A Study of the Role of Redox Potential
in Lysosomal Function

by

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PREFACE

The experimental work described in this thesis was carried out in the Department of Biochemistry, University of Natal, Pietermaritzburg, from January 1995 to December 1996, under the supervision of Professor Clive Dennison.

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

Richard Edward Meinesz

12th day of December, 1996

ABSTRACT

"Lysosomes", which include late endosomes, are traditionally defined as membrane-bound acidic organelles containing hydrolytic enzymes, and are thought to be the principle site of intracellular digestion. For their role in proteolysis, lysosomes are also thought be reducing, both for the function of the cysteine cathepsins and for the reduction of disulfide bonds in the substrate proteins. However, a number of observations raise the question of whether the intralysosomal milieu is, in fact, invariably reducing and proteolytic, or whether lysosomal function may, in effect, be regulated by a variable redox potential.

To address this question three lysosomal enzymes, cathepsins S and B and acid α-glucosidase were isolated to assess their *in vitro* response to varying concentration of the reducing agent, cysteine. The isolation procedures devised were more rapid and cost effective than reported methods and gave acceptable yields. Cathepsin S was further characterized and acid α-glucosidase was used as a source for the preparation of antibodies for possible further immunochemical and immunocytochemical studies. The effect of cysteine on these lysosomal enzymes supports the hypothesis that intralysosomal redox potential may constitute part of a regulatory mechanism for lysosomal function.

A second approach involved qualitatively assessing the *in vivo* intralysosomal redox potential using a novel double labelling indicator system. For detecting intralysosomal oxidation cells were loaded with an acetoxymethyl ester derivative which fluoresces in the oxidised form, but this dye is not selectively loaded into lysosomes. Lysosomes can, however, be visualised using a second dye which selectively accumulates in cellular compartments with low internal pH. By capturing the two separate fluorescent images and computer analyzing them it was ascertained that the intralysosomal milieu may not be invariably reducing as some lysosomes showed varying degrees of oxidation.

ACKNOWLEDGEMENTS

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ABTS	2,2'-azino-di-(3-ethyl)-benzthiozoline sulfonic acid
Ahx	6-aminohexanoyl
AMT	acetate-MES-Tris buffer
BSA	bovine serum albumin
BSA-PBS	bovine serum albumin dissolved in phosphate buffer saline
Bz	benzoyl
t-butanol	2-methylpropan-2-ol
C	N,N'-methylenebisacrylamide
C-2938	5-(and-6)-carboxy-2',7'-dichlorodifluorescein diacetate, bis(acetoxymethyl) ester
cDNA	copy deoxyribonucleic acid
Cy-SH	cysteine
Cy-S-S-Cy	cystine
dist.H$_2$O	distilled water
DMEM	Dulbecco's minimal essential medium
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
EDTA	ethylene diamine tetra-acetic acid
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
E-64	L-trans-epoxysuccinyl-leucylamido(4-guanidino)butane
Ex	excitation wavelength
FPLC	fast protein liquid chromatography
Gly-Sc	glycinaldehyde semicarbazone
GSSC	glutathione-cysteine mixed disulfide
GSSG	oxidised glutathione
GSH	reduced glutathione
HBSS	Hank's balanced saline solution
IgG	immunoglobulin G

K_{av}	availability constant
kDa	kilodaltons
MCF	Michigan Cancer Foundation
MES	2(n-morpholino)ethanesulfonic acid
mRNA	messenger ribonucleic acid
M_r	relative molecular weight
MW	molecular weight
NHMec	7-amino-4-methyl coumarin
NIH	National Institute of Health
PBS	phosphate buffered saline
PBS-Tween	Tween® 20 diluted in phosphate buffered saline
PEG	polyethylene glycol
pI	isoelectric point
®	registered trade mark
RT	room temperature
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
T	acrylamide
TEMED	N,N,N',N'-tetramethylethylenediamine
TPP	three phase partitioning
Z	benzoyloxycarbonyl

CHAPTER 1

INTRODUCTION

Lysosomes are membrane-bound intracellular digestive organelles that function principally in intracellular digestion (Bainton, 1981; Kornfeld and Mellman, 1989). Lysosomal hydrolases are capable of degrading proteins, lipids, nucleic acids and saccharides (Bainton, 1981). Materials to be digested are incorporated within the same membrane-bound compartments as the lysosomal enzymes. In this way the cell deploys its enzymes economically and fosters the co-operative activities of a voracious group of enzymes whose actions would likely prove fatal, or at least damaging, were the enzymes to be released into the cytoplasm (Holtzman, 1989). The compartmentation also facilitates the establishment of a low pH environment within the lysosome, needed for the efficient activity of some of the enzymes. Lysosomes are generally thought to have an acidic internal milieu (Ohkuma and Poole, 1978; Yamashiro and Maxfield, 1987a,b; Anderson and Orci, 1988) that is generated by proton pumps (Ohkuma *et al.*, 1982; Yamashiro *et al.*, 1983). Consistent with this is the observation that many lysosomal enzymes apparently have acidic pH optima for activity (Bond and Butler, 1987; Aronson and Kuranda, 1989). However, there is recent evidence that some lysosomal enzymes show neutral or even alkaline pH optima (Butor *et al.*, 1995; Dehrmann *et al.*, 1996). Biological materials destined for degradation in the lysosomes are either ingested by endocytosis and shuttled to the lysosomes via early and late endosomes or, in the case of large particles, are taken up by phagocytosis into specialized macrophages resulting in the formation of phagolysosomes (Mellman *et al.*, 1986). In a process called autophagy, obsolete endogenous cellular structures are transferred to and degraded in the lysosomal compartment (Dunn, 1990). However, this simple overview of lysosomes does not apply in straightforward fashion to all phenomena that are observed when studying these organelles.

The formation of lysosomes requires input from both the biosynthetic and the endocytic pathways. Newly synthesized lysosomal hydrolases are sorted in the *trans*-Golgi network by binding to mannose-6-phosphate specific receptors and are selectively transported to the endosomal compartment where uncoupling of ligand and receptor occurs. In the endosomes the newly synthesized lysosomal enzymes encounter the molecules on the endocytic route destined

for degradation. Degradative processes are likely to commence in endosomes (Peters and von Figura, 1994). From this compartment the lysosomal enzymes and their substrates are thought to be delivered to the lysosomes where degradation is completed (von Figura and Hasilik, 1986; Kornfeld and Mellman, 1989). At present there is no consensus on the exact molecular mechanisms for the formation of lysosomes. One model is based on pre-existing late endosomes and lysosomes communicating via transport vesicles (Gruenberg et al., 1989). An alternative model postulates a gradual maturation of early endosomes to late endosomes and subsequently to lysosomes by processes of continuous fusion and fission of vesicles (Stroorvogel et al., 1991). A very recent hypothesis suggests that all membrane organelles in the cell should be considered either as vesicles or compartments (Griffiths, 1996). Vesicles are defined as membrane-enclosed containers that form de novo by budding off part of a pre-existing compartment, forming discrete, sealed particles, which generally do not fuse with themselves. A compartment is defined as a complex multifunctional membrane organelle, specialized for different essential cellular functions, which can undergo homotypic or lateral fusion with themselves but not with different compartments (Griffiths, 1996).

The membrane limiting the lysosomal compartment has multiple functions. It is responsible for the acidification of the interior and the sequestration of highly active lysosomal enzymes capable of destroying cellular structures (Mellman et al., 1986). Furthermore, the lysosomal membrane mediates the transport of degradation products from the lysosomal lumen to the cytoplasm and regulates the fusion and fission events between lysosomes and other organelles (Haylett and Thilo, 1991).

The long-term goal of the project reported here was to investigate the possibility that lysosomes may be subject to redox regulation. This idea arose from two observations which are not consistent in terms of prevailing views of the lysosome. The first is that lysosomes contain a large repertoire of enzymatic activities which function optimally in an acidic, reducing environment, which was thought to be the conditions prevailing in this organelle. Second, is the existence in lysosomes of a high molecular weight form of glycogen, cross-linked via disulfide-bridged protein moieties, and which is not rapidly turned over (Geddes and Stratton, 1977b). These two observations create a paradox, if the intralysosomal milieu is both reducing and proteolytic (the conventional view), how could a molecule that has both a protein content and

disulfide linkages be <u>stored</u> in lysosomes? This led to the hypothesis that the redox potential of the internal milieu of lysosomes may be variable, and that redox potential may constitute a possible regulatory mechanism for lysosomal function.

Various investigations have pointed to a role for thiols in stimulating the rate of lysosomal degradation of disulfide-rich proteins. Griffiths and Lloyd (1979) observed an enhanced rate of ^{125}I-insulin degradation by disrupted rat liver lysosomes when reduced glutathione was added to incubation mixtures. Kooistra *et al.* (1982) found that a variety of thiols were able to stimulate the rate at which purified lysosomal enzymes could degrade ^{125}I-labelled insulin or albumin. They also showed that the degradation of bovine insulin or albumin by purified cathepsin D was thiol enhanced. Since cathepsin D is an aspartic proteinase and is thus itself not affected by thiols, and since no other enzyme was present, it was concluded that the role of thiol was most probably non-enzymic reduction of disulfide linkages, facilitating access of the proteinase to susceptible linkages in the substrate. This view was reinforced by subsequent data from Mego (1984), who showed that thiols activate proteolysis by cathepsin D only if the substrate protein contains disulfide bridges. Further insight into the role of thiols was provided by Kooistra *et al.* (1982) who showed that proteolysis proceeded optimally only if thiols were present together with proteinases in the incubation mixture. A pre-treatment with thiol did not suffice, nor was thiol able to reduce all the disulfide linkages in a protein in the absence of proteinases. Lloyd (1986) postulated that the two processes of proteolysis and disulfide reduction act synergistically to facilitate lysosomal degradation of proteins. First, thiols activate the thiol-dependent lysosomal enzymes[1] (i.e. lysosomal cysteine cathepsins), and second, thiols reduce protein disulfide linkages allowing proteins to unfold, thereby providing proteinases access to susceptible linkages within the substrate. There would therefore appear to be a synergism between disulfide reduction and enzyme-catalysed peptide bond hydrolysis, each component process opening out the substrate molecule and so permitting further attack (Lloyd, 1986).

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[1] In their isolated forms, the cysteine cathepsins require activation with a reducing agent such as cysteine, before they manifest proteolytic activity. Whether this is true of these enzymes *in vivo* is, to my knowledge, an open question.

Lloyd (1986) postulated that cysteine may be the physiological agent responsible for the intralysosomal reduction of protein disulfides. Cysteine is abundant in the cytoplasm and seems to be able to cross the lysosomal membrane of normal cells (Kooistra *et al.*, 1982). However, Kooistra *et al.* (1982) did not show whether this was an active or passive process. Lloyd (1986) suggested that cysteine may also arise in the lysosomes by degradation of cysteine-containing proteins. Like any other thiol, it could fully reduce a protein cysteine residue by two sequential reactions, but, unlike other reductants would generate a molecule of cystine in the process (Fig. 1). This cystine, arising not from the protein itself but from the reductant, could be exported from the lysosome via a cystine transporter present in the lysosomal membrane (Kooistra *et al.*, 1984; Smith *et al.*, 1987). Reduction by cysteine is plausible on thermodynamic grounds. Cysteine and protein-bound cystine must have very similar redox potentials, and a high cysteine/cystine concentration ratio in lysosomes could conceivably be maintained by the reductive effect of cytoplasmic glutathione (GSH) (Lloyd, 1986).
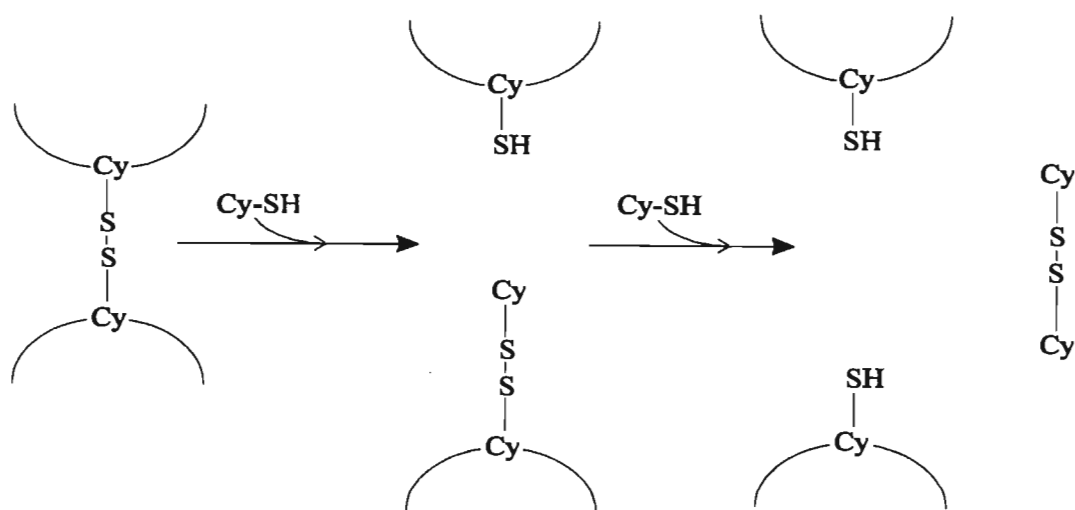
Figure 1. Diagram illustrating the reduction of a protein disulfide bridge by two consecutive reactions with cysteine (Cy-SH).

Note that cystine (Cy-S-S-Cy) is a product of this reaction sequence and that it arises from the reductant and not from the cystine residue (adapted from Lloyd, 1986).

Subsequently a lysosomal transport system, highly specific for cysteine, was discovered by Pisoni *et al.* (1990). They proposed that this cysteine specific transporter may deliver cysteine into the lysosome and aid lysosomal proteolysis by reducing protein disulfide bridges and by providing thiol for the lysosomal thiol-dependent proteinases (see Chapter 4). Lloyd (1992), however, argues from the stoichiometry of the reduction reaction that there is no need for the net import of cysteine into the lysosome for proteolysis, and that any import is likely to constitute merely an anapleurotic pathway replacing cysteine that may have been oxidised to cystine. If the role of the cysteine transporter is not to provide cysteine as an aid to disulfide reduction concomitant with proteolysis, might its role be to modulate the intralysosomal redox potential, and thereby also the activity of lysosomal enzymes, especially perhaps proteinases?

The disease cystinosis (Kooistra *et al.*, 1984) provides an interesting case in regards to lysosomal redox potential. This disease, characterised by an accumulation of cystine in lysosomes, is believed to be caused by the incompetence of the lysosomal cystine transporter. The question then arises, what effect may this have on the lysosomal redox potential and consequently on lysosomal function? Interestingly, the renal failure characteristic of the end stage of this disease (Kooistra *et al.*, 1984) is consistent with suboptimal cysteine protease activity, since renal tubules are rich in these enzymes which play a role in degrading re-absorbed proteins.

Thus, for their role in proteolysis, lysosomes are thought to be reducing both for the function of the cysteine cathepsins and for the reduction of disulfide bonds in substrate proteins. However, evidence relating to lysosomal glycogen raises the question of whether the internal milieu of lysosomes is invariably reducing. Glycogen has both large molecular size and great polydiversity, and because of the latter property, any observations on its size are of little significance without knowledge of the distribution of the different sized particles (Geddes *et al.*, 1977a). Its molecular weight distribution ranges from below 1×10^6 to greater than 100×10^6 daltons (Geddes, 1985). From electron microscopy it has been shown that glycogen is composed of single spherical particles of approximately 30 nm in diameter (β-particles) and clusters of these spheres (α-particles) which can approach 200 nm in diameter (Watson and Drochmans, 1972). The β-particles, within the α-particles, have a molecular weight of approximately 10^7 daltons (Geddes *et al.*, 1977 a,b), and they are known to be built upon a single protein backbone

(Krisman and Barengo, 1975; Calder and Geddes, 1988) in groups of two or three (Chee and Geddes, 1977; Geddes *et al.*, 1977a), which may then aggregate by disulfide bridges to form the large α-particles (Chee and Geddes, 1977; Geddes and Stratton, 1977b). A significant proportion of high molecular weight liver glycogen is compartmentalised within lysosomes (Geddes and Stratton, 1977a; Geddes *et al.*, 1983; Geddes and Taylor, 1985a,b). Apart from its lysosomal association, high molecular weight glycogen is also distinguished from its lower molecular weight counterpart by having at least double the amount of protein bound to it per gram of polysaccharide, even after extensive purification (Chee and Geddes, 1977; Calder and Geddes, 1983).

The intralysosomal glycogen is metabolised by acid α-glucosidase (EC 3.2.1.20) which is also contained within the lysosome, raising the question of how it is regulated so it does not continually digest the lysosomal glycogen. A number of interesting experiments were carried out by Calder and Geddes (1989a) on the ability of acid α-glucosidase to hydrolyse glycogen obtained from three different sources, namely, rat liver, human placenta and oyster. The main difference between the glycogen from these three sources is molecular size, with rat liver being the largest and oyster being the smallest. Determination of V_{max} values for hydrolysis by acid α-glucosidase revealed the following order of increasing activity; rat liver < human placenta < oyster. Earlier studies (Palmer, 1971a; Matsui *et al.*, 1984), gave similar results. Thus it appears that the rate of hydrolysis of glycogen depends largely upon the available surface area. Clustering of β-particles into α-particles makes less of the surface available to enzymatic attack, which is the case with the majority of liver glycogen (Chee and Geddes, 1977). Conversely single or "split" β-particles, such as oyster glycogen, are freely available to the enzyme (Calder and Geddes, 1989a).

Kinetic analysis revealed similar K_m values for the different types of glycogen. This indicates that each of the glycogens bind equally well to the enzyme. Therefore, the different hydrolysis rates reflected in the different V_{max} values must be due to different catalytic rates once the enzyme and substrate are bound. Thus the size of the glycogen molecule does not prevent enzyme binding but prevents enzymatic attack on glucosidic bonds, presumably by steric hindrance. Clustering

into α-particles must obstruct hydrolysis by not allowing the enzyme, once bound, to attack the glucosidic bonds (Calder and Geddes, 1989a).

Since lysosomal glycogen is composed mainly of protein (disulfide) cross-linked high molecular weight material (Geddes and Stratton, 1977a; Geddes *et al.*, 1983; Geddes and Taylor, 1985a,b) and the lysosomal acid α-glucosidase preferentially degrades smaller glycogen, it is likely that the first step in lysosomal glycogen breakdown is proteolysis and/or disulfide-bond reduction. This would immediately make available a high concentration of low molecular weight, accessible glycogen (Calder and Geddes, 1989c). It is known that thiols stimulate lysosomal protein breakdown (Mego, 1973; Griffiths and Lloyd, 1979; Kooistra et al., 1984) and it was shown that this occurs only if the substrate protein contained disulfide bridges (Mego, 1984). Therefore, in essence, a similar role could be envisaged for cysteine in the reduction of the disulfides between glycogen protein backbones, perhaps concomitantly with proteolysis of the protein moieties but prior to hydrolysis of the carbohydrate portion of the molecule by acid α-glucosidase (Calder and Geddes, 1989a).

Studies on post-mortem glycogenolysis may give some insight into the regulatory mechanism of lysosomal glycogen storage and acid α-glucosidase activity on the breakdown of lysosomal glycogen. After death there is a rapid loss of glycogen from the liver (Geddes and Rapson, 1973) and skeletal muscle (Bendall, 1973; Fischer and Hamm, 1980) with a corresponding increase in tissue lactate concentration (Bendall, 1973). The insufficient generation of ATP, due to the shortage of oxygen (Woods and Krebs, 1971; Bendall and Taylor, 1972), causes rapid compensatory glycogenolysis.

The molecular weight distributions of glycogens isolated from tissues post-mortem revealed marked inhomogeneity in the degradation. In percentage terms, high molecular weight glycogen was lost much more rapidly than low molecular weight material. The rapid loss of high molecular weight glycogen indicates that the lysosome is an important site for post-mortem degradation, since it has been shown that lysosomes of both liver and skeletal muscle are enriched with this material (Geddes and Stratton, 1977a; Geddes *et al.*, 1983; Geddes and Taylor, 1985a,b; Calder and Geddes, 1989b). Calder and Geddes (1989a) previously proposed that the

size of the glycogen substrate might influence its degradation, as acid α-glucosidase showed greater activity toward small glycogen molecules than towards larger material. On this basis it was proposed that an initial step in lysosomal glycogen degradation may be disulfide bond reduction and/or proteolysis. If this was the first step in post-mortem degradation then clearly the high molecular weight material would be reduced to low molecular weight soon after the onset of degradation which is observed to be the case. This suggests that as a consequence of post-mortem anoxia, lysosomes may become more reducing and proteolytic, and points to the redox potential as a possible factor regulating lysosomal function.

