after aliquoting and

storage at -70 °C. Unless otherwise stated, these cellular suspensions from *U. urealyticum* serotype 8 were as used a crude source of IgA1 protease.

N. gonorrhoeae (27631/91, a type 2 IgA1 protease-producing strain from the IB serogroup) was kindly provided by H. Young, (Department of Bacteriology, University of Edinburgh) and grown overnight (37 °C, 5% CO_2) in sterile nutrient broth medium (Oxoid No. 2) containing 10% (w/v) glucose, 10% (w/v) horse serum, 10 mg.ml^{-1} V factor (NAD or NADP) and 4 mg.ml^{-1} haemin. Cultures were harvested by centrifugation (25,000 x *g*, 20 min) and the supernatant used as a source of extracellular IgA1 protease activity. The remaining pellet was washed 3 times in PBS, resuspended in 5 ml PBS (from a 1 l culture) and used as a source of cell-associated IgA1 protease.

2.1.2 Solubilisation of IgA1 protease from *U. urealyticum*

Fresh ureaplasma harvests (100 µl) were pelleted from suspension by centrifugation (50,000 x *g*, 10 min, Beckman TL-100 centrifuge) and resuspended to their original volume in PBS:0.5% (v/v) Nonidet P-40 (NP40) (Sigma). After solubilisation (30 min, 4 °C), samples were centrifuged as before and the supernatant used as a crude source of solubilised IgA1 protease.

2.1.3 Source of IgA

Secretory IgA (S-IgA) from human colostrum was obtained from Sigma and purified human IgA1 from Calbiochem. Purified human serum IgA2 was kindly provided by M. Kerr, (Department of Pathology, Ninewells Hospital). All were reconstituted in PBS at 1 mg.ml^{-1} and stored as aliquots at -20 °C.

2.1.4 ^{125}I-labelling of IgA1

Purified IgA1 was radio-labelled by the addition of 0.5 mCi [^{125}I] ($Na^{125}I$, 100 mCi.ml^{-1}; Amersham International) to 20 µl of IgA1 (1 mg.ml^{-1} in PBS). The reaction was initiated at 20 °C with 20 µl chloramine T in PBS (0.2 mg.ml^{-1}) and terminated after 2 min with 20 µl sodium metabisulphite (0.3 mg.ml^{-1} in PBS) and an additional 40 µl PBS. Unbound label was removed by spin-column chromatography using 'medium' Sephadex G50 (Pharmacia) swollen in PBS.

[^{125}I] IgA1 was diluted in PBS to give 0.02 mg protein.ml^{-1} (containing approximately 10^6 cpm.ml^{-1}). The preparation was stored as aliquots at -20 °C.

2.1.5 Digestion reactions

5 µl of ureaplasma cell suspension (~25 µg) was incubated with either 15 µl S-IgA (1 mg.ml^{-1} in PBS) or 3 µl [^{125}I] IgA1 (in an additional 12 µl PBS) for 16 h (37 °C). Samples at zero incubation time or of IgA alone in 20 µl PBS were always included as controls. Digestion was terminated by centrifugation (12,000 x *g*, 5 min, Beckman microcentrifuge) and supernatants were assayed for the products of IgA1 protease digestion by SDS-PAGE followed by PAGE blue 83 staining or immunoblotting (for S-IgA) or by autoradiography (for [^{125}I] IgA1).

2.1.6 Preparation of SDS-Polyacrylamide gels

The method of SDS-polyacrylamide gel preparation was adapted from that described by Russell and Blair, (1977). Bio-Rad Mini Protean System gel rigs were assembled and used according to the manufacturer's instructions. 12.5% (w/v) separating gels were prepared by mixing 2 ml acrylamide: bis-acrylamide (50: 0.235 [w/v] stock solution), 3 ml 1 M Tris-HCl, pH 8.6, 80 µl 10% SDS, 2.3 ml H_2O and degassed for 15 min. 5 µl of TEMED and 40 µl of freshly prepared 10% ammonium persulphate were added to the separating gel mixture and poured between two sets of gel casting plates, leaving a 2 cm gap at the top of the gel. The running gel was overlaid with H_2O and allowed to set for at least one hour. The overlay was removed prior to addition of the top stacking gel which contained 0.4 ml acrylamide: bis-acrylamide (50: 1.3 (w/v) stock solution), 0.5 ml Tris-HCl, pH 6.8, 40 µl 10% (w/v) SDS, 0.5 ml H_2O, 40 µl 10% (w/v) ammonium persulphate and 8 µl TEMED. An appropriate comb was inserted before setting of the stacking gel.

2.1.7 Sample preparation and electrophoresis.

Samples were denatured in 1/3 their volume of '4 x denature mix', (8% (w/v) SDS, 20% (v/v) β-mercaptoethanol (Sigma), 10% (v/v) glycerol and 0.1% (w/v) bromophenol blue) by boiling for 2 min. After cooling and centrifugation

(5 min, 12,000 x *g*), supernatants were loaded into pre-formed wells and separated by applying a voltage of 150 V for 1 h using 0.19 M glycine, 0.025 M Tris, 0.1% (w/v) SDS (should be at pH 8.6) as the electrophoresis buffer. Molecular weight markers (BRL or Sigma) were loaded when required.

2.1.8 Staining with PAGE blue 83

Gels were stained for 30 min in 0.3% (w/v) PAGE blue 83 (Sigma), 46% (v/v) methanol and 7.5% (v/v) acetic acid and destained in 25% (v/v) methanol, 7.5% (v/v) acetic acid. When required, gels were dried on a vacuum gel drier (ATTA) for 1-2 h (80 °C).

2.1.9 Immunoblotting .

Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets (Gelman) was carried out on an Ancos model A semi- dry electroblotter according to the manufacturer's instructions (Ancos). Eighteen pieces of 3 MM Whatman filter paper and one sheet of nitrocellulose were cut to the same size as the gel. A 'sandwich', consisting of 6 sheets of filter paper soaked in anode buffer 1 (0.3 M Tris-HCl, pH 10.4, 20% [v/v] methanol) covered by 3 further pieces of filter paper soaked in anode buffer 2 (0.025 M Tris-HCl, pH 10.4, 20% [v/v] methanol) and overlaid by a single sheet of nitrocellulose paper soaked in H_2O, was positioned on the lower graphite plate. The gel was rinsed with H_2O and placed on top of the nitrocellulose, taking care not to trap air bubbles. The sandwich was completed with 9 pieces of filter paper soaked in cathode buffer (0.025 M Tris-HCl, 40 mM 6-aminohexanoic acid, 20% [v/v] methanol, pH 9.4) and compressed gently using a glass rod to remove air bubbles. After attaching the upper electrode, the gel was blotted for 1 h at 8 $mA.cm^{-2}$.

Pre-stained molecular weight markers (Sigma) confirmed successful transfer and nitrocellulose sheets were blocked with 10%(w/v) Marvel (Cadbury) in PBS for 2 h (20 °C). After rinsing briefly with PBS, the sheet was incubated with a 1/1000 dilution of sheep anti-human IgA (Scottish Antibody Production Unit, SAPU) in 5% (w/v) Marvel: PBS for 2 h or overnight (20 °C). Unbound antibody

was removed by washing once with PBS (10 min), twice with PBS:0.5% (v/v) NP40 (10 min each wash) and once more with PBS alone (10 min). The nitrocellulose was incubated with a 1/500 dilution of peroxidase-linked donkey anti-sheep antibody (SAPU) in 5% (w/v) Marvel: PBS for 2 h (20 °C). The nitrocellulose was washed as above, incorporating an additional final wash in PBS alone, and stained for antibody binding. The staining solution contained 60 mg 4-chloro-1-naphthol (Sigma) dissolved in 20 ml methanol and made up to 100 ml with PBS. 60 µl H_2O_2 (BDH) was added immediately before use. Staining was complete in 15-20 min and excess H_2O_2 was removed by rinsing the nitrocellulose in PBS.

2.1.10 Autoradiography

After SDS-PAGE, gels were fixed (30 min in 10% [w/v] trichloroacetic acid, 40% [v/v] methanol), dried, and exposed to Fuji X-ray film with a Philips fast tungstate intensifying screen. Autoradiograms were developed using a Kodak automatic developer and, when quantitative information was required, scanned using a Shimadzu Dual wavelength densitometer.

The extent of IgA1 digestion was estimated using the following calculation (Simpson *et al.*, 1988):

$$\frac{\text{peak area of Fc}\alpha/\text{Fab}\alpha \text{ fragments}}{\text{peak area of intact } \alpha \text{ chain + peak area of Fc}\alpha/\text{Fab}\alpha \text{ fragments}}$$

2.2 ALTERNATIVE ASSAYS FOR DETECTING IGA1 PROTEASE ACTIVITY.

2.2.1 Quantitative rocket immunoelectrophoresis

Protease-treated S-IgA and suitable control samples, as prepared in Section 2.1.5, were subjected to quantitative rocket immunoelectrophoresis (RIEP) according to the method of Lassiter *et al.*, (1989). 1% (w/v) agarose (Sigma, type 1) in Barbital buffer (25 mM sodium barbital, 5 mM barbital, 4 mM sodium azide, pH 8.6) were dissolved at 100 °C and cooled to 56 °C in a water bath. Sheep anti-human IgA (SAPU) was incorporated at 5 $\mu l.ml^{-1}$ and the agarose was poured onto standard RIEP plates. 10 µl of protease-treated samples, control samples and S-IgA

standards (0.1-1 $mg.ml^{-1}$) were electrophoresed in Barbital buffer for 18 h at 80 V. Agarose gels were washed in 0.15 M NaCl (2 x 10 min), H_2O (2 x 10 min), dried between Whatman no. 1 filter paper for 15 min and stained with PAGE blue 83 (0.5% [w/v] in 45% [v/v] ethanol, 10% [v/v] acetic acid). Destaining was in 30% (v/v) ethanol, 15% (v/v) acetic acid and the gels were allowed to air-dry.

2.2.2 High performance liquid chromatography (HPLC)

Samples for HPLC analysis were prepared by incubation of S-IgA with ureaplasma harvests or PBS alone, as described in Section 2.1.5, and by resuspending the resultant supernatant (1:5 by volume) in 0.5 M NaCl. 2 ml samples (representing 250 µg S-IgA in PBS:0.4 M NaCl) were loaded onto a Dupont gel filtration Zorbax column (GF 250) linked to an LKB HPLC system. Proteins were eluted in a mobile phase of PBS:0.4 M NaCl at a flow rate of 1 $ml.min^{-1}$ and detected spectrophotometrically at 226 nm. The localisation of intact and digested IgA α chain in 0.5 ml fractions was confirmed by SDS-PAGE (of 50 µl samples) followed by immunoblotting, as outlined previously (Section 2.1.9).

2.2.3 Continuous spectrophotometry using IgA: 10% PEG

30 µg of S-IgA (from a stock of 4 $mg.ml^{-1}$ in 0.1 M Tris-HCl, pH 7.2) were combined in a cuvette with PEG 6000 (10% [w/v] in 0.1 M Tris-HCl, pH 7.2) to give a final volume of 600 µl. A turbid suspension was produced immediately (Bleeg *et al.*, 1985). Following addition of 20 µl ureaplasma IgA1 protease preparations (either as crude cellular preparations or solubilised extracts from Tris buffer:0.5% NP40 treatment), the change in turbidity was measured at 400 nm for 1 h (37 °C), using the time course programme on a Perkin-Elmer, Lambda 5 UV/visible spectrophotometer. Controls containing Tris buffer alone or Tris buffer:0.5% NP40 to replace of ureaplasma cells or solubilised ureaplasma preparations, respectively, were included.

2.3 BIOCHEMICAL CHARACTERISATION

2.3.1 Effect of pH on IgA1 protease activity

Ureaplasma cell suspensions (5 µl) were diluted (1/5 by volume) in a citrate-phosphate-borate buffer solution ranging from pH 2-pH 10. (2 ml volumes of stock buffer solution [0.03 M citric acid, 0.03 M phosphoric acid, 0.06 M boric acid, 0.34 M NaOH] were brought to the required pH with 0.1 M HCl and adjusted to a final volume of 10 ml). After addition of S-IgA (15 µl) and exact pH determination, samples were incubated for 16 h (37 °C) and assessed for digestion products by SDS-PAGE and immunoblotting, as outlined in Section 2.1.9.

2.3.2 Effect of inhibitors on IgA1 protease activity.

The serine protease inhibitors: phenylmethylsulphonyl fluoride (PMSF), 3,4-dichloroisocoumarin (3,4-DCI), aprotinin, soy bean trypsin inhibitor (SBTI), chymostatin, di-isopropylfluorophosphate (DFP), tosyl phenylalanyl chloromethyl ketone (TPCK), tosyl lysyl chloromethyl ketone (TLCK), elastinal; the metallo protease inhibitor: 1,10-phenanthroline; the aspartyl inhibitors: bromophenacyl bromide, pepstatin; and the cysteine protease inhibitors: cystatin, leupeptin, L-trans-epoxysuccinyl-leucylamide-(4-guanidino)-butane (E64) were all obtained from Sigma. Ethylenediaminotetraacetic acid (EDTA, metallo protease inhibitor) and iodoacetic acid (IAA, cysteine protease inhibitor) were from BDH. All inhibitors were reconstituted and used at concentrations recommended by Bond (1989), as summarised in Table 2.1 (page 118).

Ureaplasma cell suspensions (5 µl made up to 25 µl with PBS) were pre-incubated for 30 min (37 °C) with 5 µl of inhibitor, prepared to give the effective concentrations described in Table 2.1. After addition of S-IgA (15 µl) and further incubation (16 h, 37 °C), the extent of digestion in the presence of inhibitor was estimated by immunoblotting, as outlined previously (Section 2.1.9). Control incubations containing the corresponding volumes of appropriate buffer or solvent were included.

2.4 DETERMINATION OF SUBSTRATE SPECIFICITY

2.4.1 IgA digestion

15 µl of IgA1, IgA2 and S-IgA (each at 1 $mg.ml^{-1}$ in PBS) were incubated with 5 µl ureaplasma cell suspension in PBS for 16 h, 37 °C, as described in Section 2.1.5. Digestion was terminated by centrifugation (12,000 x g) and supernatents were assayed for the products of digestion by immunoblotting, as described in Section 2.1.9.

2.4.2 Determination of cleavage site in S-IgA

N-terminal sequencing of Fcα fragments, following their electroblotting and excision from polyvinylidene difluoride membranes (Matsudaira, 1987), was performed in the authors presence by B. Dunbar (SERC Protein Sequencing Unit, Aberdeen University).

90 µl S-IgA was incubated with 30 µl ureaplasma cell suspension for 16 h (37 °C) and digestion products collected in the cell-free supernatant following centrifugation (12,000 x g, 5 min). To enable detection of free sulfhydryl groups (cysteine residues) in the Fcα fragments, the supernatant was dried onto the surface of an Eppendorf tube by rotary evaporation and subsequently pyridylethylated using a mixture of 4-vinylpyridine and tributylphosphine vapours (3 h, 80 °C), as described by Amons, (1987). The reaction product was treated with gel electrophoresis sample preparation buffer containing 1% (w/v) SDS (Laemmli, 1970) and run as three tracks on SDS-PAGE (12.5% [w/v] acrylamide) using a Mighty Small II apparatus (Hoefer Scientific Instruments Ltd.) as described in Applied Biosystems User Bulletin No. 25. In this instance, piperazine diacrylamide was used as a gel cross-linker instead of bis-acrylamide. The gel was electroblotted on to Immobilon-P membrane (Millipore) and stained with 0.1% (w/v) Coomassie blue (Serva) in 50%(v/v) aqueous methanol. Fcα fragments were excised with a scalpel. The first nine N-terminal amino acids of the excised band were sequenced by Edman degradation with an Applied Biosystems Model 477A pulsed-liquid

sequencer equipped with a 120A on-line PTH analyser, using the manufacturer's BLOTT 1 cycle.

2.4.3 Digestion of IgA α chains.

1 $mg.ml^{-1}$ S-IgA in 6 M guanidine:0.6 M Tris-HCl, pH 8.6 was reduced with 0.14 M β-mercaptoethanol (2 min, 100 °C, followed by 3 h, 20 °C) and subsequently alkylated with 0.15 M iodoacetamide (67 µl of a 2.1 M solution in 1 M NaOH) for 30 min in the dark (20 °C). To obtain isolated IgA α chains, 200 µl of the reduced and alkylated S-IgA (representing ~200 µg S-IgA) was separated by gel filtration using a Superose 6 column linked to a Pharmacia FPLC system. The column was eluted with 6 M guanidine at a flow rate of 0.3 $ml.min.^{-1}$, monitored spectrophotometrically at 226 nm and 1.2 ml fractions collected. Fractions corresponding to elution peaks were analysed by SDS-PAGE (12.5% (w/v), non-reducing conditions) followed by silver staining, (Bio-Rad silver stain kit). Those fractions containing IgAα chains were pooled from three separate runs, dialysed extensively against PBS and concentrated to a final volume of 100 µl using a microconcentrator (Amicon, P30).

To test whether isolated IgAα chains were cleaved by the IgA1 protease from *U. urealyticum*, 20 µl aliquots of the preparation (~20 µg) were incubated (16 h, 37 °C) with 5 µl ureaplasma cell suspension. As controls, incubation in the absence of ureaplasma cells, incubation for zero time and incubation using intact S-IgA as the substrate (25 µg in PBS) were included. Samples were examined for digestion products by SDS-PAGE and immunoblotting, as outlined in Section 2.1.9.

2.4.3 Synthetic peptide substrates

A synthetic peptide, which had been shown to be a substrate for the type 2 IgA1 protease from *N. gonorrhoeae* (Wood and Burton, 1991), was prepared to contain the following sequence of amino acids, with C terminal acetylation:

Ac-Val-Val-Ala-Pro-Pro-Ser-Pro-Gln-Ala-Asn-NH_2

The peptide was synthesised by solid phase fluorenyl methoxycarbonyl (Fmoc) polyamide chemistry in a continuous-flow system using a semi-automated Pepsynthesiser II (Cambridge Research Biochemicals, CRB). Individual amino acids were introduced from a manually-filled syringe barrel and all the operations of single residue addition and deprotection were under the control of a microcomputer (CRB software using an Apple 11e computer) with the possibility of manual override when required. Detailed operating instructions are provided by the manufacturer (CRB) and additional information on the theoretical and practical aspects of solid-phase peptide synthesis are described by Atherton and Shepard, (1989).

a) Reagents

The pepsin KB resin (0.1 m. equivalents.g^{-1}), Fmoc pentafluorophenyl ester derivatives of amino acids were from milligen whereas O-rings and filters were obtained from CRB. Dimethylformamide (DMF), ethanol, methanol, acetonitrile and trifluoroacetic acid (TFA) were all HPLC grade from Rathburn. Piperidine was purchased from Applied Biosystems and ethanedithiol, anisole, tertiary amyl alcohol (TAA) and 4-dimethylaminopyridine (DMAP) from Aldrich. Diethyl ether (DEE) and acetic acid (AA) were obtained from May and Baker and the molecular sieves and other chemicals from Sigma. All solvents were of the highest grade to avoid contamination with aqueous or amine compounds. To prevent the accumulation of dimethylamine in DMF, which can react with the amino acid Fmoc protecting groups, the solvent was stored in the presence of heat-activated molecular sieves at 20 °C for a number of weeks. To prevent blockage of the Pepsynthesiser lines and valves, DMF was filtered before use (0.2 µm nylon filter, Anachem).

b) Synthesis reactions

Prior to peptide synthesis, the main lines of the synthesiser were washed manually (10 min) with DMF (Bottle A) and the flow rate adjusted to 3 ml.$min.^{-1}$. Bottle B contained DMF:20% (v/v) piperidine in DMF. 1 g of pepsin KB resin was washed in 3 x 10 ml of DMF, packed into a 5 x 1 cm column and attached to the

Pepsynthesiser by linking the appropriate lines. The operating programme is shown below:

Pepsynthesiser operating programme:

Step		Time (min)
1	Flow A	5
2	Flow B	10
3	Flow A	15
4	call (load amino acid)	-
5	Flow S	5
6	recirculate	25
7	call	-
8	recirculate	25
9	Flow S	1
10	Flow A	5
11	recirculate	0.5
12	Flow A	5
13	call	-
14	repeat	-

The programme was initiated at step 3, a 15 min wash prior to loading the first derivatised amino acid. Each amino acid (present in five-fold excess, as calculated by the CRB programme) was reconstituted in DMF (2 ml) and centrifuged (12,000 x *g*, 3 min) to remove insoluble particles. For the initial coupling reaction, the catalyst DMAP (12 mg in 200 µl DMF) was loaded immediately prior to the first residue (step 4) and this catalyst/amino acid step was repeated, omitting the deprotection step, to ensure effective coupling. After recirculation for 1.5 h (manual override) and rinsing the loading syringe (step 9), uncoupled amino acids were washed from the column (steps, 10, 12 and 2) before removal of the Fmoc protection group ('deprotection') by 20% piperidine in DMF

(step 3). The lines were washed with DMF (15 min, step 4) and the cycle repeated until the N-terminal amino acid had been coupled and de-protected.

The continual flow of the system allowed peptide synthesis to be monitored spectrophotometrically at 320 nm (Cecil spectrophotometer) by placing an 8 µl flow cell downstream from the reaction column. Changes in absorbance were recorded on a chart recorder at a chart speed of 0.1 mm.min.$^{-1}$ with a full scale deflection (FSD) of 2. The Fmoc protecting groups absorb strongly in the UV-range which enabled the stages of amino acid coupling and deprotection to be observed.

Once synthesis was complete, the peptide was N-terminally acetylated, amino acid side-chain protecting groups were removed (de-blocking) and ammonia (NH_3) treatment of the resin released the peptide amide, as described below.

c) Acetylation

For acetylation, the peptide was treated with a 5 M excess of acetic anhydride in DMF with 0.1M equivalent of pyridine (0.5 mM acetic anhydride, 0.05 mM pyridine made up in 2 ml DMF) by using the recirculation programme (1.5 h), followed by washing with DMF (15 min).

The resin was removed from the column and washed through a scintered glass funnel with DMF, T-amyl alcohol (TAA), acetic acid (AA), and Di-ethyl ether (DEE) using 'wash protocol 1'.

Wash protocol 1:

1 x DMF	25 ml
1 x TAA	10 ml
1 x AA	10 ml
1 x TAA	10 ml
2 x DEE	25 ml

d) De-blocking

The resin was transferred to a round-bottom flask and mixed with de-blocking solvents; 40 ml TFA, 1 ml anisole, 1 ml ethanediol for 3 h (20 °C). Using a scintered glass funnel, the resin was washed with 3 x 5 ml TFA followed by 'wash protocol 1'

e) Cleavage

To produce a C-terminal amide group, the peptide was cleaved from the resin by liquid ammonia, following the method of 'Waters' (Millipore UK Ltd.). This was kindly performed by J. Walton (Department of Chemistry, University of St. Andrews).

Initially the resin was washed following 'wash protocol 2' and subsequently dried under vacuum to remove all traces of ether.

Wash protocol 2:

4 x TAA	10 ml
4 x DMF	10 ml
4 x 10% (v/v) isopropylethylamine in DMF	10 ml
2 x DMF	10 ml
2 x DAA	10 ml

Glacial acetic acid (0.4 ml) was added to 1 g resin in a pressure vessel and the mixture cooled by immersion in dry ice. Following addition of liquid ammonia (10-20 ml), the vessel was sealed and gradually warmed to 20 °C, by evaporation of the dry ice. After 16 h, the vessel was recooled with dry ice (30-60 min), the top pressure valve removed, and the ammonia allowed to evaporate. The dried peptide/resin mixture was washed in TFA, the peptide-containing solvent filtered free from resin and the TFA removed by rotary evaporation. Crystals of peptide were resuspended in 5 ml water and lyophilised. A stock solutions of peptide (20 $mg.ml^{-1}$ in PBS) was stored at -20 °C.

a) Assessment of synthetic peptide as a substrate for *U. urealyticum* IgA1 protease.

15 µl of the peptide (300 µg in PBS) was incubated with 5 µl crude ureaplasma cell suspension (16 h, 37 °C). The reaction was terminated by the addition of 0.1% (v/v) TFA (30 µl) followed by filtration through 0.2 µm filters (Millipore). The filtrates (50 µl) were loaded in 0.1% (v/v) TFA (Buffer A) onto a reverse-phase column (Ultrasphere-ODS, Altex) which was attached to a Gilson 802 HPLC system. Separation was achieved with a gradient of 0.1% (v/v) TFA in acetonitrile (Buffer B), as directed by the 'peptides' programme from a linked Apple IIe computer.

Peptides programme

Time (min)	%B
5	0
5	0
30	50
35	100
35.1	0
45	0

The flow rate was 1 $ml.min^{-1}$, eluting peptides were monitored spectrophotometrically at 226 nm and recorded using a Shimadzu chart recorder set with an FSD of 0.2.

b) Preparation of crude IgA1 protease from *N. gonorrhoeae*.

A crude preparation of IgA1 protease was obtained from *N. gonorrhoeae*, according to the method of Gilbert *et al.*, (1983). Harvest supernatants were precipitated with 60% ammonium sulphate and following 3 x 1h dialysis in 50 mM Tris-HCl, pH 8.1, were separated by gel filtration chromatography using the Superose 6 column linked to a Pharmacia FPLC system. Fractions containing IgA1 protease activity were identified by incubation with S-IgA followed by immunoblotting, as described previously (Sections 2.1.5, 2.1.9). 40 µl samples of

spent media (from before and after concentration with ammonium sulphate) and single colonies of *N. gonorrhoeae* resuspended in 15 µl PBS were examined for IgA1 protease activity by incubation with S-IgA and immunoblotting.

SECTION B
RESULTS

2.6 INITIAL DEMONSTRATION OF IGA1 PROTEASE ACTIVITY

2.6.1 SDS-PAGE and immunoblotting

Previous reports identifying an IgA1 protease in *U. urealyticum* were corroborated; overnight incubation (37 °C) of human S-IgA with ureaplasma cell suspensions resulted in partial digestion of S-IgA to produce Fabα and Fcα fragments. These were detected as two separately-migrating protein bands of relative molecular mass (Mr) 33 kDa and 28 kDa by SDS-PAGE of digestion mixtures followed by staining with PAGE blue 83 (Fig. 2.1a, lane 3). Enzyme activity was specific to the ureaplasma preparations, since incubation of S-IgA in the absence of ureaplasma suspensions or for zero time resulted in no detectable digestion products (Fig. 2.1a, lanes 1 and 2).

The additional protein bands seen in lanes 2 and 3 of Fig. 2.1a represent ureaplasma proteins. To clearly differentiate between these proteins and S-IgA digestion products and to improve the sensitivity of the detection system, the gels were Western-blotted and probed with sheep anti-human IgA (α chain) antibody followed by a peroxidase-linked anti-sheep IgG antibody and a final colour-developing reagent step (immunoblotting). By this method, the Fabα and Fcα fragments were visualised as a single band clearly separate from the undigested α chain (Fig. 2.1b, lane 3). Alternatively, if [^{125}I] IgA1 replaced S-IgA, digestion products were identified (by SDS-PAGE followed by autoradiography) as a single band of slightly higher Mr than the radiolabelled light chain (Fig. 2.1c, lane 3).

Although these SDS-PAGE-based methods allowed unequivocal detection of IgA1 protease activity in ureaplasma samples, they provided only semi-quantitative information and, for routine analysis, were time-consuming. In order to rapidly detect and quantify IgA1 protease activity a number of other assay systems were therefore examined.

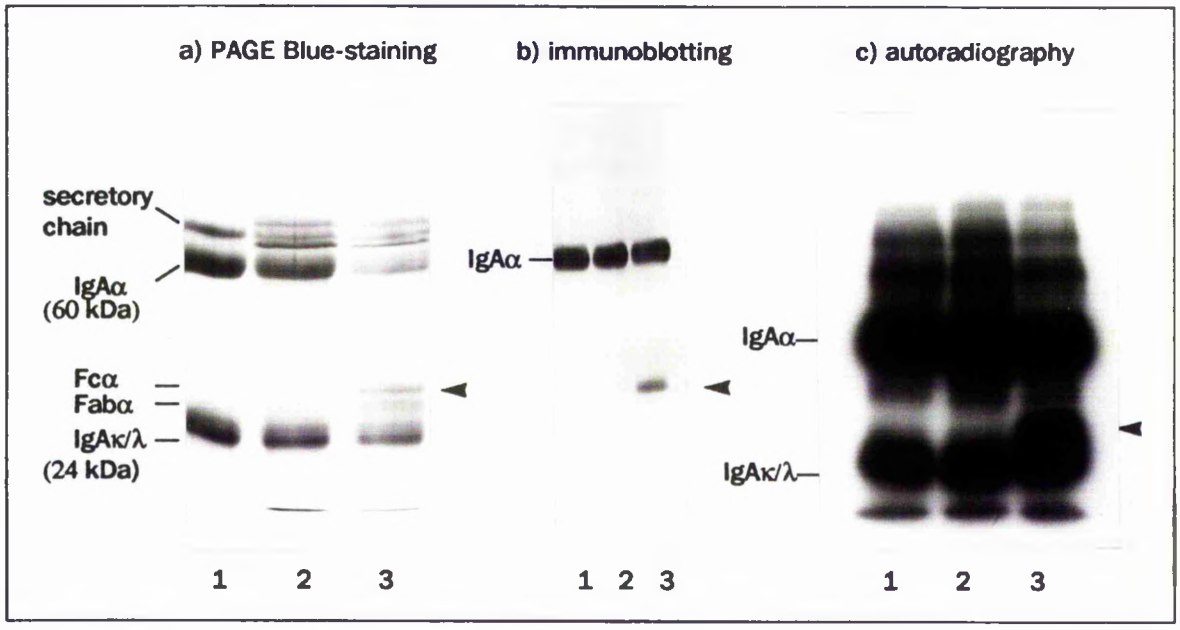


Fig. 2.1 Digestion of human secretory IgA (S-IgA) by *U. urealyticum* IgA1 protease.

S-IgA (15 µg in PBS) was incubated (37 °C) in the absence of ureaplasma cell suspension for 16 h (lane 1), with 5 µl ureaplasma cell suspension for 0 h (lane 2) or with 5 µl ureaplasma cell suspension for 16 h (lane 3), as described in Section 2.1.5. Digestion products were separated by SDS-PAGE (12.5%, reducing conditions) and identified by:

a) staining with PAGE blue 83 (Section 2.1.8),

b) immunoblotting with sheep anti-human IgA α chain antibody followed by peroxidase-linked donkey anti-sheep antibody (Section 2.1.9),

c) autoradiography, where 3 µl [^{125}I] IgA1 replaced S-IgA (Section 2.1.10).

Fabα/Fcα fragments (28 kDa and 33 kDa, respectively) are indicated by the arrows. Additional protein bands in Fig. 2.1a, lanes 2, and 3 are ureaplasma proteins.

2.7 ALTERNATIVE ASSAYS FOR DETECTING IGA1 PROTEASE ACTIVITY

2.7.1 Rocket immunoelectrophoresis

In quantitative rocket immunoelectrophoresis (RIEP), as described by Lassiter *et al.*, (1989), S-IgA digests are subjected to electrophoresis through agarose that contains anti-IgA antibody. At optimal antigen:antibody concentrations, a rocket-shaped precipitin line is formed. Digested samples should produce a larger rocket area than undigested controls, as the smaller cleaved fragments migrate faster in an electric field than intact substrate but still retain reactivity with anti-IgA antisera. As can be seen in Fig. 2.2, although ureaplasma-digested samples (lane 4) produced slightly larger rocket areas than undigested controls (lane 5), the antibody:antigen precipitation did not seem to have reached completion, even after electrophoresis for 18 h. Increasing electrophoresis times, altering running buffer composition, or varying anti-IgA antibody concentrations, failed to produce more definitive results. In addition, the long electrophoresis times made detection of digestion products slower than by SDS-PAGE.

2.7.2 Continuous spectroscopy

The monitoring of IgA1 protease activity by continuous spectrophotometry, first reported by Bleeg *et al.*, (1985), is based on the observation that IgA spontaneously forms spherical micro particles (<1 mm diameter) when added to solutions of 10% PEG. These create a turbid suspension which has a detectable absorbance at 400 nm (A_{400}) that remains constant for a number of hours. Addition of an IgA1 protease to the system results in a decrease in turbidity since Fabα and Fcα fragments are soluble in 10% (w/v) PEG (in Tris buffer). The rate of decrease of turbidity is therefore an immediate measurement of enzyme activity.

In the present study, it was initially confirmed that the A_{400} of the S-IgA:PEG suspension was proportional to S-IgA concentration but that even at maximum recommended levels of S-IgA (50 $mg.ml^{-1}$), the A_{400} only rose to 0.082.

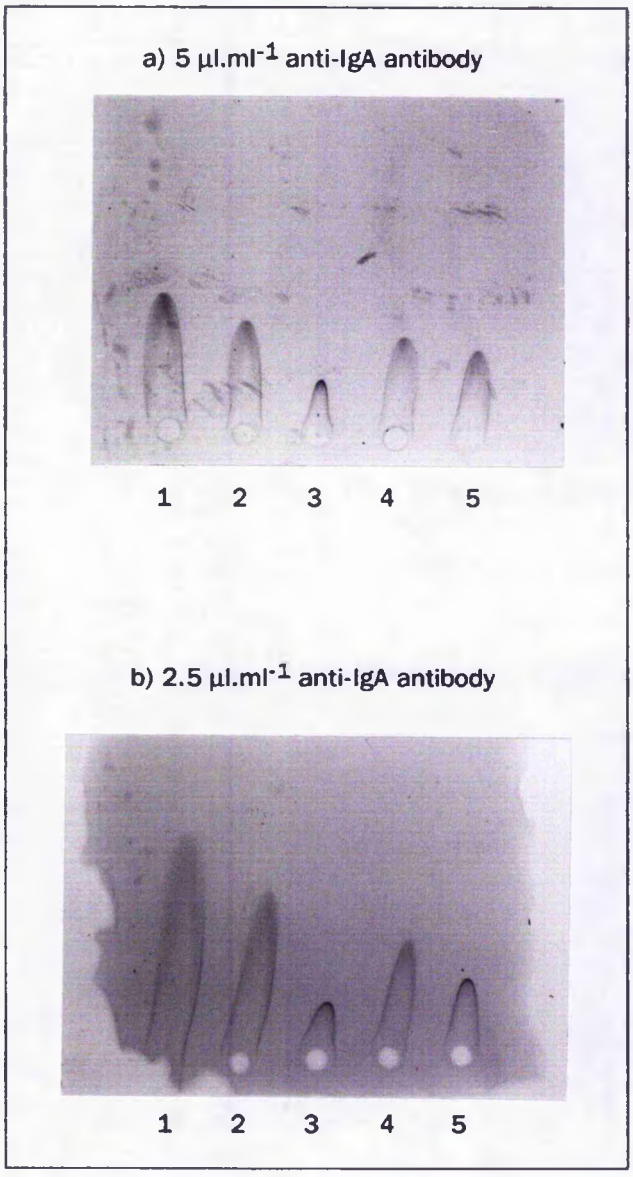
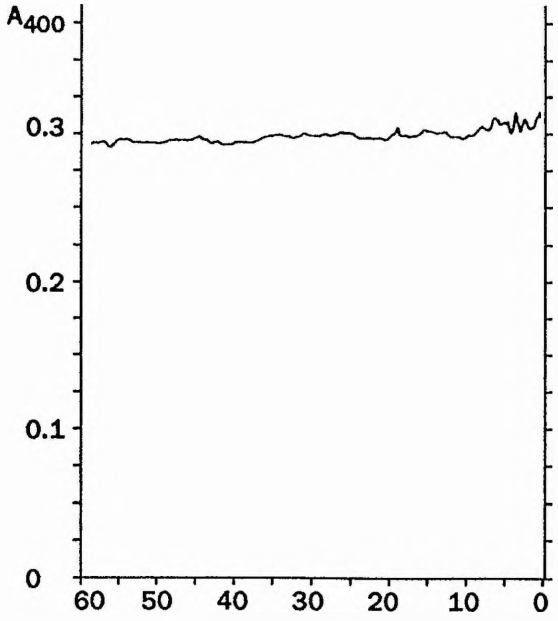


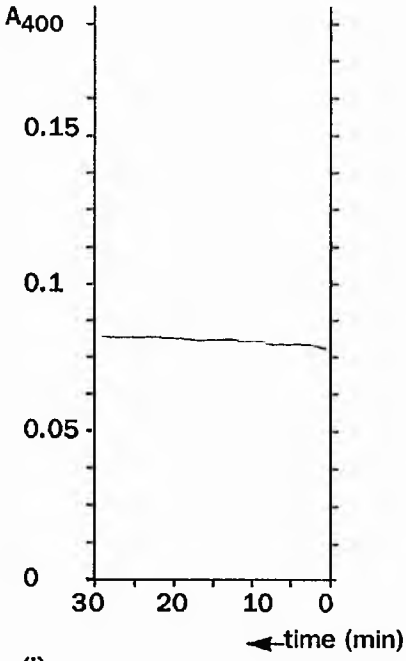
Fig. 2.2 Detection of IgA digestion products by rocket immunoelectrophoresis.
S-IgA (15 µg in PBS) was incubated (16 h, 37 °C) with either 5 µl ureaplasma cell suspension (lane 4) or PBS (lane 5). Samples were electrophoresed in 1% agarose containing anti-IgA antibody at 5 $\mu l.ml^{-1}$ (a) or 2.5 $\mu l.ml^{-1}$ (b), as described in Section 2.2.1. Immunoprecipitation rockets were visualised by PAGE blue 83 staining.
Lanes 1-3 contain IgA standards of protein concentration: 1 $mg.ml^{-1}$, 0.5 $mg.ml^{-1}$ and 0.1 $mg.ml^{-1}$ respectively.

Fig. 2.3 IgA1 protease activity measured by continuous spectrophotometry. The initial turbidity (A_{400}) of a reaction mixture containing 30 µg S-IgA in 0.1 M Tris-HCl, pH 7.2:10% PEG was recorded for an initial period of 20-30 min (i), as described in Section 2.3. The changes in A_{400} were recorded (ii) following addition of 20 µl ureaplasma cell suspension in 0.1 M Tris-HCl, pH 7.2 (a), 20 µl ureaplasma extract, solubilised with 0.1 M Tris-HCl, pH 7.2: 0.5% NP40 (b), and 20 µl 0.1 M Tris-HCl, pH 7.2: 0.5% NP40 (c).

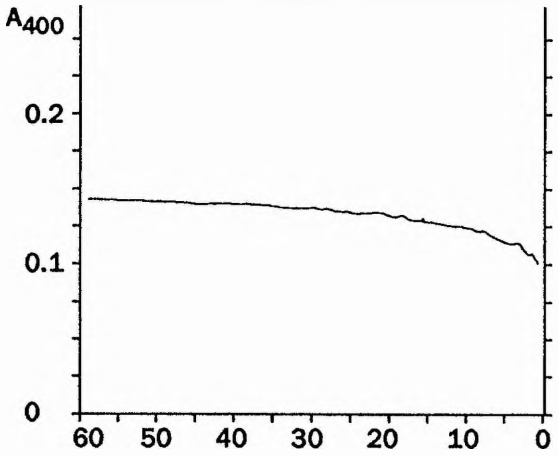
A(ii) 20 µl ureaplasma cell suspension



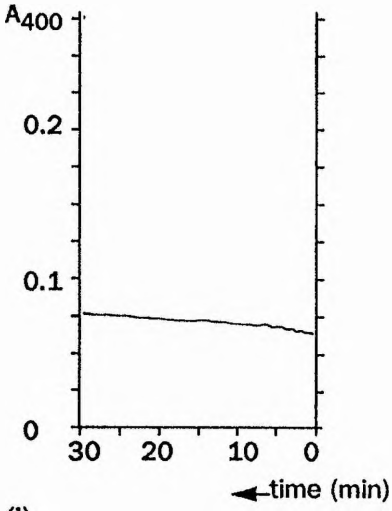
(i)



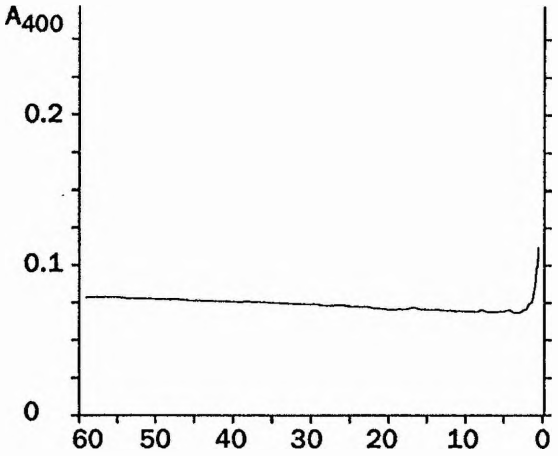
B(ii) 20 µl solubilised ureaplasma extract in Tris buffer: 0.5% NP40



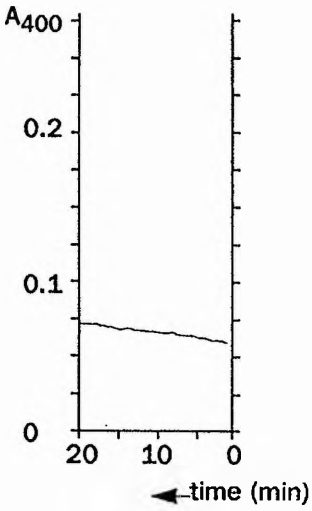
(i)



C(ii) 20 µl Tris buffer: 0.5% NP40



(i)



This was in agreement with the results of Bleeg *et al.*, (1985), who found that the A_{400} did not rise above 0.95. In contrast to these workers, however, a substantial decrease in turbidity was not detected in the presence of IgA1 proteases and, following addition of ureaplasma suspensions, the A_{400} in fact increased from 0.075 to 0.33 (Fig. 2.3). Over a period of 1 h this fell to 0.29 and after overnight incubation, followed by re-mixing, the A_{400} was 0.203. Although this suggested only minimal S-IgA digestion, it was shown by immunoblotting that the ureaplasmal IgA1 protease extensively digested S-IgA in the presence of 10% (w/v) PEG: Tris buffer.

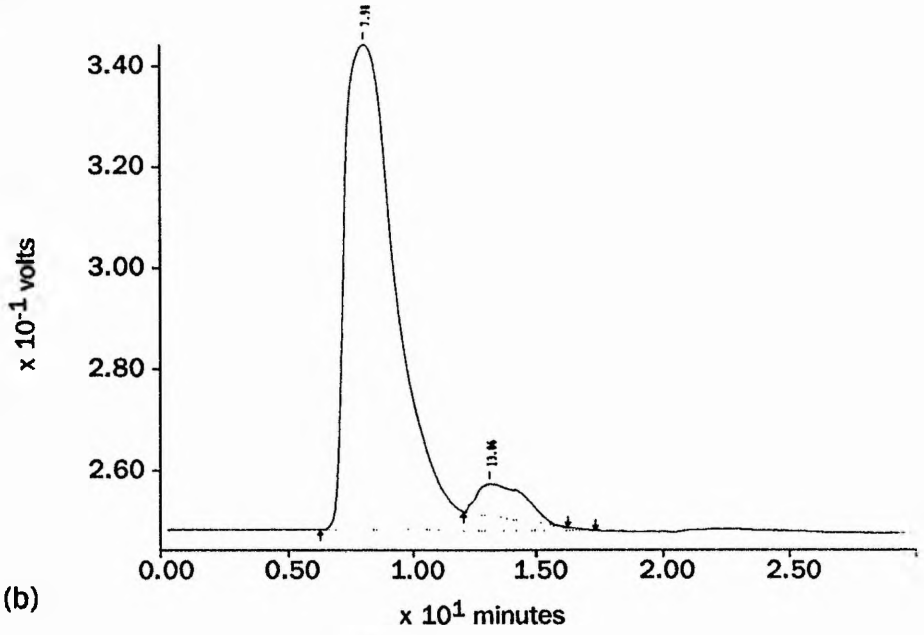
The spectrophotometric assay therefore seemed incapable of detecting this digestion but it is possible that the ureaplasma cells had interfered with the formation of the two-phase system. Absorbance by cellular suspensions certainly may have been responsible for the observed increase in A_{400}. To overcome this, soluble ureaplasma extracts, from Tris buffer:0.5% (v/v) NP40 treatment, replaced cellular suspensions. These produced a minimal increase in A_{400} above background levels but after incubation for 1 h followed by overnight (37 °C), no decrease in turbidity was recorded (Fig. 2.7b). Addition of 0.5% (v/v) NP40 alone did not appear to alter the integrity of the PEG:S-IgA suspension (Fig. 2.7c). These preliminary results suggested that the technique was not suitable for assaying ureaplasma IgA1 protease activity.

2.7.3 HPLC

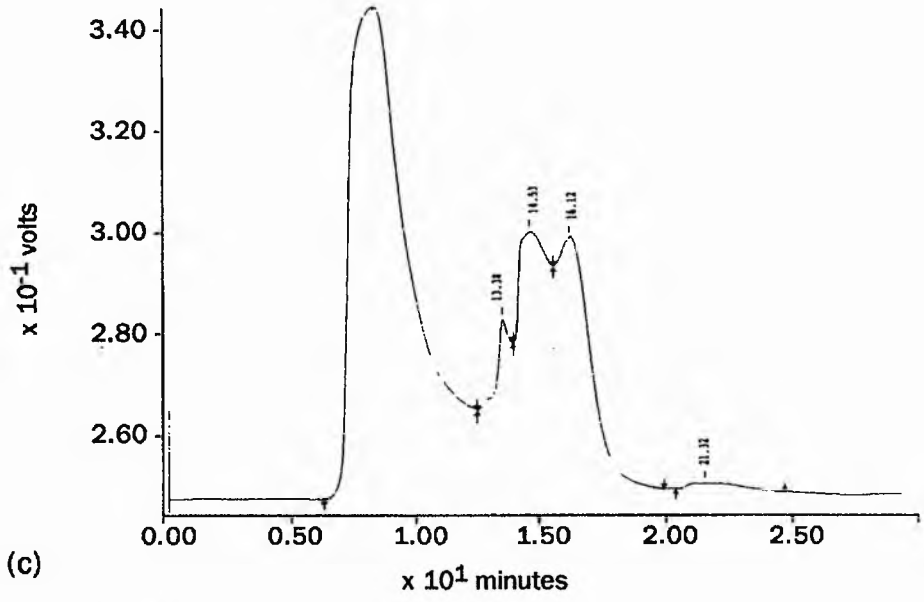
Using an HPLC system, smaller digestion products can be separated from larger undigested substrates by gel filtration chromatography and subsequently identified by spectrophotometric analysis of column eluates. By this method, ureaplasma-digested IgA eluted with an additional protein 'shoulder' (arrowed, Fig. 2.4c) not present in undigested controls (Fig. 2.4a,b). When the concentration of sample loaded onto the column was reduced (by loading 200 µl rather than 2 ml) this 'shoulder' resolved as a separate peak (peak 2, Fig. 2.5a). Analysis of fractions by SDS-PAGE and immunoblotting confirmed that peak 1 represented

Fig. 2.4 Detection of IgA1 digestion products by gel filtration (HPLC).
S-IgA (250 µg in PBS) was incubated with either 90 µl PBS for 16 h (37 °C) (a), 90 µl ureaplasma suspension for 0 h (b), or 90 µl ureaplasma suspension for 16 h (37 °C) (c). Filtered digests were diluted 1:5 in 0.5 M NaCl and 2 ml samples (250 µg S-IgA) separated on a Dupont GF 250 column (Zorbax) at a flow rate of 1 $ml.min^{-1}$ using PBS: 0.4 M NaCl as the mobile phase, as described in Section 2.2.2. Proteins were detected spectrophotometrically at 226 nm and collected as 0.5 ml fractions.
The digestion peak is indicated by an arrow.

Fig. 2.4(a)


3.40
3.20
3.00
2.80
2.60
x 10-1 volts
0.00
0.50
1.00
1.50
2.00
2.50
x 101 minutes

(b)


3.40
3.20
3.00
2.80
2.60
x 10-1 volts
0.00
0.50
1.00
1.50
2.00
2.50
x 101 minutes

(c)

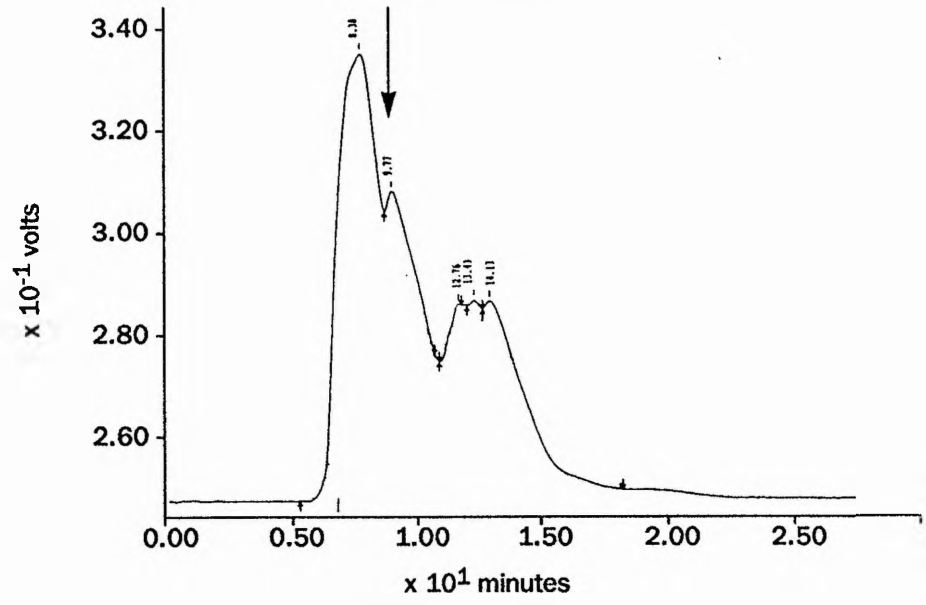
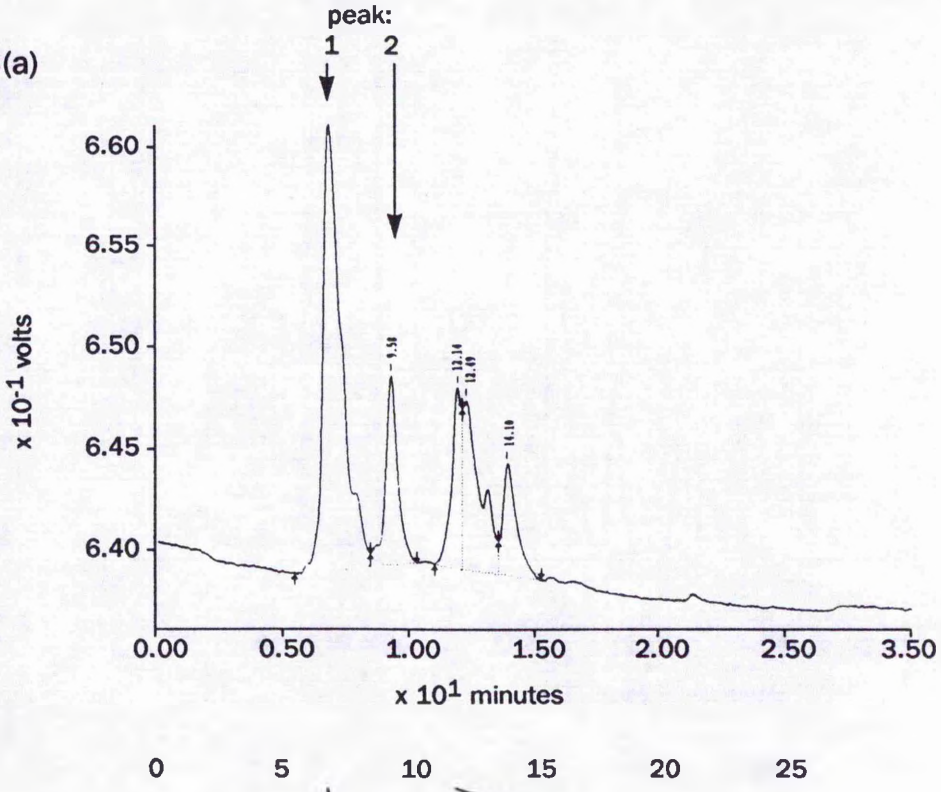
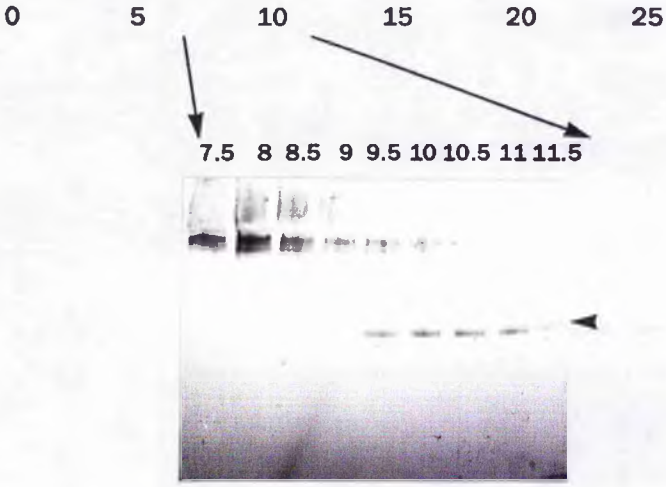

3.40
3.20
3.00
2.80
2.60
x 10-1 volts
0.00
0.50
1.00
1.50
2.00
2.50
x 101 minutes

Fig. 2.5(a)





(b)

Fig. 2.5 Analysis of digestion products identified in gel filtration (HPLC) assay.
a) Samples were prepared and analysed as described in Fig. 2.4 except that 200 µl (25 µg S-IgA) was used in the separation rather than 2 ml. The individually resolved peak eluting at fraction 7.5 is indicated by an arrow (2).

b) Protein-containing fractions (50 µl from 0.5 ml) were analysed by SDS-PAGE (12.5%, non-reducing conditions) and immunoblotting, using sheep anti-human IgA α chain antibody followed by peroxidase-linked donkey anti-sheep antibody as described in Section 2.1.9.
Fabα/Fcα fragments are indicated by the arrow.

intact S-IgA and that peak 2 represented digested S-IgA fragments (Fig. 2.5b). These protein peaks were detected with as little as 3 μg of digested S-IgA and within 10 minutes of sample loading. However, column clearance (with PBS:0.4 M NaCl) for up to 30 min was required between each sample run and when large sample numbers were involved, the method became time-consuming and labour-intensive. In addition, an HPLC machine was not available on a regular basis for such studies and it is for these reasons that a gel filtration-based assay system was not adopted as a means of identifying the ureaplasmal IgA1 protease.

In the assessment of techniques for identifying IgA1 protease activity, the immunoblotting method was used repeatedly to confirm the identity of putative digestion products. It rapidly became clear that, despite initial reservations about the method, immunoblotting was capable of unambiguous detection of IgA digestion products both reliably and reproducibly. Efforts were therefore concentrated on refining this assay system rather than investigating further the value of alternatives.

2.7.4 Refining immunoblotting techniques

By varying the concentration of S-IgA and ureaplasma cell suspension in the incubation mixture, it was found that digestion products could be detected with as low as 5 μg S-IgA when incubated with 2.5 μl ureaplasma suspension. For definitive identification of Fabα/Fcα fragments by immunoblotting, 15 μg of S-IgA combined with 5 μl of ureaplasma suspension (equivalent to 10 ml liquid culture) was used. This was an improvement on assays described by other workers where ureaplasma preparations equivalent to 100-200 ml of liquid culture were incubated with 100-400 μg of IgA substrate (Kapatais,-Zoumbos *et al.*, 1985, Kilian and Freundt, 1984). Low levels of product could be observed after two hours incubation but overnight incubation (16 h) was necessary before digestion products were unequivocally detected by immunoblotting (Fig. 2.6).

Agitation of the mixture during incubation did not improve the extent of digestion but multiple small volume digests (< 50 μl) gave proportionally more product than a single digest of larger volume. Incubation at 37 °C was optimal for

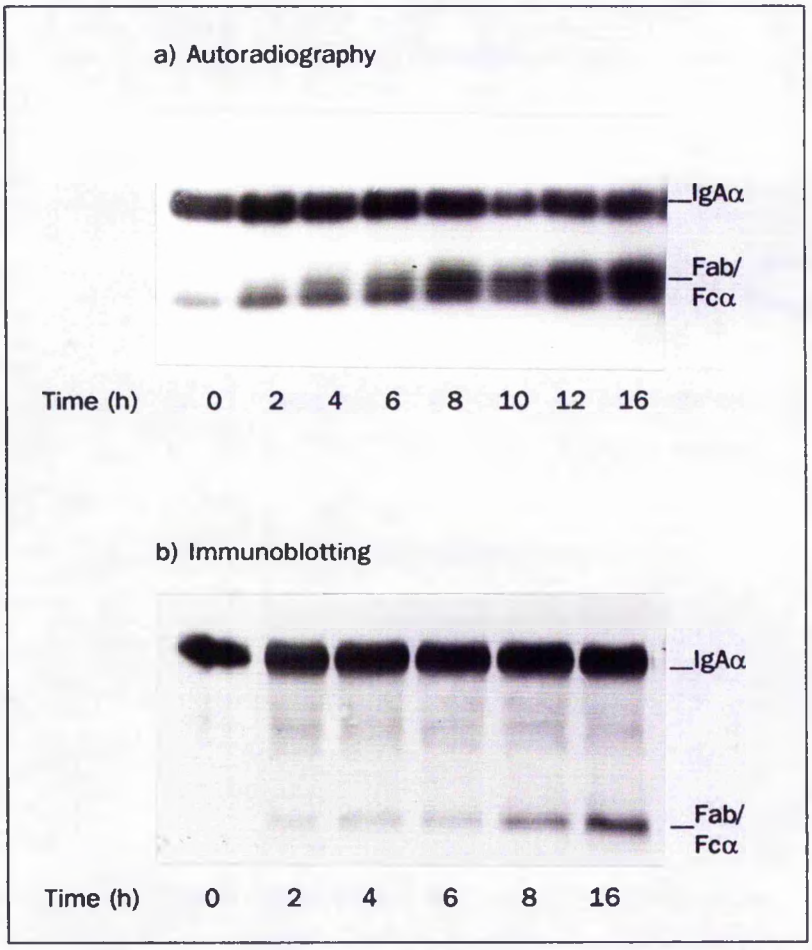


Fig. 2.6 Time course of IgA digestion by *U. urealyticum* IgA1 protease.
S-IgA (15 μg) or [^{125}I] IgA1 (3 μg) were incubated with 5 μl ureaplasma cell suspension in PBS (37 ^{o}C, 0-16 h). The reaction was terminated by addition of '4 x denature mix' and samples were analysed by SDS-PAGE (12.5%) followed by autoradiography (for [^{125}I] IgA1 samples) (a), or by immunoblotting with sheep anti-human IgA α chain antibody and peroxidase-linked donkey anti-sheep antibody (b), as described in Sections 2.1.10 and 2.1.9.

the enzymatic reaction and, as expected, exposure of the protease to temperatures above 50 °C resulted in denaturation and loss of activity. Extreme acid (<pH 3.6) or alkaline (>pH 10.5) conditions had a similar effect but the enzyme appeared stable over a wide range of pH (pH 3.6-pH 10.5) (Fig. 2.7). The activity appeared strongest between pH 3.6-8.3 and therefore PBS at pH 7 was a suitable choice of buffer for the digestion mixture. Addition of divalent cations (Mg^{2+}, Ca^{2+}, 1-10 mM) to the reaction mixture did not activate or inhibit enzyme activity.

The replacement of 14 x 14 cm polyacrylamide slab gel apparatus with Bio-Rad 'mini gel' systems substantially lowered the cost and running time of the assay. In addition, the reduction of antibody incubation times in immunoblotting to 2 h and the use of a colour-developing reagent enabled digested samples to be analysed within 8 h. The use of [^{125}I] IgA1 as a substrate did not improve the speed of the assay but was marginally more sensitive (Fig. 2.6) and used less substrate than immunoblotting (0.04 µg rather than 15 µg). Due to the hazards associated with using radioactivity, however, [^{125}I] IgA1 was only used when quantitative measurements were required.

2.8 BIOCHEMICAL CHARACTERISATION

2.8.1 Inhibition studies

The effect of inhibitors on enzyme activity is presented in Table 2.1. Of the range of compounds used that were diagnostic for the four classes of proteases, only a number of serine protease-specific inhibitors were effective in reducing or inhibiting ureaplasma IgA1 protease activity. In particular, DFP and DCI (Fig. 2.8A and B) gave clear inhibition profiles and it was established that this effect was not due to inhibitor solvent. PMSF, another diagnostic inhibitor for serine proteases, reduced enzyme activity only inconsistently. This was not due to compound instability, since each inhibitor preparation reduced trypsin activity by 100% in a standard trypsin assay. It was noted that EDTA, even at 160 mM, had no effect on ureaplasmal enzyme activity (Fig. 2.8C).

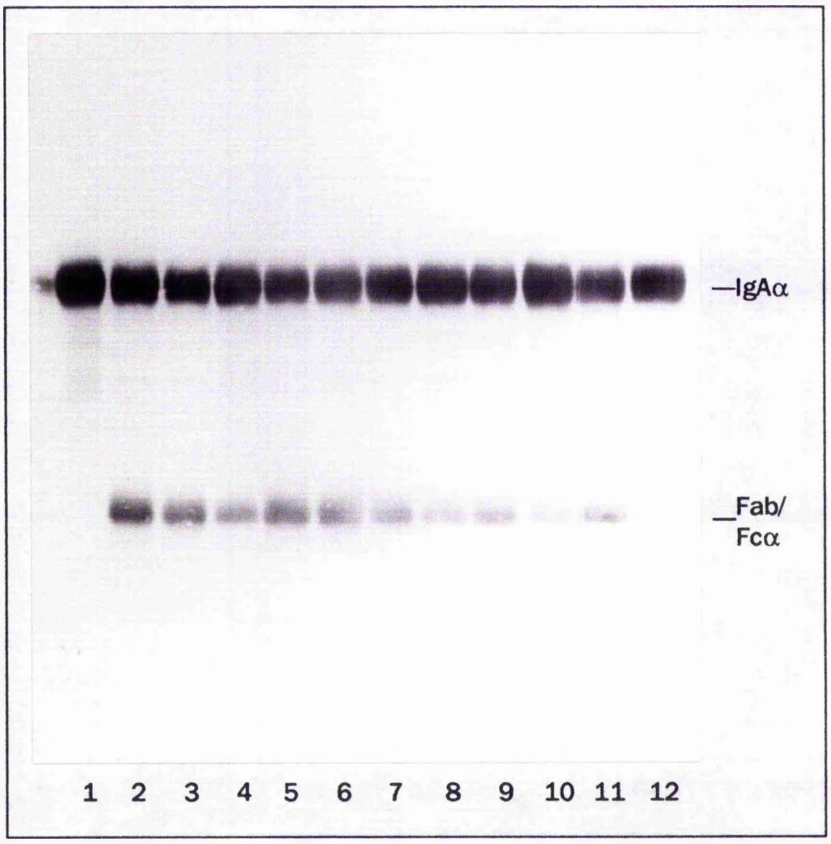


Fig. 2.7 Effect of pH on *U. urealyticum* IgA1 protease activity.
15 µl S-IgA (1 mg.ml^{-1} in PBS) was incubated with 5 µl ureaplasma cell suspensions at a range of pH values, as described in Section 2.3.1:

1 = 2.4	7 = 8.3
2 = 3.6	8 = 8.9
3 = 4.5	9 = 9.9
4 = 5.6	10 = 10.5
5 = 6.5	11 = PBS alone
6 = 7.5	12 = no IgA1 protease

Protein digests were analysed by SDS-PAGE (12.5%) and immunoblotting using sheep anti-human IgA α chain antibody followed by peroxidase-linked donkey anti-sheep antibody, as described in Section 2.1.9.

Table 2.1 Inhibition of *U. urealyticum* IgA1 protease activity.

Class	Inhibitor	Solvent	Effective conc.	Working conc.	Inhibition (+/-)
Serine	PMSF	propan-2-ol	0.1-1 mM	0.1 mM 0.5 mM 1.0 mM (propanol)	- - + -
	3,4-DCI	DMSO	5-100 µM	10 µM 50 µM 100 µM (DMSO)	+ + ++ -
	Aprotinin	H_2O	equimolar with protease	100 µM 500 µM 1 mM	- - -
	SBTI	25 mM phosphate, pH 7.0	equimolar with protease	100 µM 500 µM 1 mM	- - -/+
	DFP	propan-2-ol	100 µM	10 µM 100 µM 1 mM 10 mM (propanol)	- ++ +++ +++ -
	Chymostatin	DMSO	10-100 µM	10 µM 100 µM (DMSO)	- - -
	Elastinal	H_2O	10-100 µM	10 µM 100 µM	- -
	TPCK	methanol	10-100 µM	100 µM (MeOH)	- -
	TLCK	1 mM HCl, pH 3.0	10-100 µM	100 µM (100 µM HCl)	- -

Table 2.1 contd.

Class	Inhibitor	Solvent	Effective conc.	Working conc.	Inhibition (+/-)
Metallo	EDTA	H_2O (pH 8.6)	10 mM	1 mM 5 mM 25 mM	- - -
	1,10 Phenan-throline	Methanol	1-10 mM	5 mM (MeOH)	- -
Aspartyl	Pepstatin A	Methanol	1 µM	1 µM (MeOH)	- -
	Bromophenacyl -bromide	Methanol	0.6 mM	0.5 mM (MeOH)	- -
Cysteine	Cystatin	25 mM phosphate, pH 7.5, 20% glycerol	equimolar with protease	10 $\mu g.ml^{-1}$ 100 $\mu g.ml^{-1}$ (phos.)	- - -
	Iodoacetamide	H_2O	10-100 µM	100 µM 10 µM (H_2O)	- - -
	E64	25 mM phosphate, pH 7.5	1-10 µM	1 µM 10 µM (phos.)	- - -
Cysteine /Serine	Leupeptin	H_2O	10-100 µM	100 µM	-

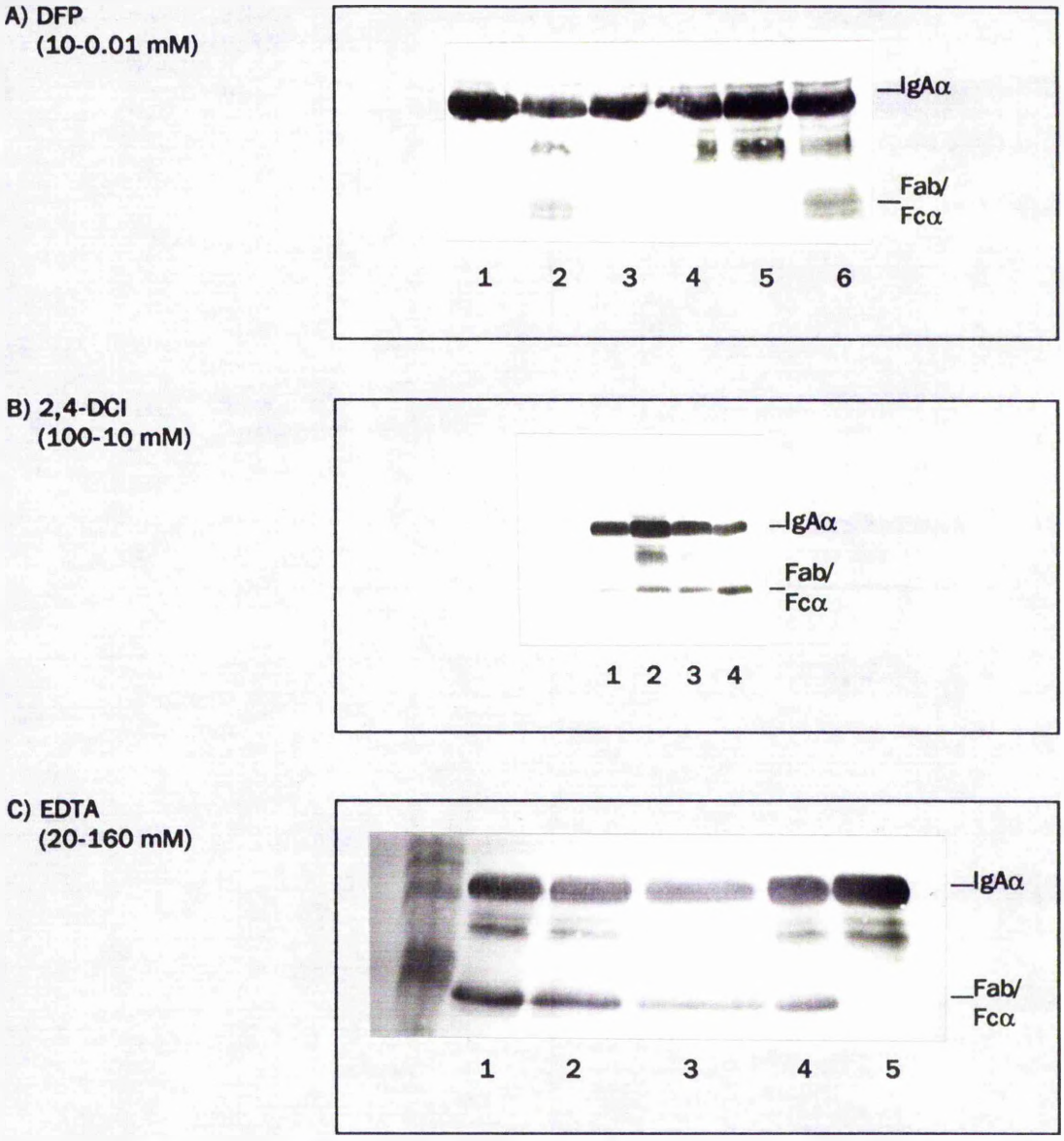


Fig. 2.8 The effect of inhibitors on *U. urealyticum* IgA1 protease activity.
Ureaplasma cell suspensions (5 µl in 25 µl PBS) were pre-incubated (30 min, 37 °C) with 5 µl inhibitor (a-c) and following addition of 15 µg S-IgA, for a further 16 h (37 °C). Controls of inhibitor solvent alone were included where appropriate. The extent of inhibition was determined by immunoblotting of digest supernatants using sheep anti-human IgA α chain antibody and peroxidase-linked donkey anti-sheep antibody, as described in Section 2.1.9. The Inhibitors used were:

a) Di-isopropylfluorophosphate in isopropanol at 10 mM, 1 mM, 0.1 mM and 0.01 mM (lanes 3-6). Lane 1, IgA alone (no IgA1 protease), lane 2, isopropanol alone.

b) 3,4-Dichloroisocoumarin in DMSO at 100 µM, 50 µM, 10 µM (lanes 1-3). Lane 4, DMSO alone.

c) EDTA (pH 8.6) at 20 mM, 100 mM, 160 mM (lanes 1-3). Lane 4, PBS alone, lane 5, IgA alone (no IgA1 protease).