A further possible regulator of lysosomal enzyme activity is pH (Calder and Geddes, 1989a). As glycogen is degraded, lactate is produced, lowering cellular pH. The pH of lysosomes is thought to be generally maintained at 1-2 units below that of the cytoplasm medium (Reijngoud $et\ al.$, 1976; Ohkuma and Poole, 1978). Therefore, as the cytosolic pH drops due to lactate production, the intralysosomal pH will also drop, and lysosomal acid α-glucosidase will become more active (Calder and Geddes, 1989c).

The above suggests that lysosomes may be regulated from "storage vesicles" to highly active "degradative vesicles", a change from one to the other occurring, for example, post-mortem. The "storage vesicle" is envisaged as being relatively oxidising and as containing α-glycogen particles, comprised of β-particles built on protein cores and linked via disulfide bonds. In the storage vesicle form, enzymes (at least the cysteine proteinases and acid α-glucosidase) are relatively quiescent. By contrast, the degradative vesicle form is seen as being more reducing and in this glycogen is rapidly degraded via disulfide-bond reduction, and by the action of reduction-activated proteinases and acid α-glucosidase.

Lysosomal glycogen storage therefore points to a varying redox potential, and pH, as two factors regulating lysosome function. To some extent these might work against each other, in the case of glycogenolysis. The reason is that the reducing strength of a given concentration of a specific redox pair increases with pH. For each increase of one pH unit, E_o' becomes more negative by 0.059 volts (Segel, 1976). With a drop in pH, therefore, the intralysosomal milieu will become less reducing.

The aim of this study was to establish whether redox potential could play any role in lysosomal regulation. This question was tackled from two different approaches. Firstly, to ascertain the effect of redox potential on lysosomal enzymes, the effect of varying concentrations of the reducing agent, cysteine, was tested on three lysosomal enzymes, two cysteine proteinases and one glycanase, which were isolated for this study. Isolated lysosomal cysteine proteinases are known to require reductive activation in order to manifest proteolytic activity, but the effect of reduction on glycanases was not known. Secondly, a method was devised for qualitatively determining the redox status of lysosomes in live, cultured cells using fluorescence microscopy. Perfection of such a method would enable differences between different lysosomes in the same cell to be determined. Also, differences between different cells, and between the same cell at different times, could be determined.

CHAPTER 2

MATERIALS AND METHODS

In this chapter methods will be described that are considered fundamental, and which were used throughout this research project. Additionally, this chapter also contains methods which could be considered relatively specialized but would hinder the intended structure of the relevant chapter. Detail has been afforded to methods where results can be either ambiguous or deceptive. In this chapter only reagents that were prepared before use are described, reagents that were used "as-is" are referred to at the relevant point in the procedure section.

2.1 Materials

Most of the common chemicals used in this study were from BDH, Merck or Boehringer Mannheim, and were of the highest purity available. Coomassie brilliant blue R-250 was from Merck. Acrylamide; N,N'-methylenebisacrylamide; and N,N,N',N'-tetramethylethylenediamine (TEMED) were from BDH. Dialysis tubing, 7-amino-4-methyl coumarin, dithiothreitol (DTT), L-trans-epoxysuccinyl-leucylamido(4-guanidino)butane (E-64), S-Sepharose, Sephacryl S-100 and Sephadex G-100 were from Sigma Chemical Co., St. Louis, Mo. USA. 2,2 Azino-di-(3-ethyl) benzthiozoline sulphonic acid (ABTS) was from Boehringer Mannheim, SA. Bz-Phe-Val-Arg-NHMec, 4-methylumbelliferyl-α-glucoside, 4-methylumbelliferone, Z-Phe-Arg-NHMec and Z-Arg-Arg-NHMec were from Cambridge Research Chemicals, UK. ELISA plates were Nunc-Immuno Maxisorp F96 plates from Weil Organisation, SA. Freund's complete and incomplete adjuvants were from Difco, Mi., USA.

2.2 Protein assays

Protein purification procedures require methods of protein quantitation which are specific, reproducible, and preferably rapid, using small amounts of sample. The protein assay used in the present study was the Bradford dye-binding assay (Bradford, 1976), as modified by Read and Northcote (1981).

2.2.1 Bradford dye-binding assay

The Bradford dye-binding assay was instituted as a rapid, sensitive, reproducible and inexpensive technique for protein quantitation, which is free of interference from most commonly used chemicals (Bradford, 1976).

The Coomassie brilliant blue G-250 dye exists in two forms: a cationic red form, which is present in the acidic solution of the Bradford reagent, and the anionic blue form. The former has an absorption maximum at 465 nm, while the latter absorbs maximally at 595 nm. The red form is converted to the blue form upon binding to protein, and the consequent shift in absorption maximum allows the spectrophotometric quantification of protein. At 595 nm the extinction coefficient of the blue protein-dye complex is far greater than that of the unbound dye, and allows for high sensitivity in protein quantification.

A major advantage of the technique is the lack of interference by most commonly used chemicals. The effects of small amounts (less than 1%) of Tris, acetic acid, sucrose, glycerol, ethylene diamine tetra-acetic acid (EDTA), Triton X-100 and sodium dodecyl sulfate (SDS) can be eliminated using an appropriate buffer blank. The potential interference caused by detergents at concentrations of 1% or greater, however, requires their removal prior to assaying.

The greatest disadvantage of the dye-binding assay is the wide variation in the dye-binding of different proteins. Extrapolation of values from standard curves to other proteins may therefore be inaccurate. To minimise such variation, Read and Northcote (1981) modified the acid/alcohol ratios in the assay solution and increased the dye concentration, using the purer Serva blue G dye. Pike (1990), however, found that under such conditions the dye precipitated over long periods, but that substitution of the dye alone sufficiently diminished variations in dye-binding. The only disadvantage of this reagent, compared to that of Read and Northcote (1981), was a non-linear response at higher protein levels (beyond 5 μg in the micro-assay procedure).

Two protein assays have been established: a macro-assay with a working range of 5-25 μg of protein (Bradford, 1976), and a micro-assay for the determination of 1-5 μg of protein (Read and Northcote, 1981). In the present study only the micro-assay was used.

2.2.1.1 Reagents

Dye reagent. Serva blue G dye (50 mg) was dissolved in 88% phosphoric acid (50 ml) and 99.5% ethanol (23.5 ml). The solution was made up to 500 ml with dist.H_2O and stirred for 30 min. The resulting solution was filtered through Whatman No. 1 filter paper and stored in an amber coloured bottle. Visual checks for precipitation were made prior to use, and if precipitation was observed, hte reagent was filtered and re-calibrated or a new batch was made up.

Standard protein solution. A 0.1 mg/ml ovalbumin solution was made up in dist.H_2O.

2.2.1.2 Procedure

Samples were diluted to 50 μl with dist.H_2O, dye reagent (950 μl) was added, and the solution was mixed and allowed to stand for 2 min for colour development. The absorbance was read at 595 nm against appropriate blanks. Plastic microcuvettes (1 ml) were used, as the dye reagent binds to glass (Bradford, 1976). The blue stain which developed on the plastic cuvettes could be easily removed with diluted sodium hypochlorite. A standard curve, from 0-50 μl (0-5 μg) of the standard protein solution was constructed. Results for protein assays were calculated from the equation:

$$y = 0.0398x + 4.8 \times 10^{-3}$$

generated by linear regression analysis of the standard curve data.

2.3 Concentration of samples by dialysis against sucrose or polyethylene glycol

It was often necessary to concentrate dilute protein samples, before subjecting them either to further purification, SDS-PAGE analysis or for inoculation procedures. Samples were concentrated by dialysis, in dialysis tubing with a M_r cut-off of 12 000, against sucrose or polyethylene glycol (PEG), which have high osmotic pressures when in solution. A concentration gradient is established between the solvent (water) in the dialysis bag, and the solute (sucrose or PEG) at the exterior surface of the membrane. Water and buffer ions will slowly move out of the dialysis bag, progressively dissolving the solute, while proteins larger than the membrane molecular cut-off limit are retained in the bag. The protein solution will therefore be concentrated.

Sucrose, which is inexpensive, was often used but a disadvantage is that it is small enough to diffuse into the dialysis bag. High levels of sucrose in a sample may interfere with certain assays or applications. When sucrose-free samples were required, they were dialysed against PEG (M_r 20 000). The molecular weight of PEG is greater than the cut-off limit of the dialysis membrane, and thus PEG will not move into the sample.

2.4 Electrophoretic techniques

2.4.1 Tris-glycine SDS-PAGE

Electrophoresis describes the migration of a charged particle under the influence of an electric field. For bands to be well resolved the starting band of sample must be as thin as possible, and the diffusion of the protein bands in the gel must be minimised. For these reasons Ornstein (1964) and Davis (1964) developed a discontinuous system, using polyacrylamide gels with consistent, defined porosities.

The Ornstein-Davis system makes use of two different buffer systems, and two different gel porosities. The sample and stacking gel contain a Tris-HCl buffer, while the electrode buffers are Tris-glycine. At the pH of the sample and stacking gel (pH 6.8), glycine is poorly dissociated and has a small negative charge and hence low mobility. The chloride ions in the sample and stacking gel have a higher mobility at pH 6.8, while the proteins have a mobility intermediate between the two ions. When an electrical potential is applied, the glycine (trailing) and chloride (leading) ions will generate and maintain a sharp interface. The voltage gradient in front of the interface is low, in contrast to the steeper voltage gradient behind the interface. Proteins in the lower voltage gradient have lower mobility than the chloride ions, whereas any protein in the higher voltage gradient will have a higher mobility than the glycine ions. Consequently all proteins will stack at a sharp interface between the leading and trailing ions. The large pore stacking gel has no molecular sieving effects, but serves to protect the sample from convective disturbances during stacking.

When the protein stack reaches the resolving gel, the increased pH of the resolving gel causes increased dissociation, and hence increased mobility, of glycine ions. The glycine ions overtake the

proteins, and form a front with the chloride ions and tracking dye. The destacked proteins are left to migrate in a uniform voltage gradient at a constant pH. The pore size of the resolving gel is smaller than that of the stacking gel, and hence molecular sieving of the proteins occurs. Proteins are therefore separated on the basis of their size, shape and intrinsic charge at the pH of the resolving gel.

Although PAGE (polyacrylamide gel electrophoresis) is useful for the separation of proteins on the basis of charge and size and for the determination of protein forms, which may vary as a result of phosphorylation, glycosylation, truncation, or by the formation of disulfides, this technique is limited in not identifying proteins solely according to MW. For this reason SDS (sodium dodecyl sulfate)-PAGE was devloped. The anionic detergent, SDS, binds to proteins at a constant ratio of 1.4 g SDS per gram protein (Reynolds and Tanford, 1970). The charge of SDS, bound to protein, is sufficient to mask the charge on the protein such that all SDS-protein complexes have the same charge to mass ratio. Proteins of different MW can therefore be separated on the basis of size alone following binding of SDS and SDS-PAGE. The principles of PAGE still apply, and hence the same buffering systems as described by Ornstein (1964) and Davis (1964), can be applied to SDS-PAGE (Laemmli, 1970).

The boiling of samples in the presence of SDS and reducing agent, prior to SDS-PAGE, ensures the reduction of disulfide bridges in proteins and complete protein denaturation and saturation with SDS. Non-reducing SDS-PAGE can, however, be used to detect protein complexes or oligomers. A number of factors may cause the anomalous migration of proteins on SDS-PAGE. The variable glyco moieties of glycoproteins do not bind SDS, and may be responsible for microheterogeneity of bands observed on SDS-PAGE (Hames, 1981). Very basic proteins may repress the overall negative charge of the SDS-protein complex, and consequently migrate to anomalous M_r values (Hames, 1981).

For the estimation of M_r values, the mobilities of the electrophoresis standard proteins relative to the mobility of the bromophenol blue tracking dye were calculated, and expressed as R_f values. A standard curve was constructed by plotting log MW of the standard proteins against their R_f values, from which the M_r of the sample proteins could be calculated.

2.4.1.1 Reagents

Solution A: monomer solution [30% (m/v) acrylamide, 2.7% (m/v) bis-acrylamide]. Acrylamide (73 g) and bis-acrylamide (2 g) were dissolved and made up to 250 ml with dist.H_2O and stored in an amber coloured bottle at 4°C.

Solution B: 4 x running gel buffer [1.5 M Tris-HCl, pH 8.8]. Tris (45.37 g) was dissolved in approximately 200 ml of dist.H_2O, adjusted to pH 8.8 with HCl, and made up to 250 ml.

Solution C: 4 x stacking gel buffer [500 mM Tris-HCl, pH 6.8]. Tris base (3 g) was dissolved in 40 ml dist.H_2O, adjusted to pH 6.8 with HCl, and made up to 50 ml.

Solutions A, B, and C were filtered through Whatman No. 1 filter paper before use.

Solution D: 10% (m/v) SDS. SDS (10 g) was dissolved in 100 ml dist.H_2O with gentle heating if necessary.

Solution E: Initiator [10% (m/v) ammonium persulfate]. Ammonium persulfate (0.2 g) was made up to 2 ml with dist.H_2O just before use.

Solution F: tank buffer [250 mM Tris-HCl, 192 mM glycine, 0.1% (m/v) SDS, pH 8.3]. Tris (15 g) and glycine (72 g) were dissolved and made up to 5 litres with dist.H_2O. Prior to use, 2.5 ml of SDS stock (solution E) was added to 250 ml.

Solution G: reducing treatment buffer [125 mM Tris-HCl, 4% (m/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, pH 6.8]. Buffer C (2.5 ml), glycerol (2 ml), 10% SDS (4 ml) (solution D) and 2-mercaptoethanol (1 ml) were made up to 10 ml with dist.H_2O. Aliquots (1 ml) were frozen and stored for no longer than 3 months.

Molecular weight markers: [1 mg standard protein/ml]. Standards for M_r determination were: lysozyme (MW 14 000), carbonic anhydrase (MW 29 000), ovalbumin (MW 45 000), bovine serum albumin (MW 68 000) and phosphorylase b (MW 97 400). A solution containing 1 mg of each of the

standard proteins in dist.H$_2$O was mixed with treatment buffer (1 ml) containing 0.005% (m/v) bromophenol blue tracking dye.

Stain stock solution [1% (m/v) Coomassie blue R-250]. Coomassie blue R-250 (1 g) was dissolved in 100 ml of dist.H$_2$O by magnetic stirring for 1 h at room temperature. The solution was filtered through Whatman No. 1 filter paper.

Stain solution [0.125% (m/v) Coomassie blue R-250, 50% (v/v) methanol, 10% (v/v) acetic acid]. Stain stock (62.5 ml) was mixed with methanol (250 ml) and acetic acid (50 ml), and made up to 500 ml with dist.H$_2$O.

Destaining solution I [50% (v/v) methanol, 10% (v/v) acetic acid]. Methanol (500 ml) was mixed with acetic acid (100 ml) and made up to 1 litre with dist.H$_2$O

Destaining solution II [7% (v/v) acetic acid, 5% (v/v) methanol]. Acetic acid (70 ml) was mixed with methanol (50 ml), and made up to 1 litre with dist.H$_2$O.

2.4.1.2 Procedure

Table 1. Preparation of resolving and stacking gels for Tris-glycine SDS-PAGE

Reagent	Running Gel (%)		Stacking gel (%)
	12.5%	7.5%	4%
Acrylamide (**A**)	6.25 ml	3.75 ml	0.94 ml
Running gel buffer (**B**)	3.75 ml	3.75 ml	
Stacking gel buffer (**C**)			1.75 ml
10% SDS (**D**)	0.15 ml	0.15 ml	70 µl
dist.H$_2$O	4.75 ml	7.25 ml	4.20 ml
Ammonium persulfate (**E**)	75 µl	75 µl	35 µl
TEMED	7.5 µl	7.5 µl	18 µl

For SDS-PAGE, the Hoefer SE 250 Mighty Small II vertical slab electrophoresis unit was assembled as described in the manufacturer's manual. Before use the notched aluminium plate and glass plate were thoroughly cleaned and clamped together using 1.5 mm polyethylene spacers to separate them at the edges. Molten 1% agarose was used to plug the bottom of the sandwich chamber before the running gel solution was poured into the space between the plates to a depth 3 cm from the top of the glass plate. The gel was overlaid with dist.H_2O, to exclude atmospheric oxygen which prevents polymerisation. Once the gel has set (appearance of interface between gel solution and water, usually about 1 h), the water was removed by inversion. Stacking gel solution was poured in, up to the notch in the aluminium plate, and a 10 well comb was inserted to form the sample application wells. Once the gel had set (about 30 min) the comb was removed and the wells were rinsed with dist.H_2O.

Tank buffer, containing SDS, was poured into the upper and lower electrode compartments. The samples for reducing SDS-PAGE were combined with an equal volume of reducing treatment buffer (solution G) and incubated in a boiling water bath for 90 s, before being placed on ice until they were loaded onto the gel. A marker dye, bromophenol blue (5 µl), which migrates with the buffer front, was added to each sample before loading onto the gels. Samples were applied into the wells, using a Hamilton microsyringe, at a concentration of at least 1 µg per band for Coomassie blue R-250, and 250 ng per band for silver staining. The gel was connected to a power pack and run at 18 mA per gel until the bromophenol blue tracker dye was about 0.5 cm from the bottom of the running gel. The gel was then disconnected from the power supply, and the plates removed. The gels were removed and placed in Coomassie blue R-250 staining solution for 4 h. Following rinsing with dist.H_2O, the gel was placed into destain I overnight and then into destain II to effect complete destaining. Gels were stored in polythene zip-seal bags and kept well hydrated until photographed.

2.4.2 Tris-Tricine SDS-PAGE

Small proteins and peptides form complexes with SDS of similar size and charge as the SDS micelles, which run at the buffer front on SDS-PAGE (Fish *et al.*, 1970). Such proteins ($M_r < 10\ 000$) therefore migrate with the buffer front and are not resolved by conventional Tris-glycine SDS-PAGE. By decreasing the mobility of protein relative to the trailing ion, using a higher pH and Tricine as a trailing ion in the stacking phase, Schägger and von Jagow (1987) were able to resolve lower MW proteins from the SDS micelles. Using this system with the recommended three sets of gel porosities,

separation of proteins of MW 1 000 - 100 000 can be achieved on a single gel (Schägger and von Jagow, 1987).

2.4.2.1 Reagents

<u>Monomer solution:</u> [49.5% (m/v) acrylamide, 3% (m/v) N,N'-methylene-bisacrylamide]. Acrylamide (120.0 g) and N,N'-methylene-bisacrylamide (3.8 g) were dissolved in dist.H_2O and made up to 250 ml. Storage was at 4°C in the dark.

<u>Gel buffer:</u> [3 M Tris-HCl, 0.3% (m/v) SDS, pH 8.45]. Tris (90.83 g) was dissolved in 200 ml dist.H_2O, and adjusted to pH 8.45 with HCl. 10% (m/v) SDS (7.5 ml) (Section 2.4.1.1) was added and the solution was made up to 250 ml.

<u>Anode buffer:</u> [0.2 M Tris-HCl, pH 8.9]. Tris (24.22 g) was dissolved in 950 ml of dist.H_2O, adjusted to pH 8.9 with HCl, and made up to 1 litre.

<u>Cathode buffer:</u> [0.1 M Tris-HCl, 0.1 M Tricine, 0.1% (m/v) SDS, pH 8.25]. Tris (12.11 g), Tricine (17.92 g) and 10% (m/v) SDS (10 ml) were made up to 1 litre with dist.H_2O and the pH checked.

<u>Treatment buffer:</u> [125 mM Tris-HCl, 4% (m/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, 0.01% (m/v) Serva blue G, pH 6.8]. Stacking gel buffer (2.5 ml), glycerol (2 ml) 10% (m/v), SDS (4 ml) and 2-mercaptoethanol (1 ml) were made up to 10 ml with dist.H_2O. Serva blue G tracking dye [0.01% (m/v)] was added. For non-reducing SDS-PAGE, 2-mercaptoethanol was omitted.

Other reagents were as described in Section 2.4.1.1.

2.4.2.2 Procedure

The procedure for SDS-PAGE (Section 2.4.1.2) was modified in the composition of the resolving and stacking gels as described in Table 2. The gels were run at 200 V with unlimited mA, and stopped when the Serva blue dye reached the bottom of the gel. The gels were stained as described in Section 2.4.1.2 or Section 2.5.