2.8.2 Serotype distribution

An IgA1 protease was expressed by all fourteen serotypes of *U. urealyticum* (Fig. 2.9) and by using the method of Simpson *et al.*, (1988) (Section 2.1.10), it was calculated that the extent of digestion was not identical for each serotype. Higher enzyme activity was found in serotypes 1, 7, 8, 10, and 13 and the lowest activity was found in serotypes 6, 9 and 12. Standardising ureaplasma protein concentrations for each serotype is subject to considerable error, however, and differences in levels of IgA1 protease activity between serotypes are therefore only presented as a possibility.

The analysis also suggested that each serotype expressed an IgA1 protease of identical substrate specificity to serotype 8, since the digestion products were of apparently identical Mr. In other bacteria, multiple IgA1 protease-types are clearly identified by SDS-PAGE of digested IgA. This is due to the extensive glycosylation in the hinge region of the molecule, which enables fragments differing by only a few amino acids to be detected by differences in their migration rates.

2.9 SUBSTRATE SPECIFICITY

2.9.1 Determination of cleavage site

Fig. 2.10A demonstrates that IgA1 but not IgA2 was susceptible to cleavage by the ureaplasma IgA1 protease. The partial digestion observed with S-IgA was therefore probably due to the fact that this is composed of a mixture IgA1 and IgA2. The small amount of intact IgA1 remaining after digestion suggested that even this isotype may be partially resistant to the ureaplasma IgA1 protease. Equally it could represent a small amount of undigested IgA2 contaminating the preparation or could be due to an inadequate time of incubation to promote total digestion.

The resistance of IgA2 to the action of the IgA1 protease suggests that the ureaplasma enzyme cleaves within the hinge of IgA1, since the absence of thirteen

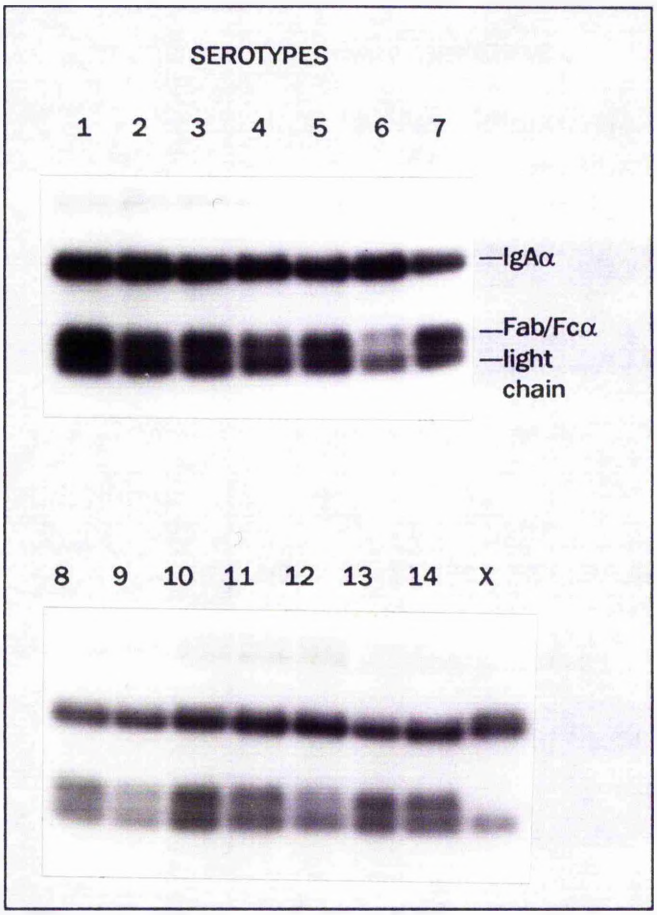


Fig. 2.9 IgA1 protease activity in 14 serotypes of *U. urealyticum*.
3 µl [^{125}I] IgA1 was incubated for 16 h (37 °C) with PBS (lane X) or with 5 µl of each serotype (lanes 1-14). Digests were examined by 12.5% SDS-PAGE (reducing conditions) followed by autoradiography, as described in Section 2.1.10.

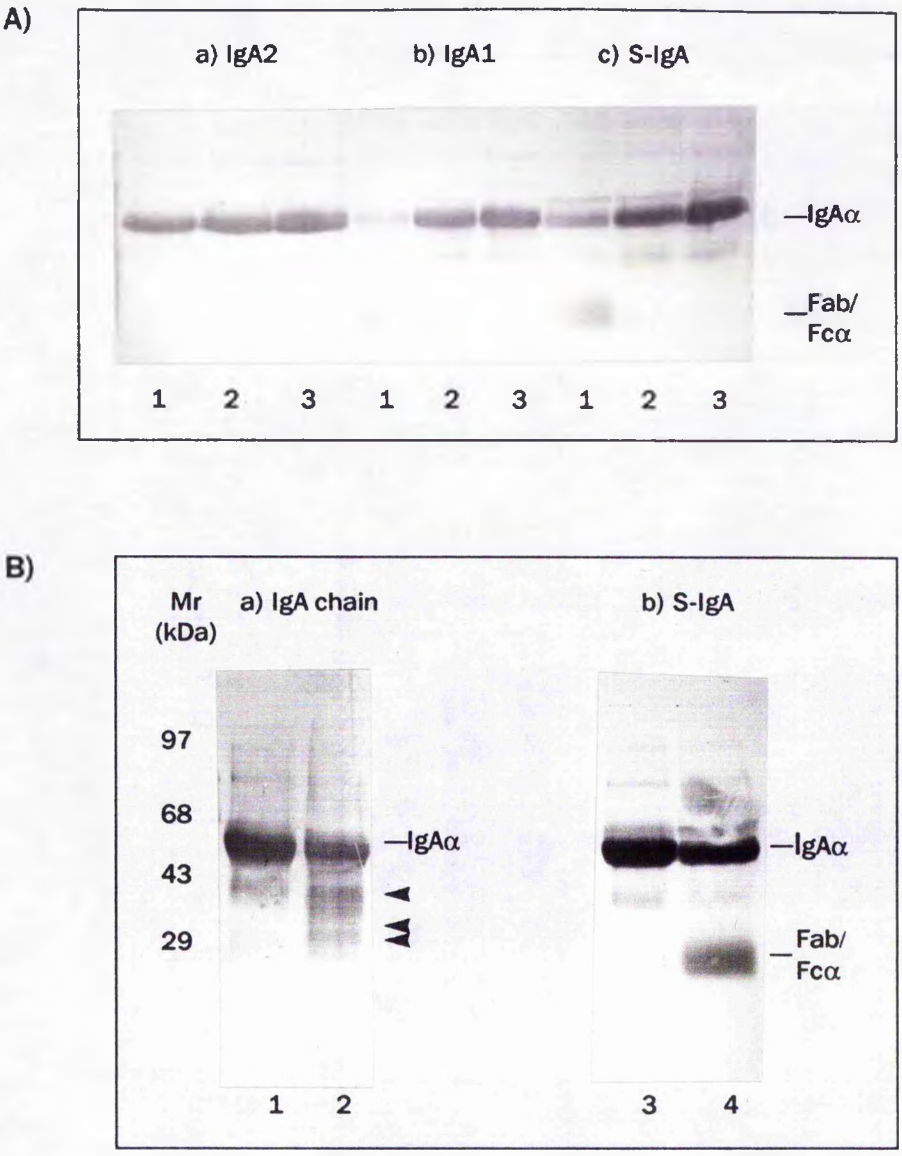


Fig. 2.10 Digestion of IgA by *U. urealyticum* IgA1 protease.

A) 15 µl aliquots of IgA2 (a), IgA1 (b) and S-IgA (c), each at 1 $mg.ml^{-1}$ in PBS were incubated with 5 µl ureaplasma cell suspension in PBS, 16 h, 37 °C (lane 1), 5 µl ureaplasma cell suspension, 0 h (lane 2), or with PBS alone, 16 h, 37 °C (lane 3). Digestion products were analysed by 12.5% SDS-PAGE (reducing conditions) and immunoblotting using sheep anti-IgA (α chain) antibody followed by HrP-labelled anti-sheep antibody, as described in Section 2.1.9.

B)Isolated S-IgA α chain fragments (20 µg), (panel a) and 25 µg S-IgA (in PBS) (panel b), were incubated (16 h, 37 °C) with 5 µl PBS (lanes 1 and 3) or with 5 µl *U. urealyticum* cellular suspensions (lanes 2 and 4).
Molecular weight markers are to the left of the figure. Possible digestion products are indicated by arrows.

amino acids in this region is the major structural difference between IgA2 and IgA1. This was confirmed by determining the point of scission in the IgA1 α chain. N-terminal amino acid sequencing of the Fcα portion of digested S-IgA reproducibly gave the following sequence:

-Thr-Pro-Ser-Pro-Ser-Cys-Cys-His-Pro-

The designation of the two cysteine residues was only possible after pyridylethylation of the Fcα fragment, since under normal conditions of Edman degradation, cysteine residues cannot be identified. Comparison with the known amino acid sequence of human IgA1 (Putnam *et al.*, 1979) revealed that cleavage had occurred between residues 235 and 236, a site located in the second repeat of the octapeptide within the hinge region of the α chain (Fig. 1.6). This was one of the two previously predicted cleavage sites for *U. urealyticum* (Kapatais-Zoumbos *et al.*, 1985) and is the confirmed cleavage site of the Type 2 IgA1 proteases from *N. gonorrhoeae*, *N. meningitidis* and *H. influenzae* (Plaut *et al.*, 1975, Mulks *et al.*, 1980a, Kilian *et al.*, 1980).

2.9.2 Synthetic peptide substrates

Having identified the site of digestion for the ureaplasma IgA1 protease, it was then possible to design a synthetic peptide that may have served as more useful substrate for assaying enzyme activity than human S-IgA or IgA1. Since the IgA1 proteases of *N. gonorrhoeae* (type 2) and *U. urealyticum* cleave the same site in the IgA1 hinge region, one of the 10-residue synthetic peptides that had been susceptible to digestion by the neisserial IgA1 protease (Wood and Burton, 1991) was synthesised.

The purified peptide molecule eluted as two distinct peaks by reverse-phase HPLC. There was no alteration in peak size or mobility following incubation with crude ureaplasma extracts, suggesting that no digestion had occurred (Fig. 2.11, panel c). The pH of the reaction mixture (pH 7 in PBS) was favourable for IgA1 protease activity, but there may have been a number of other reasons why there was no cleavage of synthetic substrate. To confirm the integrity of the

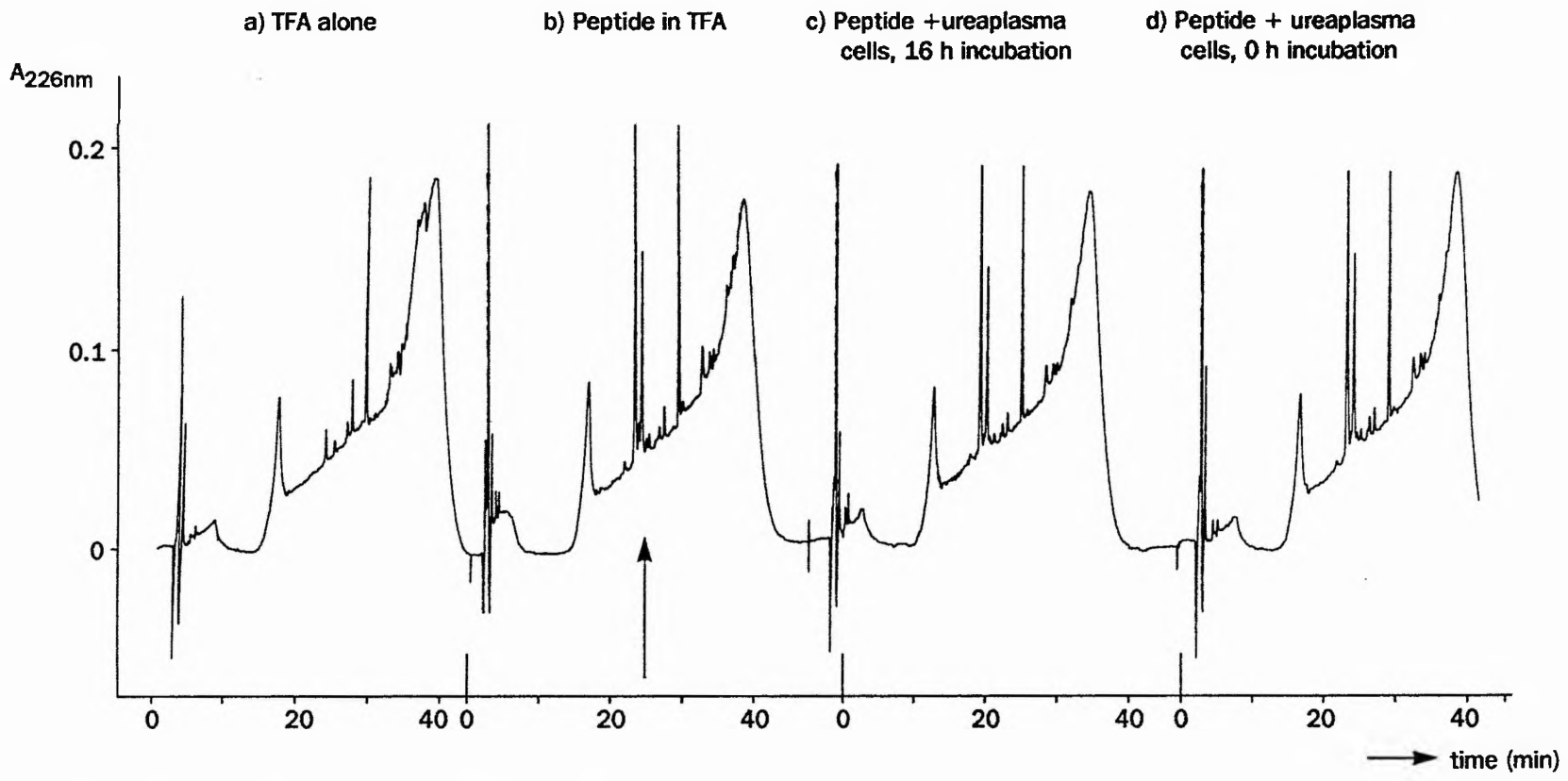


Fig. 2.11 Analysis of synthetic peptide by reverse-phase HPLC.

15 µl (300 µg in PBS) of the synthetic peptide Ac-V-V-A-P-P-S-P-Q-A-N-NH_2 in 30 µl TFA (0.1%) was analysed by reverse-phase chromatography (HPLC) using using a gradient of 0-50% TFA (0.1%) in 30 min (panel b), as described in Section 2.4.3. A sample of 15 µl PBS in 30 µl 0.1% TFA was included as a control (panel a). To assess digestion of the peptide by *U. urealyticum* IgA1 protease, 300 µg of peptide (in 15 µl PBS) was incubated (37 °C) with 5 µl crude ureaplasma cell suspension for either 0 or 16 h. After addition of 30 µl TFA (0.1%), the digest mixture was examined by reverse-phase HPLC, as before (panels c and d).

peptide, an attempt was made to prepare the type 2 IgA1 protease from *N. gonorrhoeae* to test under the same conditions. No enzyme activity was detected in neisserial pellets or spent medium using the immunoblotting method (data not shown) and further purification of the IgA1 protease was probably necessary before it could be used for this purpose. It is also possible, but very unlikely, that the bacterial strain used did not produce an IgA1 protease.

As an alternative, peptides based on the hinge region of IgA1 could have been synthesised, although such compounds had not proved to be effective substrates for other IgA1 proteases (Table 1.2). As a preliminary to such work, it was shown that isolated IgA α chains (obtained by reduction and carboxymethylation of IgA followed by gel filtration) were partially digested by the ureaplasma IgA1 protease (Fig. 2.10). It appeared that digestion was less specific than with intact IgA and a number of cleavage products were detected. Elucidation of these digestion sites may serve as a basis for future synthetic substrate design.

SECTION C
DISCUSSION

It was confirmed that *U. urealyticum* produces a classical IgA1 protease which cleaves IgA1, but not IgA2, into Fabα and Fcα fragments. There were a number of features of this process, however, that hindered progress in enzyme characterisation. The most fundamental of these was the length of time required to demonstrate the presence and levels of IgA1 protease activity. Incubation times of over ten hours were required before digestion products were identified and the detection methods themselves were time-consuming and only semi-quantitative.

Comparable incubation times have been reported by other workers examining ureaplasma IgA1 protease activity (Kilian *et al.*, 1984, Kapatais-Zoumbos *et al.*, 1985). Robertson *et al.*, (1984) found that optimal degradation occurred after 42 h (36 °C). Crude preparations of other bacterial IgA1 proteases frequently require incubation with substrate for up to 16 h (Labib *et al.*, 1978, Mulks and Knapp, 1987, Frandsen *et al.*, 1987, Tsuji *et al.*, 1989) but digestion times for partially purified or purified enzymes have been reported as low as 30 min (Plaut *et al.*, 1974b, Blake and Swanson, 1978, Mortensen and Kilian, 1984a, Halter *et al.*, 1984). The specific activity of the IgA1 protease preparation was undoubtedly higher in these cases and each reaction contained a minimum of 100 μg IgA1 substrate (Mulks *et al.*, 1980b). In the present study, where commercial S-IgA (colostrum) was used as a substrate, such concentrations were prohibitively expensive. If clinical material had been available, a high titre of monoclonal IgA1 could have been obtained from the serum of patients with a plasma cell neoplasm (Plaut, 1983). For enzyme characterisation, however, colostral S-IgA may represent the natural substrate for the ureaplasma IgA1 protease more closely than serum IgA1.

It is not known how ureaplasma IgA1 protease activity *in vitro* compares with the activity *in vivo*, but it seems likely that *in vitro* reaction conditions are suboptimal, since if IgA1 digestion is to be of benefit to *U. urealyticum in vivo*, a more rapid substrate digestion would be expected. There may be a number of

factors which operate *in vitro* to reduce enzyme activity. The reaction temperatures used (37 °C) matched those found *in vivo* and although the pH of the reaction buffer (pH 7.4) did not absolutely reflect the pH of the vagina or urinary tract (pH 5 and pH 6 respectively), it was shown that such differences in pH did not significantly influence enzyme activity. Divalent cations similarly did not affect IgA proteolysis but Blake and Eastby (1991) routinely included 10 mM $CaCl_2$ and 10 mM $MgCl_2$ into gonococcal IgA1 protease reaction mixtures, suggesting that this IgA1 protease is activated by metal ions. It is possible that the ureaplasma IgA1 protease was simply not as active in cells grown in artificial culture medium or that the medium used was suboptimal for expression. The presence of large quantities of IgA1 *in vivo* may stimulate IgA1 protease production in ureaplasmas, but it is generally regarded that IgA1 protease production in other bacteria is constitutive.

The serum component of ureaplasma growth medium, which frequently co-harvests with ureaplasma cells, may have contained inhibitors to the IgA1 protease. Bacterial IgA1 proteases have demonstrated considerable resistance to common anti-proteases such as α–2–macroglobulin and α-1-antitrypsin (Plaut, 1983) but the effect of similar serum-bound inhibitors on the ureaplasmal enzyme has not been examined. Since the enzyme may be membrane associated (Chapter 3), the cell-bound serum components may have restricted substrate access. Alternatively, the substrate itself, S-IgA, may contain antibodies inhibitory to enzyme activity (Kilian *et al.*, 1980, Gilbert *et al.*, 1983). There was no apparent difference between the rates of digestion of the commercially-obtained serum IgA1 and the S-IgA used in this study. This was only judged on a visual basis by immunoblotting, however, and accurate quantitative measurements of IgA1 protease activity were not performed.

Developing rapid and quantifiable detection methods for the ureaplasma IgA1 proteases remains a principal requirement for research on this enzyme. Although rocket immunoelectrophoresis has been used for the analysis of bacterial IgA1 proteases (Lassiter *et al.*, 1989, Kilian *et al.*, 1983a) it was found that the technique was no faster to perform than SDS-PAGE and the results obtained were

more ambiguous. It was not clear why the PEG-based spectrophotometric assay failed to detect ureaplasma IgA1 protease activity. The preparations of relatively pure *H. influenzae* IgA1 protease used in the original report by Bleeg *et al.*, (1985) may have been more suitable than the crude IgA1 protease samples obtained from *U. urealyticum* harvests. The separation of digestion products by HPLC and quantitation of digestion by measurement of peak areas demonstrated considerable potential as a quantitative method for assaying IgA1 protease activity. The time and labour required for the analysis of large sample numbers rendered the technique impractical for the purposes of this investigation. If an HPLC machine had been available on a regular basis, these problems may have been overcome by using higher flow rates and by the installation an auto-injector.

For future work, a number of other options should be considered for the quantification of ureaplasma IgA1 proteases. ELISA-based methods have been described for the measurement of gonococcal and streptococcal enzyme activity (Blake and Eastby, 1991 and Reinholdt *et al.*, 1990). As described in Chapter 1 (Section 1.7.6), the recent identification of a Cen A-IgA1 hybrid molecule which is susceptible to cleavage by the IgA1 proteases from *N. gonorrhoeae* and *H. influenzae* (Miller *et al.*, 1992) may have applications for the assay of these enzymes. It is likely that the ureaplasma IgA1 protease would also cleave this molecule, since preliminary evidence suggests that the enzyme shares a substrate specificity with the *Neisseria* and *Haemophilus* type 2 IgA1 proteases.

To examine the IgA1 substrate specificity of *U. urealyticum* IgA1 protease, Fcα digestion products were subjected to limited N-terminal amino acid sequencing. By comparison with the published sequence for IgA1 (Putnam *et al.*, 1979), the point of scission was identified as the Pro_{235}-Thr_{236} hinge region peptide bond, identical to the site cleaved by the type 2 IgA1 proteases from *N. gonorrhoeae* and *H. influenzae*. The homogenous mixture of amino acids obtained from N-terminal sequencing suggested that the enzyme cleaved at only a single bond only within the hinge region of IgA1. As was found for other bacterial IgA1 proteases digesting this site, the Pro-Thr peptide bond in the sister octapeptide of the hinge region was resistant to attack. Although the precise

reason for this is unclear, it has been suggested that bacterial IgA1 protease are conformation-dependent rather than sequence-specific enzymes (Section 1.7.6). This has been supported by the fact that synthetic peptides based on the IgA1 hinge region were not substrates for the gonococcal IgA1 type 2 IgA1 protease but peptides of similar (but not identical) sequence, based on the autocatalytic cleavage sites within the precursor molecule of the gonococcal IgA1 protease, were readily digested by the enzyme (Pohlner *et al.*, 1987).

The specificity determinants for the ureaplasma IgA1 protease may also be conformational, but a preliminary examination found that one of the synthetic peptides digested by the gonococcal enzyme (peptide B) was not susceptible to digestion by the ureaplasma enzyme. There may be a number of explanations for this, other than the peptide simply not being a suitable substrate for the enzyme. The identification of two peptide peaks from reverse-phase chromatography suggested that synthesis was only partially complete and one peak may represent a truncated version of the peptide. If this had interacted with the active site, digestion of the completed peptide may have been inhibited. For further experiments, it would be essential to purify the synthetic peptide and confirm its composition by amino acid analysis or protein sequencing. Before it is dismissed as a synthetic substrate for *U. urealyticum*, it should also be demonstrated that the peptide is susceptible to digestion by the neisserial IgA1 protease. This would require further enzyme purification than was achieved in this preliminary study and detailed protocols have been described by Plaut (1988) and Halter *et al.*, (1984).

The production of multiple digestion products following incubation of the ureaplasmal IgA1 protease with reduced and alkylated IgAα chains also suggest that substrate recognition may be different for the ureaplasmal enzyme. Using a similar approach, Kilian *et al.*, (1980) established that the *H. influenzae* type 1 enzyme only digested the single hinge-region peptide bond in isolated α chains. It is necessary to confirm the result obtained with *U. urealyticum* and if the N-terminal amino acid sequence of each fragment can be elucidated, the substrate requirements of the IgA1 protease may be defined more clearly. Furthermore,

synthetic peptides based on these sequences could serve as alternative substrates for the ureaplasmal enzyme.

To further clarify the substrate specificity and, more broadly, the function of the IgA1 protease, it would be of interest to establish whether ureaplasma proteins are substrates the enzyme, as has been demonstrated in *N. gonorrhoeae* (Shoberg and Mulks, 1991). Proteins isolated from the single ureaplasma isolate found to be devoid of IgA1 protease activity (Robertson *et al.*, 1984) may be of use in investigating a role for the enzyme in protein-processing. It is not clear whether autocatalytic cleavage of the IgA1 protease occurs during processing in *U. urealyticum*, since the enzyme produced by this organism appears to be cell-associated (discussed in Chapter 3).

An examination of IgA1 digestion products from all fourteen serotypes of *U. urealyticum* suggested that the substrate specificity for each enzyme was identical to that established for serotype 8. Although Fabα and Fcα fragments differing by only a few amino acids by are clearly identified by SDS-PAGE, observed migration patterns may nevertheless be misleading. For example, the action of streptococcal glycosidases on hinge region carbohydrate has produced distinct IgA1 degradation patterns from IgA1 proteases with identical substrate specificity (Reinholdt *et al.*, 1990). The precise cleavage sites of ureaplasma serotypes will therefore only be confirmed following N-terminal amino acid sequencing of individual digestion products. From these preliminary studies, however, it appears that *U. urealyticum* is unlike *H. influenzae*, *N. gonorrhoeae* and *N. meningitidis* where two IgA1 protease specificity types have been identified (Section 1.7.4).

The different levels of IgA digestion products obtained from apparently identical ureaplasma cell preparations (in terms of protein concentration) suggested that some serotypes express higher levels of IgA1 protease activity than others. This may have been due to experimental design rather than real differences in serotype activity, since the estimation of ureaplasma cell concentrations did not take into account the levels of medium-protein contamination present in each serotype preparation. Although each serotype was grown to the same titre, this

method does not accurately determine the number of live ureaplasma cells used in each assay.

Quantitative differences in IgA1 protease production both within and between other bacterial species have generally not been reported. Male, (1979) found that the *S. pneumoniae* IgA1 protease was more active than its *H. influenzae* counterpart and suggested that this may have been due to exposure of the IgA1 cleavage site by pneumococcal glycosidases. The effect of differential anti-IgA1 protease antibody activity in the S-IgA preparation was not taken into account in this study, however. For *N. gonorrhoeae*, the disease-associated piliated bacteria have been shown to produce more enzyme per diplococcal unit than non-piliated isolates (Blake and Swanson, 1978), but it was stressed by these workers that the quantitation of bacterial numbers per culture was subject to inaccuracy. As far as the involvement of IgA1 protease in disease is concerned, semi-quantitative estimations have identified no differences in enzyme activity between isolates associated with infected or non-infected sites (Kornfeld and Plaut, 1981). For *U. urealyticum*, it has been established that the enzyme is present in isolates from diseased as well as healthy individuals (Robertson *et al.*, 1984) but quantitative measurements of enzyme activity were not performed in this study. It would be of interest to establish whether the IgA1 protease-negative isolate of *U. urealyticum* has different pathogenic properties from the IgA1 protease-positive organisms; at present there is no definitive correlation between bacterial IgA1 protease expression and virulence although type 2 enzymes show a stronger association with disease than type 1 enzymes (Section 1.7.5).

Using a range of class-specific inhibitors, the IgA1 protease from *U. urealyticum* appeared to be a member of the serine protease class as it was inhibited by the two diagnostic serine protease inhibitors DFP and 2,4-DCI (Bond, 1989). It was not clear why an early demonstration of inhibition by PMSF was later unreproducible. Although PMSF is more unstable and less reactive than DFP, it clearly inhibited trypsin in a standard trypsin assay. The type 2 IgA1 protease from *N. gonorrhoeae* has also demonstrated insensitivity to concentrations of up to 2 mM PMSF (Blake and Swanson, 1978, Simpson *et al.*, 1988) but has been

assigned to the serine-protease class following inhibition with DFP and transition state analogues (peptidyl boronic acids) and by site-directed mutagenesis of the putative active site (Bachovchin *et al.*, 1990, Poulsen *et al.*, 1992). The inhibitory effect of DFP and peptidyl boronic acids on the *H. influenzae* IgA1 protease has also suggested that this enzymes is a serine protease (Bachovchin *et al.*, 1990). Following their sensitivity to metal chelators, reported by some but not by all workers, it has been proposed that these two enzymes may be metal-dependent (Kilian *et al.*, 1983a). The IgA1 protease from *U. urealyticum* was insensitive to EDTA but the effect of BCDS, which according to Kilian *et al.*, (1983a) is inhibitory to all IgA1 proteases, was not examined.

The mechanism of enzyme activity may be defined more clearly if a purified ureaplasma IgA1 protease was available. A pure enzyme could also be used to generate specific antisera for rapid IgA1 protease detection and for comparative immunological studies with other bacterial IgA1 proteases. For these reasons, efforts were concentrated upon purification of the ureaplasma IgA1 protease.

Chapter 3:
Purification of the IgA1 protease

SECTION A
MATERIALS AND METHODS

3.1 THE SOURCE OF *U. UREALYTICUM* IGA1 PROTEASE

3.1.1 Location of IgA1 protease activity in ureaplasma cultures.

Two identical ureaplasma liquid cultures (20 ml) were grown to very late log-phase, as described in Section 2.1.1. A 10 µl aliquot of growth medium from each culture was sampled before and after harvesting. Of the resultant ureaplasma pellets, one was resuspended in PBS to the original culture volume (20 ml) and the second was resuspended in PBS to 1/2000 x original culture volume (10 µl). (This was equivalent to resuspending a 10 l harvest into 5 ml). 5 µl of each sample was tested for IgA1 protease activity, as described in Section 2.1.5.

3.1.2 Concentration of spent medium from ureaplasma growth culture.

To concentrate the spent medium, a 1 ml sample was reduced to 50 µl (20 x concentration) using a microconcentrator (Amicon, P10, 3000 x *g*). The concentrated sample (15 µl) was analysed for IgA1 protease activity by incubation with S-IgA and immunoblotting, as described in Sections 2.1.5 and 2.1.9.

3.1.3 Fractionation of spent medium by ammonium sulphate precipitation.

Solid ammonium sulphate was slowly added to ureaplasma culture supernatants (50 ml, 4 °C) to give a 20% saturated solution (Harris and Angal, 1989). After gentle stirring (30 min., 4 °C), precipitated proteins were collected by centrifugation (10,000 x *g*, 4 °C) and resuspended in a minimum volume of distilled water (1 ml). The ammonium sulphate concentration in the remaining supernatant was increased to 30% saturation and the process repeated at 10% concentration intervals until precipitates from a range of concentrations (20%-100% saturation) were obtained. These were dialysed extensively with PBS (4 °C) and 30 µl samples assayed for IgA1 protease activity by immunoblotting or autoradiography, as described in Sections 2.1.9 and 2.1.10.

3.1.4 Solubilisation of IgA1 protease from ureaplasma cell harvests

A number of different solubilisation buffers (Table 3.1, page 144) were analysed for their ability to release the IgA1 protease from ureaplasma cell harvests. HEPES buffer (N-[2-hydroxyethyl] piperazine-N'-[2-ethane sulphonic acid]), NP40 (Nonidet-P40), Tween 20 (polyoxyethylene sorbitan monolaurate), CHAPS (3-[(3-cholamicopropyl) dimethyl ammonia]-1-propane sulphonate), Brij 35, Brij 78 and W1 (polyoxylene ether) were obtained from Sigma. Triton X-100 (TX-100) was from National Diagnostics, SDS (sodium dodecyl sulphate) from BDH and Zw3-12 (Zwittergent) from Calbiochem.

For a general assessment of each solubilisation buffer (Table 3.1A), ureaplasma cell suspensions in PBS (prepared as described in Section 2.1.1) were divided into 100 µl aliquots and harvested by centrifugation (50,000 x *g*, 10 min, 4 °C). The pellets were thoroughly resuspended in 100 µl ice-cold solubilisation buffer and placed on ice (30 min). The solubilised extract was collected by centrifugation and 10 µl aliquots were assayed for IgA1 protease activity, by incubation with IgA and immunoblotting, as described in Sections 2.1.5 and 2.1.9.

To examine the effect of repeated solubilisation steps, 100 µl aliquots of ureaplasma cell suspensions in PBS were solubilised as above. After 30 min on ice, a 10 µl aliquot (representing the original sample) was removed and the remaining suspension was separated by centrifugation (50,000 x *g*, 10 min, 4 °C). The supernatant, which contained solubilised ureaplasma cell protein (Sup. 1), was decanted and the insoluble pellet resuspended in 90 µl solubilisation buffer. A 10 µl sample (representing pellet 1) was removed and following centrifugation, as before, the resolubilised cellular material (Sup. 2) and the remaining pellet (which had been resuspended in 80 µl buffer), were collected. The solubilised extracts and resuspended pellets (10 µl) were assayed for IgA1 protease activity by incubation with S-IgA and immunoblotting, as described in Sections 2.1.5 and 2.1.9. Crude ureaplasma suspensions in PBS alone were included as controls for each immunoblotting assay.

3.1.5 Inhibition of IgA1 protease activity using specific antisera

As a preliminary to purification experiments, a number of uncharacterised monoclonal antibodies, produced from three separate hybridoma fusions and a polyclonal antiserum, all raised against *U. urealyticum* serotype 8 (Precious *et al.*, 1987) were tested for their ability to inhibit IgA1 protease activity. Ureaplasma cellular suspensions (5 µl, prepared as in Section 2.1.1) were pre-incubated (37 °C, 30 min) with each monoclonal antibody (1/250 dilution in PBS) or a polyclonal antisera (1/250 dilution in PBS) prior to incubation with S-IgA and analysis of digestion products by immunoblotting, as described in Section 2.1.5 and 2.1.9.

3.2 PURIFICATION PROTOCOLS

3.2.1 Pilot studies

The purification studies were performed using a Pharmacia FPLC system, as detailed in the manufacturers handbook (FPLC portfolio, Pharmacia). All buffers were filtered before use with 0.2 µm membranes (Anachem).

In initial pilot studies, 125 µl of ureaplasma cell suspension (representing 250 ml late log-phase culture; ~0.6 mg protein, prepared as described in Section 2.1.1) was solubilised twice (30 min, 4 °C) in 200 µl Buffer A (50 mM Tris-HCl, pH 8.0: 0.05% NP40) as described in Section 2.2.4. The resulting supernatants were pooled and filtered using 0.2 µm membranes (Anachem).

A 9 µl aliquot was removed and stored as a pre-load and 300 µl of the remainder (or the equivalent of 250 µg solubilised ureaplasma protein) was loaded on to the purification column, as described below.

a) Ion exchange chromatography

Separation by ion exchange chromatography utilised a Mono Q (anion) or Mono S (cation) column equilibrated with 50 mM Tris-HCl, pH 8.0:0.05% (v/v) NP40 (Buffer A). Proteins were selectively eluted (1 ml.min.$^{-1}$) with NaCl by increasing the concentration of Buffer B (50 mM Tris-HCl, pH 8.0:0.05% (v/v) NP40, 1 M NaCl), by a rate controlled by programme 1 or 2 (see below). The

eluted proteins were measured spectrophotometrically (280 nm) and collected as 1 ml fractions, until the recorded elution profile had reached the initial base line.

Elution programme for ion exchange chromatography:

%B	Programme 1 (steep)	Programme 2 (shallow)
0	5 min	5 min
50	15 min	35 min
100	20 min	40 min
100	25 min	45 min
0	25 min	45 min
0	30 min	50 min

b) Gel filtration chromatography

In gel filtration chromatography, proteins were separated at 0.5 $ml.min^{-1}$ on a Superose 6 column equilibrated with 50 mM Tris-HCl, pH 8.0: 0.05% (v/v) NP40. As before, eluted proteins were detected spectrophotometrically at 280 nm and 1 ml fractions were collected.

c) Identifying IgA1 protease activity

To identify IgA1 protease activity, 30µl of each fraction or a suitable volume of preload sample (~10 µl) were incubated with 4 µl S-IgA (4 $mg.ml^{-1}$, 16 h, 37 °C). Digestion products were detected by SDS-PAGE and immunoblotting, as described in Section 2.1.6 and 2.1.9. The volume of preload sample was chosen to approximate the dilution of IgA1 protease enzyme that was produced by the separation step. For example, if the enzyme was loaded onto the column in 300 µl and eluted in a single fraction of 1000 µl, assuming 100% recovery, 30 µl of the fraction should have contained the same level of enzyme activity as 9 µl preload sample. The preload sample thereby enabled a qualitative assessment of the levels of enzyme activity maintained in the purification process. The protein content of active fractions was examined by SDS-PAGE followed by PAGE-blue 83 or silver-

staining. Where necessary, the fractions were concentrated by freeze-drying or precipitation in a final concentration of 10% (w/v) trichloroacetic acid (TCA).

3.2.2 Two-step analyses

When two purification columns were used in succession, 1.0 ml ureaplasma harvest (representing 2 l log-phase culture, ~10 mg protein) was solubilised twice in 1.5 ml Buffer A. The solubilised preparations were filtered (0.2 µm nylon filters, Anachem) prior to loading onto an ion exchange column, as above. Active fractions were identified (as above), combined and concentrated to a volume of 200 µl using a microconcentrator (Amicon P10, 3000 x *g*) and loaded on to the gel filtration column, as above.

SECTION B
RESULTS

3.3 SOURCE OF *U. UREALYTICUM* IGA1 PROTEASE

The bacterial IgA1 proteases purified to date have been isolated from spent culture medium. As a preliminary to *U. urealyticum* IgA1 protease purification experiments, it was important to establish whether the spent culture medium was a more abundant source of enzyme than harvested ureaplasma cells.

3.3.1 The cellular location of the IgA1 protease

There was no evidence of extracellular IgA1 protease activity in the 'spent' medium from ureaplasma liquid cultures following incubation of an aliquot of 'spent' medium with S-IgA and analysis of digestion products by SDS-PAGE and immunoblotting (Fig. 3.1, lane 3). Furthermore, no activity was detected in the log-phase culture medium prior to harvest (lane 1), presumably as a consequence of dilution. Following centrifugation and resuspension of ureaplasma cells in PBS at 1/2000 original culture volume, a cell-associated activity was clearly identified (lane 2). In addition, when the ureaplasma cells were subsequently resuspended to their original volume in PBS (lane 4), enzyme activity was again undetectable by this method, confirming that dilution of cells was indeed a consideration. Thus, the failure to identify any extracellular IgA1 protease activity in ureaplasma 'spent' culture medium may simply be due to the enzyme being present at too low a concentration to be detected by this assay.

Following the method outlined by Kilian *et al.*, (1984), 'spent' medium was concentrated x 20 by positive pressure ultrafiltration using a microconcentrator (Amicon P10) and assayed for IgA1 protease activity, as above. The presence of a large number of medium components in the concentrated samples so distorted the electropherograms that detection of intact IgA and any specific digestion products was not possible. Because of this, immunoelectrophoresis was not attempted. The masking effect of the medium

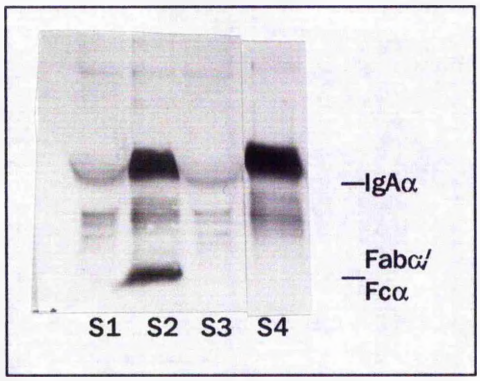


Fig 3.1 Localisation of IgA1 protease activity in *U. urealyticum* liquid cultures. Two identical ureaplasma cultures (20 ml) were grown to log-phase, as described in Section 2.1.1. 10 µl aliquots of growth medium were sampled before (S1) and after (S3) harvesting of ureaplasma cells. The resultant pellets were resuspended in either 10 µl or 20 ml of PBS and 10 µl aliquots of each were sampled (S2 and S4 respectively). The samples were analysed for IgA1 protease activity by incubation with S-IgA and immunoblotting, as described in Section 2.1.9.

proteins could not be overcome by replacing S-IgA with [^{125}I] IgA1 and examining for intact substrate and digestion products by autoradiography.

In an attempt to reduce the contamination by medium proteins, ureaplasma spent medium was fractionated by ammonium sulphate precipitation. Although there was no evidence of IgA1 protease activity in any of the ammonium sulphate fractions (Fig. 3.2A), it was possible that this was again due medium protein interference with the detection system, since the IgAα chain was masked in the immunoblot. The addition of crude IgA1 protease preparations to each sample in the assay indicated that even if digestion products are present, in some fractions (30%-60%) these will be masked by the high concentration of contaminating medium proteins (Fig. 3.2b). It was therefore not possible to determine by these methods whether *U. urealyticum* produced an extracellular IgA1 protease. It was clear, however, that 'spent' culture medium did not contain large quantities of IgA1 protease activity and for the purposes of enzyme purification, cell harvests represented the most abundant source of *U. urealyticum* IgA1 protease enzyme.

3.3.2 Solubilisation of the IgA1 protease

Prior to purification studies, it was necessary to solubilise the cell-associated ureaplasmal enzyme. Of the large number of solubilisation systems that were examined (Table 3.1), it was found that the non-ionic detergents such as Tween 20, NP40 and TX-100, at concentrations as low as 0.05% (v/v), were best able to solubilise and maintain ureaplasma IgA1 protease activity. The detergents were effective in a variety of different buffers (Tris, PBS, HEPES), although for some detergents (Tween, NP40), the presence of 1 M NaCl appeared to marginally reduce the level of enzyme activity in the solubilised preparation. For all the systems examined, a proportion of the IgA1 protease enzyme remained cell-associated after the first round of solubilisation (Fig. 3.3, Table 3.1B). Repeated detergent treatment was able to solubilise a portion but not all of this cell-associated activity. By measuring protein concentrations, it was estimated that a

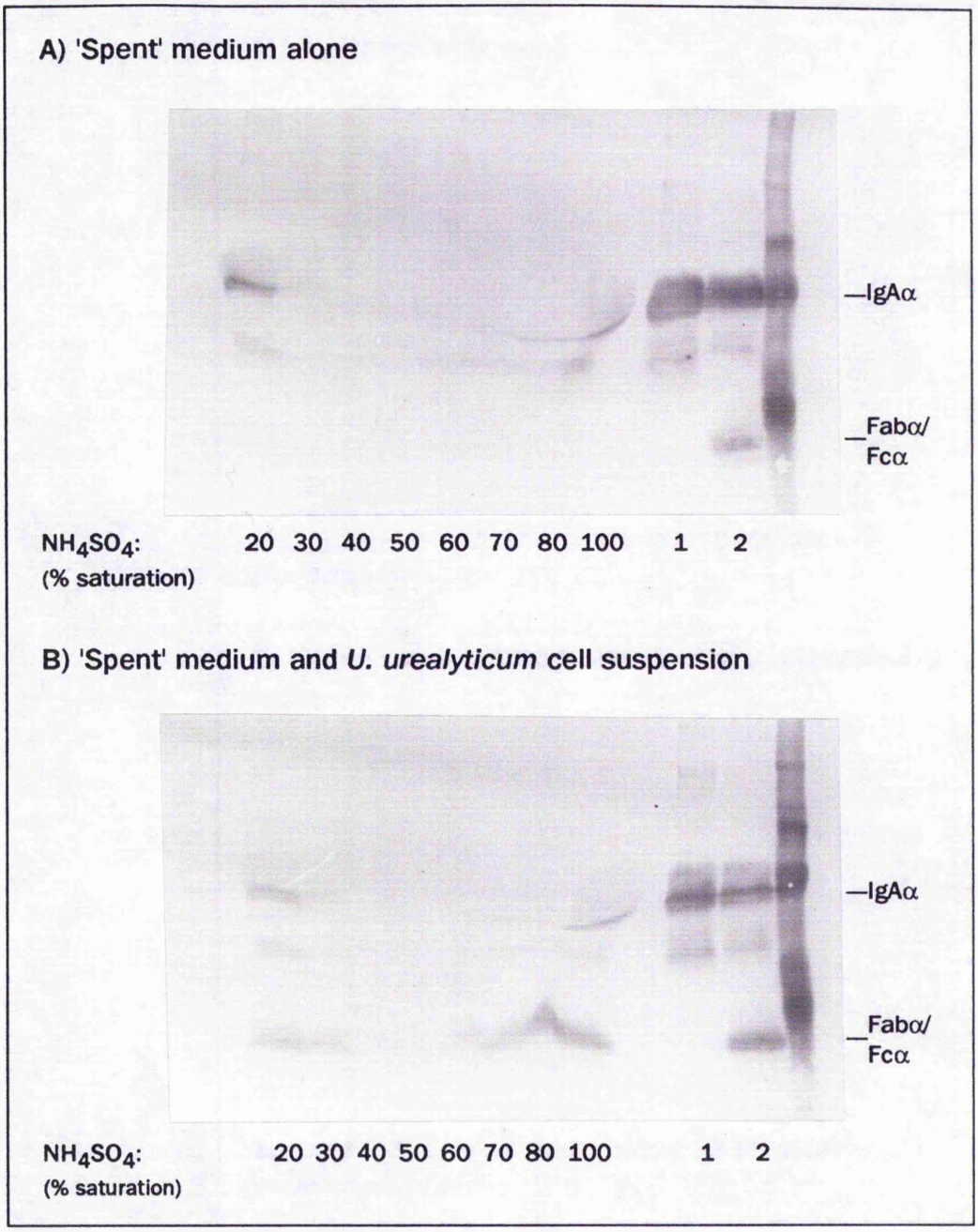


Fig. 3.2 Fractionation of *U. urealyticum* 'spent' medium by ammonium sulphate precipitation.

a) *U. urealyticum* spent medium was precipitated by increasing concentrations of ammonium sulphate (20%-100%, saturation), as described in Section 3.1.3. Each fraction was dialysed extensively with PBS (4 °C) and 30 µl samples assayed for IgA1 protease activity by incubation with S-IgA and immunoblotting.

b) To examine the effect of concentrated spent medium proteins on the detection of IgA digestion products, ureaplasma cell suspensions (5 µl) were included in each incubation reaction and the digests were analysed by immunoblotting. Reactions containing IgA alone (1) and IgA +% µl ureaplasma cell suspensions (2) were included as controls.

Table 3.1 Solubilisation of *U. urealyticum* IgA1 protease

A)

Solubilisation buffer	IgA1 protease activity in supernatant	Solubilisation buffer	IgA1 protease activity in supernatant
H_2O	-	PBS: 0.5% CHAPS	++++ (34%)
PBS	+ (9%)	PBS: 0.5% CHAPS: 0.5 M NaCl	++++ (43%)
PBS: 0.5 M NaCl	+ (12%)	PBS: 0.5 % Zw3-12	- (34%)
PBS: 0.05% NP40	++++	PBS: 0.5 % Zw3-12: 0.5 M NaCl	- (33%)
PBS: 0.1% NP40	++++	PBS: 0.5% SDS	-
PBS: 0.5% NP40	++++ (25%)	PBS: 0.5% SDS: 0.5M NaCl	-
PBS: 0.5% NP40: 0.5 M NaCl	+++ (30%)	50 mM HEPES, pH 7.5	+
PBS: 0.5% TX-100	++++ (25%)	50 mM HEPES: 0.5 M NaCl	+
PBS: 0.5% TX-100: 0.5 M NaCl	++++ (30%)	50 mM HEPES: 0.5% NP40	++++
PBS: 0.5% Tween 20	++++ (28%)	50 mM HEPES: 1 M NaCl	+
PBS: 0.5% Tween 20: 0.5 M NaCl	+++ (39%)	50 mM HEPES: 0.5 M NaCl: 0.5% NP40	++++
PBS: 0.5% BRIJ 35	++ (19%)	50 mM Tris-HCl, pH 8: 0.5% NP40	++++
PBS: 0.5% BRIJ 78	+++ (23%)	50 mM Tris-HCl, pH 8: 0.5% NP40: 0.1 M NaCl	++++
PBS: 0.5% W1	++ (20%)	15 mM Tris-HCl, pH 7.5: 0.5% NP40: 1 M NaCl	++++

(* =% of total protein solubilised, calculated by D. Smith, University of St. Andrews).

B)

	IgA1 protease activity				
Solubilisation buffer	Original sample	Sup. 1	Pellet 1	Sup. 2	Pellet 2
PBS	++++	+	++++	+	+++
PBS: 0.05% NP40	++++	++++	++++	++++	++++
PBS: 0.1% NP40	++++	++++	++++	++++	++++
PBS: 0.5% NP40	++++	++++	++++	++++	+++
PBS: 0.5% BRIJ 35	++++	++	++++	+	+++
PBS: 0.5% BRIJ 78	+++	+++	+++	+	++++
PBS: 0.5% W1	+++	++	+++	+	+++
50 mM HEPES, pH 7.5	++++	+	++++	-	++++
50 mM HEPES: 0.5 M NaCl	++++	+	++++	+	++++
50 mM HEPES: 1 M NaCl	++++	+	++++	+	++++
50 mM HEPES: 0.5 M NaCl: 0.5% NP40	++++	++++	++++	++++	++++
50 mM Tris-HCl, pH 8: 0.5% NP40	++++	++++	++++	+++	++++
50 mM Tris-HCl, pH 8: 0.5% NP40: 100 mM NaCl	++++	++++	++++	+++	++++
15 mM Tris-HCl, pH 7.5: 0.5% NP40: 1 M NaCl	++++	++++	++++	+++	++++

Fig. 3.3

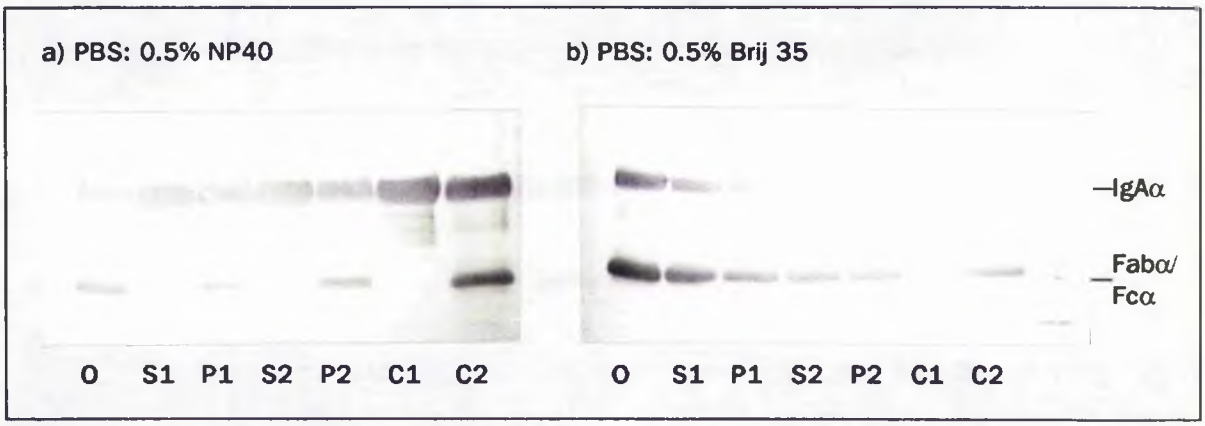


Fig. 3.4

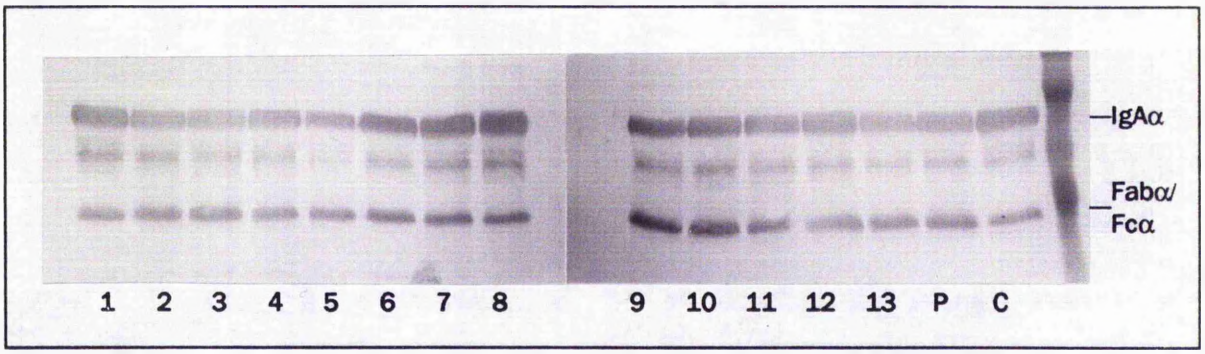


Fig. 3.3 Solubilisation of *U. urealyticum* IgA1 protease with PBS: 0.5% NP40 and PBS: 0.5% Brij 35.

Ureaplasma cellular suspensions were solubilised (2 x) with PBS: 0.5% NP40 and 0.5% Brij 35, as described in Section 3.1.4. 10 µl samples of the original solubilised preparation (O), protein-containing supernatents (S1, S2) and the remaining pellets (P1, P2) were examined for IgA1 protease activity by incubation with S-IgA followed by immunoblotting, as described in Sections 2.1.5 and 2.1.9. Incubations containing IgA alone (C1) and 5 µl of the original cellular harvest (C2) were included as controls.

Fig. 3.4 The effect of anti-*U. urealyticum* serotype 8 antibodies on IgA1 protease activity.

Cellular ureaplasma suspensions (5 µl) were pre-incubated with a range of uncharacterised monoclonal antibodies (1-13) and a polyclonal antisera (P), originally raised against *U. urealyticum* Serotype 8, as described in Section 3.1.5. The level of inhibition was estimated by incubation with S-IgA followed by immunoblotting, as described in Sections 2.1.5 and 2.1.9. Incubation in the absence of antiserum (C) was included as a control.

maximum of 40% ureaplasma protein was solubilised by each treatment (Table 3.1A). While sonication (3 x 10 sec) of ureaplasma suspensions in PBS alone resulted in a degree of enzyme release, in detergent-solubilised preparations, sonication did not appear to increase the overall level of enzyme activity. Likewise, repeated freeze-thawing (10 x) of the cellular suspension was not as effective in enzyme release as detergent treatment (data not shown). These results appear to give more evidence that the IgA1 protease in *U. urealyticum* may be membrane-associated.

3.3.3 Inhibition of IgA1 protease activity using specific antisera.

The monoclonal antibodies used were specific for *U. urealyticum* proteins by ELISA but are described as 'uncharacterised' since they have failed to identify specific ureaplasma proteins by Western blotting (experiments performed by B. Precious and A. Myles, University of St. Andrews). None of the monoclonal antibodies tested inhibited IgA1 protease activity, which suggested that none were specific for the active site of the enzyme (Fig. 3.4). Furthermore, no inhibition was seen with an anti-*U. urealyticum* rabbit polyclonal antisera, at dilutions as low as 1/250 in PBS.

In these examinations, a number of physical properties of the solubilised IgA1 protease were established which were important for the design of purification experiments. As mentioned previously, the enzyme was stable in a large number of detergent and buffer systems. It appeared to be resistant to repeated freeze-thawing and remained stable at 4 °C or 20 °C for over 8 h. Enzyme activity was not reduced by filtration (0.2 µm filter, Anachem) and could be detected by the immunoblotting assay at dilutions of up to 1/120 in PBS.

3.4 PURIFICATION PROTOCOLS

3.4.2 Pilot studies

To purify the ureaplasma IgA1 protease, a number of pilot studies were used to establish which chromatographic techniques allowed fractionation and

recovery of the enzyme. In subsequent experiments, the level of purification achieved by a series of these separation steps was examined.

a) Anion exchange chromatography

Separation by anion exchange chromatography (Mono Q) demonstrated that in 50 mM Tris-HCl, pH 8.6, the ureaplasma IgA1 protease bound to the Mono Q column and was eluted by a salt concentration of approximately 0.38 M NaCl. (Fig. 3.5). The activity of the eluted enzyme in 30 µl of the 1 ml fraction (where it had been diluted ~1:3.3 by volume) was substantially less than in 9 µl of the preload sample. This suggested that a proportion of the enzyme activity had been lost in the single purification step. As the IgA1 protease had been solubilised from ureaplasma cells using 0.05% (v/v) NP40, this non-ionic detergent was included at the same concentration in the equilibrating and eluting buffers.

Fig. 3.6 demonstrates that 0.05% (v/v) NP40 appeared to stabilise the enzyme and the total amount of IgA1 protease activity found in 30 µl of fraction 11 and fraction 12 (each 1 ml) was almost identical to that found in the 9 µl preload. In subsequent purification steps, 0.05% (v/v) NP40 was included in all the chromatography buffers. The absorbance properties of NP40 at 280 nm, however, produced erratic elution profiles which in some cases prevented the identification of peaks corresponding to IgA1 protease activity.

An examination of the selective elution of the IgA1 protease enzyme from the Mono Q column using a shallower salt gradient (from 0 M NaCl to 0.5 M NaCl in 30 min rather than 10 min) highlighted this problem (Fig. 3.7). As the concentration of Buffer B was raised, the base line of the recorder trace increased at the same rate. Although the equilibration and elution buffers contained the same concentration of NP40 (0.05%, [v/v]) it was clear that small differences in detergent concentration were producing large differences in absorbance readings at this wavelength. If the solubilisation, equilibration and elution buffers were derived from the same original stock, this effect was reduced but not completely eliminated.

It is clear from Fig. 3.7 that the shallower elution gradient improved the separation of proteins by anion exchange chromatography but the level of IgA1

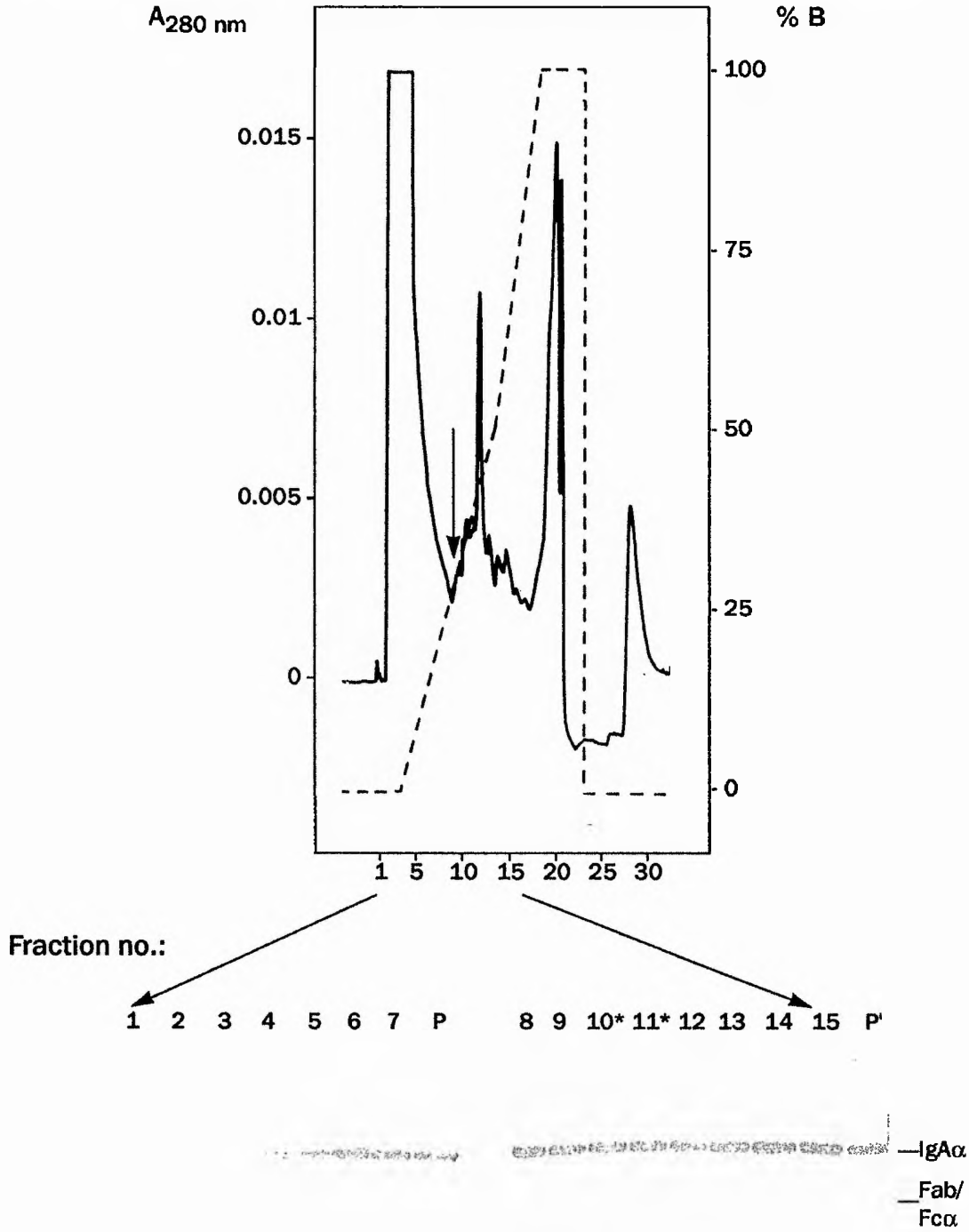


Fig. 3.5 Anion exchange chromatography of *U. urealyticum* IgA1 protease
Ureaplasma proteins were solubilised in Buffer A (50 mM Tris-HCl, pH 8.6: 0.05% NP40), as described in Section 3.1.4. 300 µl (~250 µg) were separated by anion exchange chromatography (1 $ml.min^{-1}$) using a steep gradient of 1 M NaCl in Buffer A (0-0.5 M in 10 min, 0.5-1 M in 5 min). Eluted proteins were measured spectrophotometrically at 280 nm (FSD 0.02) and collected as 1 ml fractions. To identify IgA1 protease activity, 30 µl samples and 9 µl preload (P, P' represents preload activity after ultrafiltration) were incubated with S-IgA and analysed for digestion products by immunoblotting, as described in Section 2.1.9. The arrow and asterisks indicate the position of active fractions.

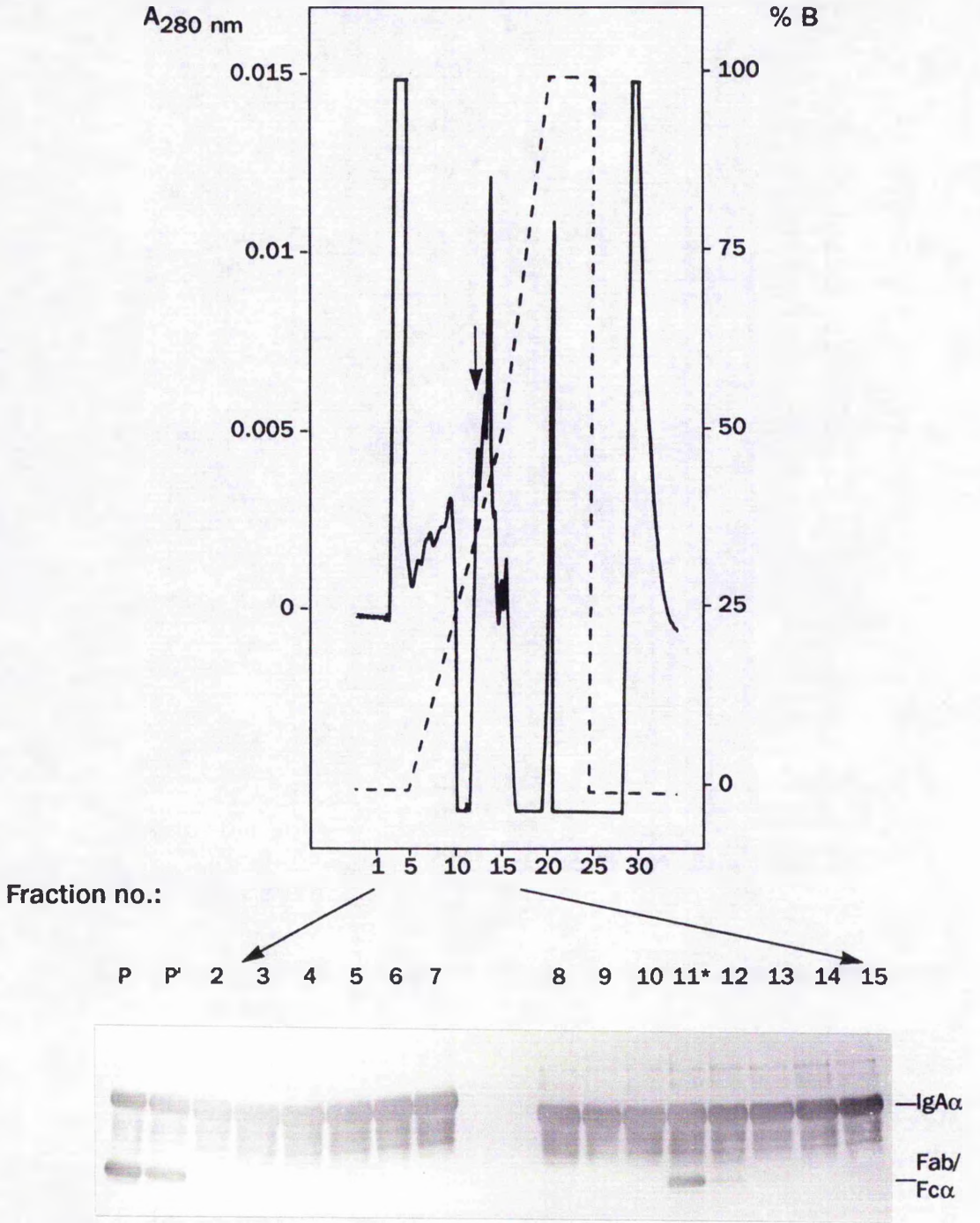


Fig. 3.6 Anion exchange chromatography of *U. urealyticum* IgA1 protease using 0.05% NP40 in the equilibration and elution buffers.

Ureaplasma proteins were solubilised in Buffer A (50 mM Tris-HCl, pH 8.6: 0.05% NP40), as described in Section 3.1.4. 300 µl (~250 µg) were separated by anion exchange chromatography (1 $ml.min^{-1}$) using a steep gradient of 1 M NaCl in Buffer A (0 M-0.5 M in 10 min, 0.5 M-1 M in 5 min). Eluted proteins were measured spectrophotometrically at 280 nm (FSD 0.02) and collected as 1 ml fractions. To identify IgA1 protease activity, 30 µl samples and 9 µl preload (P, P' represents preload activity after ultrafiltration) were incubated with S-IgA and analysed for digestion products by immunoblotting, as described in Section 2.1.9. The arrow and asterisk indicate the position of active fractions.