Table 2. Preparation of resolving and stacking gels for Tris-Tricine SDS-PAGE

Reagents	Resolving Gel	Stacking Gel
	10% T, 3% C	4% T, 3% C
Monomer (49.5% T, 3% C)	3 ml	1.5 ml
Gel buffer	5 ml	0.5 ml
dist.H_2O	14.95 ml	6.22 ml
Ammonium persulfate	50 μl	30 μl
TEMED	8 μl	12 μl

2.5 Silver staining of electrophoretic gels

A number of techniques utilise the specific chemical reduction of silver to detect protein in electrophoretic gels. The sensitivity of such techniques is comparable to that of autoradiography, in detecting nanogram amounts of proteins (Nielson and Brown, 1983).

In the presence of a reducing agent, and at high pH values, silver ions form insoluble, coloured complexes with charged amino acid side chains (Nielsen and Brown, 1983). However, the pH change, required by most silver staining procedures, causes the non-specific formation of insoluble silver salts on the gel, thereby impairing contrast (Blum *et al.*, 1987). By treating the gel with sodium thiosulfate, which complexes to and dissolves silver salts, Blum *et al.* (1987) found that the levels of background staining could be reduced. Using this technique, greater contrast could be achieved without impairing the high sensitivity of staining.

2.5.1 Reagents

<u>Fixing solution:</u> [50% (v/v) methanol, 12% (v/v) acetic acid, 0.2% (v/v) formaldehyde]. Methanol (100 ml), glacial acetic acid (24 ml) and 37% formaldehyde (0.1 ml) were made up to 200 ml with dist.H_2O.

<u>Wash solution:</u> [50% (v/v) ethanol]. Ethanol (100 ml) was made up to 200 ml with 200 ml dist.H_2O.

Pre-treatment solution: [0.02% (m/v) $Na_2S_2O_3.5H_2O$]. $Na_2SO_3.5H_2O$ (0.1 g) was made up to 500 ml with dist.H_2O.

Impregnation solution: [0.2% (m/v) $AgNO_3$, 0.03% (m/v) formaldehyde]. $AgNO_3$ (0.4 g) and 37% formaldehyde (0.15 ml) were made up to 500 ml with dist.H_2O.

Development solution: [6% (m/v) Na_2CO_3, 0.0004% (m/v) $Na_2S_2O_3.5H_2O$, 0.02% (v/v) formaldehyde]. Na_2CO_3 (12 g), pre-treatment solution (4 ml) and 37% formaldehyde (0.1 ml) were made up to 200 ml with dist.H_2O.

Stop solution: [50% (v/v) methanol, 12% (v/v) acetic acid]. Methanol (50 ml) and glacial acetic acid (12 ml) were made up to 100 ml with dist.H_2O.

2.5.2 Procedure

All steps were carried out on a shaker, at RT and in clean glass containers. After electrophoresis, the gel was soaked in fixing solution (1 h), and wash solution (3 x 20 min) to neutralise the gel and allow for subsequent treatment with the acid labile $Na_2S_2O_3$. The gel was treated with pre-treatment solution (1 min), rinsed with dist.H_2O (3 x 20 s), and soaked in impregnation solution (20 min). After rinsing with dist.H_2O (3 x 20 s), to remove excess $AgNO_3$ from the gel surface, the gel was immersed in development solution until bands were evident against a lightly stained background (<10 min). The gel was rinsed in dist.H_2O (2 x 2 min), treated with stop solution (10 min), and stored in a sealed plastic bag, in the dark, until photographed.

2.6 Protein fractionation using three phase partitioning (TPP)

Protein folding and protein solubility are dependent on hydration, and hence agents which change the properties of water can affect folding and precipitation. Kosmotropic agents tend to promote the structure of water, whereas chaotropic agents disrupt the structure of water. The kosmotropes, sulfate and t-butanol, cause the ordering of water molecules solvating them, thereby minimising the surface area of any protein in the solution, and stabilising the protein. Tertiary butanol, which is

completely miscible with water, forms a separate phase upon the addition of sufficient ammonium sulfate. In addition, any protein present in the aqueous phase may precipitate out, depending on the concentration of ammonium sulfate, due to hydration effects. Three phases are therefore formed: a lower aqueous phase, an upper t-butanol phase, and an interfacial t-butanolated protein phase (Pike and Dennnison, 1989; Dennison *et al.*, 1996).

Since kosmotropes tend to tighten and stabilise protein conformations, the proteins are precipitated in a protected form (Dennison *et al.*, 1996). However, multimeric proteins in which the subunits are linked by disulfide bonds, such as haemoglobin, tend to be denatured by the TPP process (Pike and Dennison, 1989). TPP has an additional advantage in removing contaminants which are soluble in organic solvents, such as lipids, phenolics and pigments, into the t-butanol phase. Furthermore, TPP derived protein precipitates contain low levels of salts and t-butanol, and once redissolved in an appropriate buffer, can be applied directly to ion exchange chromatography columns. TPP has been found to be superior to conventional ammonium sulfate precipitation in the isolation of a number of proteinases in this laboratory. These include cathepsin L (Pike and Dennison, 1989), cathepsin D (Jacobs *et al.*, 1989) and trypanosome proteinases (Troeberg *et al.*, 1996). In the present study, TPP was utilised during the purification of porcine liver acid α-glucosidase, and bovine spleen cathepsins S and B.

TPP was effected by the addition and mixing in of 2-methylpropan-2-ol (t-butanol) to 30% (v/v of final volume).

The volume of t-butanol to be added was calculated as follows:

$$\frac{x}{x + y} = 0.3$$

where x = volume of t-butanol

y = volume of pH 4.2 supernatant

The t-butanol was first warmed to 30°C (above its crystallisation temperature of about 25°C). Solid $(NH_4)_2SO_4$ (% (m/v) based on the volume of the pH 4.2 supernatant and t-butanol) was dissolved with stirring. The mixture was centrifuged (8000 x g, 10 min, 4°C) in a swing-out rotor and the supernatant t-butanol and subnatant aqueous phases decanted, leaving behind the

third phase of interfacial protein precipitate which was discarded. Further $(NH_4)_2SO_4$ was added to the aqueous and solvent phases to bring the concentration to a % (m/v) (based on the volume of pH 4.2 supernatant and t-butanol) and was dissolved with stirring. The solution was again centrifuged as before and the interfacial protein layer collected by decanting the super- and subnatants. The precipitate was redissolved in an appropriate buffer at approximately one-tenth of the volume of the acid supernatant. The solution was centrifuged (15 000 x g, 10 min, 4°C) and filtered through Whatman No. 4 filter paper to remove undissolved protein.

2.7 Hydroxyapatite chromatography

Crystalline calcium hydroxyphosphate, $[Ca_5(PO_4)_3(OH)]_2$, is known as hydroxyapatite, or hydroxylapatite (Gorbuoff, 1985), and its particular usefulness in protein isolations is that it binds proteins by a unique mechanism, different from MEC and simple ion-exchange, and it can therefore separate proteins which may not be separable by other means (Bernardi, 1971; 1973; Gorbuoff, 1985). Hydroxyapatite forms blade-like crystals and because the protein binds to the surface of the crystals, rather than within a gel lattice, the protein binding capacity is relatively low. For this reason, hydroxyapatite is best suited for use as one of the final steps in a purification.

Blade shaped crystals are not optimal for chromatography and they tend to be brittle, thus generating "fines" which block the column and limit its life to three or four runs. Several manufacturers have attempted to overcome this by making spherical forms of hydroxyapatite, e.g. macro-prep ceramic hydroxyapatite from Bio-Rad and HA ultragel from Pharmacia. However, if the hydroxyapatite is used at the end of a purification, the fact that the classical crystals have a limited life is of lesser consequence.

2.7.1 The mechanism of hydroxyapatite chromatography

The separating mechanism of hydroxyapatite is summarised in reviews by Gorbunoff (1985) and by Dennison (pers. comm.). Hydroxyapatite crystals have positive surface charges, due to their constituent calcium ions, and negative charges due to their phosphate groups. The net charge

can be varied by the buffer - it is negative in phosphate buffer, neutral in NaCl and positive in $CaCl_2$ or $MgCl_2$.

Positive amino groups of proteins bind electrostatically to negative charges on the hydroxyapatite, and are thus influenced by net charge.

$$\text{Hydroxyapatite - } PO_4^- \cdots\cdots H_3^+N \text{ - Protein}$$
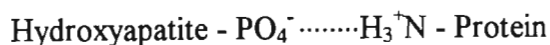
Negative carboxyl groups, on the other hand, bind by complexing with calcium in the hydroxyapatite.

$$\text{Hydroxyapatite - Ca - OOC - Protein}$$
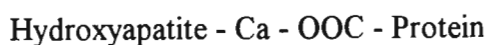
The retention of acidic (negatively charged) proteins is thus affected by the net charge on the hydroxyapatite in a manner opposite to that of basic (positively charged) proteins. $CaCl_2$ and $MgCl_2$ increase the binding of acidic proteins by the formation of salt bridges between protein carboxyl groups and hydroxyapatite phosphate sites.

$$\text{Hydroxyapatite - } PO_4^- \cdots\cdots {}^-OOC \text{ - Protein}$$
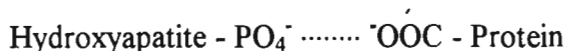
Basic proteins may be eluted from hydroxyapatite by negative ions such as F^-, Cl^-, and HPO_4^{2-}, which compete with its negative phosphate sites, or by Ca^{2+} or Mg^{2+} ions, which specifically complex with its phosphate sites and neutralise their charges. Acidic (negative) proteins may be eluted by displacement of their carboxyl groups from hydroxyapatite complexing sites by ions, such as phosphate or F^-, which form stronger complexes with calcium.

Most proteins contain both amino and carboxyl groups and phosphate is effective in eluting both types. Consequently, a common means of eluting proteins from hydroxyapatite is by the application of a phosphate gradient - often K-phosphate, because Na-phosphate has limited solubility at low temperatures. Gorbunoff (1985) discussed alternative approaches, where the effects of $CaCl_2$ and $MgCl_2$, and NaCl and KCl, can additionally be exploited in elution schemes. As previously mentioned, using this procedure, separations may be achieved which are not possible using other chromatographic systems and hydroxyapatite is thus a valuable technique.

2.8 Generation and isolation of antibodies

Antibodies were raised in rabbits following inoculation with antigen emulsified with an adjuvant. The adjuvant facilitates the slow release of antigen as the emulsion breaks down, and hence

prolongs the exposure of the antigen to the immune system of the experimental animal. The initial inoculation was with the antigen emulsified with Freund's complete adjuvant, which contains killed *Mycobacterium tuberculosis* cells in order to enhance the primary immune response by attracting neutrophils and macrophages to the inoculation site. In subsequent inoculations, the antigen of interest was emulsified with Freund's incomplete adjuvant to further stimulate the selected B-cell clone, which produces antibodies specific to the antigen. Rabbits were inoculated at 2, 4, 8, and 12 weeks after the first inoculation, to ensure high levels of specific antibody production.

IgG may be purified from serum by a simple and convenient method that makes use of the protein precipitating properties of polyethylene glycol (PEG), a water soluble polymer (Polson *et al.*, 1964). Polyethylene glycol is a mild precipitating agent which operates on a steric exclusion mechanism, whereby proteins are concentrated in the extrapolymer space, until they exceed their solubility limit. It was found that the concentration of the polymer required to precipitate a protein is a function of the charge of the protein, and hence is dependent on the pH of the protein solution (Polson *et al.*, 1964). The protocol used to purify IgG from rabbit serum was that of Polson *et al.* (1964).

2.8.1 Reagents

Borate buffered saline, pH 8.6. A sodium borate solution was prepared by dissolving boric acid (2.16 g), NaCl (2.19 g), NaOH (0.7 g) and 37% HCl (0.62 ml) in 950 ml dist.H_2O, checking and adjusting the pH if necessary with HCl or NaOH, and making the solution up to 1 litre.

100 mM Na-phosphate buffer, 0.02% (m/v) NaN_3, pH 7.6. $NaH_2PO_4.H_2O$ (13.8 g) and NaN_3 (0.2 g) were dissolved in 950 ml of dist.H_2O, titrated to pH 7.6 using NaOH, and made up to 1 litre.

2.8.2 Procedure for the isolation of IgG from rabbit serum

Rabbits were bled from the marginal ear vein into scrupulously clean, dry, 10 ml test glass tubes, and the blood was allowed to clot overnight at 4°C. The serum was drawn off the clot and diluted 1:2 with borate buffer. Solid polyethylene glycol (M_r 6 000) was added to the diluted serum to 14% (m/v), dissolved by gentle stirring, and the solution was centrifuged (12 000 x g,

10 min, RT). The pellet was redissolved in the original serum volume of sodium phosphate buffer. PEG was once more added to a concentration of 14% (m/v), dissolved, and centrifuged (12 000 x g, 10 min, RT). The pellet was redissolved in half the initial serum volume of sodium phosphate buffer containing 60% glycerol, and stored at -20°C. The A_{280} of a 1:40 dilution of IgG solution in 100 mM phosphate buffer was determined and the concentration of IgG in the undiluted solution was calculated [extinction coefficient of IgG, E_{280} (1 mg/ml) = 1.43 (Hudson and Hay, 1980)].

2.9 Enzyme Linked Immunosorbent Assay (ELISA)

Immunoassays use the specific interaction of an antibody with an antigen to provide information about antibody or antigen concentrations in unknown samples. In principle, the labelling by chemical conjugation of an enzyme to either antibody or antigen allows the detection of immune complexes formed on a solid phase. This forms the basis of the enzyme-linked immunosorbent assay (ELISA), first introduced by Engvall and Perlmann (1971). One of the simplest and most commonly used ELISAs, for the detection of antibodies, is a three layer system. Briefly, antigen is coated to the plastic surface of the wells of polystyrene microtitre plates, and the primary antibodies to be quantified allowed to form a complex with the immobilised antigen. After excess antibody has been washed away, the degree or amount of reactivity is quantified with an appropriate detection system. In an ELISA this takes the form of an enzyme conjugated to a secondary antibody which will recognise the primary antibody bound to the immobilised antigen. The enzyme reacts with a substrate which yields a coloured product which can be measured spectrophotometrically.

During the course of this work, an ELISA was used to evaluate the progress of polyclonal antibody production during the immunisation of rabbits with bovine liver acid α-glucosidase.

2.9.1 Reagents

Phosphate buffered saline (PBS), pH 7.2. NaCl (8 g), KCl (0.2 g), $Na_2HPO_4.2H_2O$ (1.15 g) and KH_2PO_4 were dissolved in 1 litre of dist.H_2O.

0.5% (m/v) Bovine serum albumin-PBS (BSA-PBS). BSA (0.5 g) was dissolved in 100 ml of PBS.

<u>0.1% PBS-Tween</u>. Tween 20 (1 ml) was made up to 1 litre in PBS.

<u>0.15 M citrate-phosphate buffer, pH 5.0</u>. A solution of citric acid.H_2O (21.0 g/l) was titrated with a solution of $Na_2HPO_4.2H_2O$ (35.6 g/l) to pH 5.0.

<u>Substrate solution   [0.05% (m/v) 2,2'-azino-di-(3-ethyl)-benzthiozoline sulphonic acid (ABTS) and 0.0015% (v/v) H_2O_2 in citrate-phosphate buffer]</u>. ABTS (7.5 mg) and H_2O_2 (7.5 µl) were dissolved in citrate-phosphate buffer, pH 5.0 (15 ml), for one ELISA plate.

<u>Stopping buffer (citrate-phosphate-0.1% (m/v) NaN$_3$)</u>. NaN$_3$ (0.1 g) was made up to 100 ml in citrate-phosphate buffer.

<u>Carbonate coating buffer</u>. NaHCO$_3$ (0.21 g) was dissolved in 45 ml of dist.H_2O, titrated to pH 6.0 with HCl , and made up to 50 ml.

2.9.2 Procedure

Wells of microtitre plates (Nunc Immunoplate) were coated with antigen (150 µl) at a predetermined dilution (1 µg/ml as determined by a checkerboard ELISA) in PBS overnight at room temperature. Wells were blocked with BSA-PBS (200 µl) for 1 h at 37°C and washed 3 times with PBS-Tween. Serial two fold dilutions of the primary rabbit antiserum in BSA-PBS (1 mg/ml) was added (100 µl), incubated for 1 h at 37°C and excess antibody washed out 3 times with PBS-Tween. A 1:500 dilution of sheep anti-rabbit IgG-horseradish peroxidase (HRPO) conjugate in BSA-PBS, was added (120 µl) and incubated for 1 h at 37°C. The ABTS substrate (150 µl) was added and incubated in the dark for optimal colour development (usually 10-20 min). The enzyme reaction was stopped by the addition of 50 µl of 0.1% (m/v) NaN$_3$ in citrate-phosphate buffer and the absorbance read at 405 nm in a Bio-Tek EL307 ELISA plate reader. Titration curves were constructed from the spectrophotometric values.

CHAPTER 3

ISOLATION OF THREE LYSOSOMAL ENZYMES, CATHEPSINS S AND B AND ACID α-GLUCOSIDASE

3.1 Introduction

Lysosomes, which are membrane-bound intracellular organelles, have important functions in mammalian cells under physiological as well as pathological conditions. The lysosomes contain many different enzymes, mostly acid hydrolases. Collectively, these enzymes are capable of degrading virtually all large cellular molecules, such as nucleic acids, proteins, polysaccharides, and lipids, to low molecular weight products. In this study attention will be focused on the lysosomal proteinases, especially cathepsins S and B, and the enzyme responsible for digestion of lysosomal glycogen, acid α-glucosidase.

Proteinases are classified into two groups, endo- and exopeptidases, according to their proteolytic cleavage sites. They are further subclassified into four groups, the serine, cysteine, aspartic, and metalloproteinases, based on groups constituting the active site. In some highly specialised cells, such as leukocytes, serine- and metalloproteinases have been demonstrated, but cysteine and aspartic proteinases are ubiquitously present in the lysosomes of various tissue cells. Lysosomal cathepsins B, C, H, L and S are cysteine proteinases and cathepsin D is an aspartic proteinase. Proteolytic enzymes have a variety of biological functions ranging from peptide biosynthesis to protein degradation. Cysteine proteinases have been implicated in many diverse cellular events, including receptor internalisation, protein turnover, neuronal degradation and regeneration during development, and antigen processing (Katunuma and Kominami, 1983; Bond and Butler, 1987). They have also been proposed to be involved in selective cleavage of protein precursors to release regulatory peptides (Devi, 1991). Since cysteine proteinases play a vital role in normal cellular protein metabolism, it is understandable that they have also been implicated in a number of disease states, including cancer, arthritis, and Alzheimer's disease (Katunuma and Kominami, 1983; Tryggvason et al., 1987; Golde et al., 1992).

The mammalian lysosomal cysteine proteinases cathepsins B [EC 3.4.22.1], H [EC 3.4.22.16], L [EC 3.4.22.15] and S [EC 3.4.22.27] belong to the group of closely related proteins of the papain superfamily (Barrett, 1986). These enzymes are considered to play an important role in the initial or terminal stages of protein degradation (Bond and Butler, 1987). Whereas cathepsins B, H, and L have been extensively studied (Takio *et al.*, 1983; Ritonja *et al.*, 1985, 1988; Meloun *et al.*, 1988), there is still limited knowledge about cathepsin S.

Acid α-glucosidase is a γ-amylase with specificity for glycogen and several natural and synthetic oligoglucosides (Hoefsloot *et al.*, 1988). Its importance in lysosomal glycogen turnover has been emphasised by the lysosomal glycogen storage disease glucogenosis type II (Pompe's disease), an autosomal recessive disease, where a deficiency of lysosomal acid α-glucosidase causes an accumulation of glycogen in lysosomes (Hers, 1963).

This chapter describes the isolation of the three lysosomal enzymes cathepsins S, B and acid α-glucosidase. The aim was to streamline the currently known isolation procedures for these three enzymes to make it simple and cost effective to obtain them for studies described in Chapter 4. Cathepsin S, the least studied of these enzymes, was further characterized to obtain more knowledge on its activity profile, cathepsin B was isolated primarily for further studies described in Chapter 4, and acid α-glucosidase was isolated for these studies and was also used to raise anti-α-glucosidase antibodies in rabbits for envisioned immunocytochemical studies to confirm the intracellular location of the enzyme.

3.2 Cathepsin S

Mature active cathepsin S is a single-chain polypeptide comprising 217 amino acid residues (24 000 kDa). The NH_2-terminal prepro extension is 114 amino acids long in the human (Wiederanders *et al.*, 1992; Shi *et al.*, 1992) and 112 in the rat (Patanceska and Devi, 1992) enzymes, respectively. The only potential glycosylation sites are located at position N^{-11}-T^{-9} in the human enzyme and at position N^{-13}-S^{-11} in the rat enzyme (Shi *et al.*, 1994). Thus, they are located in the propeptide region, which indicates that maturation of procathepsin S occurs after delivery to the final compartment, the lysosome (Kirschke and Wiederanders, 1994).