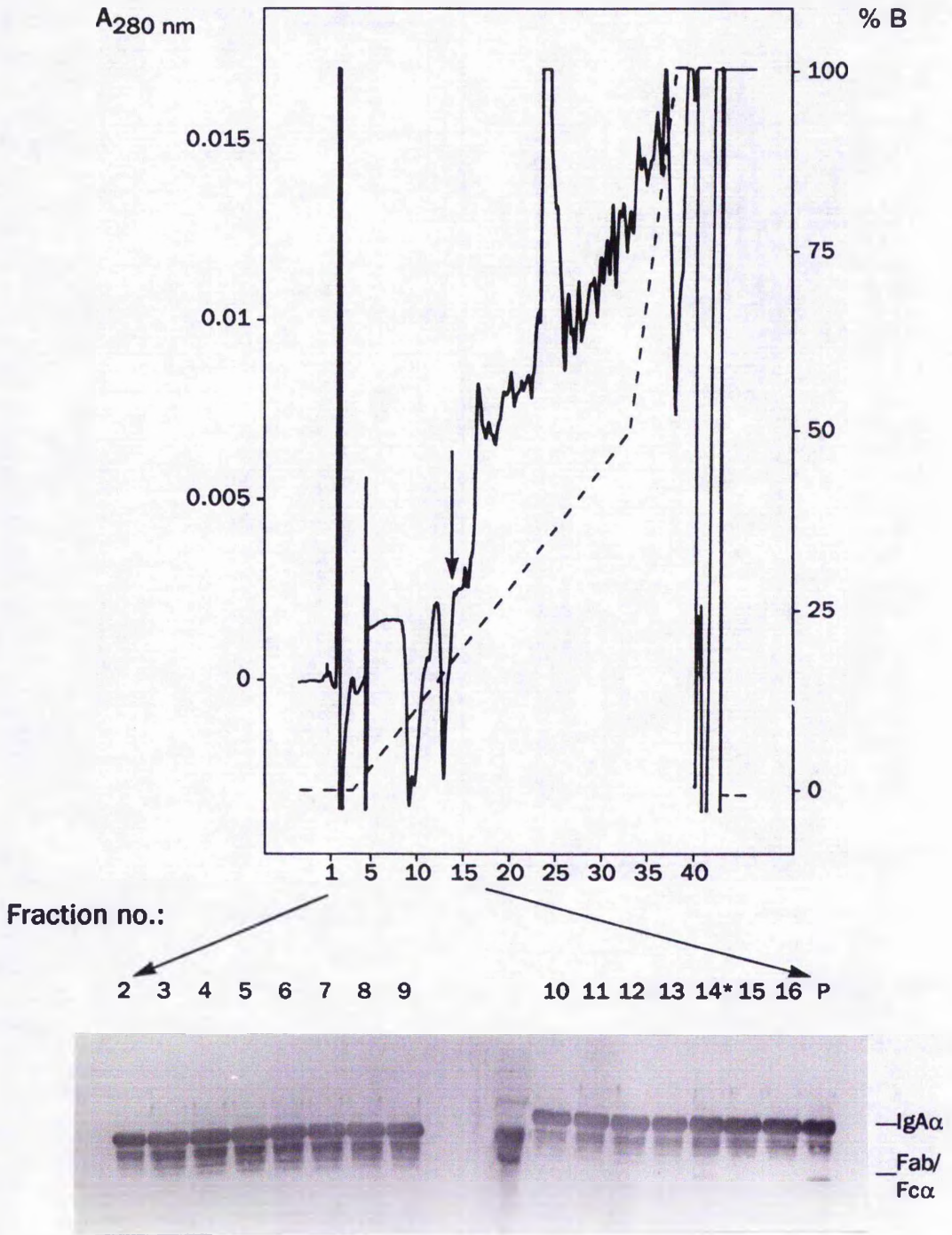


Fig. 3.7 Anion exchange chromatography of *U. urealyticum* IgA1 protease using a shallow gradient of NaCl.

Ureaplasma proteins were solubilised in Buffer A (50 mM Tris-HCl, pH 8.6: 0.05% NP40), as described in Section 3.1.4. 400 µl (250 µg) were separated by anion exchange chromatography (1 $ml.min^{-1}$) using a shallow gradient of 1 M NaCl in Buffer A (0 M-0.5 M in 30 min, 0.5 M-1 M in 5 min). Eluted proteins were measured spectrophotometrically at 280 nm (FSD 0.02) and collected as 1 ml fractions. To identify IgA1 protease activity, 30 µl samples and 12 µl preload (P) were incubated with S-IgA and analysed for digestion products by immunoblotting, as described in Section 2.1.9. The arrow and asterisk indicate the position of active fractions.

protease activity recovered from the Mono Q column was substantially less than when a steeper NaCl gradient had been used (Fig. 3.6). The enzyme had also eluted at approximately 0.28 M NaCl rather than 0.38 M NaCl. It is possible that under these elution conditions, the IgA1 protease was separated from a putative stabilising factor which both conferred a charge to the molecule and was essential for maximum enzyme activity.

b) Gel filtration chromatography

An initial study examining the purification of the IgA1 protease by gel filtration chromatography demonstrated that the ureaplasma enzyme could be recovered from a Superose 6 column (see Fig. 3.8). It was not possible to detect an elution peak corresponding to IgA1 protease activity since the chromatographic trace was apparently off-scale at this point. This phenomenon was found reproducibly and may and have been caused by the NP40-containing buffers. As with ion exchange, enzyme activity was reduced by a single gel filtration step, although in this case it may have been because the activity was distributed between four separate fractions. In addition, the level of enzyme activity was at the limit of the detection system. For this reason, subsequent purification attempts used a higher concentration of solubilised ureaplasma proteins.

3.4.3 Two-step analyses

To examine the purification of the IgA1 protease enzyme by cation-exchange chromatography (Mono S), harvested cells from a 5 l ureaplasma culture were solubilised in 0.05% (w/v) NP40, as described in Section 3.1.4. In initial experiments, the sample was eluted with 50 mM Tris-HCl, pH 8.6, 1 M NaCl, 0.05% (v/v) NP40, the buffer that had been used for anion exchange chromatography. As expected, the IgA1 protease did not bind to the Mono S column under these conditions but eluted in fractions 2, 3, and 4 (Fig. 3.9). This step served to separate the enzyme from a proportion of ureaplasma proteins that bound to the column (not all visible from the recording trace) without removing the enzyme from the putative stabilising factor that appeared to be necessary for maximum enzyme activity. The fractions were pooled, concentrated 30 x by ultrafiltration and

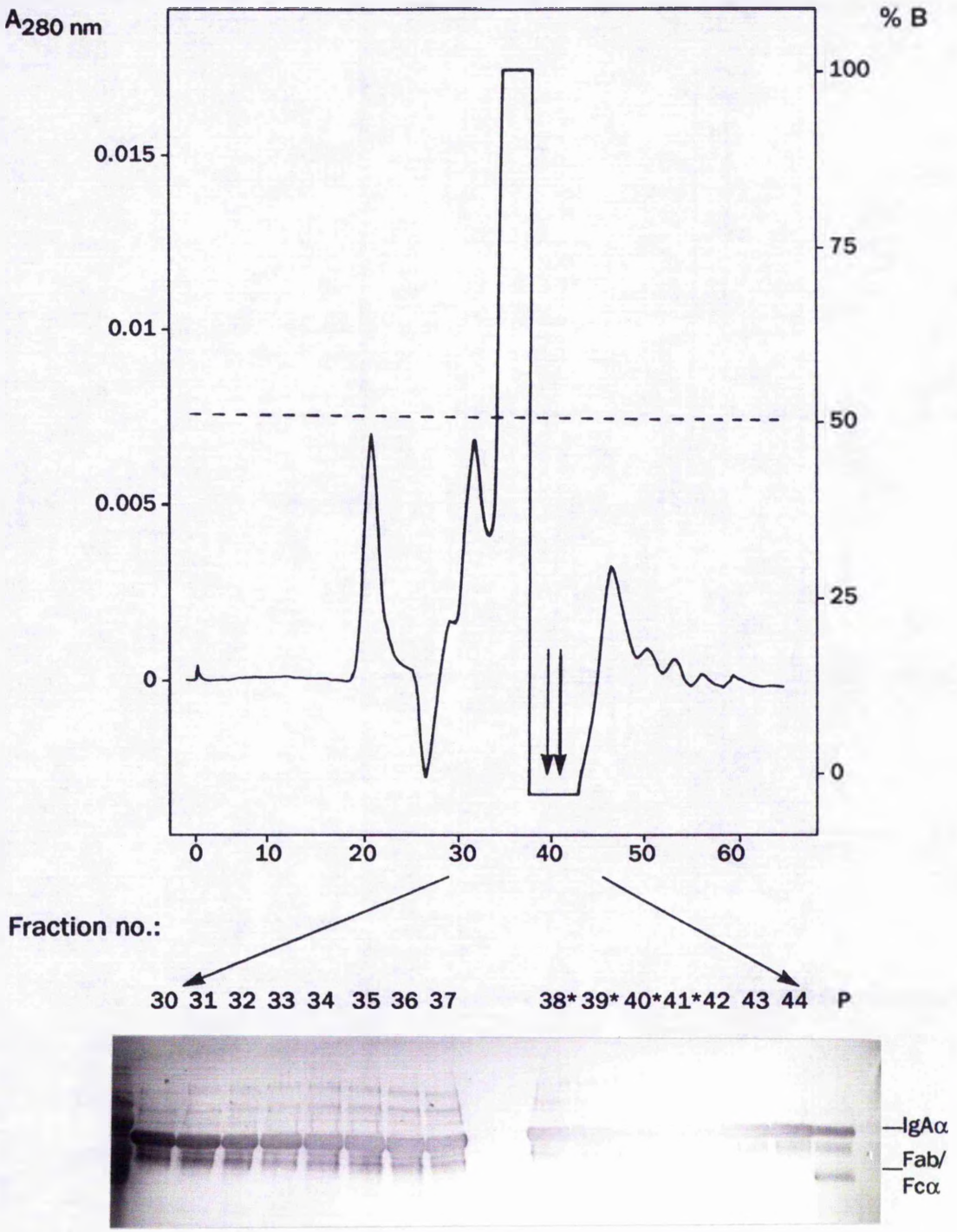


Fig. 3.8 Gel filtration chromatography of *U. urealyticum* IgA1 protease
Ureaplasma proteins were solubilised in Buffer A (50 mM Tris-HCl, pH 8.6: 0.05% NP40), as described in Section 3.1.4. 400 µl (400 µg) were fractionated using a Superose 6 column (0.5 ml.min^{-1}) with Buffer A as the mobile phase. Eluted proteins were measured spectrophotometrically at 280 nm (FSD 0.02) and collected as 0.5 ml fractions. To identify IgA1 protease activity, 30 µl samples and 24 µl preload (P) were incubated with S-IgA and analysed for digestion products by immunoblotting, as described in Section 2.1.9. The asterisks indicate the position of active fractions. Protein peaks corresponding to IgA1 protease activity (arrowed) are not visible, due to negative absorbance reading at this position.

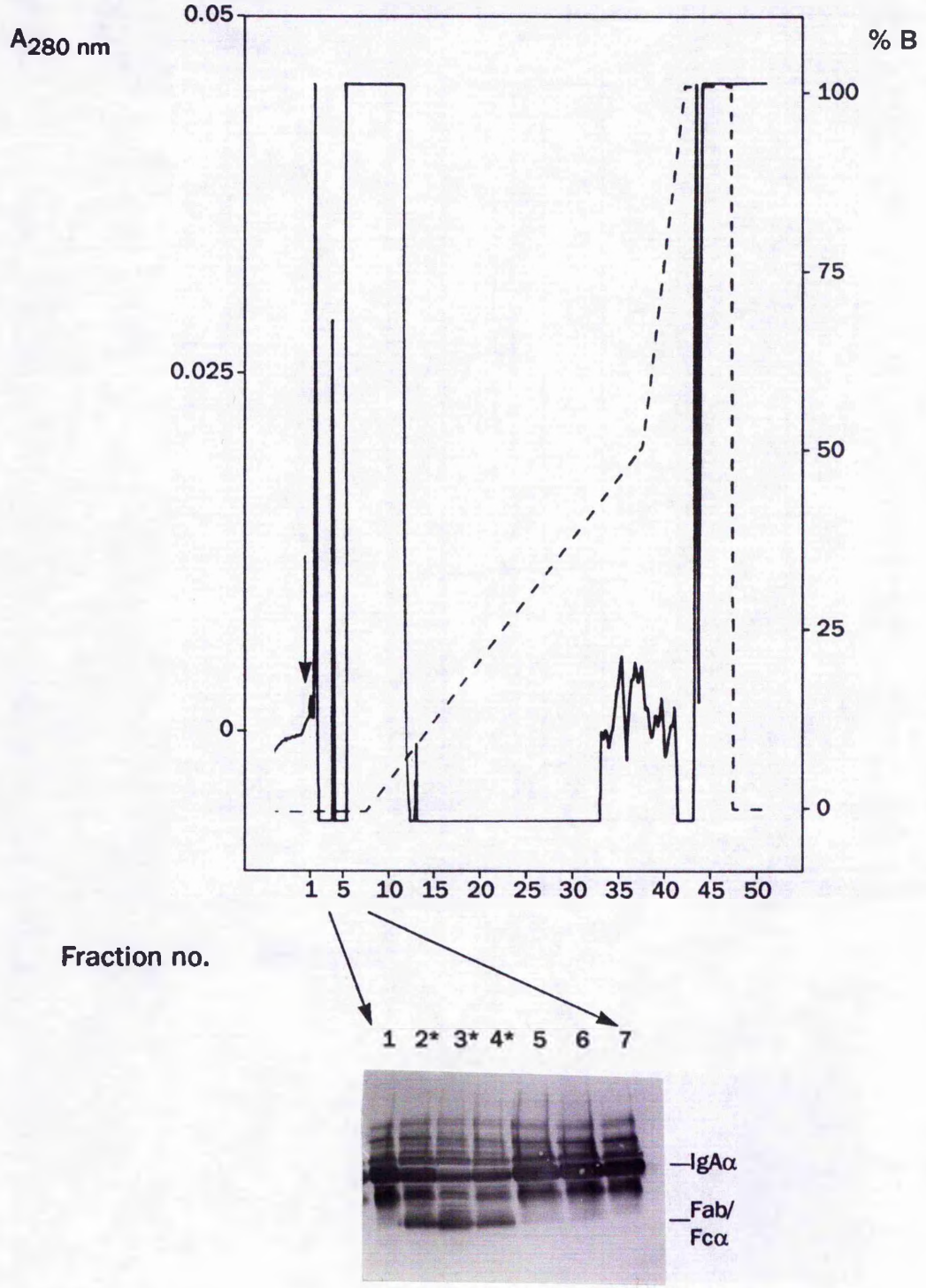


Fig. 3 9 Cation exchange chromatography of *U. urealyticum* IgA1 protease
Ureaplasma proteins were solubilised in Buffer A (50 mM Tris-HCl pH 8.6: 0.05% NP40), as described in Section 3.1.4. 2 ml (1.25 mg) were separated using a Mono S column (1 ml.min^{-1}) with a steep gradient of 1 M NaCl in Buffer A (0 M-0.5 M in 10 min, 0.5 M-1 M in 5 min). Eluted proteins were measured spectrophotometrically at 280 nm (FSD 0.05) and collected as 1 ml fractions. To identify IgA1 protease activity, 30 µl samples were incubated with S-IgA and analysed for digestion products by immunoblotting, as described in Section 2.1.9. The arrow and asterisks indicate the position of active fractions.

separated by gel filtration using the Superose 6 column. The IgA1 protease activity eluted in fractions 18 and 19 but when their activity was compared to the pre-load sample, only a small proportion of enzyme activity appeared to have been lost by this separation (Fig. 3.10). When these fractions were concentrated by TCA (10% [w/v]) precipitation and examined by SDS-PAGE, however, it was clear that the level of purification achieved had been minimal (Fig. 3.11).

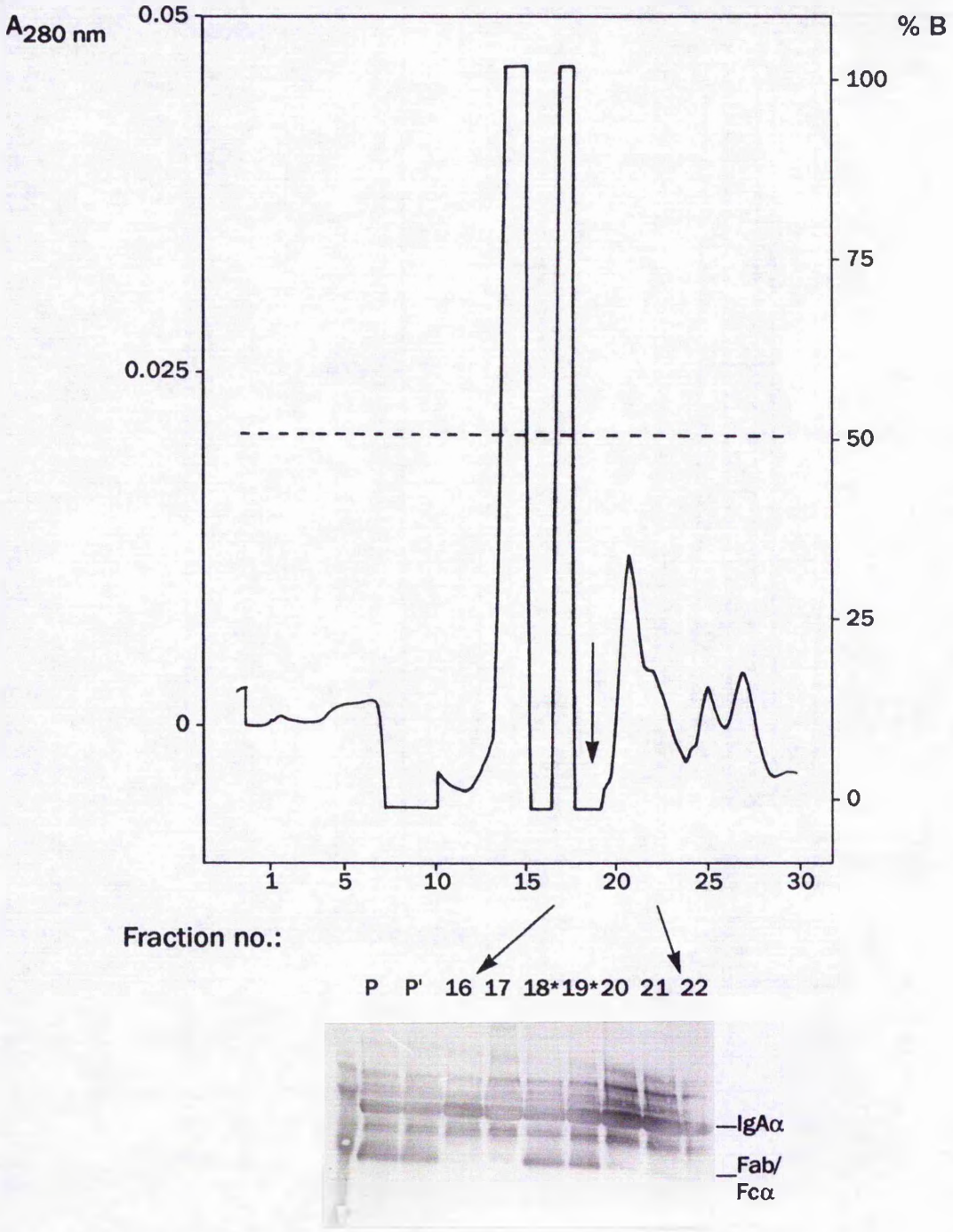


Fig. 3.10 Gel filtration chromatography of *U. urealyticum* IgA1 protease following elution from cation exchange column.

IgA1 protease-active fractions from cation exchange chromatography were pooled and concentrated by ultrafiltration. 200 µl were separated using a Superose 6 column (0.5 ml.min^{-1}) with Buffer A (50 mM Tris-HCl, pH 8.6: 0.05% NP40) as the mobile phase. Eluted proteins were measured spectrophotometrically at 280 nm (FSD 0.05) and collected as 1 ml fractions. To identify IgA1 protease activity, 30 µl samples and 6 µl preload (P, P' represents preload activity after ultrafiltration) were incubated with S-IgA and analysed for digestion products by immunoblotting, as described in Section 2.1.9. The asterisks indicate the position of active fractions. Protein peaks corresponding to IgA1 protease activity (arrowed) are not visible.

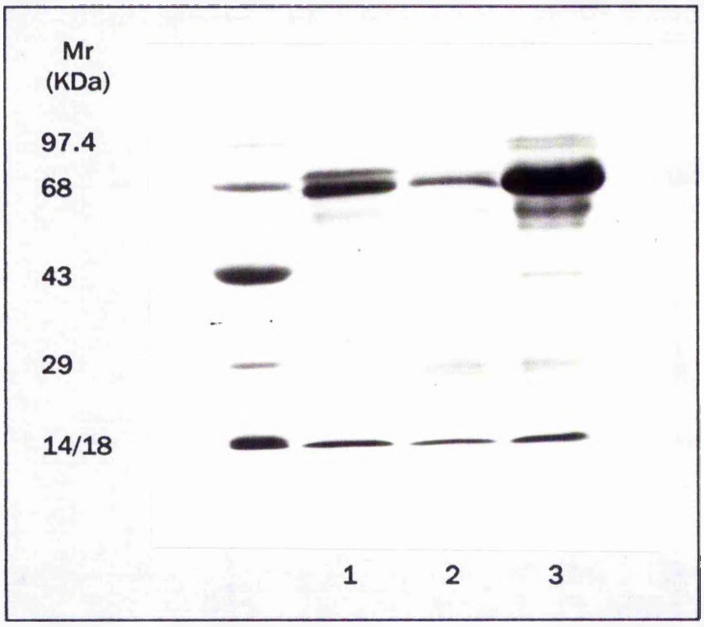


Fig. 3.11 The protein content of IgA1 protease-active fractions from cation exchange and gel filtration chromatography.

IgA1-protease active fractions (650 µl) from a cation exchange column (lane 1) and a gel filtration column (fractions 18 and 19, lane 2 and 3) were concentrated by TCA precipitation (10%[w/v]) and analysed by SDS-PAGE (12.5%) followed by staining with PAGE-blue 83, as described in Sections 2.1.6-2.1.8. Molecular weight markers are to the left of the figure

SECTION C
DISCUSSION

Contrary to a single publication reporting extracellular IgA1 protease production by *U. urealyticum* (Kilian *et al.*, 1984), it was not possible to detect the enzyme activity in ureaplasma 'spent' medium. The harvested ureaplasma cells, however, contained relatively high levels of IgA1 protease activity. This was the reverse of the situation found with other bacterial IgA1 proteases. For *S. pneumoniae*, *N. gonorrhoeae* and *S. sanguis*, virtually all the IgA1 protease activity has been isolated from cell-free supernatants and only minimal quantities are associated with the cell pellets (Blake and Swanson, 1978, Plaut *et al.*, 1978b, Mulks, 1985). Mulks (1985) estimated that in *N. gonorrhoeae* type 2, the cell-associated levels were less than 2.5% of the total IgA1 protease activity and Blake and Swanson (1978) reported that IgA1 protease activity in sonicated gonococcal pellets was virtually non-existent. Apart from a recent report identifying a proportion of cell-bound IgA1 protease activity in *H. influenzae* (Pohlner *et al.*, 1991), one of the few unifying features of all bacterial IgA1 proteases is their extracellular location (Kilian and Reinholdt, 1986). It was important therefore to establish whether the enzyme from *U. urealyticum* was also secreted into the extracellular environment.

The inability to detect even cell-bound IgA1 protease activity in ureaplasma liquid cultures prior to harvesting highlighted the importance of dilution effects on IgA1 protease detection. Thus, an extracellular enzyme may have been present in the 'spent' medium but at levels too low to be identified by the assay system used. Concentration of 'spent' medium by ultrafiltration resulted in additional problems, since high levels of serum proteins interfered with the detection of enzyme digestion products by SDS-PAGE, even when using radiolabelled substrate. Differential fractionation of spent medium using ammonium sulphate did not allow detection of IgA1 protease activity since all fractions contained high concentrations of medium proteins and these may have masked Fabα/Fcα digestion products.

For other bacterial IgA1 proteases, such difficulties in identifying cell-free IgA1 protease activity have not been reported, either because the levels of extracellular enzyme are higher in these cases or because bacterial growth medium is not as complex as the ureaplasma growth medium. Horowitz and Gal (1991) have recently described a method of growing *U. urealyticum* in media dialysed-free of serum components. Attempts to cultivate ureaplasmas by such a method in this laboratory have not proved successful, mainly due to contamination problems (D Smith, personal communication). If the nutrients required for ureaplasma growth are able to pass through a semi-permeable dialysis membrane, it may be more convenient to grow *U. urealyticum* on solid media covered by sterile dialysis membranes, as described for the growth of *S. sanguis* (Higerd *et al.*, 1977). Under these conditions, bacterial products of high molecular weight, such as the IgA1 protease are separated from large proteins in the medium agar and the enzyme may then be harvested by rinsing the dialysis membrane into a small volume of buffer and removing bacteria by centrifugation.

Alternatively, SDS-PAGE could be replaced by an assay which incorporates the removal of growth medium components before quantifying the extent of IgA1 digestion. ELISA-based methods (Reinholdt and Kilian, 1983, Blake and Eastby, 1991) and those exploiting IgA receptors on type 4 group A streptococci (Lindahl *et al.*, 1981) allow the digested substrate to be separated from growth medium components by immunological methods.

It would be even more convenient to establish the presence of extracellular IgA1 protease activity directly by selectively removing the enzyme from the 'spent' medium. If a specific antibody was available, the IgA1 protease could have been removed by affinity chromatography or immune precipitation. However, neither a rabbit polyclonal antiserum or a number of previously uncharacterised monoclonal antibodies raised against *U. urealyticum* appeared to react with the IgA1 protease active site, as indicated by their failure to inhibit enzyme activity. This suggests that the active site of the ureaplasma enzyme, or regions associated with the active site, were not immunogenic to the rabbit host. A number of other IgA1 proteases have failed to elicit an immune response in host

animals, even when administered in a partially purified state (Kilian and Reinholdt, 1986, Frandsen *et al.*, 1987). For *S. mitior*, a high titre of neutralising antiserum could only be obtained from a patient with *S. mitior*-associated endocarditis. Patients demonstrating a good serological response to *U. urealyticum* may likewise be a source of anti-ureaplasma IgA1 protease antiserum, but such patients were not available for this study.

An antibody raised against another IgA1 protease with similar substrate specificity or classification, such as the type 2 IgA1 proteases from *Haemophilus* or *Neisseria* could have been used. This approach did not seem feasible, since antibody inhibition tests have suggested that antigenic relationships between IgA1 protease active sites do not exist between genera (Kilian *et al.*, 1983a). However, Pohlner *et al.*, (1991) have recently isolated a monoclonal antibody that reacts with both *H. influenzae* and *N. gonorrhoeae* IgA1 proteases by Western blotting. It would be of value to examine whether this antibody also cross-reacts with the ureaplasma enzyme, since an examination for extracellular enzyme activity by Western blotting may then be possible.

One other consideration is that the ureaplasmal IgA1 protease is released extracellularly but that the soluble enzyme is either degraded by hydrolytic enzymes or that it aggregates in the growth medium and therefore co-pellets with ureaplasma cells upon harvesting. Although problems with extracellular IgA1 protease degradation have generally not been reported by workers in this field, there are two instances where precautions have been taken to reduce the action of degradative enzymes. Lindler and Stutzenberger (1983) incorporated p-chloromercuribenzoate (PCMB) into the growth medium of *S. sanguis* (at 31 °C) to inhibit non-specific streptococcal proteases and Simpson *et al.*, (1988) grew type 1 *N. gonorrhoeae* at 35 °C to reduce cell lysis and increase extracellular enzyme yields. If these cultures were harvested in late log phase, IgA1 protease activity was only found in the cellular fraction, suggesting that the cell-bound enzyme was more resistant to cellular hydrolytic enzymes than its extracellular counterpart (Simpson *et al.*, 1988). If the same is true for the ureaplasmal IgA1 protease,

harvesting of cells in early log-phase or the incorporation of general protease inhibitors may increase the levels of IgA1 protease found in the medium.

Aggregation of extracellular IgA1 proteases has been reported for *H. influenzae* grown in human milk (Plaut *et al.*, 1992). It is thought that S-IgA specific for the *H. influenzae* IgA1 protease causes this effect since it can be abrogated by the addition of an IgA1 protease from *S. sanguis*. Although specific anti-IgA1 proteases are unlikely to be present in the horse serum contained within ureaplasma growth medium, there may be other components that encourage aggregation. The release of the enzyme from cell pellets following detergent treatment, however, suggests that the IgA1 protease is more than loosely associated with ureaplasma cells and may indeed be membrane-bound. The minimal amount of enzyme activity released on cellular sonication may represent membrane fragments too small to be harvested by centrifugation. Isolation of purified ureaplasma membranes, or localisation of enzyme activity using immunogold-labelled specific antibodies, for example, is required before the site of enzyme activity is confirmed.

If none of these approaches reveal extracellular IgA1 protease activity in ureaplasma 'spent' medium, it may be concluded that *U. urealyticum* expresses a cell-associated enzyme only. In this case, the mechanism of transport and processing of the ureaplasma IgA1 protease is likely to be different from that described in *N. gonorrhoeae* (Section 1.8.1). Either a totally different transport mechanism is involved, or the final stage of enzyme release from the transmembrane β portion of the precursor IgA1 protease molecule does not occur in this organism. This could be due to a lack of susceptible cleavage sites in the precursor molecule or because of a different substrate specificity for the ureaplasma enzyme. The apparent failure of the IgA1 protease to digest one of the synthetic peptides based on the autoproteolytic sites found in *N. gonorrhoeae* tentatively supports the latter theory but, as discussed in Chapter 2, this is only one of many explanations for this negative result. To gain insight the IgA1 protease transport mechanisms operating in *U. urealyticum* it may be necessary to sequence and express the *iga* gene.

Analysis of the *iga* gene from *H. influenzae* identified putative leader and helper sequences, analogous to those found in the *N. gonorrhoeae iga* gene (Grundy *et al.*, 1987a) but only one of the predicted autoproteolytic cleavage sites was found to be susceptible to cleavage, suggesting that the mechanism of IgA1 protease secretion in these two species is similar but not identical. In contrast, no such sequence motifs have been identified in the streptococcal IgA1 protease and the mechanism of enzyme secretion in this organism is unclear. It may that a number of accessory genes or proteins are required, as is the case with other gram positive extracellular enzymes (Gilbert *et al.*, 1991).

A cell-bound IgA1 protease may also confer particular advantages to *U. urealyticum* for colonisation and establishment *in vivo*. In addition to interfering with the protective function of S-IgA1, the enzyme may combine with IgA1 on the luminal surface of mucosal cells and aid ureaplasma adherence. It has been proposed that bacterial IgA1 proteases may serve as adhesins Mulks (1985), but such a function only seems relevant for a cell-associated enzyme. Subsequent digestion of IgA1 may of course reduce binding and the relative advantages of adhesion verses IgA1 digestion are not clear. A cell-bound enzyme may also only act upon closely associated ureaplasma-specific secretory IgA1 and digestion of S-IgA specific for neighbouring IgA1 protease-negative microorganisms may not occur. The wide-spread effects of extracellular IgA1 protease activity on the general mucosal flora, as proposed by Kilian and Reinholdt, (1987) (Section 1.10.5), may therefore not be relevant in the case of ureaplasma colonisation.

Whatever the explanation for failing to identify the presence of extracellular IgA1 protease activity in 'spent' culture medium, it was clear that for purification purposes the harvested ureaplasma cells served as a more abundant and convenient source of IgA1 protease activity. To obtain a soluble preparation of the enzyme from ureaplasma cell harvests, however, was a problem not encountered in the purification of other bacterial IgA1 proteases.

Ureaplasma preparations were partially solubilised by a number of ionic and non-ionic detergents but the enzyme was only stable in the latter, as had been found with the gonococcal IgA1 protease (Blake and Swanson, 1978). Using an

excess of solubilisation buffer or repeated re-extractions, it was not possible to solubilise more than 40% of the total ureaplasma protein. This does not necessarily imply that only 40% of the IgA1 protease had been released from the cells, since the solubility of different ureaplasma proteins is likely to vary, but the high levels of enzyme activity remaining in cell pellets after solubilisation suggested that a proportion of the enzyme had indeed remained insoluble. Difficulties in solubilising ureaplasma cells have been found by other workers (D. Thirkell, personal communication) and this may be due to the compacting of cells produced by the high *g* forces used in centrifugation, or to a peculiar, but as yet unknown, membrane structure.

It is well documented that research on *U, urealyticum* is hindered by the low cell yields obtained from large volumes of expensive culture medium (O'Brien and Barile, 1983) and failure to obtain more than 40% of this protein in a soluble form renders purification of individual proteins virtually impossible. It is probably for this reason that only one ureaplasma protein has been purified to homogeneity, the urease enzyme, and this was only possible by affinity chromatography using a specific anti-urease monoclonal antibody (Thirkell *et al.*, 1989b). As discussed earlier, a monoclonal antibody specific for the ureaplasma IgA1 protease is not presently available and it was therefore necessary to resort to conventional methods for enzyme purification.

Only a limited number of purification strategies were attempted, since in every case there were difficulties in detecting eluted IgA1 protease activity, particularly when more than one chromatographic step was used. Thus, the level of enzyme activity recovered from an anion exchange separation was reduced below detectable limits by a subsequent gel filtration step. Although a cation exchange separation in combination with gel filtration allowed recovery of enzyme activity, this was at the expense of purity, presumably because the IgA1 protease eluted in the 'flow-through' of the cation exchange column, thereby producing minimal purification. Before assessing other methods for IgA1 protease purification, a number of fundamental problems in the whole process need to be addressed. These include the inadequate assay for the detection of enzyme activity

in chromatographic fractions, the losses incurred at each purification step and the low levels of starting material obtained from ureaplasma harvests.

In the purification of other bacterial IgA1 proteases, enzyme activity has generally been assayed by SDS-PAGE using [^{125}I] IgA1 (Halter *et al.*, 1984, Simpson *et al.*, 1988) or by ELISA (Mortensen and Kilian, 1984a, Blake and Eastby, 1991), both of which rely on the detection of IgA1 digestion products. As discussed in Chapter 2, the recent identification of alternative synthetic substrates for IgA1 proteases may enable a cheap, more rapid and quantitative assay to be developed. Combined with protein estimations this would allow enzyme yields and specific activities to be monitored during the purification procedure. Although quantitative measurements of IgA1 protease activity were not performed during preliminary attempts to purify the enzyme from *U. urealyticum*, it was clear that substantial losses in enzyme protein or activity were sustained in each chromatographic step.

A loss in enzyme activity may have been caused by the oxidation of free sulphydryl groups, particularly if this occurred within or close to the active site of the IgA1 protease. Simpson *et al.*, (1988) stored gonococcal IgA1 protease extracts in Tris-acetate buffer (pH 7.5) containing 1 mM dithiothreitol (DTT) and Mortensen and Kilian (1984a) reported that 100 mM DTT prevented loss in activity of the IgA1 protease from *B. melaninogenicus*. It may have been prudent to include such reducing agents into the purification buffers in this study. Alternatively, the enzyme may be susceptible to proteolysis, which could have been reduced by conducting chromatography at low temperatures (4 °C) or by the addition of a cocktail of protease inhibitors. EDTA (50-100 mM) has been included in a number of IgA1 purification protocols to inactivate metal-dependent proteases (Simpson *et al.*, 1988; Blake and Eastby, 1991; Mortensen and Kilian, 1984a). 10-20% (v/v) glycerol can also increase the stability of proteins by reducing the 'water activity' of the buffer and thereby minimising denaturation losses (Harris and Angal, 1989). Halter *et al.*, (1984) included 10% (v/v) glycerol in purification buffers used for the isolation of the type 2 enzyme from *N. gonorrhoeae*. However, when a sample of solubilised ureaplasma enzyme was stored under the same conditions as the

preparation undergoing purification (20 °C in chromatographic buffer), there was no apparent loss in activity for several hours, suggesting that denaturation by oxidation or proteolysis was not occurring under these circumstances. It seemed more likely, therefore, that the process of purification was simply separating the IgA1 protease from factors required to maintain its stability.

Inclusion of NP40 into equilibration and elution buffers appeared to increase the stability of the ureaplasma IgA1 protease during purification in all systems. If the *U. urealyticum* IgA1 protease is membrane bound, the non-ionic detergent may serve to shield hydrophobic portions of the enzyme and maintain its solubility in solution. Since NP40 only partially reduced the losses in enzyme activity it is possible that some dissociation between detergent and protein had occurred during the purification process, thereby inducing protein precipitation. It is also possible that the enzyme was simply more unstable in dilute protein solutions due to the dissociation of subunits or to adsorption onto container surfaces. The bacterial IgA1 proteases characterised to date, however, do not appear to have a subunit structure and the inclusion of 0.05% (v/v) NP40 should have minimised adsorption losses. Alternatively, the substantial loss of ureaplasma IgA1 protease activity incurred during gel filtration chromatography suggests that a low molecular weight ligand may be required for enzyme activity. Simpson *et al.*, (1988) proposed a similar explanation for the loss of gonococcal enzyme activity following a gel filtration step. Reconstitution experiments with the eluted fractions may confirm such a requirement for the ureaplasma IgA1 protease.

In addition to all these potential improvements, it is of perhaps of primary importance to increase the initial yield of IgA1 protease from *U. urealyticum*. It is generally regarded, however, that IgA1 proteases are highly active enzymes that are only released in small quantities (Plaut, 1983). Thus large starting cultures have only generated sufficient quantities of pure bacterial IgA1 protease for immunochemical characterisation and detailed biochemical or structural studies have not been possible (Blake and Eastby, 1991). With the additional problems inherent in isolating a membrane-associated enzyme it seems likely that

purification of the IgA1 protease from *U. urealyticum* is not readily be possible, even if improvements are made in cultivation and cellular solubilisation.

For these reasons, efforts were directed towards isolation of the *iga* gene from *U. urealyticum* with a view to expressing the enzyme in larger quantities from transformed bacteria.

Chapter 4:
Identification of the *iga* gene in *U. urealyticum*

SECTION A
MATERIALS AND METHODS

A number of basic cloning techniques were used routinely for the genetic analysis of *U. urealyticum*, many of which are based on methods described by Sambrook *et al.*, (1989). These are described in general in Appendix I to the Materials and Methods, Section 4.4. Appendix II, Section 4.5, details the preparation of bacterial growth media. As in previous chapters, all buffers were sterilised by autoclaving and H_2O refers to sterile double-deionised water obtained from a Milli Q water purification system (ELGA).

4.1 PREPARATION OF *U. UREALYTICUM* GENOMIC DNA LIBRARY

4.1.1 Source and cultivation of bacteria

U. urealyticum was grown according to the method described in Section 2.1.1. *M. hominis* (PG21) was obtained from D. Taylor-Robinson (Clinical Research Centre, Harrow, UK) and was grown in 70% (v/v) PPLO broth (Difco), incorporating 20% (v/v) donor horse serum (NBL), 10% (v/v) fresh yeast extract, 0.01% (w/v) phenol red, 0.2% (w/v) L-arginine hydrochloride and 1000 $U.ml^{-1}$ penicillin at a starting pH of 7.0. Cultures were incubated (37 °C) to pH 8 and harvested as previously described for *U. urealyticum*.

4.1.2 Isolation of genomic DNA

Freshly harvested pellets from 5 l ureaplasma or mycoplasma cultures were resuspended in 2 ml TE buffer and solubilised with SDS (0.5 %, [w/v]), proteinase K (Gibco BRL, 100 $\mu g.ml^{-1}$) and RNAase (Boehringer/Mannheim, 0.5 $\mu g.ml^{-1}$) in a 10 ml polypropylene tube for 2 h (50 °C). After addition of a further 2 ml TE buffer, ureaplasma DNA was purified from the cellular digest by phenol:chloroform extraction, using an adaptation of the method described in Section 4.4.1, that was suitable for the extraction of genomic DNA. Initially, the viscous solution was mixed (10 min) with an equal volume of phenol by gentle inversion of the tube. After centrifugation (2,000 x *g*, 10 min, 20 °C), the aqueous

phase was transferred, with a wide bore pipette, to a fresh tube, extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1, by vol.) and separated by centrifugation, as above. Traces of phenol were removed from the resulting aqueous phase by re-extraction in an equal volume of chloroform:isoamyl alcohol (24:1, by vol.), and re-centrifugation. The final aqueous phase was precipitated in a fresh tube with 0.1 x vol. 3 M NaAc (pH 5.2) and 2.5 x vol. ethanol (-20 °C, 30 min). The ureaplasma DNA was visible as a white precipitate which was collected by 'spooling' onto a Pasteur pipette. After washing in 70% (v/v) ethanol and air drying, the DNA was resuspended in 100 µl sterile TE buffer and its purity/concentration estimated by spectrophotometry, as described in Section 4.4.2.

DNA from *U. urealyticum* serotype 1 was kindly provided by B. Precious (University of St. Andrews, UK).

4.1.3 Partial digestion of genomic DNA

An initial study, to determine the concentration of restriction enzyme necessary to partially digest *U. urealyticum* DNA, was based on a protocol described by Sambrook *et al.*, (1989). 180 µl ureaplasma genomic DNA (33 µg, prepared as in Section 4.2.2 and stored at 4 °C), was combined with 100 µl *Eco*RI restriction enzyme buffer (10 x: 0.5 M Tris-HCl, pH 8, 0.1 M $MgCl_2$, 1 M NaCl) and H_2O to give a final volume of 1 ml. After equilibration (2 h, 4 °C), with intermittent gentle stirring, the solution was dispensed into ice-cold Eppendorf tubes; 60 µl (containing ~2 µg ureaplasma DNA) into tube 1 and 30 µl (containing ~1 µg ureaplasma DNA) into each of tubes 2-10. 10 U (1 µl) *Eco*RI restriction enzyme was added to tube 1 and serial 1/2 dilutions made to tube 10 by sequential mixing, removal and transfer of 30 µl. The samples were heated to 37 °C and incubated for 1 h. The reaction was terminated by addition of EDTA (pH 8) to a final concentration of 10 mM and the digestion products examined by gel electrophoresis, using 0.3% (w/v) agarose on a 1%(w/v) agarose support, followed by ethidium bromide staining, as described in Section 4.4.5.

Under these conditions, 2.5 U *Eco*RI produced a suitable range of digestion fragments (2-10 kbp) and ten identical reactions containing 2.5 U *Eco*RI were prepared, as before, to make a stock of partially digested ureaplasma DNA. To ensure that the correct size range of fragments had been produced by this method, an aliquot of the digestion mix was analysed by gel electrophoresis. The remainder was pooled, phenol:chloroform extracted and the aqueous phase centrifuged through a gel filtration column (250 x *g*) (Pharmacia 400) to remove any small fragments of DNA (<400 bp). The DNA was concentrated by ethanol precipitation and resuspended in TE buffer at 0.1 $\mu g.ml^{-1}$.

4.1.4 Ligation and packaging into λ ZAP bacteriophage vector

The ligation and packaging of ureaplasma DNA into the λ ZAP cloning vector and the subsequent transfection of host bacteria were performed using a λ Zap Cloning Kit (λ ZAP II, Gigapack II Plus). The protocols described were adapted from those provided by the manufacturer (Stratagene).

In the ligation reaction, 1.6 µl partially-digested ureaplasma DNA (0.16 µg) was combined with 1 µl pre-digested and phosphatase-treated λ arms (1 µg), 0.9 µl H_2O, 0.5 µl 10 x ligation buffer (5 M Tris-HCl, pH 7.5, 0.1 M $MgCl_2$, 0.1 M DTT, 10 mM ATP, 250 $\mu g.ml^{-1}$ BSA) and 1 µl T4 DNA ligase (both from NEB). As a control, 1.6 µl (0.4 µg) of pRheo:*Eco*RI test insert (2.8 kbp) replaced ureaplasma DNA. The reactions were incubated for 2 h at 14 °C.

For packaging, 4 µl of the ligation mix was added to ice-cold 'freeze thaw extract' followed by 15 µl 'sonic extract' (both extracts were provided in the Gigapack II Plus packaging kit). After brief centrifugation (1 sec, 1,200 x *g*),the mixture was incubated for 2 h at 20 °C. The reaction was terminated by addition of 500 µl SM buffer (86 mM NaCl, 8 mM $MgSO_4$, 50 mM Tris-HCl, pH 7.5, 0.01% [w/v] gelatine) and 20 µl chloroform. The solution was centrifuged briefly to remove sediment (12,000 x *g*, 2 min) and the supernatant stored as 'packaging extract' at 4 °C. As a control, 1 µl (~0.2 µg) of wild-type λ DNA (c1857 Sam7) was packaged following the same protocol.

The success of the ligation and packaging procedure was assessed by transfection of XL1-Blue cells with 'packaging extract', as described below.

XL1-Blue cells (Tn10 $(tet)^R$) were grown from a 15% (v/v) glycerol stock on LB-tet agar plates (see Appendix II). A single colony was transferred to 50 ml LB broth containing 0.2% (w/v) maltose:10 mM $MgSO_4$ and grown with agitation for 4-6 h (37 °C), or 16 h (30 °C), to an OD_{600} of 0.45-0.55. The bacterial culture (10 ml) was harvested by centrifugation (1,000 x *g*, 10 min), resuspended in 0.5 x vol. $MgSO_4$ and stored for up to 3 days (4 °C) until required ('plating bacteria'). Immediately before use, the 'plating bacteria' were diluted to an OD_{600} of 0.5.

The 'packaging extract' was diluted in SM buffer (neat, 10^{-1}, 10^{-2}) and 1 µl samples were pre-incubated (15 min, 37 °C) with 200 µl 'plating bacteria'. 3 ml aliquots of melted top NZYDT agar (see Appendix II) were equilibrated to 48 °C in a water-bath. Working quickly, 50 µl of 5-bromo-4 chloro-3-indolyl-β-D-galactoside (X-gal; 250 $mg.ml^{-1}$ in DMF), 15 µl of isopropylthio-β-galactoside (IPTG; 0.5 M in H_2O, filter sterilised) and the 201 µl of phage-associated bacteria were mixed with the top agar and immediately poured onto pre-warmed NZYDT agar plates (see Appendix II). After incubation for 6-8 h, recombinant and wild-type phage appeared on the bacterial lawn as white and blue plaques respectively. If the titre of recombinants was greater than 1 x 10^6 pfu.µg $arms^{-1}$, the library was amplified and screened for specific ureaplasma DNA inserts.

To amplify the library, 100 µl packaging extract (calculated to give ~50,000 recombinant bacteriophage) was incubated with 600 µl plating cells (15 min, 37 °C). The phage-bound bacteria were combined with 6.5 ml aliquots melted top agar (48 °C) and immediately poured onto pre-warmed NZYDT plates (15 cm diameter). Following incubation (6-8 h, 37 °C), the plates were overlaid with 10 ml SM buffer and stored overnight with gentle shaking (4 °C). The bacteriophage suspension was removed, the plates were rinsed with a further 2 ml SM buffer and chloroform was added to 5% (v/v). After 15 min (20 °C), the cell debris was removed by centrifugation (4,000 x *g*) and the supernatant stored (4 °C) as 1 ml aliquots containing chloroform (0.3 %, [v/v]). The titre of the amplified library was

estimated, as described above, except that the phage suspension was diluted from 10^{-3}-10^{-6} in SM buffer.

4.1.5 Screening the Library

When screening for ureaplasma inserts, a suitable number of bacteriophage plates, each containing approximately 10^4 plaques, were prepared as described previously, except that X-gal and IPTG were omitted from the top agar. After growth for 6-8 h, when plaques should just have been touching, the plates were stored at 4 °C for 2 h (but no longer than 48 h) to prevent the top agar from sticking to the filter. Circular 'Hybond N' filters (Amersham) were placed on each bacteriophage plate and after 2 min at 20 °C, plaque transfer was complete. The orientation of the filter was recorded and duplicate plaque-transfers (4 min, 20 °C) were made where possible. Transferred phage DNA was denatured by placing the filters, plaques-uppermost, on Whatman 3 MM paper soaked in denaturing buffer (1.5 M NaCl, 0.5 M NaOH) (2 min) and neutralised by transferring the filters to neutralising buffer (1.5 M NaCl, 0.5 M Tris-HCl, pH 8)-soaked 3 MM paper (5 min). After rinsing in wash buffer (0.2 M Tris-HCl, pH 7.5, 2 x SSC) (30 sec), the filters were dried (1 h, 20 °C) and fixed by UV irradiation (3 min).

The filters were probed with radiolabelled DNA by the method described for Southern blotting (Section 4.2.7).

4.2.1 Plasmid preparation

A derivative of pBR322, pIP503, containing the *iga* gene from *N. gonorrhoeae* (Pohlner *et al.*, 1987) was kindly provided by J. Pohlner (Max-Planck-Institut fur Biologie, Tubingen, Germany). The plasmid was propagated in *E. coli* strain HB101 (as below), where it could be selected by virtue of its ampicillin resistance gene (Fig. 4.1). The preparation of growth medium (LB) and appropriate antibiotics (LB-amp, LB-strep) for the growth and selection of HB101 transformants is described in the Appendix II.

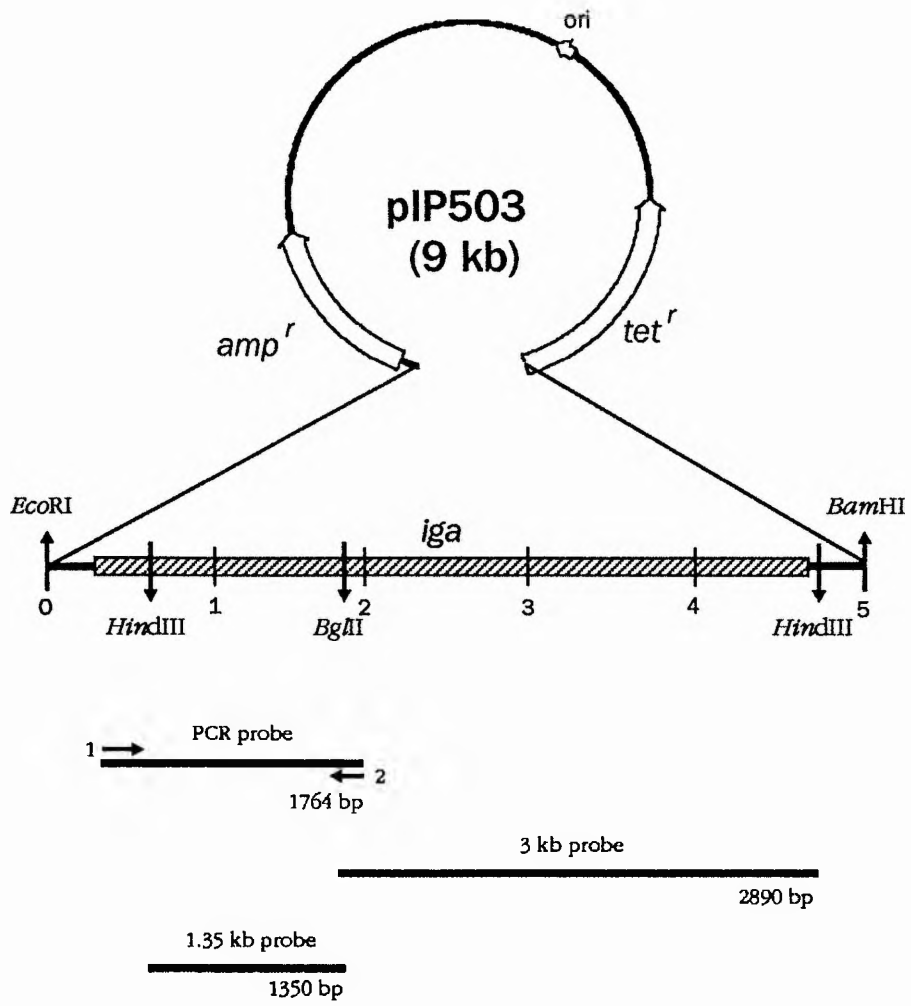


Fig. 4.1 The production of *iga* probes from pIP503

Plasmid pIP503 was constructed by the insertion of a 5 kb fragment, encoding the IgA1 protease from *N. gonorrhoeae* (*iga* gene, shaded region), into the *Eco*RI (0) and *Bam*HI (375) restriction sites within pBR322 (Halter *et al.*, 1984, Pohlner *et al.*, 1987). For the generation of *iga* gene probes from pIP503, an N-terminal portion of the gene was amplified using PCR primers corresponding to nucleotide bases 184-213 (primer 1) and complementary to 1950-1921 (primer 2, reverse primer). Restriction enzyme digestion of the plasmid at *Hin*dIII and *Bgl*II sites generated two additional probes of approximately 1.35 kb and 3 kb.

4.2.2 Transformation of *E. coli* HB101

E coli were made competent for plasmid transformation by the 'calcium chloride' method, based on the method described by Sambrook *et al.*, (1989). Single colonies of strain HB101 from LB-strep plates were grown overnight with agitation in 1 ml LB-strep broth (37 °C). The culture was reinoculated into 50 ml LB-strep broth and grown, with agitation, to an OD_{600} of 0.45-0.55 (1-2 h). The bacteria were transferred to a 50 ml polypropylene tube and chilled on ice (10-15 min). Following centrifugation (1000 x *g*, 10 min) and complete removal of supernatant, the pellet was resuspended in 25 ml of ice cold 50 mM $CaCl_2$: 10 mM Tris-HCl, pH 8. and left on ice (15-30 min). After a final centrifugation step (as before) the pellet was drained free of supernatant and resuspended in 2-3 ml 50 mM $CaCl_2$:10 mM Tris-HCl, pH 8. The competent cells were stored at 4 °C and for maximum transformation efficiencies, used after 24 h.

For transformation, 10-50 ng plasmid DNA in a final volume of 100 µl (in H_2O) was combined with 0.3 ml competent cells in ice-cold 'Sterilin' tubes (10 ml). After 30 min (on ice), the cells were 'heat shocked' at 42 °C for 90 sec and transferred to an ice bath for 1-2 min. To allow expression of antibiotic resistance, the transformed cells were grown in 0.7 ml LB broth (37 °C, 25 min), before 50-100 µl aliquots were plated onto LB-amp agar (7 cm diameter). Control transformations, containing no DNA, were plated onto LB-amp and LB agar. After overnight incubation (37 °C), the presence of colonies on the test plates, but not the control LB-amp plates, indicated successful transformation.

4.2.3 Mini-preparation of plasmid DNA ('Mini prep')

To confirm the presence and nature of the transformed DNA, small-scale plasmid purification's ('mini-preps') were prepared by the 'alkali lysis method'. Single colony transformants were grown overnight (37 °C) in 2 ml LB-amp broth. 1 ml of each overnight culture was harvested by centrifugation (12,000 x *g*, 30 sec, 4 °C) and following complete removal of the supernatant, the pellet was dispersed by vortexing in 100 µl ice-cold resuspension buffer (Solution 1: 50 mM glucose,

25 mM Tris-HCl, pH 8, 10 mM EDTA, pH 8). 200 µl lysis buffer (Solution 2: 0.2 M NaOH, 1% [w/v] SDS) was added to disrupt the bacterial cell wall and following gentle mixing, genomic DNA was precipitated by addition of 150 µl ice-cold acetate buffer (Solution 3: 5 M potassium acetate, 11.5 ml glacial acetic acid, 28.5 ml H_2O). After 5 min at 0 °C (ice), the copious white precipitate was removed by centrifugation (12,000 x *g*, 5 min, 4 °C), and the plasmid-containing supernatant decanted and re-centrifuged to remove final traces of genomic DNA. The supernatant was extracted with an equal volume of phenol:chloroform and plasmid DNA recovered from the aqueous phase by ethanol precipitation and centrifugation, as described previously (Section 4.4.1). The pellet was resuspended by vortexing in 50 µl TE buffer containing 20 $\mu g.ml^{-1}$ DNAase-free pancreatic RNAase (Boehringer/Mannheim). Typically, 3 µl of this 'mini prep' DNA was used to confirm the nature of the plasmid by restriction endonuclease digestion (*Hin*dIII, *Bgl*II, see Fig. 4.1) followed by agarose gel electrophoresis (Sections 4.4.3, 4.4.5).

pIP503 was stored within HB101 cells as agar plate cultures (renewed every month), frozen glycerol stocks (15% [v/v] glycerol) and as purified DNA from a medium scale preparation, as described below. For the preparation of glycerol stocks, single colonies were grown overnight (37 °C) in 5 ml LB-amp broth. 0.85 ml aliquots were thoroughly mixed with 0.15 ml sterile glycerol and stored at -70 °C in screw-top Eppendorf tubes.

4.2.4 Medium scale plasmid preparation ('Medium prep')

A single colony transformant of HB101 was inoculated into 100 ml LB-amp (in a 500 ml flask) and grown overnight with agitation (37 °C). The log-phase culture was divided between two 50 ml 'Corex' tubes (2 x 30 ml) and the bacterial cells harvested by centrifugation (12,000 x *g*, 10 min, 20 °C). The pellets were washed in Solution 1, pooled and resuspended in 4 ml Solution 1 containing 10 $mg.ml^{-1}$ lysozyme and 20 $\mu g.ml^{-1}$ RNAase (Sigma). After 15 min at 20 °C, with intermittent vortexing, the cells were alkali-lysed with 8 ml Solution 2 and left at 20 °C until the cloudy suspension cleared (~10 min). 6 ml Solution 3 was added

and after immediate vortexing, the genomic DNA was allowed to precipitate (5 min., 20 °C). The plasmid-containing supernatant was separated from the flocculent precipitate by centrifugation (as above), followed by filtration through cheese-cloth. The filtrate was divided between two 'Corex' tubes and recovered by addition of an equal volume of isopropanol followed by centrifugation, as before. The pellet was dried under vacuum, resuspended in 5 ml TE buffer, containing DNAase-free RNAase (1 $\mu g.ml^{-1}$), and incubated (37 °C) for 30 min. Plasmid DNA was extracted with phenol:chloroform, as described in Section 4.4.1, except that an initial phenol-extraction step followed by centrifugation (12,000 x *g*, 10 min, 20 °C) preceded phenol:chloroform extraction and ethanol precipitation. The DNA pellet was dried under vacuum, resuspended in 3 x 200 µl of H_2O, pooled and stored at -20 °C. The concentration of plasmid was determined spectrophotometrically at 260 nm and its integrity examined by agarose gel electrophoresis (Sections 4.4.2, 4.4.5).

Portions of the *N. gonorrhoeae iga* gene, for use as specific probes in hybridisation studies, were generated from pIP503 by either PCR or by restriction endonuclease digestion, as described in the following sections.

4.2.5 Preparation of DNA probes

For restriction endonuclease digestion, 2.5 µg of pIP503 was digested with *Hin*dIII (20 U) and *Bgl*II (20 U) in 1 x KGB buffer, as described in Section 4.4.3 (Fig. 4.1). For PCR, oligonucleotide primers corresponding to nucleotides 184-213 (primer 1) and 1950-1921 (reverse primer 2) of the published *iga* gene sequence (Fig. 4.2) were synthesised as follows:

Primer 1 (left): 5'-GCATTGGTGAGAGACGATGTCGATTATCAA-3'

Primer 2 (right): 5'-GATCCAGTCGTTATTTTCTGCTACACCGTT-3'

Using 200 ng pIP503 DNA as a template and thermal cycler Programme A, the PCR reaction was performed as described in Section 4.4.4.

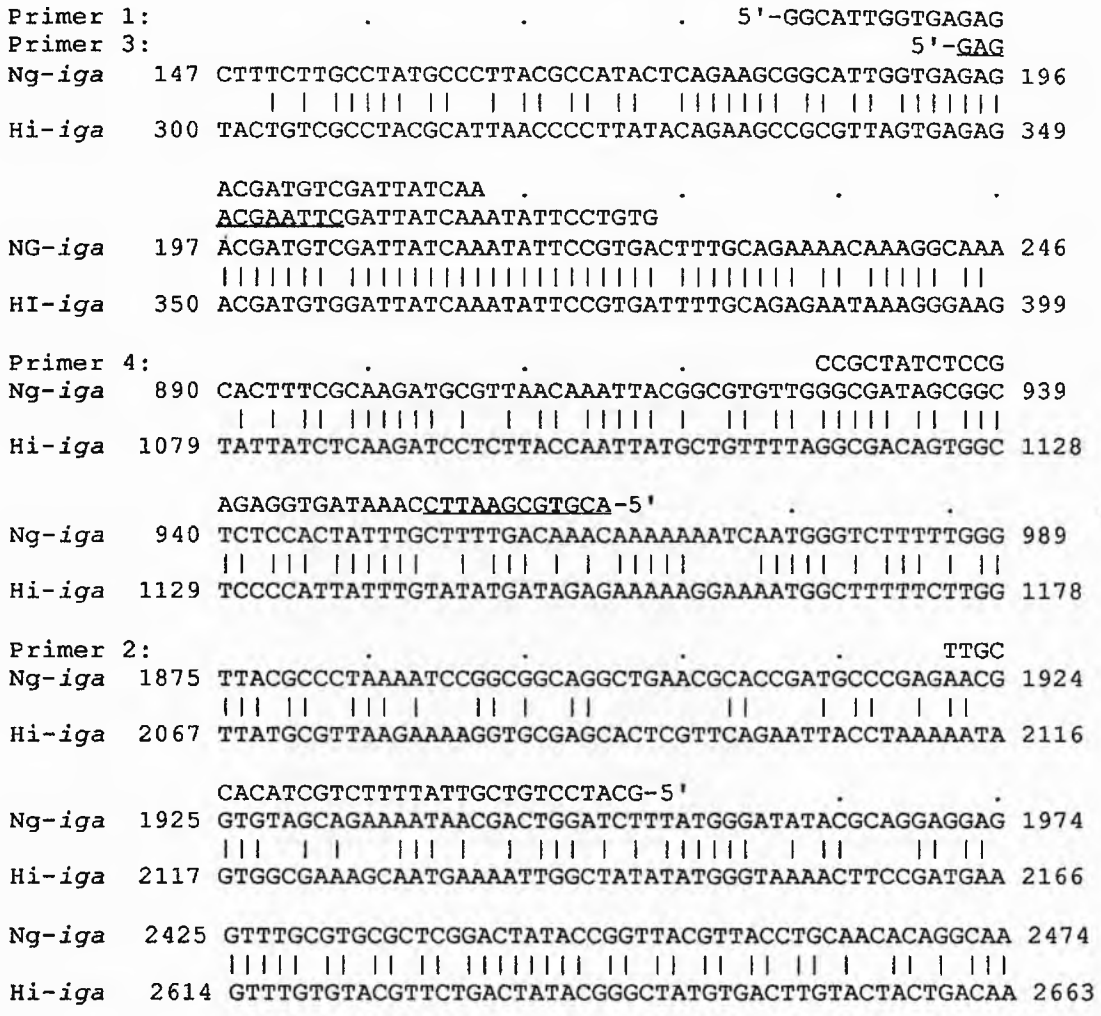


Fig. 4.2 Design of PCR primers for amplification of the *iga* gene from *N. gonorrhoeae* and *U. urealyticum*.

The *iga* sequences from *N. gonorrhoeae* (Pohlner *et al.*, 1987) and *H. influenzae* (Poulsen *et al.*, 1989) were aligned using the 'GAP' programme from the 'GCG' sequence analysis package. To amplify a portion of the *iga* gene from *N. gonorrhoeae*, primers were selected to give a ~2 kbp PCR product from the N-terminal region of the gene (primer 1 and reverse primer 2). In order to identify the *iga* gene within *U. urealyticum*, a region of high conservation between the *iga* genes from *H. influenzae* and *N. gonorrhoeae* (nucleotides 205-224) and a second region thought to code for the serine protease active site in these enzymes (nucleotides 928-948) were chosen for the design of primers 3 and 4. To aid cloning of amplification products, the primers incorporated an additional tail (underlined) containing the *Eco*R1 cleavage site (GAATTC). The region between nucleotides 2429-2464, encoding two cysteine residues, was originally proposed as the gonococcal IgA1 protease active site (Pohlner *et al.*, 1987).

The plasmid restriction digests (~2.5 μg), or PCR amplification products (~5 μg), were electrophoresed in 1% (w/v) low melting-point agarose (SeaPlaque GTC, FMC BioProducts), as described in Section 4.4.5, except that NT-TBE (0.09 M Tris-borate, 0.2 mM EDTA, pH 8.0) replaced TBE buffer. DNA was visualised by UV irradiation (< 1 min), the required band excised with the minimum excess of agarose and transferred to a pre-weighed Eppendorf tube. After addition of H_2O (3 ml per g of gel), the gel slice was melted (65 °C, 2 min) and either used immediately or stored at -20 °C. DNA fragments were radio-labelled directly from gel slices by random priming using a T7 Quick-Prime kit, according to the manufacturers instructions (Pharmacia). The equivalent of 200 ng DNA (<25 μl) was made up to a total volume of 37 μl with H_2O, heated to 95-100 °C for 7 min and transferred to a water bath (37 °C) for a further 10 min. This was combined with 10 μl labelling buffer (provided with the kit and containing random oligonucleotides, dATP, dGTP, dTTP) and 2 μl [α^{32}P] dCTP (3000 Ci.mmol^{-1}). The reaction was initiated by addition of 1 μl T7 DNA polymerase and incubated at 37 °C (5-15 min). Radiolabelling was terminated by addition of 0.2 M EDTA, pH 8 (5 μl) and unincorporated nucleotides were removed by centrifugation through a 'medium' Sephadex G50 (10 x 0.5 cm bed, height x diameter) column (500 x *g*, 2 min). The efficiency of labelling was estimated by precipitation of a portion of the eluted probe with 10% (w/v) TCA and the remainder was either used immediately or stored (-20 °C) for up to one week. Before use in hybridisation analysis, the double-stranded DNA was denatured (95 °C, 3 min) and snap-frozen (ice, 5 min) to produce a single-stranded probe.

4.2.6 Southern Blotting

DNA from *U. urealyticum* (1 μg), *M. hominis* (1 μg), and pIP503 (1-10 ng) was digested with suitable restriction enzymes (*Eco*RI, *Hin*dIII, *Bgl*II, see results section) and analysed by agarose gel electrophoresis (0.7% [w/v]), followed by ethidium bromide staining. λ:*Hin*dIII markers were included for size comparisons. The gel was prepared for Southern blotting by an initial depurination step (immersion in 200 ml, 0.2 M HCl, 15 min, 20 °C), which introduced nicks into high

molecular weight DNA and improved transfer. After rinsing with H_2O, the double-stranded DNA was denatured by soaking the gel in several volumes of denaturing solution (0.6 M NaCl, 0.2 M NaOH), for 2 x 20 min (20 °C) and neutralised by gel-immersion in several volumes of neutralising buffer (0.6 M NaCl, 1 M Tris-HCl, pH 8.0), 2 x 20 min (20 °C). The Southern blot was assembled by placing the gel on a wick of Whatman 3 MM paper, followed by a 'Hybond N' filter (Amersham) and a stack of absorbent paper (4-5 cm high), cut to the same size of the gel. The assembly was compressed using a 500 g weight placed on a glass plate, as detailed by Sambrook *et al.*, (1989). The wick was in contact with a tank of 10 x SSC (salt:sodium citrate buffer; 1.5 M NaCl, 0.15 M $Na_3C_6H_5O_7.2H_2O$) and DNA was transferred from gel to filter by capillary action. Once transfer was complete (8-16 h), as confirmed by ethidium bromide staining of the gel, the filter was rinsed, air-dried and UV irradiated (3 min) to immobilise bound DNA. For prehybridisation, the filters were immersed in 15 ml (0.2 $ml.cm^{-2}$) 'hybridisation' buffer (6 x SSC, 0.5% [w/v] SDS, 0.1% BSA, 0.1% Ficoll, 0.1% polyvinyl pyrollidine and 100 $\mu g.ml^{-1}$ denatured, sonicated, calf-thymus DNA, [Sigma]) and agitated in hybridisation chambers (Techne) for 1 h at the required temperature (42 °C-55 °C). This was replaced by a further 15 ml hybridisation buffer containing radiolabelled probe (2 x 10^9 $cpm.\mu g^{-1}$, 10-20 ng $ml.^{-1}$), prepared as described in Section 4.2.5) and the filters agitated at the same temperature (12-16 h). The filters were washed using the following general protocol:

Wash 1:	6 x SSC:0.1% (w/v) SDS (50 ml)	42 °C-55 °C	2 x 15 min
Wash 2:	2 x SSC:0.1% (w/v) SDS (50 ml)	42 °C-55 °C	30 min
Wash 3:	0.2 x SSC:0.1% (w/v) SDS (50 ml)	42 °C-55 °C	10 min

The stringency of hybridisation and washing was varied by altering the temperature of the hybridisation reaction and by altering the temperature and salt

concentration of the wash solution. Details of the stringencies used for individual experiments are presented in the results section.

The filters were blotted to remove excess liquid, wrapped in cling film and subjected to autoradiography for 1-5 days.

4.4 PCR-BASED ANALYSIS

4.3.1 PCR amplification of the *iga* gene from *U. urealyticum*

PCR primers 1 and 2, originally synthesised to amplify a portion of the *iga* gene from *N. gonorrhoeae* (see Fig. 4.2), were used to PCR-amplify a portion of the *iga* gene within *U. urealyticum*. Two additional primers (3 and 4), that flanked regions of conservation between the *iga* genes from *N. gonorrhoeae* and *H. influenzae* (see Fig. 4.2), were also designed for this purpose. For ease of cloning, these contained additional bases (underlined) at their 5' termini adjacent to an *Eco*RI site (GAATTC):

Primer 3 (left): 5'-GAGACGAATTCGATTATCAAATATTCCGTG-3'

Primer 4 (right): 5'-GGACGTGAATTCCAAATA($^{G}_{A}$)TGGAGAGCC($^{G}_{A}$)CTATCGCC-3'

Primer 4 was degenerate, since at two sites a mixture of guanidine and adenine nucleotides were included in the synthesis reaction. These correspond to sites of non-conservation between the *N. gonorrhoeae* and *H. influenzae iga* gene (nucleotide bases 946 and 936) where a thymidine in *H. influenzae* replaces the cytidine in *N. gonorrhoeae*. Since the ureaplasma genome shows a strong A+T bias, it is more likely that a thymidine rather than a cytidine is present at this position.

PCR amplification was performed as described in Section 4.4.4, using 1 μg ureaplasma genomic DNA as a template and thermal cycler Programme B. Amplified products were purified by electroelution from 1.5% (w/v) agarose gels (Section 4.4.6) and reamplified using identical primers and thermal cycler Programme B. The products were purified once more from agarose gels and the

purified DNA resuspended in a minimum volume of H_2O (~20 ng.μl^{-1}). A proportion of this (100 ng) was radiolabelled with [α^{32}P] ATP (Section 4.2.5) and used to probe Southern blots of DNA from *U. urealyticum* and *M. hominis* (55 °C, 2 x SSC:0.1% [w/v] SDS washes, as described in Section 4.2.6). If the amplified fragment was confirmed to be ureaplasma DNA, the remainder was cloned into the filamentous bacteriophage M13 for the production of single-stranded DNA required for DNA sequencing.