The enzyme is unevenly distributed between organs. Cathepsin S protein has been detected in high concentrations in spleen and lung (Kirschke *et al.*, 1989), and similar results have been described by Qian *et al.* (1989) for cathepsin S mRNA. Petanceska and Devi (1992) presented results on the expression of high levels of cathepsin S mRNA in ileum, brain, thyroid, and ovary. In a subsequent study Patanceska *et al.* (1996) revealed, through *in situ* hybridization analysis of the adult rat brain, spleen and lung, that cathepsin S mRNA is preferentially expressed in cells of mononuclear-phagocytic origin.

The isoelectric points of bovine cathepsin S have been measured in the range 6.3-7.0 (Kirschke *et al.*, 1989). The use of synthetic peptide substrates and low M_r inhibitors allows the characterisation of substrate-binding sites. Cathepsin S does not prefer bulky hydrophobic residues in the S2 and S3 binding subsites as cathepsin L does. Instead, less bulky hydrophobic residues increase the specificity constant k_{cat}/K_m (Kirschke and Wiederanders, 1994). Bulky hydrophobic residues such as phenylalanine in the P2 position of peptidyl acylhydroxamate-type irreversable inhibitors (Brömme and Demuth, 1994) reveal high k_2/K_i (8400 M^{-1} sec^{-1}) values in comparison to glycine, for example, in this position (780 M^{-1} sec^{-1}) (Brömme and Kirschke, 1993), indicating strong hydrophobic interactions at the S2 subsite of the cathepsin S active centre (Kirschke and Wiederanders, 1994).

Cathepsin S is active toward proteins and synthetic peptide substrates over the range of pH 5.0 to 8.0 (Xin *et al.*, 1992; Kirschke *et al.*, 1989; Brömme *et* al., 1989, 1993). The stability of active enzyme above pH 7.0 (Brömme *et al.*, 1993) is a remarkable property of cathepsin S. It is capable of hydrolysing protein substrates (Kirschke *et al.*, 1989) as fast as cathepsin L. It shows collagenolytic (Kirschke *et al.*, 1989) and elastinolytic (Xin *et al.*, 1992) activities. Cathepsin S also has the ability to degrade soluble proteins as well as insoluble elastin at acid and neutral pH values. The action of cathepsin S on the oxidised B chain of insulin is very similar to that of cathepsin L with one exception: the Tyr^{26}-Thr^{27} bond is resistant to cathepsin S, even after 10 h incubation (Brömme *et al.*, 1989).

3.2.1 Cathepsin S assay

Cathepsin S activity was assayed using the synthetic substrate Z-Phe-Arg-NHMec for routine assays and Bz-Phe-Val-Arg-NHMec for more specific assays. Both substrates liberate the intensely fluorescent 7-amino-4-methylcoumarin group upon hydrolysis (Barrett and Kirschke, 1981; Kirschke and Wiederanders, 1994). These substrates are, however, also cleaved by other proteinases such as cathepsin B and L and no peptide derivative has been described so far that is specific for cathepsin S at acidic pH values. However, by preincubating at pH 7.5 for 1 h at 40°C, the activities of cathepsins L, B, and H are completely abolished, whereas cathepsin S retains 60-70% of its activity (Kirschke *et al.*, 1989).

3.2.1.1 Reagents

Buffer/activator [0.1 mM K-phosphate, 5 mM Na$_2$EDTA, 0.02% (m/v) NaN$_3$, 5 mM dithiothreitol, pH 7.5]. The buffer was made up to 4/3 times the final (nominal) concentration to accommodate dilution in the assay. KH$_2$PO$_4$ (9.1 g), Na$_2$EDTA (0.93 g) and NaN$_3$ (0.13 g) were dissolved in 450 ml dist.H$_2$O, adjusted to pH 7.5 with KOH, and made up to 500 ml. Immediately before use, dithiothreitol was added to the buffer to 5 mM (i.e. 0.51 g/500 ml).

1 mM Z-Phe-Arg-NHMec substrate stock solution. Z-Phe-Arg-NHMec (1 mg) was dissolved in DMSO (1.5 ml), divided into 50 μl aliquots and stored at -4°C. When required, 10 μl was diluted to 800 μl with dist.H$_2$O, giving a working strength of 12.5 μM.

10 mM Bz-Phe-Val-Arg-NHMec substrate stock solution. Bz-Phe-Val-Arg-NHMec (7.18 mg) was dissolved in DMSO (1 ml), divided into 50 μl aliquots and stored at -4°C. When required, 10 μl was to diluted 8 ml with dist.H$_2$O, giving a working strength of 12.5 μM.

Stopping reagent [100 mM Na-monochloroacetate, 30 mM Na-acetate, 70 mM acetic acid, pH 4.3]. Na-monochloroacetate (5.83 g), Na-acetate (2.04 g), and glacial acetic acid (2.0 ml) were dissolved in 450 ml dist.H$_2$O, adjusted to pH 4.3 with NaOH, and made up to 500 ml (made as described by Kirschke and Wiederanders, 1994).

Diluent [0.01% Triton X-100, 100 mM K-phosphate, 1 mM Na$_2$EDTA, pH 7.5]. Triton X-100 (10 µl), KH$_2$PO$_4$ (1.36 g) and Na$_2$EDTA (0.19 g) were dissolved in 95 ml dist.H$_2$O, adjusted to pH 7.5 with KOH and made up to 100 ml.

1 mM 7-amino-4-methylcoumarin standard. 7-amino-4-methylcoumarin (1.8 mg) was dissolved in DMSO (10 ml). The standard was used as a 0.5 µM solution by diluting stock solution (5 µl) in assay buffer/activator (10 ml).

3.2.1.2 Procedure

Microassay. The enzyme sample was diluted to 100 µl, and 500 µl of buffer/activator was added, mixed in and preincubated for 60 min at 40°C. Thereafter, 60 µl of this solution was placed in a Flouronunc maxisorp microtitre plate well, and 25 µl of substrate solution was added and mixed in. After 10 min at 37°C the fluorescence of the free aminomethylcoumarin was determined in a fluorescent microplate reader (Cambridge Technology, Model 7620) with excitation at 370 nm and emission at 460 nm. The enzyme activity units were taken as equivalent to the arbitrary fluorescence values.

Macroassay. The enzyme sample was diluted to 150 µl, and 750 µl of buffer/activator was added, mixed in and preincubated for 60 min at 40°C. Thereafter, 150 µl of substrate solution was added, mixed in and incubated for 10 min at 37°C. Stopping reagent (2 ml) was added and the solution vortexed. The fluorescence of the free aminomethylcoumarin was determined in a Hitachi F-2000 spectrofluorimeter with excitation at 370 nm and emission at 460 nm. Continuous assays in a temperature controlled cell were also carried out by eliminating the addition of the stopping reagent.

According to Barrett and Kirschke (1981), the reading given by the 0.5 µM standard can be taken to be equal to 1000 arbitrary enzyme units, which in turn is equal to 0.1 mU of enzyme activity. The reading given by the 0.5 µM standard was 18 904 and thus milliunits of activity in a sample could be calculated as follows:

$$\text{mUnits activity} = \frac{\text{fluorescence intensity x 0.1 mU}}{18\ 904}$$

The above procedure was employed for the assay of cathepsin S in the presence of cathepsins L, B, and H. If these enzymes were absent, an assay buffer/activator of pH 6.5 and a preincubation time of 5 min. was used.

3.2.2 Purification of cathepsin S

Cathepsin S has been purified from bovine (Xin, *et al.*, 1992; Brömme, *et al.*, 1989), rabbit (Maciewicz and Etherington, 1988), rat, and human (Kirschke and Wiederanders, 1994, Lemere *et al.*, 1995) tissues, and from *Saccharomyces cerevisiae* expressing the human enzyme (Brömme *et al.*, 1993). The amino acid sequences have been reported from the bovine (Wiederanders *et al.*, 1991; Ritonja *et al.*, 1991), rat (Petanceska and Devi, 1992), and human (Wiederanders *et al.*, 1992; Shi *et al.*, 1992) enzymes. cDNAs have been cloned from bovine (Wiederanders *et al.*, 1991), rat (Petanceska and Devi, 1992), and human (Wiederanders *et al.*, 1992; Shi *et al.*, 1992) cathepsin S.

The purification methods used to date for the isolation of cathepsin S from a number of different organs and species are very similar. The methods generally include an acid autolysis step, ammonium sulfate fractionation, ion exchange, molecular exclusion chromatography, affinity chromatography and chromatofocusing (Kirschke *et al.*, 1986; 1989; Dolenc, *et al.*, 1992; Xin *et al.*, 1992; Kirschke and Wiederanders, 1994). However, the above procedures are both long and costly, so in the present study an attempt was made to develop a method that not only incorporated fewer steps but also techniques that were relatively cost effective.

3.2.2.1 Reagents

Homogenisation buffer [50 mM Na-acetate, 150 mM NaCl, 1 mM Na$_2$EDTA, pH 4.5]. Glacial acetic acid (2.86 ml), NaCl (8.77 g) and Na$_2$EDTA (0.37 g) were dissolved in 950 ml dist.H$_2$O, adjusted to pH 4.5 with diluted NaOH, and made up to 1 litre.

Buffer A [20 mM Na-acetate, 1 mM Na$_2$EDTA, 0.02% (m/v) NaN$_3$, pH 5.0]. Glacial acetic acid (2.29 ml), Na$_2$EDTA (0.74 g) and NaN$_3$ (0.4 g) were dissolved in 1.9 litres of dist.H$_2$O, adjusted to pH 5.0 with diluted NaOH, and made up to 2 litres.

Buffer B [100 mM Na-acetate, 500 mM NaCl, 1 mM Na$_2$EDTA, 0.02% NaN$_3$ pH 5.5]. Glacial acetic acid (11.44 ml), NaCl (58.44 g), Na$_2$EDTA (0.74 g) and NaN$_3$ (0.4 g) were dissolved in 1.9 litres of dist.H$_2$O, adjusted to pH 5.5 with dilute NaOH, and made up to 2 litres.

Buffer C [20 mM K-phosphate 1 mM Na$_2$EDTA, 0.02% NaN$_3$, pH 6.5]. KH$_2$PO$_4$ (2.72 g), Na$_2$EDTA (0.37 g) and NaN$_3$ (0.2 g) were dissolved in 950 ml of dist.H$_2$O, adjusted to pH 6.5 with diluted NaOH, and made up to 1 litre.

S-Sepharose fast flow. S-Sepharose was prepared by diluting 90 ml of the supplied hydrated gel in 180 ml of buffer A, and packing the resulting slurry into a glass column under gravity. The column bed was initially regenerated with two column volumes of 2 M NaCl in buffer A and equilibrated with five column volumes of buffer A, before use. In between purification procedures, the column was regenerated with one column volume of 2 M NaCl in buffer A. On occasions, one column volume of 1 M NaOH was used to remove precipitated proteins and lipids. In either case the column was re-equilibrated with five column volumes of buffer A, before use.

Sephacryl S-100 HR. Sephacryl S-100 was prepared by diluting 500 ml of the supplied hydrated gel in 1000 ml of buffer B, and packing the resulting slurry into a glass column under gravity. The column was initially equilibrated with five column volumes of buffer B, before use. In between purification procedures, the column was re-equilibrated with two column volumes of buffer B.

<u>Hydroxyapatite</u>. Hydroxyapatite was prepared by diluting 10 ml of the supplied hydrated gel in 20 ml of buffer C, and packing the resulting slurry into a glass column under gravity. The column was initially regenerated with two column volumes of 1 M KH_2PO_4 in buffer C. The gel was equilibrated with five column volumes of buffer C, before use. In-between purification procedures, the column was regenerated with two column volumes of 1 M KH_2PO_4 in buffer C, and re-equilibrated with buffer C.

<u>Bovine spleen</u>. Fresh bovine spleen was obtained from Cato Ridge Abattoir. After removing the capsule, the spleen was diced into 2 x 2 cm cubes and frozen at -70°C for at least 3 days before use.

3.2.2.2 Procedure

The spleen was prepared essentially according to Kirschke *et al.* (1989). Frozen, diced spleen was allowed to thaw overnight at 4°C and homogenised at a ratio of 1:1 (spleen mass to buffer) in a Waring blendor with buffer A for 3 min. The homogenate was centrifuged (9000 x g, 20 min, 4°C), the supernatant decanted and adjusted, with stirring, to pH 4.2 with diluted glacial acetic acid and centrifuged as before.

Three-phase partitioning was effected on the pH 4.2 acid supernatant using a 15 to 30% ammonium sulfate cut as described in Section 2.6. The sample was loaded directly onto a column (2.5 x 18.5 cm = 90.8 ml) of S-Sepharose, equilibrated with buffer A, and eluted with a 0-300 mM NaCl gradient in buffer A, all at 50 ml/h. Fractions corresponding to the active peak were pooled and concentrated by dialysis against sucrose to approximately 1-5% of the volume of the molecular exclusion column used in the next step.

The concentrated sample from ion exchange chromatography was applied to a calibrated column of Sephacryl S-100 (2.5 x 84 cm = 412 ml), equilibrated with buffer B and run at 25 ml/h. Active fractions in the M_r range 18 000-25 000 were pooled and concentrated against sucrose. The sample from gel filtration was loaded onto a column of hydroxyapatite (Section 2.7) (1.0 x 5 cm = 3.9 ml), equilibrated in buffer C, and eluted with a 400 mM KH_2PO_4 in buffer C, all at 18 ml/h. The active peak eluted at approximately 180 mM K-phosphate and was concentrated using an Ultrafuge ultrafiltration centrifuge filter.

3.2.2.3 Results

The purification of cathepsin S is summarized in Table 3.

Table 3. The purification of cathepsin S from bovine spleen

	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purifi-cation (fold)	Yield (%)
Homogenate	490	10 173	7342	0.72	(1)	(100)
TPP (15-30%)	31	134	6874	52.1	72.4	93.6
S-Sepharose	8.5	29.2	1821	62.4	86.6	24.8
Sephacryl S-100	9.8	0.168	260.8	1552	2156	3.6
Hydroxyapatite	1.0	0.106	224.4	2117	2940	3.0

Freezing of tissue prior to homogenization is essential to disrupt lysosomes and facilitate the release of lysosomal enzymes. Using TPP early on in the purification, it was possible to obtain a fraction from the acid supernatant selectively enriched in cathepsin S. Contaminant enzymes such as cathepsins H and L were removed in the ion exchange step (Fig. 2), as they eluted after cathepsin S. However, cathepsin B eluted in the same fraction and was removed in the subsequent Sephacryl S-100 (Fig. 3) step which gave good resolution of these two enzymes. The cathepsin S was subsequently fractionated on hydroxyapatite (Fig 4), which eluted a small contaminant peak of unbound material, while a larger cathepsin S active peak was eluted with the step gradient.
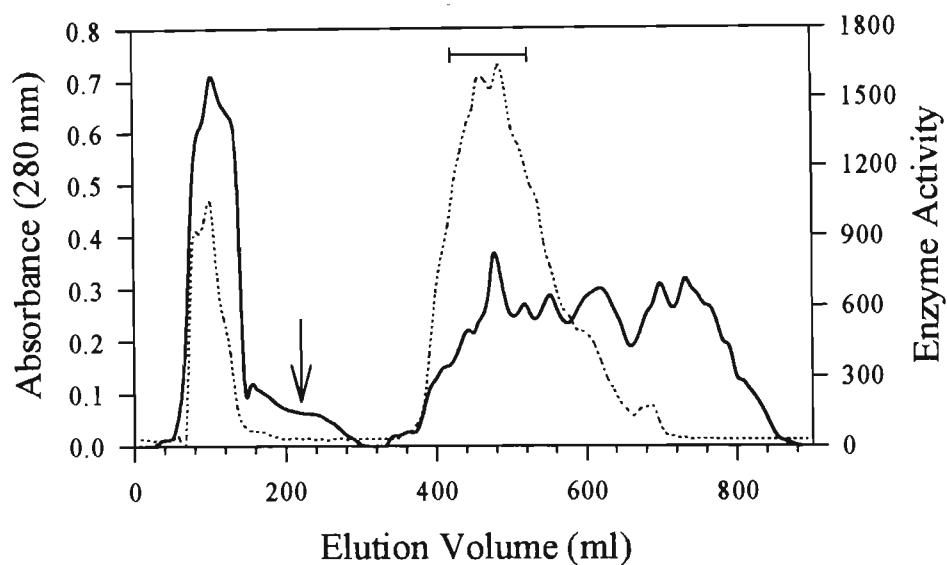
Figure 2.   Chromatography of the 15-30% pH 4.2 TPP fraction on S-Sepharose.

Column, 2.5 x 18.5 cm (91 ml bed volume), equilibrated in 20 mM Na-acetate, pH 5.0, containing 1 mM Na_2EDTA, 0.02% NaN_3 and eluted with a 0-300 mM NaCl gradient in the same buffer applied at ↓; flow rate, 50 ml/h (10 cm/h); fractions, 8.3 ml (10 min). (–) A_{280}, (···) cathepsin S activity (units) against Z-Phe-Arg-NHMec, (├─┤) pooled fraction.
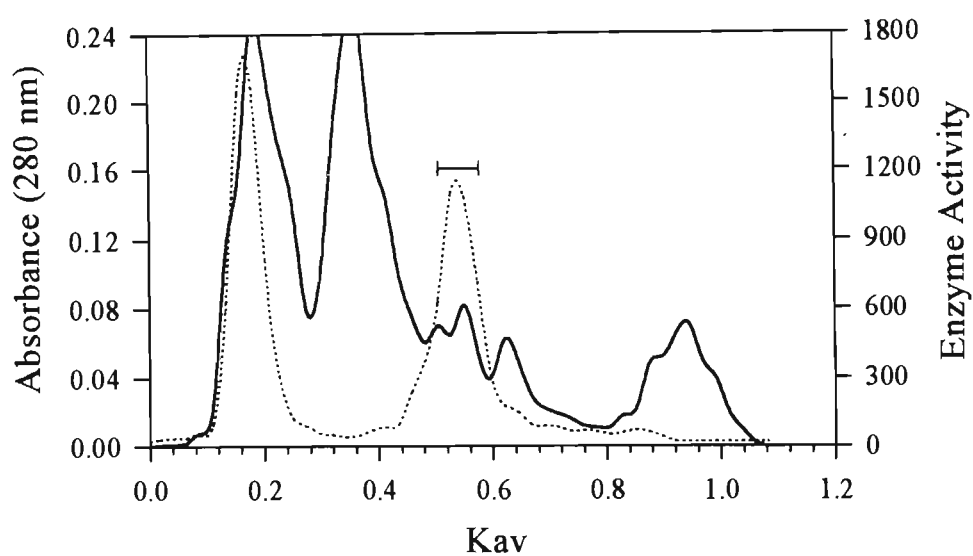
Figure 3. Chromatography of the pH 5.0 S-Sepharose fraction of cathepsin S on Sephacryl S-100.

Column 2.5 x 84 cm (412 ml bed volume); buffer, 100 mM Na-acetate, pH 5.5, containing 500 mM NaCl, 1 mM Na$_2$EDTA, 0.02% NaN$_3$; flow rate, 25 ml/h (5 cm/h); fractions, 5 ml (12 min). (–) A$_{280}$, (···) cathepsin S activity (units) against Z-Phe-Arg-NHMec, (├─┤) pooled fraction.
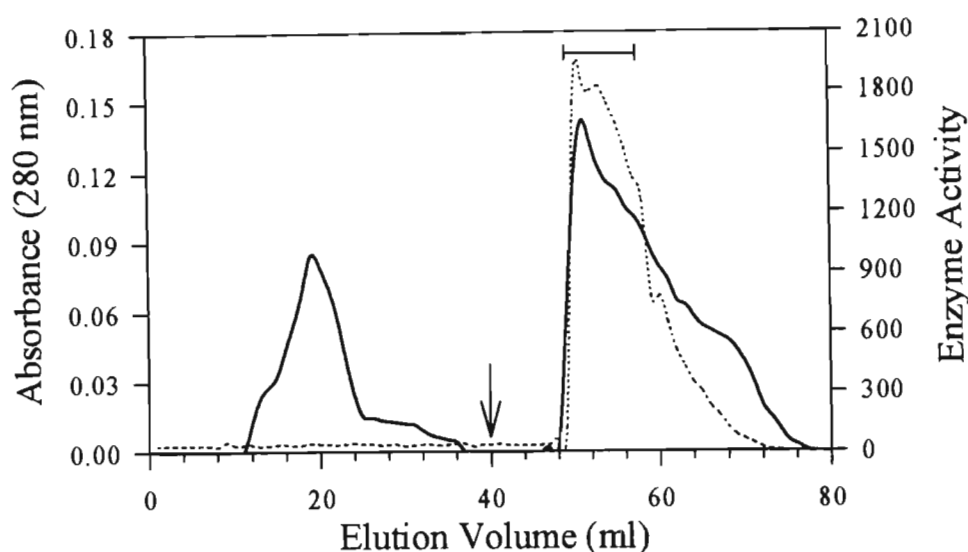
Figure 4.   Affinity chromatography of the pH 5.5 Sephacryl S-100 fraction on hydroxyapatite.
Column, 1.0 x 5.0 cm (3.9 ml bed volume), equilibrated in 20 mM KH_2PO_4, pH 6.5, containing 1 mM Na_2EDTA, 0.02% NaN_3 and eluted with 400 mM K-phosphate, pH 6.5, containing 1 mM Na_2EDTA, 0.02% NaN_3 applied at ↓; flow rate, 18 ml/h (4.6 cm/h); fractions, 1 ml (3.3 min). (−) A_{280}; (···) cathepsin S activity (units) against Z-Phe-Arg-NHMec, (⊢⊣) pooled fraction.