4.3.2 Cloning of PCR products into M13

The preparation of PCR inserts for cloning into M13 depended upon which primers had been used to generate the amplified fragments. Those produced from primers 3 and 4 were precipitated from the PCR reaction mixture (as described in Section 4.4.4) *Eco*RI-digested (1 h, 37 °C), purified by electroelution (Section 4.4.6) and resuspended in the minimum volume of H_2O. Those amplified using primers 1 and 2 were Klenow-treated and 5' phosphorylated in preparation for blunt-ended ligation into *Sma*I-digested M13, as detailed below. 100-200 ng of ethanol-precipitated PCR product (Section 4.4.4) was phosphorylated (30 min, 37 °C) with T4 polynucleotide kinase (NEB, 5 U) in a reaction containing 10 µl of 10 x kinase buffer (0.5 M Tris-HCl, pH 7.6, 0.1 M $MgCl_2$, 50 mM DTT, 1 mM spermidine, 1 mM EDTA) and H_2O made up to 50 µl. To fill-in any recessed 3' termini, 5 U of the Klenow fragment from *E. coli* DNA polymerase I (NEB) and 0.5 mM of each dNTP (from a stock of 2 mM) was added to the phosphorylation mixture. After 15 min, 37 °C the reaction was terminated by heat inactivation (65 °C, 10 min) and the products purified by electroelution.

The M13 vector (RF M13mp18, NBL) was prepared for ligation by digestion with either *Eco*RI (10 U, 1 h, 37 °C) or *Sma*I (10 U, 1 h, 30 °C), and linearised molecules were purified by electroelution. To prevent vector recircularisation during the ligation reactions, 1-2 µg linearised M13 was incubated (30 min, 37 °C) with 1 U of calf intestinal alkaline phosphatase (CIAP, Boehringer/Mannheim) and 2 µl of 10 x CIAP buffer (30 mM triethanolamine, 3 M NaCl, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, pH 7.6) in a final volume of 20 µl. The reaction

was terminated by addition of 10 mM ethylene glycol-bis N, N, N', N'-tetra acetic acid (EGTA, pH 8) followed by transfer to 68 °C (45 min). Any remaining CIAP was removed by phenol:chloroform extraction (Section 4.4.1) and following ethanol precipitation, vector DNA was resuspended a minimum volume of H_2O.

a) Ligation of insert and vector

Prior to ligation, the concentration of vector and insert DNA was estimated by agarose gel electrophoresis and ethidium bromide staining. In the ligation reaction between cohesive termini, approximately 100 ng of vector DNA was combined with insert DNA (at molar ratios of 3:1, 1:1, 1:3, 1:10; vector:insert), 1 µl 10 x ligase buffer (0.5 M Tris-HCl, pH 7.8, 0.1 M $MgCl_2$, 0.2 M DTT, 0.5 $mg.ml^{-1}$ BSA, 100 mM ATP) and H_2O in a total volume of 9 µl. To separate cohesive termini, the mixture was heated to 45 °C (5 min) and cooled on ice before addition of 0.1 Weiss unit T4 DNA ligase and incubation at 16 °C (2-4 h). For blunt-ended ligation, a total of 200 ng of vector was combined with insert DNA at the same molar ratios with 1 µl 10 x ligase buffer, 0.1 Weiss unit ligase enzyme in a final volume of 10 µl and incubated for 2-4 h (16 °C). 1-2 µl (10-20 ng) of each ligation mixture was used to transform competent *E. coli* JM101 cells.

b) Preparation of competent *E. coli* JM101 cells (Hanrahan method)

All of the solutions required for the production and transformation of competent of JM101 cells (M9 minimum agar, SOB media, FSB solution, H agar, H-top agar) are described in Appendix II.

A single colony of *E. coli* strain JM101, grown on minimum agar, was dispersed by vortexing in 1 ml SOB:20 mM $MgSO_4$ medium and transferred to 50 ml SOB:20 mM $MgSO_4$ in a 250 ml tissue culture flask. The bacteria were grown in an orbital shaker (37 °C, 200 rpm) to a density of 4-7 x 10^7 $cells.ml^{-1}$ (OD_{600} = 0.45-0.55, 3-5 h). Bacterial cells were cooled on ice in a 50 ml polypropylene tube (10 min) and collected by centrifugation (1000 x *g*, 4 °C, 10 min). Following complete removal of supernatant media, the pellet was resuspended gently in 1/3 x original vol. ice cold transformation buffer (FSB) and left on ice (10 min). After a further centrifugation step (as before) the supernatant-free pellet was disrupted gently in 1/12.5 x original vol. FSB, DMSO was added to 3.5% (v/v) and

the mixed suspension stored on ice (5-15 min). A further 3.5% (v/v) DMSO was added and the solution stored on ice for an additional 5 min. Competent cells were dispensed (100 µl) into pre-cooled Eppendorf tubes, snap-frozen in an ethanol-dry ice bath and stored (-70 °C) until required.

To prepare 'plating cultures', a single colony of JM101 was selected from a minimum agar plate and grown in 100 ml LB broth for 6-8 h (37 °C) with agitation. The cells were cooled on ice and stored for up to one week (4 °C).

c) Transformations

For transformation, competent JM101 cells were thawed in a water bath (20 °C) and transferred to ice (10 min). 10-20 ng recombinant M13 DNA was mixed with 50 µl competent cells in ice-cold Eppendorf tubes and stored on ice (30 min). The tubes were transferred to a circulating water bath (42 °C) 'heat-shocked' for exactly 90 sec and immediately returned to ice (1-2 min). Following addition of 195 µl SOB, aliquots of the transformed bacteria (25 µl, 125 µl) were combined with 100 µl 'plating culture', mixed with 4 ml melted H-top agar (46 °C), containing freshly-added X-gal (40 µl, 20 $mg.ml^{-1}$ in DMF), and IPTG (4 µl, 200 $mg.ml^{-1}$), and immediately poured onto H agar plates. After 5 min, the plates were inverted and incubated overnight (8-12 h) to allow growth of the bacterial lawn and M13 plaque-production. Blue plaques were formed by wild-type M13 and white/colourless plaques by recombinant M13.

d) Isolation single-stranded DNA

Recombinant plaques (representing ~10^6-10^8 pfu) were isolated using a Pasteur pipette, transferred to TE buffer (100 µl) and vortexed to release bacteriophage. For the preparation of M13 single-stranded DNA templates, 20 µl of the plaque suspension was combined with 2 ml LB broth containing 20 µl 'plating culture' and grown (6-8 h) with agitation (37 °C). 1 ml of the transfected culture was collected by centrifugation (12,000 x *g*, 5 min, 20 °C), to produce a bacterial pellet containing double stranded RF M13 and a supernatant containing single-stranded M13 bacteriophage (~10^{12} $pfu.ml^{-1}$). The supernatant was removed, centrifuged (as before), to remove traces of bacterial pellet and gently resuspended in 200 µl 2.5 M NaCl containing 20% (w/v) PEG 8000. After 15 min

(20 °C), the bacteriophage particles were harvested by centrifugation (14,000 x *g*, 5 min, 4 °C) and after complete removal of PEG supernatant by recentrifugation, were resuspended by vigorous vortexing in 100 µl TE buffer. Bacteriophage DNA was recovered by phenol-extraction and ethanol-precipitation (as described in Section 4.4.1), except that the first extraction step was with phenol alone. Following resuspension in 20 µl TE buffer, the concentration of single-stranded DNA was estimated by agarose gel electrophoresis and ethidium bromide staining.

4.3.3 Sequencing reactions.

The single-stranded templates (1 µg) were sequenced by the dideoxy chain-termination reaction using a 'Sequenase' (Version 2.0) sequencing kit (United States Biochemical) and [α^{35}S] dATP (1000 Ci.mmol^{-1}) (Amersham). Sequencing reactions were performed exactly as described by the manufacturers protocol. The radiolabelled fragments (2-3 µl) were loaded onto denaturing polyacrylamide gels (6% [w/v] acrylamide:bis; 19:1, 7M urea in 1 x TBE buffer), prepared using wedge spacers in a 'Sequi-Gen' nucleic acid sequencing gel apparatus, following the manufacturers instructions (Bio-Rad). Electrophoresis was carried out in 1 x TBE buffer at 10,000 V (55 °C) until the first blue dye front (xylene cyanol) had migrated from the gel (4-6 h). The gels were dried on to 3 MM Whatman filter paper and autoradiographed (24-72 h).

Deduced nucleotide sequences were analysed using the Genetics Computer Group (GCG) sequence analysis soft-ware package. For translation and comparison with other *iga* gene sequences the 'Translate' and 'Pile-up' programmes were used. For more general data base search comparisons, the 'FastA' programme allowed access to the Gen Bank, EMBL and the PIR Nucleic data bases.

4.4 APPENDIX I: GENERAL CLONING TECHNIQUES

4.4.1 Purification of DNA by phenol:chloroform extraction

Re-distilled phenol was equilibrated to pH >7.8 with 100 mM Tris-HCl, pH 8 and stored as aliquots (-20 °C). DNA-containing solutions were mixed in 10 ml polypropylene tubes with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1, by vol.), and separated by centrifugation (12,000 x *g*, 5 min, 20 °C). The aqueous phase was removed to a fresh tube, re-extracted with an equal volume of chloroform:isoamyl alcohol and centrifuged as before. DNA was precipitated from the aqueous phase by the addition of 0.1 x vol. 3 M sodium acetate (pH 5.2) followed by 2.5 x vol. ethanol. After 10-30 min (4 °C), the DNA was pelleted by centrifugation (12,000 x *g*, 10 min, 4 °C), washed with 1 ml 70% (v/v) ethanol, allowed to air-dry and resuspended in the required volume of H_2O or TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

4.4.2 Estimating DNA concentration, [DNA]

The optical density (OD) of an appropriate dilution of the DNA preparation (generally 1/500 in TE buffer) was measured using the 'scan' programme (220 nm - 320 nm) on a Perkin-Elmer, Lambda 5 spectrophotometer. An absorbance unit of 1 indicated a concentration of:

50 $\mu g.ml^{-1}$ for single stranded DNA,

40 $\mu g.ml^{-1}$ for double stranded DNA,

20 $\mu g.ml^{-1}$ for oligonucleotides.

For pure preparations of DNA, the absorbance ratio at 260 nm and 280 nm (260:280) should be between 1.8-2.0. A lower ratio indicates protein or phenol contamination and accurate quantitation by this method is not possible.

In these circumstances, or when the sample contained low levels of DNA (<250 $ng.ml^{-1}$), preparations were examined by agarose gel electrophoresis followed by ethidium bromide staining and DNA concentration was estimated from the intensity of fluorescence emitted (see Section 4.4.5).

4.4.3 Restriction endonuclease digestion.

The restriction enzymes and their appropriate 10 x buffers were obtained from Gibco (BRL). A typical reaction contained 0.1-5 µg DNA, 2 µl 10 x buffer, 1-10 units of restriction enzyme (<1/10 total volume) and H_2O, to give a final volume of 20 µl. Digestion was for 1 h at 37 °C (unless otherwise stated) and was terminated by addition of 0.5 M EDTA (pH 8), to a final concentration of 10 mM. When simultaneous digestion with two restriction enzymes was required, the reaction was performed in the appropriate KGB buffer (Stock solution 2 x KGB: 200 mM potassium glutamate, 50 mM Tris-acetate [pH 7.5], 20 mM magnesium acetate, 100 $\mu g.ml^{-1}$ bovine serum albumin [BSA], 1 mM β mercaptoethanol), as described by Sambrook *et al.*, (1989).

4.4.4 Polymerase chain reaction (PCR)

Taq polymerase and 10 x Taq buffer (0.1 M Tris-HCl, pH 8, 15 mM $MgCl_2$, 0.5% [v/v] Tween 20, 0.5% [v/v] NP40) were obtained from Northumbria Biologicals Limited (NBL). Deoxyribonucleotide triphosphates (dNTPs; dATP, dTTP, dGTP, dCTP, [100 mM], Pharmacia) were prepared to give a single stock solution containing 2 mM of each dNTP. Oligonucleotide primers were kindly synthesised by I. Armitt (University of St. Andrews) using an Applied Biosystems 381A DNA synthesiser.

The primers were reconstituted in a solution containing 200 µl H_2O, 20 µl 3M sodium acetate, 2.2 µl magnesium acetate and precipitated with 600 µl ethanol. After centrifugation (12,000 x *g*, 5 min), they were again reconstituted, precipitated and collected by centrifugation (as above). The final pellets were air-dried and resuspended in 100 µl H_2O. Oligonucleotide concentration was determined spectrophotometrically at 260 nm (assuming the mass of one base to be 330 Daltons) and a stock solution (100 µM) of each primer was prepared. The Taq buffer, dNTPs and oligonucleotide primers were all aliquoted and stored (-20 °C) separately from sources of template DNA and PCR amplification products. Amplification reactions, prepared in a laminar-flow hood using PCR-designated Gilson pipettes contained the following components:

H_2O	26 µl
10 x Taq buffer	4 µl
dNTPs (2 mM each)	5 µl
oligonucleotide primer 1 (100µM)	1 µl
oligonucleotide primer 2 (100 µM)	1 µl
DNA template (200 ng)	3 µl
[mineral oil	50 µl]

Heat, 94 °C; 5 min

Taq mix	10 µl
[Total volume	50 µl]

A negative control containing no template DNA and a positive control containing homologous DNA template were included where possible. The reaction mixtures were overlaid with mineral oil, to prevent evaporation and pre-heated to 94 °C, to ensure complete denaturation of template DNA. Amplification was initiated by addition of 10 µl Taq mix (1 µl Taq buffer, 8.5 µl H_2O, 0.5 µl (2.5 U) Taq enzyme) and controlled by a programmable thermal cycler, set to one of the following programmes:

	Programme A:	Programme B:
Step 1 (denaturation)	94 °C, 1.5 min	94 °C, 1.0 min
Step 2 (annealing)	55 °C, 1.5 min	40 °C, 1.5 min
Step 3 (elongation)	72 °C, 2.0 min	65 °C, 3.0 min
No. cycles	30	40
Final step	72 °C, 10 min	60 °C, 5 min

After amplification was complete, the reaction products were removed from beneath the paraffin layer, extracted with an equal volume of chloroform,

ethanol-precipitated and resuspended in 20 µl of H_2O or TE buffer. PCR products were either used as DNA probes in Southern blotting or for cloning purposes.

4.4.5 Agarose gel electrophoresis

Samples were separated by horizontal agarose gel electrophoresis using submersible gel tanks (Gibco/BRL) with a capacity for 20 ml agarose. The concentration of agarose (Sigma) reconstituted in Tris-borate buffer (TBE, 0.09 M Tris-borate, 2 mM EDTA, pH 8) ranged from 0.7-2 % (w/v), depending on the size of DNA to be analysed. DNA-containing samples (50-1000 ng) were mixed with 1:6 (v/v) gel loading buffer (0.25% [w/v] bromophenol blue, 0.25% [w/v] xylene cyanol, 30% [v/v] glycerol in H_2O) and loaded directly into well slots. The amount of DNA applied to each well depended on the number of fragments in the sample and their respective sizes. For simple populations of DNA, such as PCR products or plasmid preparations, 50-500 ng were loaded into a 0.5 cm well slot. If restriction digests of ureaplasma or mycoplasma genomic DNA were being examined, loading was increased to 1 µg. Phage λ:*Hin*dIII (Boehringer/Mannheim) or øX174:*Hae*III (New England Biolabs, NEB) digests were included when appropriate as DNA size markers. Samples were electrophoresed in TBE buffer for 1 h at constant voltage (75-100 V), stained in TBE buffer: 1 $\mu g.ml^{-1}$ ethidium bromide (5 min) and DNA was visualised using a UV transilluminator. For quantitative estimations, standard DNA solutions (5-100 ng) containing a single species of DNA, approximately the same size as the as the test DNA, were loaded and the intensities of fluorescence compared by photography following UV irradiation.

4.4.6 Recovery of DNA fragments from agarose gels

DNA fragments (>100 ng) were purified from agarose gels (1.5%) by electroelution, using 7 cm x 10 cm flat-bed slab gel rigs. After UV examination for the minimum time required, a narrow well was cut immediately in front of the DNA band of interest, about 2 mm wider on either side of the band. DNA was eluted into the well with short pulses of 100 V and DNA-containing buffer (TBE)

was removed after each pulse. For DNA of approximately 5 kbp, pulses of 1 x 50 sec, followed by 7 x 30 sec were suitable. For smaller fragments, such as a 500 bp PCR product, a further 3-9 x 40 sec pulses were required. The progress of electroelution was monitored by UV examination. DNA was purified from pooled, eluted buffer fractions by extraction with phenol alone, phenol:chloroform and by ethanol precipitation, as previously described in Section 4.4.1. The final pellet was resuspended in 20 µl H_2O.

4.5 APPENDIX 2: BACTERIAL GROWTH MEDIA

a) Luria-Bertani medium (LB)

Bacto-tryptone (Difco)	10 g
Yeast extract	5 g

The components were dissolved in 800 ml 0.17 M NaCl, adjusted to pH 7.0 with NaOH, made up to 1 l and autoclaved.

For LB agar, Bacto-agar (Difco) was added to 1.5%.

b) Antibiotic-containing media

If antibiotic-containing agar plates were required, the agar was equilibrated to 50 °C following autoclaving and antibiotic added to the recommended concentration (see Table 4.1). The plates could be stored at 4 °C for up to one week and before use, were dried for 15 min in a UV cabinet at 45 °C.

Table 4.1. Preparation of antibiotics

Antibiotic	Stock solution	Working concentration
Ampicillin (amp)	25 $mg.ml^{-1}$ in H_2O, filter sterilised.	50-100 $\mu g.ml^{-1}$
Streptomycin (strep)	2.5 $mg.ml^{-1}$ in H_2O, filter sterilised	25-50 $\mu g.ml^{-1}$
Tetracycline (tet)	12.5 $mg.ml^{-1}$ in 50% ethanol, filter sterilised	12.5-15 $\mu g.ml^{-1}$

c) M9 minimal medium

5 x M9 salts	200 ml
1 M $MgSO_4$	2 ml
1 M $CaCl_2$	0.1 ml
20 % glucose	20 ml

The components were dissolved in sterile deionised H_2O made up to 1 l. For M9 minimum agar, noble agar was added to 1.5%.

c) NZYDT

NZYDT media contained 21.1 g NZYDT (Gibco) in a total volume of 1 l. For NZYDT agar, Noble agar was added to 2%(base agar) or 0.7% (top agar).

d) SOB

Bactotryptone	20 g
bacto yeast extract	5 g
NaCl	0.5 g
KCl, 250 mM	10 ml

The components were dissolved in deionised H_2O (950 ml) and adjusted to pH 7 with 5 M NaOH. The volume was made up to 1 l with deionised H_2O and the solution sterilised by autoclaving. Just before use $MgCl_2$ (2 M) was added to a concentration of 20 mM.

For the preparation of SOC, glucose was added to 20 mM.

e) FSB

Potassium acetate (1 M stock, pH 7.5)	10 mM
$MnCl_2.4H_2O$	45 mM
$CaCl_2.2H_2O$	10 mM
KCl	100 mM
Hexamminecobalt chloride	3 mM
Glycerol	10%

The components were mixed in 800 ml H_2O, the pH adjusted to 6.4 with 0.1 N HCl and the volume made up to 1 l. After filter sterilisation (0.45 µm filter), the solution was stored as aliquots (40 ml) in tissue-culture flasks (4 °C).

f) H-agar

Tryptone (Merck)	10 g
NaCl	5 g
Noble agar (Difco)	10 g

The components were made up to 1 l with deionised water and autoclaved.

For H-Top agar, Noble agar was reduced to 6 $g.l^{-1}$.

SECTION B
RESULTS

4.5 PREPARATION OF *U. UREALYTICUM* GENOMIC DNA LIBRARY

In order to identify the *iga* gene in *U. urealyticum*, a genomic DNA library was constructed in the Lambda-based vector, λ ZAP (Short *et al.*, 1988). This was selected as a suitable cloning vehicle as it combined a high efficiency of cloning with ease of library screening, amplification and storage. Positively-selected DNA inserts could be excised with helper phage and recircularised to generate subclones in a pBluescript phagemid vector. This had the potential to generate single-stranded DNA suitable for sequencing reactions.

4.5.1 Isolation of genomic DNA

Ureaplasma DNA was extracted from cell harvests using a method based on that described by Razin *et al.*, (1983). In initial extractions, it was found that very low yields of genomic DNA (100 µg) were purified from large ureaplasma cultures (10 l) and that the extracted DNA did not remain stable upon storage at 4 °C. It had been reported for *Acholeplasma laidlawii* (*A. laidlawii*) that DNAase enzymes released during the process of DNA extraction substantially degraded acholeplasma genomic DNA and that addition of 20 mM EDTA into the cell-lysis buffer could alleviate this effect (Razin *et al.*, 1983). Increasing EDTA concentration to 20 mM in the TE buffer at all stages of ureaplasma DNA extraction appeared to improve yields to a limited extent and substantially reduced the DNA degradation observed after prolonged storage. As a precaution, a stock of ureaplasma DNA (for use in Southern blotting) was stored at -20 °C. Preparations required for the construction of a gene library, however, were stored at 4 °C, to avoid the shearing of high molecular DNA upon freeze-thawing. Higher yields of DNA were also obtained by using freshly harvested cell pellets rather than frozen pellets, although other workers had found frozen preparations to be satisfactory (Razin *et al.*, 1983, J. Willoughby, personal communication). A reduction in the

number of phenol:chloroform extractions and collection of ethanol-precipitated DNA by centrifugation (12,000 x *g*, 30 min) rather than by 'spooling' also resulted in improved yields, but generally at the expense of DNA purity, as measured by the spectrophotometric absorbance at 260 nm and at 280 nm (260:280). By optimising extraction conditions, the maximum yield of genomic DNA obtained from 5 l of freshly harvested cells was approximately 300 µg, which is comparable to yields reported by Bak and Black (1968) and by Razin *et al.*, (1983).

4.5.2 Ligation and packaging reactions

For the construction of the genomic library, ureaplasma DNA was partially digested with *Eco*RI and fragments within the range 1-10 kbp were ligated to the λ ZAP vector. This was packaged to produce a stock of λ phage containing the whole ureaplasma genome. By using the blue-white selection property of λ ZAP, it was estimated that the number of insert-containing phage (white plaques) was approximately 7.5×10^5 pfu.ml^{-1}, which was within the range estimated by the manufacturer (6×10^5 -6×10^6 pfu.ml^{-1}). The number of wild-type phage (blue plaques) was approximately 3×10^4 pfu.ml^{-1}, which at 1/25 the number of recombinant phage, was within the background levels predicted by the manufacturers (1/10-1/100 the number of white plaques). For long-term storage, the library was amplified to give a stock of approximately 5×10^9 pfu.ml^{-1}. Although this was at a slightly lower titre than recommended by the manufacturer (10^{10}-10^{13} pfu.ml^{-1}), it was nevertheless at an acceptable level. To confirm that the library contained inserts of ureaplasma DNA, radiolabelled DNA from this organism was used to probe a filter containing phage DNA obtained from a 'plaque lift'. A large proportion of plaques were detected using this probe whereas plaque lifts from a second library containing control DNA inserts (pRheo-*Eco*RI) gave no strongly hybridising plaques.

4.6 SCREENING THE LIBRARY

To establish whether heterologous DNA probes could be used to identify the *iga* gene within the ureaplasma DNA library, preliminary hybridisation studies

were performed. A suitable range of ureaplasma genomic DNA fragments were generated by digestion with frequently cutting restriction enzymes (*Eco*RI, *Bgl*II, *Sca*I) and transferred to 'Hybond N' filters by Southern blotting. In addition, DNA from *M. hominis,* which does not elaborate an IgA1 protease, was included as a control.

4.6.1 Preparation of probes

Heterologous DNA probes were derived from a plasmid containing the neisserial *iga* gene, pIP503, which had been propagated in *E. coli* strain HB101. 1 mg purified plasmid DNA was obtained from 60 ml bacterial culture by an adaptation of the 'alkali lysis' method. To identify regions within the *iga* gene that may have been conserved between bacterial genera, nucleotide sequences from *N. gonorrhoeae* and *H. influenzae iga* genes were obtained from the GenBank data base and compared using the 'GAP' programme within the GCG sequencing analysis package. An area of DNA homology was located towards the 5' end of the gene, in particular between nucleotide bases 189-225 (Fig. 4.2, page 177). To generate a suitable DNA probe, which contained this region but not the adjacent leader sequence which might have been present in a number of ureaplasma secretory proteins, oligonucleotide primers were synthesised, as described in Fig. 4.1 and 4.2 (pages 173, 177). When used in a PCR reaction, containing 1 µg pIP503 as template DNA, these generated approximately 5 µg of amplified DNA (1.76 kbp). The identity of the PCR product was confirmed by digestion with specific restriction enzymes (*Hae*III, *Hin*dIII, *Hin*fI) which gave the predicted fragment sizes when analysed by agarose gel electrophoresis. The DNA was radiolabelled by 'random priming', using [α^{32}P] ATP, to give a probe of high specific activity (2 x 10^9 cpm.μg^{-1}). This was used to probe Southern blot filters at a concentration of 10-20 ng.ml^{-1} in SSC-based hybridisation solutions.

4.6.2 Southern blotting

Fig. 4.3 shows the result of hybridisations and washes (down to 2 x SSC) conducted at a range of temperatures (42 °C-57 °C). Under low stringency

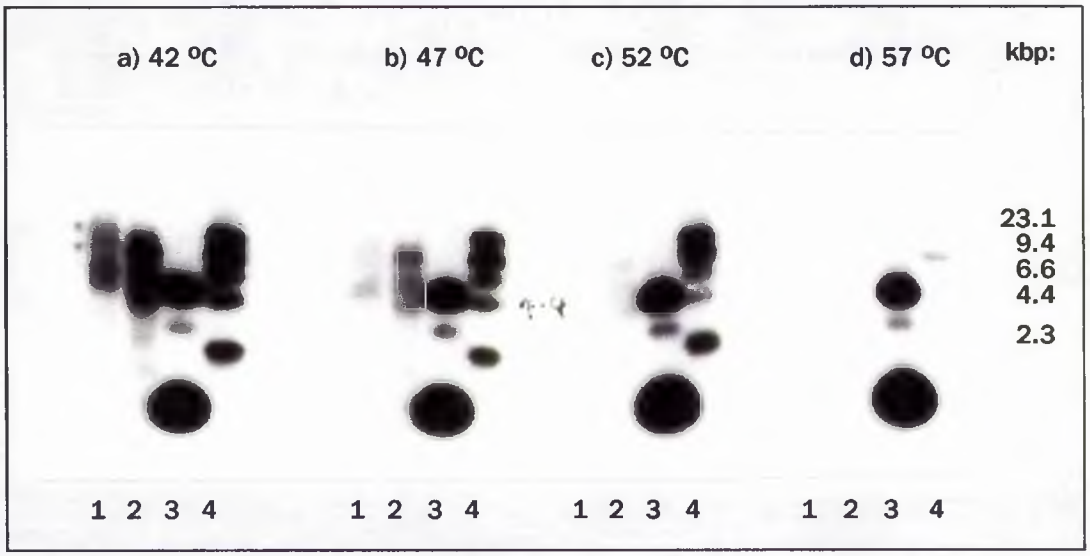


Fig. 4.3 Southern blot hybridisation of genomic DNA from *U. urealyticum* and *M. hominis* using a PCR probe derived from *N. gonorrhoeae iga* gene.

Genomic DNA from *U. urealyticum* serotype 8 (1 µg, lane 1) and *M. hominis* (1 µg, lane 2) was digested with *Eco*RI. Plasmid DNA from pIP503 (containing the *iga* gene from *N. gonorrhoeae*) was digested with *Hin*dIII and *Bgl*II (8 ng, lane 3). The digestion products were analysed by 0.7% agarose gel electrophoresis and λ/*Hin*dIII markers (125 ng, lane 4) were included for size comparisons. Following Southern transfer, filters were hybridised (16 h) with a PCR-derived probe (10 $ng.ml^{-1}$), containing a portion of the *iga* gene from *N. gonorrhoeae,* as described in Section 4.2.6. The filters were washed with 2 x SSC:0.1% SDS and autoradiographed (6 h). Hybridisations and washes were conducted at 42 °C (a), 47 °C (b), 52 °C (c) and 57 °C (d).

DNA size markers are to the right of the figure.

conditions (42 °C, 47 °C) there was detectable binding of the probe to ureaplasma DNA but also substantial levels of binding to control *M. hominis* DNA and λ markers. As the stringency was increased (52 °C, 57 °C), there was an equivalent reduction in hybridisation to mycoplasma and ureaplasma DNA but, even at 57 °C, the probe continued to bind to λ DNA. This suggested that there may have been some unexpected homology between the PCR probe and λ DNA. Comparing the sequence of the probe with λ DNA using the Staden package, no obvious area of homology was revealed. Incorporation of λ DNA (50-100 μg) into the hybridisation reactions appeared to reduce probe binding to both λ DNA and *M. hominis* DNA but not to ureaplasma DNA, suggesting that the hybridisation to the ureaplasma DNA was specific and that binding to λ DNA and *M. hominis* DNA was non-specific (see Fig. 4.4). This was perhaps a surprising result, since carrier DNA (calf-thymus DNA at 100 $\mu g.ml^{-1}$) had been incorporated routinely into the hybridisation solutions to reduce non-specific binding, although it is possible that additional non-specific carrier DNA was required.

Alternatively, and contrary to the comparative sequence analysis, it is possible that there was a degree of specific binding to λ DNA. To ensure that this was not a feature particular to the PCR probe used, two additional probes (3 kb and 1.35 kb) were generated from digestion of pIP503 with *Hin*dIII and *Bgl*II (see Fig. 4.1). The fragments were purified from agarose gel electrophoresis and radiolabelled, as described previously. The 1.35 kb probe represented an area of the *iga* gene contained within the PCR fragment, but the 3 kb probe spanned the second half of the gene not found within the PCR probe. This region was of interest as it contained a nucleotide sequence conserved between the *H. influenzae* and *N. gonorrhoeae iga* genes (Fig. 4.2). This region had been originally proposed as the active site of the enzyme (Pohlner *et al.*, 1987), when it was thought that all IgA1 proteases were metallo-enzymes. Although the enzymes from these two genera have subsequently been shown to be serine-type enzymes, this sequence may still represent a region of conservation between IgA1 proteases.

When probe hybridisation and washes (2 x SSC:0.1% SDS) were conducted at 47 °C , both probes bound to λ DNA and *M. hominis* DNA in

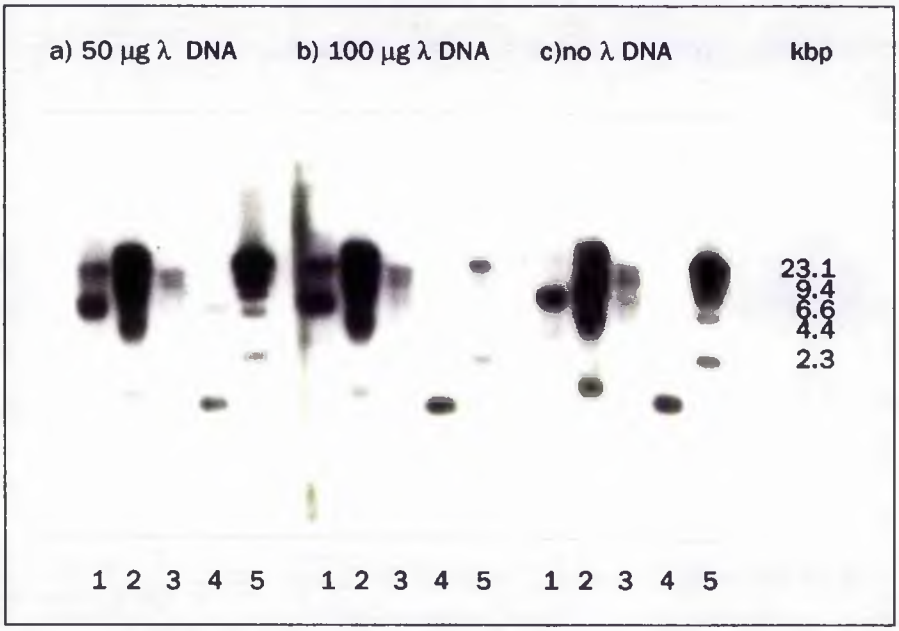


Fig. 4.4 The effect of incorporating λ DNA into Southern blot hybridisation of genomic DNA from *U. urealyticum* and *M. hominis*.

Genomic DNA from *U. urealyticum*, serotype 8 (1 µg, lane 1), *U. urealyticum*, serotype 1 (1.5 µg, lane 2), and *M. hominis*, PG50 (1 µg, lane 3) was digested with *Eco*RI. Plasmid DNA from pIP503 (containing the *iga* gene from *N. gonorrhoeae*) was digested with *Hin*dIII and *Bgl*I (0.8 ng, lane 4). The digestion products were analysed by 0.7% agarose gel electrophoresis and λ/*Hin*d III markers (125 ng, lane 5) were included for size comparisons. Following Southern transfer (Section 4.2.6), the filters were hybridised (16 h, 47 °C) with a PCR-derived probe (10 ng.ml^{-1}, containing a portion of the *iga* gene from *N. gonorrhoeae*) in the presence of 50 µg λ DNA (a), 100 µg λ DNA (b) and no λ DNA (c). The filters were washed with 2 x SSC, 0.1% SDS (47 °C) and subjected to autoradiography (16 h).
DNA size markers are to the right of the figure.