On non-reducing Tris-tricine SDS-PAGE (Fig. 5) the progression of the isolation can be seen. Lane b of Fig. 4 shows the proteins in the homogenate supernatant, lanes c and d show the supernatant after acid precipitation and TPP, and lanes e and f show the TPP fraction after ion exchange and molecular exclusion chromatography. Note the purification after molecular exclusion chromatography, with the removal of all higher molecular weight proteins. Lane g shows a single protein band corresponding to 14 kDa. Cathepsin S samples have never been analysed on Tris-tricine SDS-PAGE, therefore this anomalous behaviour has not been previously noted.
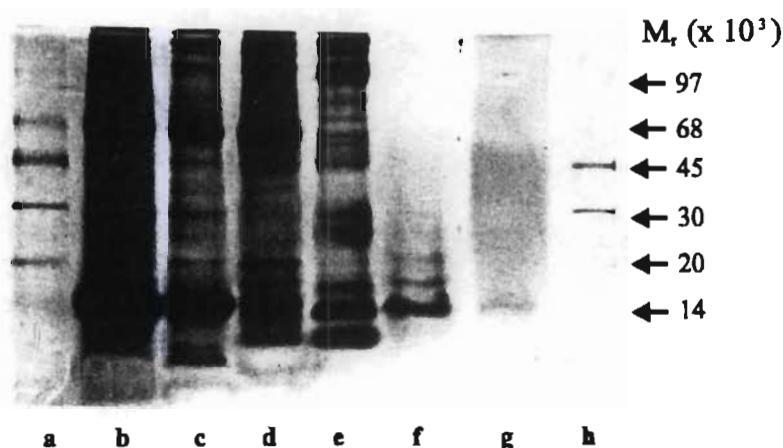
Figure 5. Non-reducing Tris-Tricine SDS-PAGE of cathepsin S isolated from bovine spleen. Samples were boiled in non-reducing treatment buffer and loaded onto a 10% Tris-Tricine gel. (b) Spleen homogenate fraction; (c) pH 4.2 acid precipitate supernatant; (d) TPP fraction; (e) S-Sepharose fraction; (f) Sephacryl S-100 fraction; (g) hydroxyapatite fraction; (a, h) MW markers (phosphorylase b, M_r 97 000; BSA, M_r 68 000; ovalbumin, M_r 45 000; carbonic anhydrase, M_r 30 000; trypsin inhibitor, M_r 20 100; lysozyme, M_r 14 000). Visualised using silver staining.

3.2.3 pH Optimum of cathepsin S using constant ionic strength AMT buffers

In a single component buffer of constant molarity, the ionic strength changes with the pH. Since cysteine cathepsins are sensitive to ionic strength, this can give an erroneous impression of the pH optimum (Dehrmann et al., 1995; 1996). To determine the pH optimum of cathepsin S, therefore, the activity was assayed against Bz-Phe-Val-Arg-NHMec in a constant ionic strength AMT buffer (Ellis and Morrison, 1982), to remove the possible influence of ionic strength on the activity of the enzyme. The assay used was based on that described by Dehrmann et al. (1995).

3.2.3.1 Reagents

Acetate-MES-Tris (AMT) buffers (50 mM acetate, 50 mM MES and 100 mM Tris, 5 mM Na_2EDTA). The buffers were made up to 4/3 times the final (nominal) concentration to accommodate dilution in the assay. Glacial acetic acid (0.953 ml), MES (3.26 g), Tris (4.04 g) and Na_2EDTA (0.62 g) were dissolved in 200 ml of dist.H_2O. The solution was divided into 10 aliquots (20 ml), each of which was titrated to a pH value in the range 4.0-8.5 using HCl or NaOH, and diluted to 25 ml.

<u>10 mM Bz-Phe-Val-Arg-NHMec substrate stock solution</u>. Bz-Phe-Val-Arg-NHMec (7.18 mg) was dissolved in DMSO (1 ml), divided into 50 µl aliquots and stored at -4°C. When required, 27 µl was diluted to 8 ml with dist.H_2O, giving a working strength of 34 µM.

<u>Diluent [0.01% (v/v) Triton X-100, 100 mM K-phosphate, 1 mM Na_2EDTA, pH 7.5]</u>. Triton X-100 (10 µl), KH_2PO_4 (1.36 g) and Na_2EDTA (0.19 g) was dissolved in 95 ml dist.H_2O, adjusted to pH 7.5 with KOH and made up to 100 ml.

<u>1 M dithiothreitol (DTT)</u>. DTT (154.2 mg) was dissolved in 1 ml dist.H_2O

3.2.3.2 Procedure

AMT buffers of constant ionic strength were prepared according to Ellis and Morrison (1982) with an ionic strength of 0.1 and at pH 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, and 8.5. All solutions were equilibrated to 37°C, except the diluted enzyme solution which was kept on ice. Each assay consisted of 750 µl buffer, 4 µl DTT, and 96 µl of appropriately diluted enzyme. This was allowed to activate for 2 min at 37°C, followed by the addition of 150 µl of substrate solution. The change in fluorescence was measured continuously at 37°C in a temperature-controlled Hitachi F-2000 recording spectrofluorometer, with excitation at 370 nm and emission at 460 nm. Data was sampled at 10 s intervals over a 10 min period and activity was determined from the slope of the plot of fluorescence intensity versus time. This was expressed in terms of relative activity.

3.2.3.3 Results

The profiles shown in Fig. 6 illustrate the activity of cathepsin S over a wide pH range, firstly, in the presence of constant ionic strength AMT buffers, and secondly, the profile reported by Kirschke *et al.* (1989) who used a range of buffers of varying ionic strength. The graph indicates an optimum of pH 6.5 with constant ionic strength AMT buffers which is similar to the previously reported results of Kirschke *et al.* (1989). However, cathepsin S shows remarkable stability in the presence of constant ionic strength AMT buffers as it remains over 55% active over the entire pH range, whereas the results of Kirschke *et al.* (1989) show a large drop in activity either side of the pH optimum.
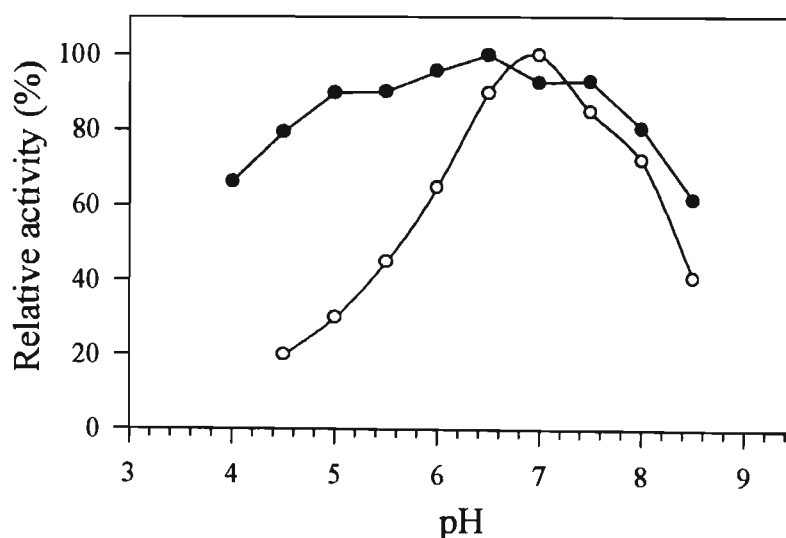
Figure 6.   pH-Activity profiles of bovine spleen cathepsin S.

Cathepsin S isolated in this study (•), was assayed with Bz-Phe-Val-Arg-NHMec in buffers of constant ionic strength as described in Section 3.2.3. Cathepsin S pH-activity profile of Kirschke *et al.* (1989) (o), was assayed against Z-Phe-Arg-NHMec in Na-acetate (pH 4.5-5.5), Na-phosphate (pH 6.0-7.5), and Tris/HCl pH (7.5-8.5) buffers.

3.2.4 Active site titration of cathepsin S with E-64

3.2.4.1 Reagents

Buffer/activator [0.1 mM K-phosphate, 5 mM Na_2EDTA, 0.02% NaN_3, 5 mM dithiothreitol, pH 6.5]. The buffer was made up to 4/3 times the final (nominal) concentration to accommodate dilution in the assay. KH_2PO_4 (9.1 g), Na_2EDTA (.93 g) and NaN_3 (0.13 g) were dissolved in 450 ml dist.H_2O, adjusted to pH 6.5 with KOH, and made up to 500 ml. Immediately before use, dithiothreitol was added to the buffer to 5 mM (i.e. 0.51 g/500 ml).

10 mM Bz-Phe-Val-Arg-NHMec substrate stock solution. As in Section 3.2.1.

Diluent [0.01% Triton X-100, 100 mM K-phosphate, 1 mM Na_2EDTA, pH 6.5]. Triton X-100 (10 µl), KH_2PO_4 (1.36 g) and Na_2EDTA (0.19 g) were dissolved in 95 ml dist.H_2O, adjusted to pH 6.5 with KOH and made up to 100 ml.

10 mM E-64 stock solution. E-64 (1.8 mg) was dissolved in DMSO (500 µl).

3.2.4.2 Procedure

Active site titration of cathepsin S with the inhibitor E-64 was done essentially as described by Barrett and Kirschke (1981). Enzyme (8 µl), of an appropriate dilution, was added and incubated together with the inhibitor (5 µl of a 1-10 µM solution) for 30 min at 37°C in the presence of the pH 6.5 assay buffer/activator (39 µl). After 30 min, the mixture was diluted to 200 µl by the addition of Triton X-100 diluent. Aliquots (150 µl) were assayed against Bz-Phe-Val-Arg-NHMec using the continuous assay at pH 6.5 as described in Section 3.2.1 (macroassay). From this a graph of fluorescence versus the concentration of E-64 was plotted and the concentration of E-64 which halted the enzyme reaction (i.e. where the graph intercepts the x-axis) is designated [E_o].

3.2.4.3 Results

In order to calculate the percentage of active enzyme present in the isolated sample {to calculate [E_o] and hence k_{cat} (Section 3.2.5)}, active site titration of cathepsin S by E-64 was carried out. This revealed that 42% of the purified sample was active enzyme. [E_o], the absolute concentration of active enzyme, was calculated to be 1.85 μM.

3.2.5 Determination of K_m, V_{max} and k_{cat} values

3.2.5.1 Reagents

Buffer/activator [0.1 mM K-phosphate, 5 mM Na$_2$EDTA, 0.02% NaN$_3$, 5 mM dithiothreitol, pH 6.5]. As described in Section 3.2.4.

10 mM Bz-Phe-Val-Arg-NHMec substrate stock solution. As described in Section 3.2.1.

Diluent [0.01% Triton X-100, 100 mM K-phosphate, 1 mM Na$_2$EDTA, pH 6.5]. As described in Section 3.2.4.

3.2.5.2 Procedure

To determine K_m and V_{max}, a semicontinuous assay in a fluorescent microplate reader (Cambridge Technology, Model 7620) at 37°C was used as described by Dehrmann et al. (1995). For each determination, buffer/activator (75 μl) plus appropriately diluted enzyme (12.5 μl) was added to each of 14 wells of a white Fluoronunc maxisorp microtitre plate and incubated for 2 min. Serial two-fold dilutions of Bz-Phe-Val-Arg-NHMec were added to duplicate wells in a row of seven, giving final substrate concentrations ranging from 50 to 0.78125 μM. After 2, 4, and 6 min, the wells were read, with excitation at 370 nm and emission at 460 nm. Readings at three different times provides a check on the linearity of the progress curve. Dehrmann et al. (1995) established that before 2 min the reaction rate increases with time, but the reaction was linear between 2 and 6 min. K_m and V_{max} values were determined using the direct linear plot of Eisenthal and Cornish-Bowden (1974), implemented using the programme HYPER obtained from Dr. J.S. Easterby (University of Liverpool). [E_o] values were established as described in Section 3.2.5, and the relationship

$$k_{cat} = V_{max}/[E_o]$$

was used to calculate k_{cat} from V_{max} values.

3.2.5.3 Results

The measurement of K_m and V_{max} for bovine spleen cathepsin S against the synthetic substrate Bz-Phe-Val-Arg-NHMec at pH 6.5 was carried out as described in Section 3.2.5. From the K_m (3.91 μM), V_{max} (5.39 μM.s^{-1}), and $[E_o]$ (1.85 μM, Section 3.2.4), k_{cat} (2.9 s^{-1}) was calculated, using the equation in Section 3.2.5.2.

3.3 Cathepsin B

Cathepsin B [EC 3.4.22.1] is an abundant lysosomal cysteine proteinase which plays a role in a variety of physiological processes, such as general intracellular protein turnover (Barrett and Kirschke, 1981), bone resorption (Delaisse et al., 1991), cartilage proteoglycan breakdown (Buttle and Saklatvala, 1992) and antigen processing (Guagliardi et al., 1990). However, it has also been implicated in pathological states such as arthritis (Mort et al., 1984), muscular dystrophy (Gopalan et al., 1987; Katunuma and Kominami, 1987) and tumour metastasis (Sloane et al., 1990).

Human cathepsin B was the first lysosomal cysteine proteinase whose crystal structure was elucidated (at a 2.15 Å level) by X-ray crystallography (Musil et al., 1991). The enzyme shows a two-domain structure, the L domain containing residues 13-147 and 251-254 and the R domain containing residues 1-12 and 148-250. Complete amino acid sequences of cathepsin B have been determined for rat (Takio et al., 1983), human (Ritonja et al., 1985), and bovine (Meloun et al., 1988) cathepsin B. From cDNA clones encoding for mouse and human (Chan et al., 1986) cathepsin B, it has been demonstrated that this enzyme is synthesised as a large-M_r precursor containing signal and pro-sequences. The precursor undergoes multiple processing steps, the most important of which results in the removal of the 62 residue pro-region to yield the active, single-chain form of the enzyme (Chan et al., 1986).

Mature cathepsin B appears to be unique with respect to other members of the cysteine proteinase family in that it possesses both endopeptidase and exopeptidase (carboxydipeptidase) activities (Aronson and Barrett, 1979; Barrett and Kirschke, 1981). The optimal activity of cathepsin B against most substrates was originally thought to occur in slightly acidic media (Barrett and Kirschke, 1981), while at pH values above 7.0 its activity was shown to fall sharply and was thought to be due to irreversible inactivation (Barrett, 1973). The action of cathepsin B on biological peptides at acidic pH was shown to occur predominantly through its carboxydipeptidase activity (Takahashi *et al.*, 1986). The active-site model which was drawn based on these observations (Takahashi *et al.*, 1986) has largely been confirmed with data on the crystal structure of cathepsin B (Musil *et al.*, 1991). Due to the occluding loop which partly covers the active-site cleft of human cathepsin B, and the presence within this loop of two pH-sensitive histidine residues, the active-site of cathepsin B can easily accommodate C-terminal carboxyl groups of peptides and proteins (Musil *et al.*, 1991).

Cathepsin B is also capable of endoproteolytic attack (Barrett and Kirschke, 1981), and it appears that this catalytic mechanism may predominate at physiological pH if the enzyme is properly stabilised. Proof for this might be the observed speed at which the enzyme autolyses *in vitro* at pH 7.2 if no exogenous protein substrate is present, e.g. extracellular matrix components like fibronectin (Buck *et al.*, 1992). Indeed, inactivation of cathepsin B at pH > 7.0 does not seem to be due to the irreversible unfolding of the enzyme as might appear from *in vitro* studies using synthetic substrates (Barrett and Kirschke, 1981). The fact that at pH > 7 human cathepsin B undergoes a time-dependent autodegradation, which can be slowed by the presence of substrates such as fibronectin or even blocked by the addition of E-64 (Buck *et al.*, 1992), argues against the contention that at pH > 7.0 cathepsin B is irreversibly denatured. Some earlier work (Willenbrock and Brocklehurst, 1985) showing that the specificity constant (k_{cat}/K_m) of cathepsin B for a dibasic naphthylamide substrate was about 4 fold higher at pH 8.0 than at pH 6.0 is also in good agreement with this more recent finding (Buck *et al.*, 1992). Depending on the substrates used, at least seven separate ionisation groups were shown to be involved in substrate binding and hydrolysis by cathepsin B (Hasnain *et al.*, 1992). Altogether these results demonstrate that the pH-dependent catalytic activity of cathepsin B is extremely complex and depends not only on the composition of the medium and the nature and conformation of the

substrate, but also on the presence or absence of stabilising factors (Keppler and Sloane, 1996). Dehrmann *et al.* (1995; 1996) showed that the activity of cathepsin B and that of cathepsin L is affected by specific buffer ions as well as pH, ionic strength and the presence of protein substrates. The stability is also affected by the activation state of the enzyme, which may in part explain the markedly different results which have been previously reported. Dehrmann *et al.* (1996) found the pH optimum of cathepsin B to be pH 7-7.5.

3.3.1 Cathepsin B assay

Cathepsin B was originally assayed with Bz-DL-Arg-NPhNO$_2$ or Bz-Arg-2-NNap as substrates (Barrett, 1972; 1976). However, it was found that these substrates were not only insensitive, but also susceptible to cathepsin H, so the results obtained were not specific (Barrett and Kirschke, 1981). As has been found with the serine proteinases, substrates containing longer peptide sequences are much more selective and specific than the blocked amino acid derivatives. Thus naphthylamide substrates containing the -Arg-Arg- sequence, introduced by McDonald and Ellis (1975), were found to be extremely sensitive to cathepsin B, and resistant to cathepsin H and L (Kirschke *et al.*, 1980). The most sensitive, safe, and convenient leaving group for substrates of the cysteine proteinases is 7-amino-4-methylcoumarin, and just as Z-Arg-Arg-NNap is specific for cathepsin B, so is Z-Arg-Arg-NHMec which has proven to be an almost ideal substrate for cathepsin B (Barrett and Kirschke, 1981).

3.3.1.1 Reagents

Buffer/activator [0.1 M Na-phosphate, 4 mM Na$_2$EDTA, 0.02% NaN$_3$, 5 mM dithiothreitol, pH 6.0]. NaH$_2$PO$_4$ (6.90 g), Na$_2$EDTA (0.93 g) and NaN$_3$ (0.1 g) were dissolved in 450 ml dist.H$_2$O, adjusted to pH 6.0 with NaOH and made up to 500 ml. Immediately before use, dithiothreitol was added to 5 mM (i.e. 0.51 g/500 ml).

1 mM Z-Arg-Arg-NHMec substrate stock solution. Z-Arg-Arg-NHMec (1.1 mg) was dissolved in DMSO (1.5 ml), divided into 100 μl aliquots and stored at -4°C. When required, 100 μl was diluted to 2.5 ml with dist.H$_2$O, giving a working strength of 40 μM.

<u>Diluent [0.1% Brij]</u>. Brij (0.1 g) was dissolved in 95 ml dist.H_2O, and made up to 100 ml.

<u>1 mM 7-amino-4-methylcoumarin standard</u>. 7-amino-4-methylcoumarin (1.8 mg) was dissolved in DMSO (10 ml). The standard was used as a 0.5 μM solution by diluting stock solution (5 μl) in assay buffer/activator (10 ml).