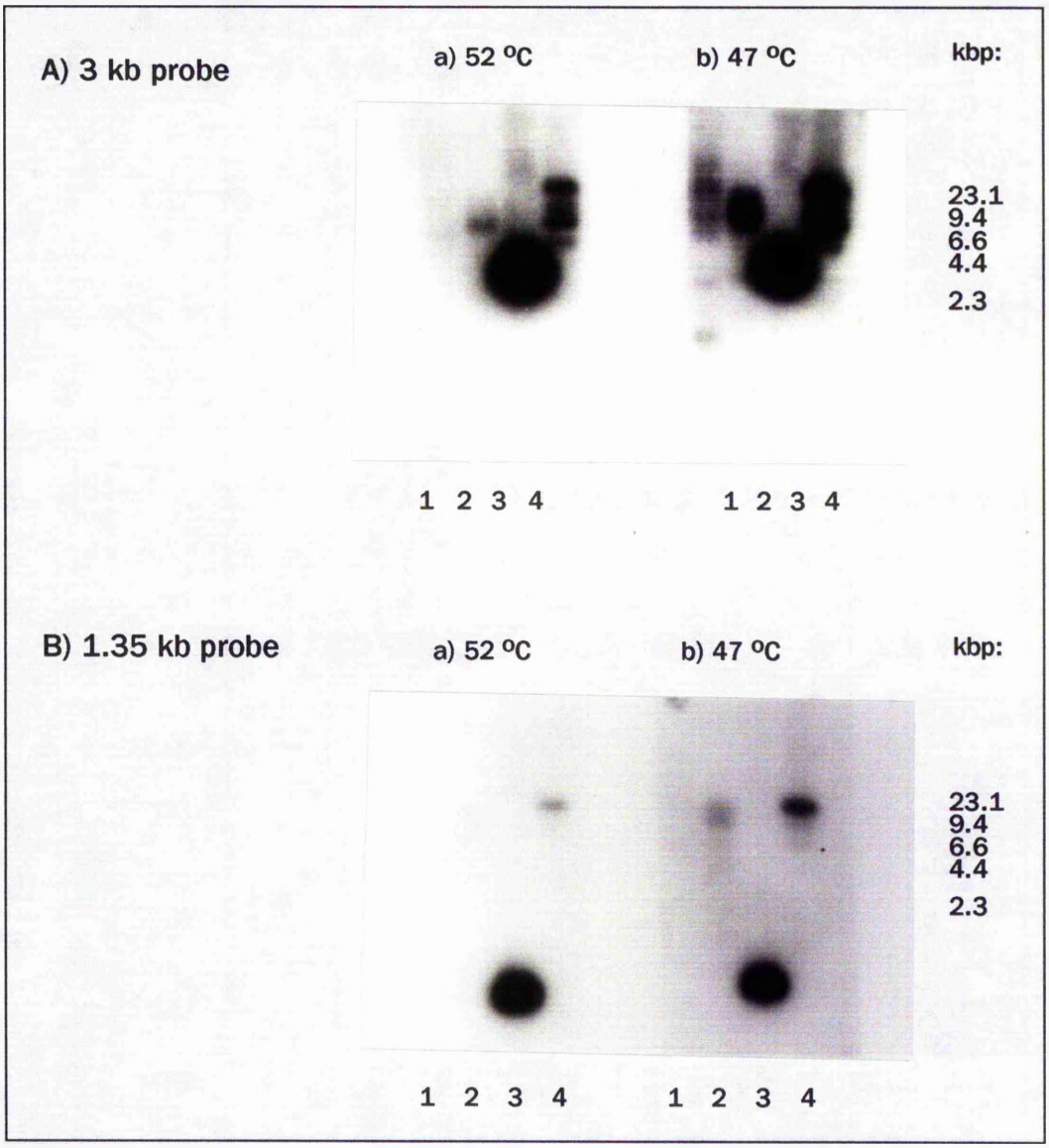


Fig. 4.5 Southern blot hybridisation of genomic DNA from *U. urealyticum* and *M. hominis* using two probes derived from restriction digests of plasmid pIP503.

Genomic DNA from *U. urealyticum* serotype 8 (1 µg, lane 1) and *M. hominis* PG50 (1 µg, lane 2) was digested with *Eco*RI. Plasmid DNA from pIP503 (containing the *iga* gene from *N. gonorrhoeae*) was digested with *Hin*dIII and *Bgl*II (8 ng, lane 3). The digestion products were analysed by 0.7% agarose gel electrophoresis and λ/*Hin*dIII markers (125 ng, lane 4, see right of figure) were included for size comparisons. Following Southern transfer, filters were hybridised (16 h) with a 3 kb plasmid-derived probe (6 $ng.ml^{-1}$, panel A), or a 1.35 kb plasmid-derived probe (2 $ng.ml^{-1}$, panel B) (Section 4.2.6). The filters were washed with 2 x SSC, 0.1% SDS and autoradiographed (72 h). Hybridisations and washes were conducted at either 52 °C (a) or 47 °C (b), as indicated.

addition to ureaplasma DNA. At 52 °C, there was minimal binding to *U. urealyticum* DNA, but some binding to λ DNA markers and *M. Hominis* DNA remained. Under conditions of low stringency (47 °C), the hybridisation pattern produced by the 3 kb probe appeared to be different from that seen with the PCR probe (Fig 4.3 and Fig. 4.5). This was not consistent with both probes binding to the same genomic fragment(s) containing the *iga* gene and suggested that they were hybridising non-specifically to different regions of ureaplasma DNA. It is possible that all of these bands represent distinct regions of the *iga* gene and that the two probes were binding to different regions of the gene. It is very unlikely, however, that the gene would comprise of so many different regions at such high molecular weight.

It was clear, therefore, that the conditions employed for these hybridisation studies were not able to detect any homology between the ureaplasma *iga* gene and the neisserial *iga* gene. The experimental conditions were altered by using a different solid-phase DNA supports and by hybridising in formamide-based solutions rather than salt-based solutions. 'Hybond N' filters were replaced by nitrocellulose filters, as there had been reports that nitrocellulose reduced some of the non-specific binding found with 'Hybond N' (D. Kirk, personal communication). It was found that nitrocellulose simply reduced the overall level of hybridisation and did not appear to encourage specific binding in favour of non-specific binding (Fig. 4.6). In this experiment, the concentration of markers present on filter B was inadvertently higher than on filter A. This highlighted a fundamental problem in hybridisation studies; a small increase in filter-bound DNA concentrations can change apparently non-specific binding into apparently specific binding. This may have been important when comparing hybridisation levels to *M. hominis* DNA and *U. urealyticum* DNA, particularly since the standardisation of genomic DNA concentrations measured by spectrophotometric methods is subject to inaccuracy.

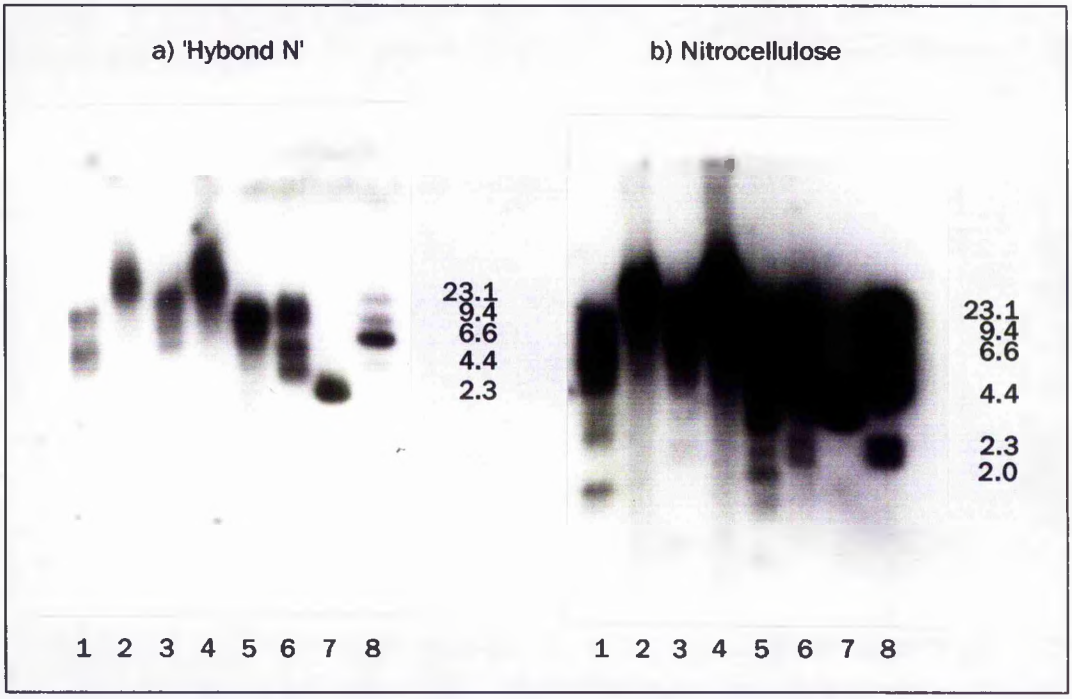


Fig. 4.6 A comparison of nitrocellulose and 'Hybond N' in Southern blot hybridisations of genomic DNA from *U. urealyticum* and *M. hominis*

Genomic DNA from *U. urealyticum,* serotype 8 (1 µg, lanes 1and 3) and *M. hominis,* PG50 (1 µg, lanes 5 and 6) was digested with *Eco*RI (lanes 1 and 5) or *Bgl*II (lanes 3 and 6). Plasmid DNA from pIP503 (containing the *iga* gene from *N. gonorrhoeae)* was digested with *Bam*HI and *Eco*RI (0.8 ng, lane 7). The digestion products were analysed by 0.7% agarose gel electrophoresis and λ/*Hin*dIII markers (lane 8, ~75 ng [a], ~175 ng [b]) were included for size comparisons. Following Southern transfer to 'Hybond N' (a) or nitrocellulose (b), the filters were hybridised (16 h, 47 °C) with a PCR-derived probe (10 $ng.ml^{-1}$), containing a portion of the *iga* gene from *N. gonorrhoeae.* The filters were washed with 2 x SSC, 0.1% SDS (47 °C) and subjected to autoradiography (22 h). DNA size markers are to the right of the figure.

4.6.3 Varying hybridisation conditions

The hybridisation conditions using formamide-based solutions were exactly as described by Koomey and Falkow (1984), for detecting nucleotide sequence homology between the *iga* genes from *N. gonorrhoeae* and *H. influenzae*. By this method, no further indication of homology between the neisserial and the ureaplasmal *iga* gene was indicated (data not shown). It was of interest that these workers only detected specific homology between the genes of *H. influenzae* and *N. gonorrhoeae* at conditions of apparently low stringency (25% formamide, 37 °C, washed in 5 x SSC, 0.1% SDS, 37 °C) but, upon sequencing, a number of regions sharing a high degree of homology were identified between the two genes. This suggested that Southern blotting and hybridisation studies were only useful for the identification of heterologous genes showing a high degree of conservation at the nucleotide level. Such a finding was also reported by Dybvig and Woodard (1992), who by hybridisation studies found no apparent homology between the *recA* genes from *B. subtilis* and *M. pulmonis*, but later sequence analysis revealed a 65% nucleotide homology of between the two genes (Dybvig *et al.*, 1992). This homology had not allowed identification of the gene by hybridisation, but was sufficient for a portion of the gene to be amplified by PCR using degenerate primers, directed towards predicted regions of nucleotide conservation.

4.7 PCR-BASED ANALYSIS

As synthetic oligonucleotides directed against the *N. gonorrhoeae iga* gene were already available, these were used as primers in preliminary PCR reactions using genomic ureaplasma DNA as the template. The annealing step was conducted at low temperatures to allow for mismatches between the primer and target sequences. At an annealing temperature of 40 °C and elongation at 60 °C, two regions of ureaplasma DNA, approximately 1.2 kbp and 400 bp, were amplified at low but detectable levels (Fig. 4.7). The 1.2 kbp fragment may have represented the portion of the *iga* gene corresponding to the 1.7 kbp fragment

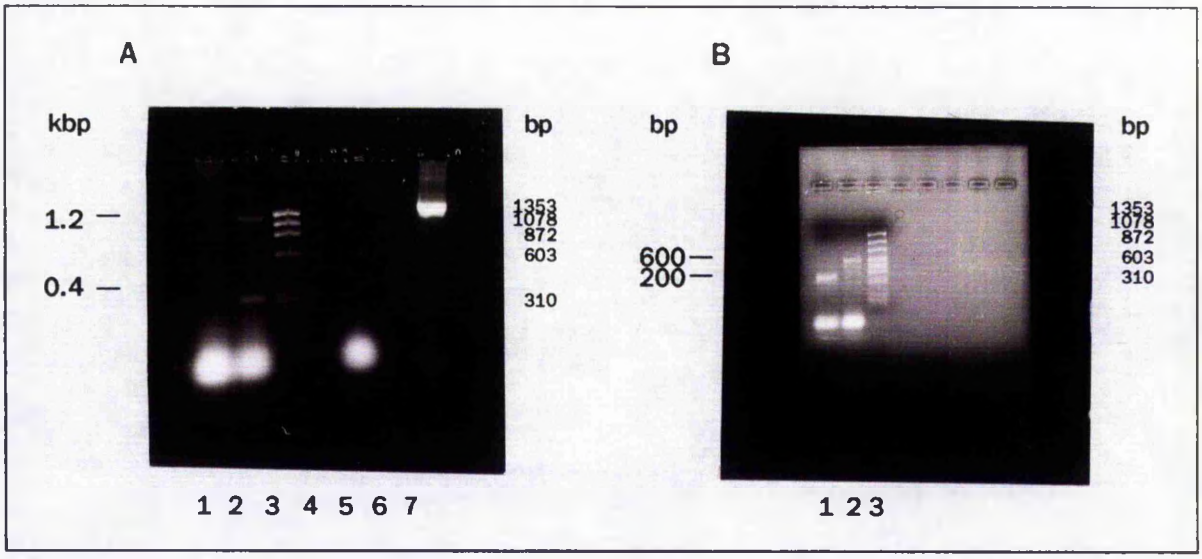


Fig. 4.7 Gel electrophoresis of reamplified DNA produced from PCR of *U. urealyticum* genomic DNA

A) PCR of *U. urealyticum* genomic DNA using synthetic primers 1 and 2 (as described in Section 4.4) gave two amplification products of approximate size 1.2 kb and 0.4 kbp. These were gel-purified, PCR-reamplified using the same primers and 1/10 of the product was analysed by gel electrophoresis (lanes 1 and 2 respectively). PCR reactions using no DNA template (lane 5) or DNA from plasmid pIP503 (containing the *iga* gene from *N. gonorrhoeae*, lane 7) were included as negative and positive controls.

B) PCR of *U. urealyticum* genomic DNA using synthetic primers 3 and 4 (as described in Section 4.4) gave two amplification products of approximate size 600 bp and 200 bp. These were gel-purified, PCR-reamplified with the same primers and 1/10 of the product was analysed by gel electrophoresis (lanes 1 and 2 respectively).

In each case DNA from ø X174:*Hae*III digest (lane 3) provided size markers, as indicated to the right of the figures. The gels were stained with ethidium bromide (1 $\mu g.ml^{-1}$) and photographed using Polaroid film.

amplified from *N. gonorrhoeae*. The two bands were gel-purified and reamplified using the same primers and standard PCR conditions (annealing at 55 °C, amplification at 72 °C), since the primers should have been homologous with the flanking regions of the first PCR products. The quantity of amplified DNA was not as high as expected but after a third round of amplification sufficient DNA was produced to use in hybridisation and cloning experiments (Fig. 4.7). It was not clear why an additional band of 300 bp was produced after reamplification of the 1.2 kbp band.

A proportion of each fragment was radiolabelled with [α^{32}P] dCTP and used as a probe in Southern blotting (Fig. 4.8). Using hybridisation temperatures of 55 °C, the specific homology between both of the probes and ureaplasma DNA was clear. Under these conditions, there was little or no binding to mycoplasma DNA or λ markers. The 1.2 kbp probe, however, did not hybridise with DNA isolated from serotype 1 which had already been demonstrated to express an IgA1 protease enzyme (Section 2.8.2). It was also clear that the two products (1.2 kbp, 400 bp) had not been amplified from the same region of DNA, as restriction fragments of different sizes were highlighted by the two different probes. This cast doubt upon the specificity of the PCR and suggested that the primers may not have bound specifically to the ureaplasma *iga* gene but bound randomly to the template DNA. Nevertheless, the 400 bp fragment (which had hybridised with ureaplasma serotypes 1 and 8) was blunt-end ligated into the *Sma*I site of M13 and transformed into competent preparations of *E. coli*, strain JM101. Recombinant phage appeared as colourless plaques on a bacterial lawn containing X-gal and IPTG. By virtue of the life cycle of M13, single stranded DNA was purified from liquid cultures of JM101 transfected with these phage and sequenced using the dideoxy chain-termination method. The 1.2 kbp amplification product repeatedly degraded during purification and therefore was not cloned into M13 or sequenced.

The sequence obtained from the 400 kbp fragment is presented in Fig. 4.9. The A+T content is 30 %, suggesting that it is ureaplasma DNA, in agreement with the results obtained from Southern Blotting. Using the 'translate facility' in the GCG package, an obvious open reading frame could not be found (Fig. 4.9). This

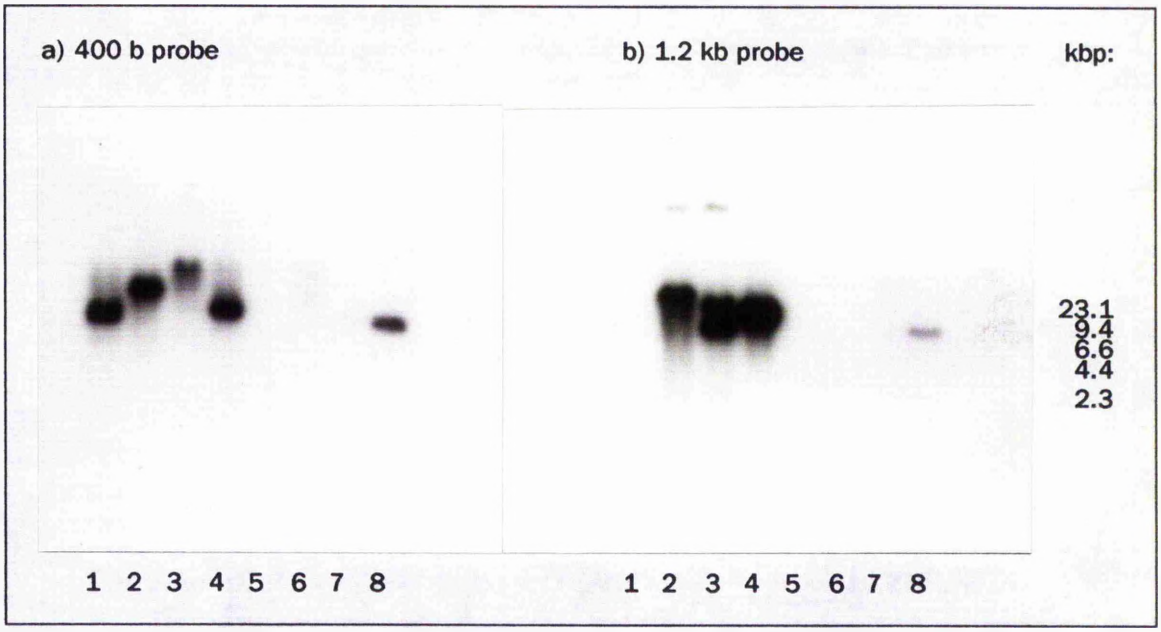


Fig. 4.8 Southern blot hybridisation of genomic DNA from *U. urealyticum* and *M. hominis* using PCR-derived probes amplified from ureaplasma template DNA.

Genomic DNA from *U. urealyticum* serotype 1 (1 µg, lane 1), *U. urealyticum* serotype 8 (1 µg, lanes 2-4) and *M. hominis* PG50 (1 µg, lane 5) was digested with *Eco*RI (lanes 1, 2, 5), *Sca*I (lane 3) and *Bgl*II (lane 4). Plasmid DNA from pIP503 (containing the *iga* gene from *N. gonorrhoeae*) was digested with *Bam*HI and *Eco*RI (200 ng, lane 8). Lane 6 contained size markers (λ/*Hin*dIII, 125 ng) as indicated on the right of the figure. Following gel electrophoresis (0.7% agarose) and Southern blotting, filters were hybridised (16 h, 55 °C) with a 400 bp PCR probe (10 ng.ml^{-1}, panel a), or a 1.2 kbp PCR probe (10 ng. ml^{-1}, panel b). The filters were washed with 2 x SSC, 0.1% SDS and autoradiographed (1 h).

Fig. 4.9 Sequence obtained from *U. urealyticum* PCR-amplified DNA.
The PCR product amplified from *U. urealyticum* genomic DNA using primers 1 and 2 (as described in Section 4.4), was cloned into the filamentous bacteriophage phage vector, M13. Single-stranded transcripts were sequenced by the dideoxy chain-termination reaction and translated into three reading frames (a, b, c) using the 'translate' programme in the GCG sequence analysis package. Since there is no apparent open reading frame, it is assumed that the product is a result of non-specific amplification of ureaplasma DNA.

suggested that the sequence did not represent a coding region. In the event that there had been some mistakes in interpretation of the sequence, so preventing identification of an open reading frame in the translational analysis, the sequence was compared with the FastA data base within the GCG programme. No clear homology between the ureaplasma sequence and any other published sequence within this data base was found.

It was unfortunately not possible to obtain more than one transformant containing this sequence. This was partly due to the failure to obtain large quantities of the DNA necessary for blunt ended ligation. In addition the rates of transformation were very low for these constructs; only five plaques were obtained from a ligation reaction containing 10 ng DNA. Using wild-type M13, transformation rates of 1×10^6 pfu.μg^{-1} DNA were produced which suggested that the transformation had been efficient but that the ligation reactions had been very inefficient. The primers had not originally been designed either for heterologous amplification reactions or for subsequent cloning steps. They had demonstrated, however, that a PCR-based method was a feasible approach for the identification ureaplasma genes.

It was for this reason that two new PCR primers were synthesised (Fig 4.2). Primer 3 was similar to primer 1 but contained terminal *Eco*RI restriction enzyme sites, enabling the amplified product to be ligated more efficiently into *Eco*RI sites within M13 DNA. Primer 4 also contained an *Eco*RI site but was based on different area of nucleotide homology found between the *iga* genes of *H. influenzae* and *N. gonorrhoeae.* It has been recently proposed that this area codes for the serine protease active site (Poulsen *et al.,* 1992) and as the ureaplasma enzyme also appears to be a serine protease (Section 2.8.1), it is possible that this sequence has also been conserved within the ureaplasma gene.

Using the newly designed primers, two amplification products of 600 bp and 200 bp were obtained by PCR following the protocol described earlier (Fig. 4.7). Despite repeated attempts to clone these products into M13 for sequence analysis this proved impossible, possibly because of the small quantities

of DNA obtained from PCR. Their relationship to the *iga* gene from *U. urealyticum* is at present unknown.

SECTION C
DISCUSSION

To identify the *iga* gene within *U. urealyticum,* only a limited number of options were available. As ureaplasmas utilise the translational stop codon UGA to code for the amino acid tryptophan, it was very unlikely that an active enzyme would be expressed from the λ ZAP library. A number of *E. coli* repressor strains have been used to express whole mycoplasma proteins from genes with UGA codon usage (Dybvig, 1990), but these are not suitable for expression of a whole gene library. In *M. pneumoniae,* a number of genes encoding the adhesin (P1) and related adhesion proteins (HMW 3 and P 30) have been identified from fusion proteins in λgt11 libraries using specific monoclonal antibodies (Dallo *et al.,* 1990, Inamine *et al.,* 1988, Ogle *et al.,* 1991). Although inserted DNA sequences in λ ZAP could be expressed as fusion proteins, there was no means of identifying portions of the IgA1 protease as no specific antiserum to this enzyme was available. Furthermore, since the ureaplasma IgA1 protease had not been purified, synthetic DNA probes based on amino acid sequences could not be designed. Willoughby *et al.,* (1991) and Su *et al.,* (1987) have used such an approach to identify the urease and P1 gene in *U. urealyticum* and *M. pneumoniae,* respectively.

It was known, however, that in terms of substrate specificity and enzyme classification the ureaplasma IgA1 protease was similar to the type 2 enzymes in *N. gonorrhoeae* and *H. influenzae.* Hybridisation studies by Koomey and Falkow (1984), had previously identified a significant degree of nucleotide homology between the *iga* gene from these two species. Similar cross-genera relationships have been used to isolate a number of other mycoplasma and ureaplasma genes. The ATPase gene in *M. hominis* PG50, the gyrase gene in *M. pneumoniae* and the urease gene in *U. urealyticum* were all identified by hybridisation experiments using heterologous DNA probes obtained from the genes within *E. coli, B. subtilis* and *Providencia stuartii,* respectively (Rasmussen and Christiansen, 1987, Colman *et al.,* 1990, Blanchard and Barile, 1989).

Hybridisation of Southern-blotted ureaplasma genomic DNA using probes derived from the *N. gonorrhoeae* type 2 *iga* gene, however, revealed no obvious DNA duplexes. Substantial efforts were made to ensure that this result was not due to unsuitable experimental conditions. Thus, three different molecular probes spanning the whole gonococcal *iga* gene were used under a variety of hybridisation and washing conditions. It was possible that the weakly-hybridising ureaplasma DNA fragments observed under low stringency conditions represented the *iga* gene from this organism but there were equivalent levels of probe-hybridisation to control DNA from *M. hominis* and λ DNA markers. It is not clear why the gonococcal probes bound to λ DNA at hybridisation temperatures as high as 55 °C, but the reduction in binding observed in the presence of carrier λ DNA suggested that there may have been some homology between the two species of DNA. Nucleotide sequence comparisons between the gonococcal and λ DNA and did not support this proposal and it is likely that the observed reaction was simply a result of the high concentration of filter -bound λ DNA in combination with the low stringency hybridisation conditions used.

When comparing nucleotide homology between the *iga* genes from *N. gonorrhoeae* and *H. influenzae*, Koomey and Falkow (1984) had also reported that gonococcal probes bound non-specifically to Southern blot-transferred DNA under conditions of low stringency. In this case, however, the non-specific binding was to *N. gonorrhoeae* DNA itself, and it therefore may have been due to a repetitive element in the *iga* gene probes binding to similar sequences in genomic DNA. This effect nevertheless called into question the validity of the DNA duplexes observed between the gonococcal probe and *H. influenzae* genomic DNA. It was only because two gonococcal probes derived from different regions of the *iga* gene bound to the same *H. influenzae* DNA restriction fragments that a specific reaction was suspected. This was confirmed by cloning *H. influenzae* DNA fragments of the same size range into a pBR322-based plasmid vector and identification of IgA1 protease activity in 5% of *E. coli*-expressed clones.

In contrast, a number of *U. urealyticum* genomic DNA fragments were highlighted by the two different gonococcal probes used in this study. While this

could have been due to the ureaplasma *iga* gene containing more than one restriction site for the particular enzymes used, the size and number of these fragments made this explanation unlikely. It was concluded therefore, that the hybridisation observed with ureaplasma DNA was non-specific.

This proposal was indirectly supported by similar hybridisation experiments using a DNA probe obtained from the ATPase gene in *M. gallisepticum* (experiments performed by P. McCready, University of St. Andrews, UK). The filter-bound DNA and the methods employed were identical to those developed for the IgA1 protease studies. Under relatively stringent conditions (55 °C hybridisation, 40 °C washes in 0.2 x SSC:0.5% SDS), distinct hybridisation was observed between the mycoplasma gene probe and the ureaplasma DNA. There was only minimal binding to *M. hominis* and λ DNA. The same restriction fragment was detected by two probes derived from different regions of the ATPase gene. These results confirmed that suitable materials and methods had been developed for the IgA1 protease study but it was clear that if there had been meaningful homology between the *iga* genes it would not have been necessary to try such a variety of hybridisation and washing conditions. Furthermore, although the homology between the ATPase genes was obvious by Southern blotting, the hybridisation of the probe to the ureaplasma λ library 'plaque lifts' was less apparent, and prolonged exposure of autoradiograms was required. This emphasised that even if the weakly hybridising bands had represented the ureaplasma *iga* gene, the levels of binding were probably insufficient to subsequently identify the gene within the genomic λ library. Work on the ATPase gene nevertheless confirmed that a functional *U. urealyticum* genomic DNA library had been generated.

Although it was a reasonable assumption that genes encoding an enzyme with shared biochemical characteristics would have sufficient sequence homology to form filter-bound DNA duplexes, this has not been demonstrated for the streptococcal *iga* genes. The IgA1 proteases from *S. sanguis* and *S. pneumoniae* are both metallo enzymes with identical substrate specificity but *S. sanguis iga* gene probes showed no detectable hybridisation with chromosomal DNA from

S. pneumoniae, even under low stringency conditions (Gilbert *et al.*, 1988). The *iga* gene sequence for *S. pneumoniae* is not yet available and it may be the case that regions encoding streptococcal IgA1 protease functions are conserved between the species but that the overall level of nucleotide conservation is not sufficient to allow the formation of stable hybrids. The same may be true for the ureaplasmal and neisserial *iga* genes. In order to investigate this, degenerate oligonucleotide primers directed towards potential regions of nucleotide conservation between *iga* genes were used in PCR to amplify a portion of the gene from ureaplasma genomic DNA. If this had been successful, the amplified product could then have been used as a 100% homologous probe to isolate the whole ureaplasma *iga* gene from the genomic library.

A similar approach had been used by Dybvig *et al.*, (1992) to identity the *recA* gene from *M. pulmonis*. These workers found no apparent homology between the *recA* genes from *B. subtilis* and *M. pulmonis* by hybridisation studies but were able to identify the gene within *A. laidlawii* by such methods (Dybvig and Woodard, 1992). A comparison of the nucleotide sequences from *E. coli*, *B. subtilis* and *A. laidlawii recA* genes revealed regions of nucleotide conservation which were used to design a number of degenerate synthetic oligonucleotide primers. By using various combinations of these primers in PCR, a 323 bp region was amplified which, when sequenced, was identified as a portion of the *M. pulmonis recA* gene. Comparative sequence analysis revealed a 65% nucleotide identity with the *recA* genes from *B. subtilis*, which had not been detected by hybridisation studies.

Using the same PCR-based approach, it was not possible to identify the *iga* gene within *U. urealyticum*. This could have been due to a number of reasons. It was not that the condition of the template DNA was unsuitable for PCR, since a portion of this DNA was used to amplify a region of the urease gene using the specific oligonucleotide primers described by Willoughby *et al.*, (1991). (Experiment performed by I. Leith, University of St. Andrews). In the *iga* study, the synthetic primers chosen for PCR may not have been complementary for any regions within the ureaplasma gene. Since the IgA1 protease from *U. urealyticum*

appeared to have identical substrate specificity and reaction mechanism to the type 2 genes from *N. gonorrhoeae* and *H. influenzae*, it was predicted that nucleotide regions conserved between these genera, particularly in the putative active site, may also have been conserved with ureaplasmas. This may not necessarily be the case.

Future work should concentrate on designing alternative degenerate primers, particularly since Dybvig *et al.*, (1992) used various combinations of five degenerate primers to optimise PCR amplification of the *recA* gene. These could be based on the 370 bp cleavage site specificity determinant (CSD) identified within the *H. influenzae* type 2 *iga* gene (Grundy *et al.*, 1990). Although the precise nucleotide sequences controlling the cleavage specificity of type 2 IgA1 proteases have not been clearly defined, it has been shown that this region is more strongly conserved between type 2 gonococcal and *Haemophilus* genes than between the type 1 and type 2 *Haemophilus* genes and therefore may be conserved within the ureaplasma gene.

As an adaptation of the PCR-based method, it may also be possible to amplify the *iga* gene from the ureaplasma genomic λ library using one primer directed towards a known λ sequence, such as the multiple cloning site and another directed towards a region within the *iga* gene, such as the putative serine protease active site. By this approach, it is only necessary to predict one conserved sequence between *iga* genes.

It ultimately may not be possible to isolate the *iga* gene from *U. urealyticum* by the prediction of regions of nucleotide conservation. *iga* genes have already demonstrated considerable divergence within the genus *Streptococcus* and since it has been proposed that ureaplasmas are evolving more rapidly than other prokaryotes (Woese, 1987), it is likely that there is yet further divergence within the genus *Ureaplasma*.

To isolate the ureaplasma *iga* gene, it may therefore be necessary to return to protein purification experiments to obtain specific tools for gene identification such as a monoclonal antibody or a portion of amino acid sequence.

Concluding Remarks

To investigate the IgA1 protease from *U. urealyticum*, a variety of different approaches have been employed. Using crude enzyme preparations, it has been established that the enzyme cleaves between a Thr-Pro peptide bond within a stretch of IgA1 heavy chain amino acids that are absent in the hinge-region of IgA2. All fourteen serotypes have an apparently identical substrate specificity and enzyme activity levels are comparable, if not identical, between each serotype. By using a range of class-specific inhibitors, it has been suggested that the enzyme is a serine protease.

In order to confirm and extend these findings, attempts were made to purify the enzyme by conventional chromatography. Due to low levels of starting material and losses sustained during the separation steps, the attempts proved unsuccessful. By examining the localisation of the IgA1 protease in *U. urealyticum*, however, it was established that a large proportion, if not all of the enzyme activity, is cell-associated rather than extracellularly expressed. The failure of the enzyme to digest a synthetic peptide, based on the site cleaved by the gonococcal IgA protease during its processing and secretion, supported this proposal.

Expression of the *iga* gene in a bacterial host may have served to provide large quantities of ureaplasmal IgA1 protease for purification purposes. Sequence analysis of the *iga* gene may also have confirmed the proposed reaction mechanism and cellular location of the ureaplasmal enzyme. To identify the *iga* gene within a ureaplasma genomic DNA library, heterologous DNA probes derived the *N. gonorrhoeae iga* gene were used in Southern blotting hybridisation studies. Although the ureaplasma enzyme shared a substrate specificity and serine protease class with the type 2 enzyme from this organism, there was insufficient nucleotide homology between the two *iga* genes to allow stable duplex formation and gene identification. In a PCR-based approach, using synthetic primers based on predicted conserved regions between *iga* genes, amplification of specific sequences was also not achieved.

The progress achieved in any one of the areas described above has been highly dependant upon the degree of success achieved in each of the other areas.

Thus, in the absence of a purified enzyme or *iga* gene sequence, biochemical characterisation was limited. Likewise, gene isolation has been hampered by the lack of specific antibody or protein sequence and protein purification would have been greatly facilitated by bacterial gene expression.

To further characterise the enzyme, advancement in one of these areas is necessary. It is possible that additional synthetic primers and a variety of PCR reaction conditions may allow amplification of specific ureaplasma *iga* sequences. Alternatively, purification of the IgA1 protease followed by limited protein sequencing may provide more suitable probes for *iga* gene isolation.

To repeat the purification attempts, a more rapid and quantifiable assay is required. The HPLC-based method showed promise as a quantitative assay for future use, but the recent cloning and expression of IgA in mammalian tissue culture cells may provide a cheaper and more rapid means of identifying IgA1 protease activity.

As far as IgA1 proteases in general are concerned, characterisation of the *U. urealyticum* IgA1 protease has confirmed that a high level of diversity exists between this group of enzymes. Although it was thought that all IgA1 proteases were extracellularly expressed, this does not appear to be the case for the ureaplasmal enzyme. Recent reports have indicated that bacterial IgA1 proteases also show a degree of cell-association (Pohlner *et al.* 1991). The distinct nature of the ureaplasma enzyme is highlighted by the apparent lack of nucleotide conservation between genomic DNA and the gonococcal *iga* gene. Since the two enzymes show similar properties, a significant role for the IgA1 protease is suggested.

If the small and simple ureaplasma has only retained the essential genetic information required for growth and survival, expression of the IgA1 protease by all fourteen serotypes further attests to its significance. While the function of the IgA1 protease in *U. urealyticum* (or any bacteria) is unclear, it seems likely that enzyme expression may contribute to the virulence of this organism. Since a species-specific enzyme has been identified in the canine ureaplasma, progress in

elucidating this function by animal-based pathogenicity studies may be possible. These preliminary studies serve as a basis for such future work.

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