3.3.1.2 Procedure

<u>Microassay</u>. The enzyme sample, diluted to 10 μl, and 75 μl of buffer/activator was added to wells of a white Fluoronunc maxisorp microtitre plate and preincubated for 2 min at 37°C. Thereafter, 25 μl of substrate solution was added and mixed in. After exactly 10 min at 37°C the fluorescence of the free aminomethylcoumarin was determined in a fluorescent microplate reader (Cambridge Technology, Model 7620) with excitation at 370 nm and emission at 460 nm. The enzyme activity units were taken as equivalent to arbitrary fluorescence values.

<u>Macroassay</u>. The enzyme sample, diluted to 125 μl, and 750 μl of buffer/activator was added and preincubated for 2 min at 37°C. Thereafter, 125 μl of substrate solution was added and mixed in. The fluorescence of the free aminomethylcoumarin was determined continuously for 10 min in a Hitachi F-2000 spectroflourimeter with excitation at 370 nm and emission at 460 nm. This was used to give units of activity for a 10 min assay and to assess linearity of the reaction.

According to Barrett and Kirschke (1981), the reading given by the 0.5 μM standard can be taken to be equal to 1000 arbitrary enzyme units, which in turn is equal to 0.1 mU of enzyme activity. The reading given by the 0.5 μM standard was 19 652 and thus milliunits of activity in a sample could be calculated as follows:

$$\text{mUnits activity} = \frac{\text{fluorescence intensity x 0.1 mU}}{19\ 652}$$

3.3.2 Purification of cathepsin B

The isolation of cathepsin B has mostly relied on specific techniques such as covalent chromatography using an aminophenylmercuric acetate coupled to Sepharose (Barrett and Kirschke, 1981), or the method of Rich *et al.* (1986) which uses a specialised affinity resin, Agarose-Ahx-Gly-Phe-Gly-Sc, which is currently not commercially available. Consequently, in the present study it was decided to explore the possibility of developing an alternative quick, cost effective method for the isolation of cathepsin B from bovine spleen.

3.3.2.1 Reagents

Homogenisation buffer [50 mM Na-acetate, 150 mM NaCl, 1 mM Na_2EDTA, pH 4.5]. As described in Section 3.2.2.1.

Buffer A [20 mM Na-acetate, 1 mM Na_2EDTA, 0.02% NaN_3, pH 5.0]. As described in Section 3.2.2.1.

Buffer B [100 mM Na-acetate, 500 mM NaCl, 1 mM Na_2EDTA, 0.02% NaN_3 pH 5.5]. As described in Section 3.2.2.1.

3.3.2.2 Procedure

Cathepsin B was isolated by a slight modification of the method used for cathepsin S, as described in Section 3.2.2. The TPP cut used was 30 to 40% and the cathepsin B active peak from Sephacryl S-100 molecular exclusion chromatography eluted between 25 to 30 kDa.

3.3.2.3 Results

The isolation of cathepsin B from bovine spleen is summarized in Table 4.

Table 4. The purification of cathepsin B from bovine spleen

	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purifi-cation (fold)	Yield (%)
Homogenate	490	10 173	77 221	7.59	(1)	(100)
TPP (30-40%)	31	23.7	754.3	31.8	4.2	0.98
S-Sepharose	4.8	0.79	668.2	845.8	111	0.87
Sephacryl S-100	1.0	0.007	35.7	5 100	672	0.05

The procedure followed the same steps as the purification of cathepsin S (Section 3.2.2). However, a different TPP cut was used (30-40%), which selectively precipitated the cathepsin B activity. The enzyme eluted from Sephacryl S-100 as a pure fraction, so no further steps were required.
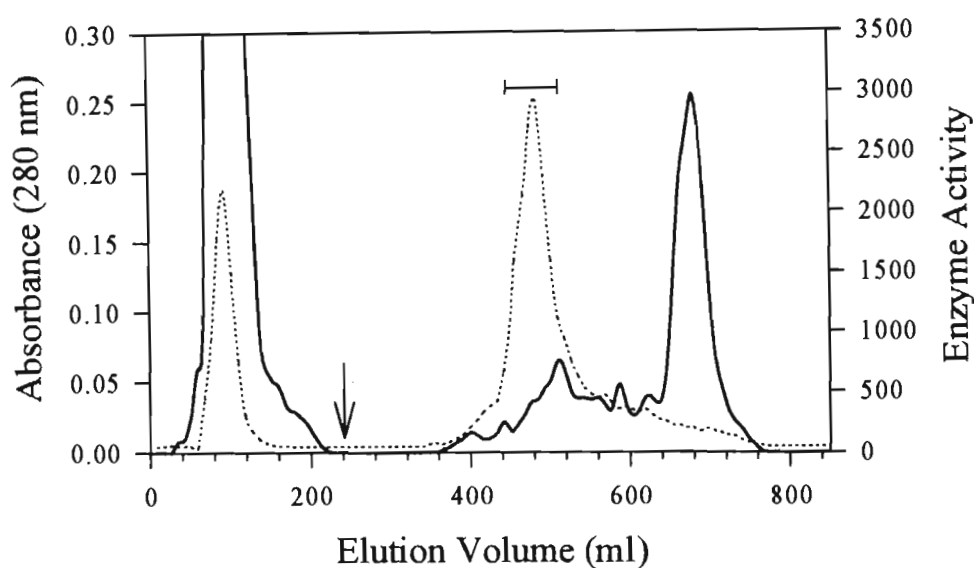
Figure 7.   Chromatography of the pH 4.2 TPP fraction of cathepsin B on S-Sepharose.
Column, 2.5 x 18.5 cm (91 ml bed volume), equilibrated in 20 mM Na-acetate, pH 5.0, containing 1 mM Na_2EDTA, 0.02% NaN_3 and eluted with a 0-300 mM NaCl gradient in the same buffer applied at ↓; flow rate, 50 ml/h (10 cm/h); fractions, 8.3 ml (10 min). (–) A_{280}, (···) cathepsin B activity (units) against Z-Arg-Arg-NHMec, (⊢⊣) pooled fraction.
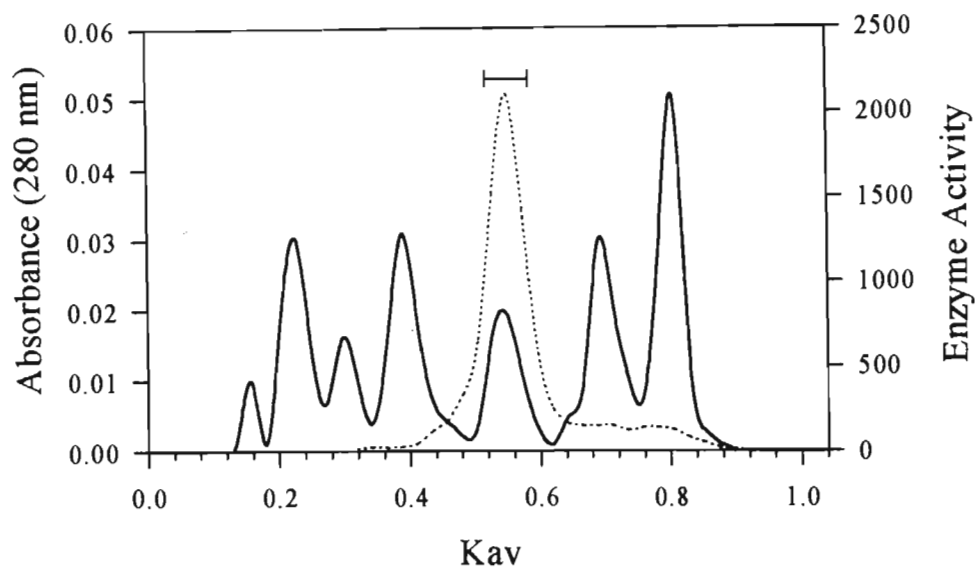
Figure 8.   Chromatography of the pH 5.0 S-Sepharose fraction of cathepsin B on Sephacryl S-100.
Column. 2.5 x 84 cm (412 ml bed volume); buffer, 100 mM Na-acetate, pH 5.5, containing 500 mM
NaCl, 1 mM Na$_2$EDTA, 0.02% NaN$_3$; flow rate, 25 ml/h (5 cm/h); and fractions, 5 ml (12 min).
(–) A$_{280}$; (···) cathepsin B activity (units) against Z-Arg-Arg-NHMec, (⊢⊣) pooled fraction.

The progression of the isolation was analysed by non-reducing Tris-tricine SDS-PAGE (Fig. 9).
Lane f shows a single protein band of 28 kDa which corresponds to previously reported results
for the MW of cathepsin B (Barrett, 1973; Barrett and Kirschke, 1981).
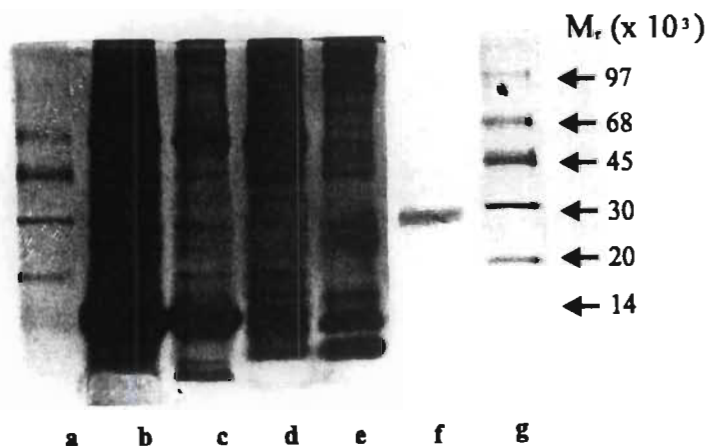
Figure 9. Tris-Tricine SDS-PAGE (Section 2.4.2) of cathepsin B fractions isolated from bovine spleen.
Samples were boiled in non-reducing treatment buffer and loaded onto a 10% Tris-Tricine gel.
(b) Spleen homogenate fraction; (c) pH 4.2 acid precipitate supernatant; (d) TPP fraction;
(e) S-Sepharose fraction; (f) Sephacryl S-100 fraction; and (a, g) MW markers (phosphorylase b, M_r
97 000; BSA, M_r 68 000; ovalbumin, M_r 45 000; carbonic anhydrase, M_r 30 000; trypsin inhibitor, M_r
20 100; lysozyme, M_r 14 000). Visualised using silver staining.

3.4 Acid α-glucosidase

Acid α-glucosidase catalyses the hydrolysis of both α-1,4 and α-1,6 glycosidic linkages from the
non-reducing terminus of various substrates (Hoefsloot *et al.*, 1988; Onodera *et al.*, 1989).
Mammalian α-glucosidase acts not only on substrates of low molecular weight, such as maltose,
but also on high molecular weight substrates, such as glycogen (Matsui *et al.*, 1984). In general,
mammalian α-glucosidases are classified into two groups, acid α-glucosidase (optimum pH 4-5),
and neutral α-glucosidase (optimum pH 6-7) according to their pH optima. The essential
function of acid α-glucosidase in lysosomes is the degradation of glycogen to glucose which is
transported out of the lysosomes and reutilized in the cytoplasm (Mancini *et al.*, 1990; Jonas
et al., 1990). The importance of acid α-glucosidase in the lysosomal degradation of glycogen is
emphasized by the pathological conditions which ensue when the enzyme is deficient. Acid
α-glucosidase deficiency was discovered in 1963 (Hers, 1963) as the primary defect in
glycogenosis type II (Pompe's disease), until then known only as an inherited, generalised,
glycogen storage disease with a fatal outcome in the first two years of life (Hers, 1963; Hers and
De Barsy 1973).

For the active site of acid α-glucosidase, two conflicting proposals have been made. Jeffrey *et al.* (1970a,b) have suggested that acid α-glucosidase from rat liver lysosomes may have at least two catalytically active binding sites, one which can bind low molecular weight substrates such as maltose, and the other which can bind α-glucans such as glycogen. Palmer (1971a,b) suggested similar "multiple substrate binding sites" for rabbit muscle acid α-glucosidase. On the other hand, there are many reports that acid α-glucosidases catalyse the hydrolysis of both maltose and α-glucan at a single active site (Matsui and Chiba, 1983a,b; Matsui *et al.*, 1984). Further evidence based on results of kinetic approaches and the effects of pH and temperature on the hydrolysis of maltose and shell fish glycogen (Onodera *et al.*, 1989), and active site-directed inhibition and site-directed mutagenesis (Hermans *et al.*, 1991) pointed to a single catalytic site with Asp-518 as the catalytic base and a second aspartic or glutamic acid residue acting as the proton donor.

3.4.1 Acid α-glucosidase assay

Acid α-glucosidase activity was assayed using the synthetic substrate 4-methylumbelliferyl-α-glucoside. This substrate liberates the fluorescent 4-methylumbelliferone group upon hydrolysis. The amount of substrate cleaved is equivalent to the amount of the highly fluorescent 4-methylumbelliferone anion generated. Quenching the reaction with alkaline (pH 10.5) glycine:ammonium hydroxide solution accomplishes two things; first it prevents further enzyme-catalysed hydrolysis of the substrate and second, it converts 4-methylumbelliferone to its more fluorescent anion form (Daniels and Glew, 1984).

3.4.1.1 Reagents

Assay buffer [0.1 M Na-acetate, pH 4.5]. Glacial acetic acid (2.86 ml) was added to 450 ml dist.H_2O, adjusted to pH 4.5 with NaOH and made up to 500 ml.

20 mM 4-methylumbelliferyl-α-glucoside stock solution. 4-methylumbelliferyl-α-glucoside (0.067 g) was dissolved in 10 ml of dist.H_2O, divided into 500 μl aliquots and stored at -4°C. When required, it was diluted to a working strength of 0.2 mM with dist.H_2O (0.1 ml to 10 ml).

Stopping reagent [50 mM glycine/NaOH, pH 10.5]. Glycine (3.75 g) was dissolved in 450 ml dist.H$_2$O, adjusted to pH 10.5 with NaOH and made up to 500 ml.

10 mM 4-methylumbelliferone standard. 4-methylumbelliferone sodium salt (198.2 mg) was dissolved in 10 ml DMSO. A standard curve was constructed by diluting the 4-methylumbelliferone standard solution 1:1000 with water, pipetting 5 to 100 μl (0.05 to 1 nmol) into assay tubes, and adding 2.9 ml stopping reagent. The fluorescence was measured in a Hitachi F-200 spectrofluorimeter, with excitation at 365 nm and emission at 450 nm and plottted as a function of 4-methylumbelliferone concentration.

3.4.1.2 Procedure

The enzyme sample (10 μl) was added to 30 μl of assay buffer followed by 10 μl of substrate solution, mixed thoroughly and incubated for 10 min at 37°C. Stopping reagent (2.95 ml) was added and the solution vortexed. The fluorescence of the free 4-methylumbelliferone was determined in a Hitachi F-2000 spectrofluorimeter, with excitation at 365 nm and emission at 450 nm.

3.4.2 Purification of acid α-glucosidase

Several of the mammalian acid α-glucosidases have been purified, including those from rat liver (Auricchio and Bruni, 1967; Auricchio *et al.*, 1968; Jeffrey *et al.*, 1970a,b), dog liver (Torres and Olivarria, 1964), bovine liver (Bruni *et al.*, 1970), human liver (Koster *et al.*, 1976; Murray *et al.*, 1978), porcine liver (Matsui and Chiba, 1983a), human kidney (Auricchio and Bruni, 1967; Auricchio *et al.*, 1968), human placenta (Martiniuk *et al.*, 1984) and rabbit muscle (Palmer, 1971a,b; Matsui *et al.*, 1984). The properties of many of these enzymes have been described, including their pH dependence, activity towards various substrates and inhibition by various agents (Calder and Geddes, 1989a).

Acid α-glucosidase can be purified on the basis of its affinity to dextran gel filtration media such as Sephadex (Auricchio *et al.*, 1968; Bruni *et al.*, 1970), which may act as an analogue of the natural substrates, such as glycogen. Employing this principle, Hasilik and Neufeld (1980 a,b)

purified this enzyme from human placenta. They found that the enzyme contained two components, with molecular masses of 76 kDa and 70 kDa. Tashiro *et al.* (1986) also reported that acid α-glucosidase purified from pig liver, using Sephadex G-100 as an affinity medium, contained two components. In the present study the procedure for the isolation of acid α-glucosidase was based on that of Tashiro *et al.* (1986).

3.4.2.1 Reagents

<u>Homogenisation "buffer"</u> [1% NaCl, 0.1% Na$_2$EDTA, 2% n-butanol]. NaCl (10 g) and Na$_2$EDTA (1 g) were dissolved in 950 ml of dist.H$_2$O. Just before use, 20 ml of n-butanol was added and the volume made up to 1 litre.

<u>Equilibration buffer</u> [20 mM Na-acetate, 0.02% NaN$_3$, pH 5.8]. Glacial acetic acid (2.29 ml) and NaN$_3$ (0.4 g) were added to 1.9 litres of dist.H$_2$O, adjusted to pH 5.8 with NaOH and made up to 2 litres.

<u>Sephadex G-100</u>. Sephadex G-100 was prepared by adding 30 g of the supplied xerogel to 500 ml of dist.H$_2$O. This was boiled in a water bath for 1 h and allowed to cool to room temperature over 24 h. The resulting slurry was packed into a glass column under gravity. The column bed was equilibrated with five column volumes of equilibration buffer before use. In between purification procedures, the column was regenerated with one column volume of 0.25% maltose in equilibration buffer.

<u>Bovine liver</u>. Fresh bovine livers were obtained from Cato Ridge Abattoir, cut into small pieces (2 x 2 cm cubes) and frozen at -70°C for at least 3 days before use.

3.4.2.2 Procedure

The liver was prepared essentially according to Tashiro *et al.* (1986). The diced liver was allowed to thaw overnight at 4°C and homogenised in a Waring blendor with homogenisation buffer. A ratio of 1:2 (liver mass to buffer) was used and the liver was homogenised for 3 min in 100 g batches. The homogenate was centrifuged (6000 x g, 30 min, 4°C), the supernatant

decanted and adjusted, with stirring, to pH 4.2 with diluted glacial acetic acid and centrifuged as before. Three-phase partitioning was effected on the pH 4.2 acid supernatant using a 15 to 25% ammonium sulfate cut as described in Section 2.6. The resulting pellet was redissolved in 15 ml equilibration buffer.

The sample obtained from three-phase partitioning was loaded onto a Sephadex G-100 column (5 x 17 cm = 320 ml; flow rate 75 ml/h), equilibrated with equilibration buffer. The column was washed with 1 column volume of equilibration buffer and the adsorbed material was eluted with 0.25% maltose in equilibration buffer. Fractions corresponding to the active peak were pooled and concentrated by dialysis against PEG 20 000.

3.4.2.3 Results

The purification of acid α-glucosidase is summarized in Table 5.

Table 5. Purification of acid α-glucosidase from porcine liver

	Vol. (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Homogenate	725	55522	613.35	0.011	(1)	(100)
Acid Ppt.	480	16604	386.08	0.023	2.09	63
TPP (15-25%)	14.5	618.5	195.65	0.316	28.72	32
Sephadex G-100	30.4	2.004	35.17	17.55	1595.5	0.06

This method proved to be highly reproducible and very quick. TPP fractionation of the acid supernatant provided an enriched fraction which was subsequently separated by affinity chromatography on Sephadex G-100 (Fig. 10). A very large peak of unbound material eluted, while a small acid α-glucosidase active peak eluted after the addition of maltose to the elution buffer.
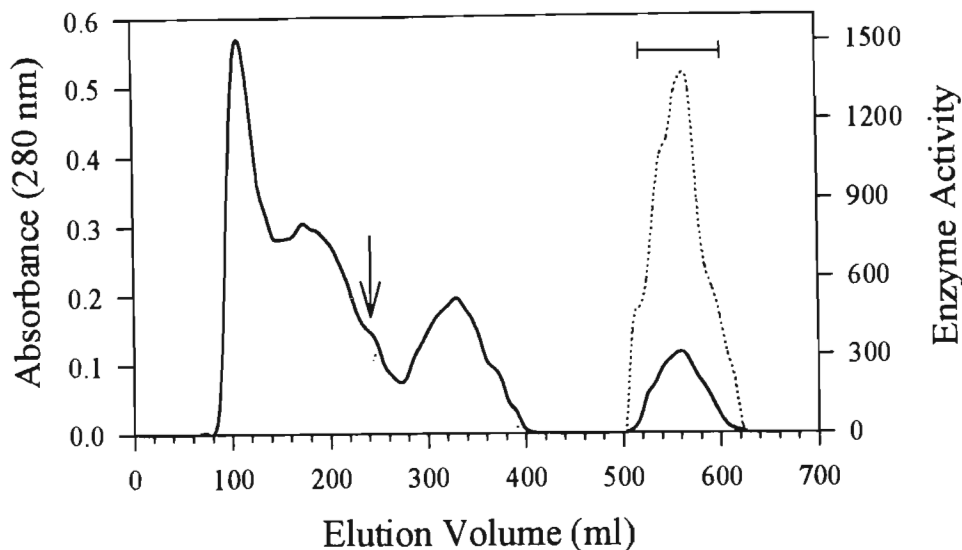
Figure 10. Affinity chromatography of the pH 4.2 TPP fraction of porcine liver acid α-glucosidase on Sephadex G-100.

Column; 5 x 17 cm (334 ml bed volume), equilibrated in 20 mM Na-acetate, pH 5.8, containing 0.02% NaN$_3$ and eluted with 20 mM Na-acetate, pH 5.8, containing 0.25% maltose and 0.02% NaN$_3$ applied at ↓; flow rate, 75 ml/h (3.8 cm/h); fractions, 7.5 ml (6 min). (−) A$_{280}$; (···) acid α-glucosidase activity (units), (⊢−⊣) pooled fraction.

The progression of the isolation was analysed by reducing Tris-glycine SDS-PAGE (Fig. 11). Lanes c and d show the supernatants after acid precipitation and TPP. Note the degree of purification achieved after the TPP step and the concomitant disappearance of the very prominent band corresponding to haemoglobin. Acid α-glucosidase was purified as a mixture of the higher (74 kDa) and the lower molecular weight forms (66 kDa) and the double protein band is clearly visible in lane e of Fig. 11.
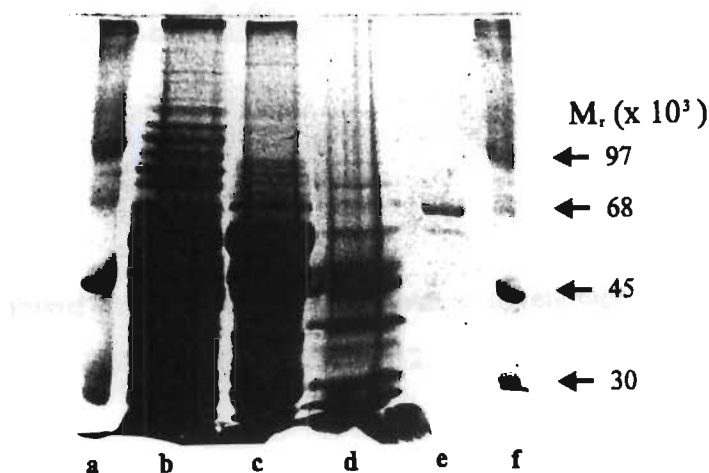
Figure 11. Reducing Tris-glycine SDS-PAGE of acid α-glucosidase fractions isolated from bovine liver. Samples were boiled in reducing treatment buffer and loaded onto a 7.5% glycine gel. (b) Homogenate fraction; (c) acid precipitate supernatant; (d) TPP fraction; (e) Sephadex G-100 fraction; and (a, f) MW markers (phosphorylase b, M_r 97 000; BSA, M_r 68 000; ovalbumin, M_r 45 000; carbonic anhydrase, M_r 30 000). Visualised using Coomassie Blue R-250.

3.4.3 Production of anti-bovine liver acid α-glucosidase antibodies

3.4.3.1 Reagents

Freund's complete and incomplete adjuvant. Freund's adjuvants were from Difco, Mi., USA.

3.4.3.2 Procedure

Antibodies against bovine liver acid α-glucosidase were raised in rabbits by immunising rabbits subcutaneously at 2 sites on the back, with a total of 100 μg of bovine liver acid α-glucosidase emulsified in a 1:1 ratio with Freund's complete adjuvant. Further inoculations were administered, in the same manner, in Freund's incomplete adjuvant, using the same dose, after two weeks and then at 4-week intervals. Blood was collected from the marginal ear vein at 7, 9 and 12 weeks and rabbit anti-sheep liver α-glucosidase (IgG) antibodies were purified from the serum by precipitation with PEG (Section 2.7.2). The antibodies were partially characterized by ELISA (Section 2.7.3).

3.4.3.3 Results

ELISA analysis showed that the antibody level was relatively high at week 7, and increased by a relatively small amount at weeks 9 and 12 (Fig. 12).
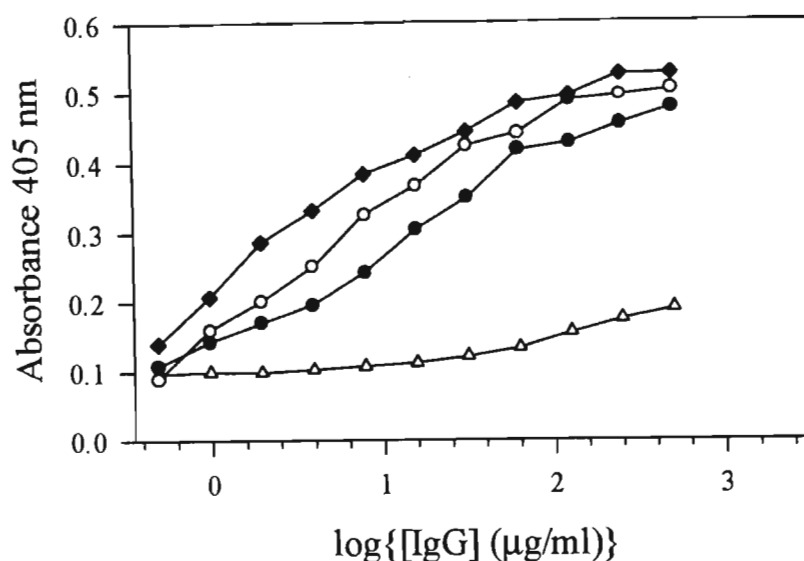
Figure 12. ELISA of the progress of immunisation of rabbits with bovine liver acid α-glucosidase.

Bovine acid α-glucosidase was coated at 1 µg/ml, incubated with serial two-fold dilution of rabbit anti-bovine acid α-glucosidase antiserum followed by sheep anti-rabbit IgG-HRPO (Section 2.9). Antiserum from weeks 7 (●), 9 (O), 12 (◆) and pre-immune (Δ).

3.5 Discussion

The isolations of cathepsins S and B and acid α-glucosidase were undertaken for further studies on the effect of the reducing agent cysteine on their activity (Chapter 4). The isolation protocols of cathepsins S and B were extensively modified from those previously reported, and were aimed at streamlining the isolations and making them more cost effective. Cathepsin S, which is the least studied of the three enzymes, was also further characterized, while acid α-glucosidase was also used as a source of immunogen for the preparation of antibodies for possible further immunochemical and immunocytochemical studies.

Isolation of cathepsin S

All other published procedures for cathepsin S purification appear to be more labour and time intensive than that developed in this study. The most commonly used method of Kirschke *et al.* (1986; 1989), and the slightly modified version of Kirschke and Wiederanders (1994), involve the following steps to yield an electrophoretically pure sample: overnight autolysis at 4°C (Kirschke *et al.*, 1986) or 1 h autolysis at 40°C (Kirschke and Wiederanders, 1994), ammonium sulfate fractionation, overnight dialysis, ion exchange and molecular exclusion chromatography, covalent chromatography (Kirschke *et al.*, 1986) or chromatofocusing (Kirschke and Wiederanders, 1994), hydrophobic and ion exchange chromatography. Dolenc *et al.* (1992) and Maciewicz and Etherington (1988) have also developed cathepsin S isolation procedures which differ from the above procedures but also comprise a large number of time consuming and costly steps. With the aim of reducing the length and cost of cathepsin S isolation, yet still obtaining an acceptable yield, a number of different approaches were explored, leading to the final purification protocol reported here.

Firstly, the autolysis step was by-passed as no apparent increase in yield could be found and it was felt that after homogenisation of the spleen and the release of active proteinases it may do more damage than good. The ammonium sulfate fractionation step was replaced with TPP, which has been successfully employed in the rapid isolations of both cathepsin L (Pike and Dennison, 1989) and D (Jacobs *et al.*, 1989). One advantage of TPP is that the resulting fraction has a much lower salt concentration than after ammonium sulfate fractionation (Pike and Dennison, 1989), and can therefore be applied directly to an ion exchange column, without a prior desalting step. Ion exchange and molecular exclusion chromatography steps were used essentially as described by Kirschke *et al.* (1989) except that S-Sepharose and Sephacryl S-100 were used instead of CM-Sephadex C-50 and Sephacryl S-200. Two peaks of cathepsin S-like activity eluted from Sephacryl S-100 (Fig. 3), one at a much higher molecular weight, which has been noted before by Xin *et al.* (1992). They concluded that the high M_r fraction consisted of cathepsins B and S noncovalently associated with other proteins. Analysis of the Sephacryl S-100 fraction on SDS-PAGE (Fig. 5) showed only three bands of protein still remaining, so a single step was sought to remove the two contaminating proteins. A variety of techniques were explored, including preparative isoelectric focusing and Resource S high resolution ion exchange chromatography. None of these removed both the contaminant proteins. Finally hydroxyapatite

was used and the contaminate proteins passed through giving an inactive breakthrough peak, while a cathepsin S active fraction was eluted using a step gradient (Fig. 4). This fraction proved to be electrophoretically pure (Fig. 5).

Analysis of the pure enzyme on Tris-tricine SDS-PAGE (Fig. 5) showed a band corresponding to M_r 12 000, which was exactly half the value previously reported for the enzyme on Tris-glycine SDS-PAGE (Kirschke *et al.*, 1986; 1989; Xin *et al.*, 1992). However, the purified cathepsin S sample was subsequently run on a calibrated Sephacryl S-100 column and eluted at M_r 20 000 which was the same as previously reported (Kirschke *et al.*, 1989; Kirschke and Wiederanders, 1994). Therefore, it was concluded that cathepsin S showed anomalous behaviour on Tris-tricine SDS-PAGE. The sample was run on Tris-glycine SDS-PAGE, but resolution was not good enough at the lower molecular weight range so the molecular weight of the cathepsin S fraction could not be accurately assessed.

This isolation protocol employed fewer steps than any previously reported and also used cost effective techniques. For example, the three chromatographic steps used are less expensive than chromatofocusing (Kirschke *et al.*, 1989; Kirschke and Wiederanders, 1994) which uses expensive polybuffers and also requires a further hydrophobic chromatography step to remove the polybuffer from the cathepsin S fraction. Xin *et al.* (1992) also showed that the ampholines may become irreversibly adsorbed to cathepsin S. The fact that fewer steps were used to isolate the enzyme means that it was in contact with other potentially damaging enzymes for the shortest possible time and so there is less chance that artefacts, created by enzyme digestion, could occur.

The effectiveness of each step used in this purification can be assessed by looking at what each achieved. TPP not only gave a low salt fraction, so eliminating the need for a dialysis step, but with the TPP protocol being optimised for cathepsin S a very selective fraction was obtained. The following cation exchange step was crucial for the removal of cathepsins H and L which both eluted later than cathepsin S. Molecular exclusion removed cathepsin B, which eluted just before cathepsin S. This sample only contained cathepsin S activity, but SDS-PAGE analysis indicated two contaminating proteins. These were removed using hydroxyapatite chromatography, which showed specific affinity for cathepsin S.

The yield of cathepsin S from bovine spleen (0.2 mg/kg) was lower than that reported by Kirschke *et al.* (1989), Dolenc *et al.* (1992) and Kirschke and Wiederanders (1994), who recovered between 1 and 3 mg/kg, but was very similar to those of Kirschke *et al.* (1986) and Xin *et al.* (1992) (0.2 to 0.4 mg/kg). The speed and cost effectiveness of this protocol was considered to be an acceptable trade-off for reduced yields. One of the main reasons for a low yield was the molecular exclusion chromatography step. In other purification protocols it has been reported that cathepsin S and B elute together on both the ion exchange and molecular exclusion columns (Kirschke *et al.*, 1989). However, in this protocol it was possible to separate these two enzyme activities by molecular exclusion by changing from Sephacryl S-200 to Sephacryl S-100, which is better able to separate lower molecular weight proteins. The activities of cathepsin S and B eluted close together; therefore, in order not to contaminate the sample a very selective fraction was taken, thus reducing the yield of cathepsin S.

One property of cathepsin S by which it was thought to differ from all other lysosomal cysteine proteinases is in its stability at neutral pH values (Kirschke *et al.*, 1989). However, recently this supposed defining characteristic of cathepsin S has been shown to be less definitive with the discovery that the stability of cathepsin B is affected by its activation state and by the buffer ionic strength (Dehrmann *et al.*, 1995; 1996; Turk *et al.*, 1994). Previously it was reported that cathepsin B exhibited optimal activity in slightly acidic media (pH 6.0) (Barrett and Kirschke, 1981). Dehrmann *et al.* (1996), however, showed that previous pH profiles of cathepsin B did not take into consideration the effect of ionic strength on the activity of the enzyme over a pH range. They showed that ionic strength had a marked effect on enzyme stability and activity, and that this was affecting the apparent pH activity profile. If the molarity of, say, a phosphate buffer is kept constant, then the ionic strength will increase with pH, as shown by the Henderson-Hasselbalch equation,

$$pH = pK_a + \log \frac{[\text{salt}]}{[\text{acid}]}$$

where the ionic strength is a function of [salt]. Increasing ionic strength lowers the stability and activity of cathepsin B and the apparent pH optimum and pH stability is thus biased towards the low side in anionic buffers of constant molarity. Cathepsin B activity measured in the presence of a constant ionic strength buffer showed an optimum of pH 7-8 (Dehrmann *et al.*, 1996). Therefore, the pH activity profile of cathepsin S was determined in the presence of constant ionic

strength buffers to see if this changed the pH profile from those reported. The results indicate an optimum of pH 6.5 which was similar to previous reports (Kirschke *et al.*, 1989; Kirschke and Wiederanders, 1994). However, the enzyme showed greater stability over the pH range as it was still over 50% active at both pH extremes, whereas Kirschke *et al.* (1989) showed an almost complete loss of enzyme activity at these pH values (Fig. 6).

The percentage of active enzyme in the purified sample, revealed by titration with E-64, showed that the cathepsin S preparation contained 42% active enzyme molecules. This compares well with the results of Kirschke and Wiederanders (1994) who reported 30 to 40% active enzyme. However, with the inclusion of an affinity chromatography step (activated thiol-Sepharose 4B or activated thiopropyl-Sepharose 6B), Kirschke and Wiederanders (1994) removed the inactive molecules and the resulting preparation was 100% catalytically active.

K_m (3.91 μM) and V_{max} (5,39 μM.s^{-1}) were determined using the direct linear plot of Eisenthal and Cornish-Bowden (1974) (Section 3.2.5). K_m is characteristic of an enzyme/substrate couple and thus can be used to identify a particular enzyme. V_{max} varies with the total concentration of enzyme present and so is, by itself, not very useful. The constant k_{cat}, called the turnover number, is often applied to enzyme catalysed reactions. It represents the maximum number of substrate molecules which can be converted to products per molecule enzyme per unit time and can be derived from V_{max} and [E$_o$] values. The turnover number, k_{cat}, was calculated to be 2.9 s^{-1}. These results differ somewhat from those of Brömme *et al.* (1989), who reported a K_m value of 8.1 μM and k_{cat} of 13 s^{-1} for bovine cathepsin S. However, their assay conditions differed, in activation time and heat treatment of the enzyme, from those used in this study.

Isolation of cathepsin B

Cathepsin B has been purified using a variety of isolation protocols. Barrett and Kirschke (1981) described two different methods, one for human liver and the other for rat liver cathepsin B. However, these protocols used a large number of steps which included specific covalent chromatography steps. A more recent protocol described by Rich *et al.* (1986), which was widely used, also contains a specific isolation step, use of the affinity resin Agarose-Ahx-Gly-Phe-Gly-Sc, but this resin is no longer commercially available. A co-purification of four cathepsins including cathepsin B has been developed by Maciewicz and Etherington (1988) using

a number of specific and expensive column chromatographic steps on an FPLC system. So a simple purification method which did not contain such limiting factors, was needed.

The purification of cathepsin S in this study gave insight into a possible cathepsin B isolation procedure. Monitoring of the cathepsin B activity during the cathepsin S isolation procedure showed that it eluted from S-Sepharose in the same fraction as cathepsin S, whereas on Sephacryl S-100 the cathepsin S and B activities separated. This fact, and that the cysteine proteinases cathepsins L and H had been removed in the ion exchange step, led to the cathepsin S protocol being considered a suitable starting point for a cathepsin B isolation. The TPP cut was optimised for cathepsin B, followed by the same ion exchange (Fig. 7) and molecular exclusion chromatographic steps (Fig. 8) as used for the isolation of cathepsin S. The cathepsin B activity which eluted from Sephacryl S-100 was found to be electrophoretically pure (Fig. 9). In this study a co-purification of cathepsins S and B was performed as the optimal cathepsin S and B TPP cuts only overlapped slightly and, by taking a narrower cathepsin B cut, the two enzymes could be purified from the same starting material. In this procedure the optimal cathepsin S TPP cut of 15 to 30% was used and a 30 to 40% TPP cut for cathepsin B, although, the optimal TPP cut for cathepsin B was 25 to 40%. Thus, a higher yield of cathepsin B could be obtained from this procedure if it was not done in conjunction with the cathepsin S isolation. A purification of cathepsin B was carried out using this optimum procedure, but the results are not shown here.

The yield of cathepsin B, which was obtained from the dual purification, was low (Table 4), but, as explained above this was a trade-off for the easy and quick method by which it was isolated. Cathepsin B was isolated purely for further studies to be described in Chapter 4, so quantity was not as essential as the need for easily obtainable, pure enzyme. This cathepsin B purification protocol does not contain any techniques which select specifically for the enzyme, such as affinity chromatography and, therefore, the purity of the sample can be easily affected by small changes in any of the techniques used. For example, if the TPP cut is varied the subsequent purification techniques may be unable to remove the different contaminants. This is also true when selecting fractions to pool from the ion exchange step, as the contaminants picked up by pooling too broad an activity peak may also not be removed in the following molecular exclusion step. The fractions from the molecular exclusion step were assayed on SDS-PAGE before being pooled so as to minimise contamination.

Isolation of acid α-glucosidase

Current published procedures for the isolation of acid α-glucosidase almost all incorporate Sephadex G-100 affinity chromatography. This technique was first discovered when acid α-glucosidase activity was observed to be 'retarded' on dextran-type gels and it was realised that this would constitute a method of separating acid α-glucosidase from other proteins of similar molecular weight that were not retarded (Auricchia and Bruni, 1967). Retardation of acid α-glucosidase on dextran-type gels, which contain α-linked glucose units, is due to the gel acting as a substrate analogue (Auricchio *et al.*, 1968). Due to the consequent affinity between the enzyme and the gel, the enzyme elutes later than one column volume, though in a broad peak. However, addition of a competing substrate to the elution buffer, after one column volume, causes the enzyme to elute in a sharper peak of activity (Tashiro *et al.*, 1986).

The protocol used in this study was a modification of that of Tashiro *et al.* (1986). This commonly used method involves the preparation of a lysosomal extract, ammonium sulfate fractionation and Sephadex G-100 affinity chromatography. The method is neither costly nor time consuming, but slight modifications were made to streamline the procedure. The preparation of a lysosomal extract was by-passed as it did not increase the yield of enzyme, and was a relatively time consuming procedure. However, an acid precipitation step was added as this was quick and removed a large portion of contaminating proteins, thus increasing the effectiveness of the following TPP step. TPP, which was optimised for acid α-glucosidase, was used in place of ammonium sulfate fractionation so that the sample could be directly applied to the Sephadex G-100 affinity column, without a prior desalting step. The acid α-glucosidase activity was subsequently eluted using maltose in the buffer (Fig. 10). Acid α-glucosidase has a higher affinity for maltose than for the dextran gel so by adding it to the elution buffer the enzyme preferentially binds maltose, and thus no longer interacts with the Sephadex G-100 column.

The yield of acid α-glucosidase (6.7 mg/kg) was very similar to that of Tashiro *et al.* (1986) (5.4 mg/kg) so the changes made to their protocol, if anything, improved the yield. This, on its own, vindicates the removal of the lysosomal extract step used by Tashiro *et al.* (1986) and underscores the effectiveness of TPP as a fractionation technique. Therefore, the isolation of

acid α-glucosidase has been further streamlined in time rather than in a reduction in the number of steps, with a resulting increase in yield of enzyme.

The purified sample of acid α-glucosidase contained two distinct components at 74 kDa and 66 kDa on SDS-PAGE (Fig. 11). Hasilik and Neufeld (1980 a,b) purified the enzyme from human placenta and also found two components, with molecular masses of 76 kDa and 70 kDa. Tashiro *et al.* (1986) reported that acid α-glucosidase purified from pig liver contained two components. Nakasone *et al.* (1991) studied the heterogeneity of pig lysosomal acid α-glucosidase by isolation of the enzyme from different organs, and separated the enzyme activities using their differing affinity for Sephacryl S-200. They concluded that the different molecular weight components may originate from different cells, as the macrophage enzyme seemed to consist of only a 76 kDa component and the hepatocyte enzyme of both 76 kDa and 67 kDa components. However, the question arises as to whether the 67 kDa molecule represents a normal mature form of the enzyme. From amino acid analysis of the 76 kDa and 67 kDa components, the 67 kDa molecule was considered to be a product of the 76 kDa mature form (Nakasone *et al.*, 1991). Nakasone *et al.* (1991) concluded that in macrophages, which contain only a 76 kDa component, the processing or degradation of acid α-glucosidase is different from that in other cells.

Following the successful isolation of acid α-glucosidase, the objective was to raise anti-acid α-glucosidase antibodies that would be suitable for immunocytochemical studies of the enzyme. Acid α-glucosidase was able to elicit an immune response in rabbits (Fig. 12). The enzyme elicited a fairly rapid immune response, this being fairly high after 7 weeks and subsequently only increasing slightly up to 12 weeks. An attempt was also made to raise antibodies in chickens, but both chickens died during the inoculation procedure. This could be due to an auto-immune response, but this would have to be studied further. The anti-acid α-glucosidase antibodies produced require further characterization by Western blot analysis before use in immunocytochemistry.

CHAPTER 4

LYSOSOMAL REDOX POTENTIAL

4.1 Introduction

The intralysosomal cysteine/cystine ratio, and thus the redox potential that this generates, may in part regulate lysosomal function in the cell (Chapter 1). However, there are a number of questions surrounding this hypothesis. Firstly, whether cysteine is in fact the intralysosomal reducing agent (Lloyd, 1986) and, if it is, is the cysteine/cystine ratio within the lysosome actively regulated by specific membrane transporters (Pisoni *et al.*, 1990) or is their ratio maintained by cysteine and cystine molecules liberated from protein breakdown (Lloyd, 1986; 1992). Secondly, what is the effect of varying cysteine concentrations on lysosomal cysteine proteinases, in this case especially cathepsins B, L and S, and the lysosomal enzyme responsible for glycogen hydrolysis, acid α-glucosidase?

Observations on the metabolic disease cystinosis, characterized biochemically by a high cystine concentration in the lysosomes and normal concentrations in the cytoplasm and extracellular fluids, provided some insight into the identity of the intralysosomal reducing agent. The cystine of cystinotic fibroblasts may be depleted by incubation *in vitro* with the thiol cysteamine, and it was found that the rate of re-accumulation of cystine correlates with the presence of cystine-containing proteins in the culture medium (Thoene and Lemons, 1982). This and earlier evidence (Thoene *et al.*, 1977; Thoene and Lemons, 1980) indicates that cystine accumulates in lysosomes of cystinotic cells as a consequence of lysosomal digestion of exogenous and/or endogenous cystine containing proteins. This data on cystinotic cells was consistent with the metabolic defect being one of cystine reduction, perhaps through an under-production of the physiological reducing agent (Kooistra *et al.*, 1982). Further work, however, showed that the defect in cystinosis is the absence of a cystine-transport system, normally present in the lysosomal membrane (Kooistra *et al.*, 1984; Smith *et al.*, 1987). Thus, cystine leaves normal lysosomes as cystine, without prior reduction to cysteine (Lloyd, 1986).

Mego (1984) drew attention to a paradox presented by these results. If cystine residues in proteins are reduced during proteolysis (Chapter 1), why do cells need a cystine-transport system

in the lysosomal membrane and why does its absence (in cystinosis) cause profoundly elevated intralysosomal concentrations of cystine? Lloyd (1986) suggested the resolution of this paradox lay in the identity of the reducing agent responsible for intralysosomal reduction. The first candidate is cysteamine (Thoene et al., 1976; Kooistra et al., 1982). This substance is a normal component of the cytoplasm, albeit in low concentration, is able to cross the lysosomal membrane in both its reduced and oxidised (cystamine) forms (Kooistra et al., 1982), and reduces cystine in cystine-loaded isolated lysosomes (Gahl et al., 1985). However, this hypothesis fails to explain why cystine accumulates in cystinotic cells, as cysteamine is present in normal concentrations in these cells (Orloff et al., 1981). The second candidate for physiological reductant is reduced glutathione (GSH). However, it appears that GSH cannot cross the lysosomal membrane (Mego, 1984; Gahl et al., 1985). A third candidate, cysteine, is abundant in the cytoplasm and can penetrate the lysosomal membrane in normal and cystinotic cells (Kooistra et al., 1982; Gahl et al., 1982). Cysteine may also arise in the lysosomes by degradation of cysteine-containing proteins (Lloyd, 1986). Like any other thiol, it could by two sequential reactions fully reduce a protein cystine residue, but unlike other reductants would in the process generate a molecule of cystine. This cystine, arising not from the protein itself but from the reductant, would still require a cystine transporter for efflux from the lysosome.

The above observations not only provide a strong case for cysteine being the intralysosomal reducing agent, but also for the existence of a lysosomal membrane cystine-transporter for efflux of cystine from the lysosome. Following the development of the above argument, Pisoni et al. (1990) discovered a cysteine-specific lysosomal transport system which they claim provides a major route for the delivery of cysteine into the lysosomal compartment. Therefore, it appears that the lysosomal membrane may contain two highly specific transport systems, which Pisoni et al. (1990) concluded were involved in regulating the flow of cystine and cysteine between the intralysosomal space and the cytosol (Fig. 13). However, Lloyd (1992) disagreed with the conclusion drawn by Pisoni et al. (1990) and argued, from the stoichiometry of the reduction reaction, that there is no need for the net import of cysteine into lysosomes for proteolysis. Lloyd (1992) proposed that if the reducing agent is free cysteine, the cysteine consumed will be exactly balanced by the cysteine liberated when the disulfide moieties in the protein are reduced and its peptide linkages hydrolysed by the cathepsins. There will also be an equivalence between the cystine content of proteins entering the lysosomes by endocytosis or autophagy and the

cystine released from the lysosomes via the cystine porter. Lloyd (1992) therefore concluded that there is no requirement for a net influx of cysteine and if there was an inward flow it is likely to constitute an anapleurotic pathway, serving only to maintain the cysteine concentration within lysosomes by replacing any cysteine that undergoes autoxidation to cystine. Lloyd (1992) implied that this does not weaken his argument (Lloyd, 1986) that cysteine is the physiological reducing agent for disulfide reduction in lysosomes, but that direct experimental evidence is still lacking. If the role of the cysteine transporter is not to provide cysteine as an aid to disulfide reduction concomitant with proteolysis, might its role be to modulate the intralysosomal redox potential, and thereby also the activity of lysosomal enzymes, especially proteinases?

It seems possible that lysosomal proteolytic activity may be regulated by changes in the lysosomal thiol-disulfide status. It may be observed that the two known transporters are thought to operate in the same sense in terms of altering the intralysosomal redox potential (Fig. 13), i.e. importing cysteine will favour reduction by increasing the cysteine/cystine ratio, and exporting cystine will have a similar effect. Presumably balancing this is a net import of cystine into the lysosome in the form of imported proteins and the possible efflux of cysteine through systems e and f (Pisoni *et al.*, 1990). A decreased cysteine/cystine transporter activity would lead to an increase in redox potential and a concomitant slowing of proteolysis (and glycogenolysis). Moreover, for each unit increase in pH, E'_o becomes more negative by 0.059 volts (i.e. $E'_o = E_o - 0.059pH$) (Segel, 1976), thus an increase in pH will increase the reducing potential at a given cysteine/cystine ratio. Thus, the activity of lysosomal enzymes may be regulated by both pH and redox potential and possibly their interaction. The intralysosomal redox potential may thus constitute part of a regulatory mechanism with links to the lysosomal pH and the cellular ATP/ADP status perhaps via an ATPase proton pump, amongst others.

Determination of the *in vivo* redox potential of lysosomes and the possibility that this potential may vary, poses a number of experimental difficulties. Redox states of cerebral mitochondria during metabolic stress have been determined (Vannucci and Brucklacher, 1994) using the $NAD^+/NADH$ ratio as a reflection of the redox state, but this approach is not applicable to lysosomes. Lysosomes have not been reported to have intrinsic redox reporter molecules such as NAD^+. Consequently, in this study, use was made of an exogenous, lysosomotropic, redox-sensitive, fluorescent dye.
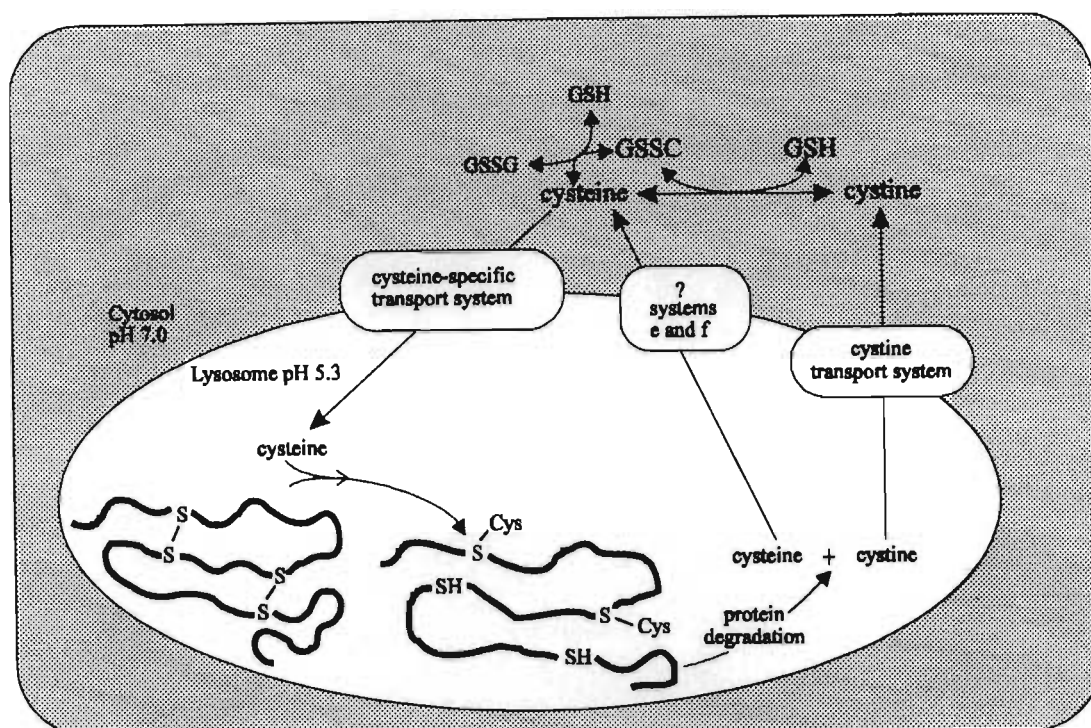
Figure 13. Schematic diagram showing (1) the interconversions between cystine and cysteine in different cellular compartments, (2) the role of transport systems in maintaining this cycle, (3) and the role of cysteine in supporting lysosomal proteolysis. Systems e and f possibly facilitate lysosomal cysteine exodus. Shaded area represents the cytosol and the clear area represents the lysosomal compartment (adapted from Pisoni *et al.*, 1990).

The effect of cysteine on the lysosomal cysteine proteinases may give insight into how lysosomal proteolytic activity may respond to different redox potentials. It is known that in their isolated forms the cysteine cathepsins require activation with a reducing agent, such as cysteine, before they manifest proteolytic activity. Whether this is true of the enzymes *in vivo* is, to my knowledge, an open question. The response of acid α-glucosidase to reducing conditions, especially in the context of its role in glycogen turnover, may also be relevant, as the rapid breakdown of glycogen could be initiated by reduction (Chapter 1). The possible effect of cysteine concentration on the activity of acid α-glucosidase might cast light on the question of whether redox potential affects lysosomal enzymes in general, or only the cysteine cathepsins.

This chapter involves assessing the effect of cysteine on the activity of the three lysosomal enzymes isolated as described in Chapter 3, followed by qualitatively determining the *in vivo* intralysosomal redox potential using specific fluorescent dyes.

4.2 Materials

Cell culture components, including insulin, hydrocortisone, Penicillin G/Streptomycin (10 000 units and 10 mg/ml, resp.), Dulbecco's Minimal Essential Medium (DMEM): Hams F-12 Medium, trypsin-EDTA solution (X1), Hanks balanced saline solution (HBSS), and cysteine.HCl were from Sigma Chemical Co., St Louis, Mo. USA; Fungizone (250 μg/ml) and horse serum were from Gibco, Paisley, UK, and epidermal growth factor (EGF) was from Upstate Biotechnology Incorporated, Lake Placid, NY USA. LysoTracker™ Red and C-2938 were from Molecular Probes Inc., Europe BV.

4.3 The effect of cysteine concentration on three lysosomal enzymes

The effect of increasing cysteine concentrations on the lysosomal cysteine proteinase cathepsin L has previously been described by Dennison *et al.* (1992) (Fig. 16). To similarly determine the effect of cysteine concentration on cathepsins S and B and acid α-glucosidase, activity was assayed against their specific artificial substrates in the presence of a constant ionic strength buffer containing a range of cysteine concentrations. The assay buffers had constant ionic strength to remove the possible effect this could have on activity, and was achieved by balancing the ionic strength of the cysteine in each buffer with sodium chloride. The assay used for cathepsins S and B was a modification of that of Barrett and Kirschke (1981), and for acid α-glucosidase a modification of the method of Geddes and Taylor (1985a).

4.3.1 Cathepsin S assay

4.3.1.1 Reagents

Buffer/activator [0.1 M K-phosphate, 5 mM Na_2EDTA, 0 - 200 mM cysteine, pH 6.5]. The buffers were made up to 4/3 times the final (nominal) concentration to accommodate dilution in the assay. KH_2PO_4 (0.91 g), Na_2EDTA (0.12 g) and the appropriate amounts of cysteine and NaCl were dissolved in 40 ml dist.H_2O, adjusted to pH 6.5 with NaOH and made up to 50 ml. The buffers were stored in light-proof bottles under nitrogen. These buffers were always made up immediately prior to use.

<u>10 mM Bz-Phe-Val-Arg-NHMec substrate stock solution</u>. Bz-Phe-Val-Arg-NHMec (7.18 mg) was dissolved in DMSO (1 ml), split into 50 μl aliquots and stored at -4°C. When required, it was diluted to a working strength of 34 μM (27 μl to 8 ml).

<u>Diluent [0.01% Triton X-100, 0.1 M K-phosphate, 1 mM Na$_2$EDTA, pH 6.5]</u>. Triton X-100 (10 μl), KH$_2$PO$_4$ (1.36 g) and Na$_2$EDTA (0.19 g) were dissolved in 95 ml dist.H$_2$O, adjusted to pH 6.5 with KOH and made up to 100 ml.

4.3.1.2 Procedure

The assay was carried out in assay buffer/activator containing a range of cysteine concentrations, 0, 12.5, 25, 50, 75, 100, 150, 200 mM (final concentration) with NaCl being added so that the buffers had equivalent ionic strengths (i.e. [cysteine] + [NaCl] = 200 mM). All solutions were equilibrated to 37°C, except the diluted enzyme solution which was kept on ice. Each assay consisted of 750 μl buffer/activator and 100 μl of appropriately diluted enzyme. This was activated for 2 min at 37°C, followed by the addition of 150 μl of the substrate solution. The change in fluorescence was measured continuously at 37°C in a temperature-controlled Hitachi F-2000 recording spectrofluorometer, with excitation at 370 nm and emission at 460 nm. Data was sampled at 10 sec intervals over a 10 min period and activity was determined from the slope of the plot of fluorescence intensity versus time. This was expressed in terms of a percentage of the maximal activity.

4.3.2 Cathepsin B assay

4.3.2.1 Reagents

<u>Buffer/activator [0.1 M Na-phosphate, 4 mM Na$_2$EDTA, 0-200 mM cysteine, pH 6.0]</u>. The buffers were made up to 4/3 times the final (nominal) concentration to accommodate dilution in the assay. NaH$_2$PO$_4$ (6.90 g), Na$_2$EDTA (0.93 g) and the appropriate amounts of cysteine and NaCl were dissolved in 40 ml dist.H$_2$O, adjusted to pH 6.0 with NaOH and made up to 50 ml. The buffers were stored in light-proof bottles under nitrogen. These buffers were always made up immediately prior to use.

1 mM Z-Arg-Arg-NHMec substrate stock solution. Z-Arg-Arg-NHMec (1.1 mg) was dissolved in DMSO (1.5 ml), split into 100 µl aliquots and stored at -4°C. Just before use the stock was diluted to 40 µM with dist.H$_2$O (0.1 ml to 2.5 ml).

Diluent [0.1% Brij]. Brij (0.1g) was dissolved in 95 ml dist.H$_2$O, and made up to 100 ml.

4.3.2.2 Procedure

The procedure was the same as described for cathepsin S (Section 4.3.1.2).

4.3.3 Acid α-glucosidase assay

4.3.3.1 Reagents

Buffer/activator [0.1 M Na-acetate, 0-200 mM cysteine, pH 4.5]. The buffers were made up to 4/3 times the final (nominal) concentration to accommodate dilution in the assay. Glacial acetic acid (3.81 ml) and the appropriate amounts of cysteine and NaCl were dissolved in 40 ml dist.H$_2$O, adjusted to pH 4.5 with NaOH and the volume made up to 500 ml. The buffers were stored in light-proof bottles under nitrogen. These buffers were always made up immediately prior to use.

20 mM 4-methylumbelliferyl-α-glucoside stock solution. 4-methylumbelliferyl-α-glucoside (0.067 g) was dissolved in 10 ml of dist.H$_2$O, split into 500 µl aliquots and stored at -4°C. When required, it was diluted to a working strength of 0.2 mM with dist.H$_2$O (0.1 ml to 10 ml).

4.3.3.2 Procedure

The procedure was the same as for cathepsin S (Section 4.3.1.2), except the change in fluorescence for the 4-methylumbelliferyl-α-glucoside substrate was measured with excitation at 365 nm and emission at 450 nm.

4.4 Qualitative measurement of the redox potential of acidic compartments *in vivo*

The measurement of lysosomal redox potential within cultured cells requires a redox indicator system which can penetrate the cell membrane, accumulate specifically in lysosomes, and remain there to give a quantitative indication of their redox status. However, no single indicator system meets all these criteria. Therefore, in order to study lysosomal redox potential, a double labelling system was devised. For detecting intralysosomal oxidation the cells were loaded with the acetoxymethyl ester derivative, 5-(and-6)-carboxy-2',7'-dichlorodifluorescein diacetate, bis(acetoxymethyl) ester (Molecular Probes, C-2938). This dye is membrane permeable and after hydrolysis forms a carboxyfluorescein, which fluoresces in the oxidised form (Fig. 14). C-2938 is, however, not selectively loaded into lysosomes, but may also accumulate in other intracellular organelles such as mitochondria. Lysosomes can, however, be visualized using a second dye, LysoTracker Red (Molecular Probes, L-7528) (I), which is a membrane permeable, acidotropic probe for labelling and long-term tracing of acidic organelles in living cells. Acidotropic probes selectively accumulate in cellular compartments with low internal pH. Using this double labelling system, the intralysosomal oxidation status in cultured cells may be visualized (This system was devised in conjunction with Dr A.M.C. Dreijer, Technical assistance representative, Molecular Probes Inc., Europe).
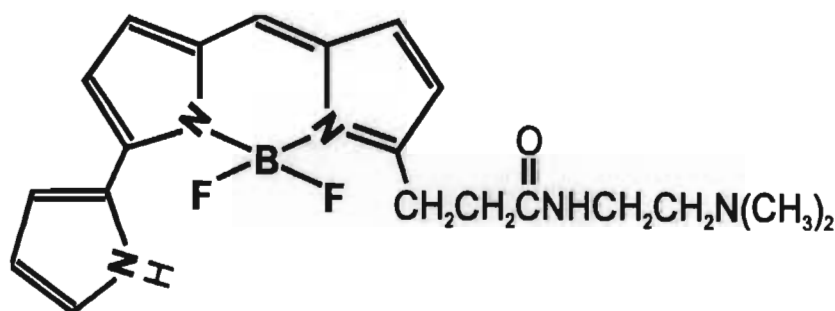
I
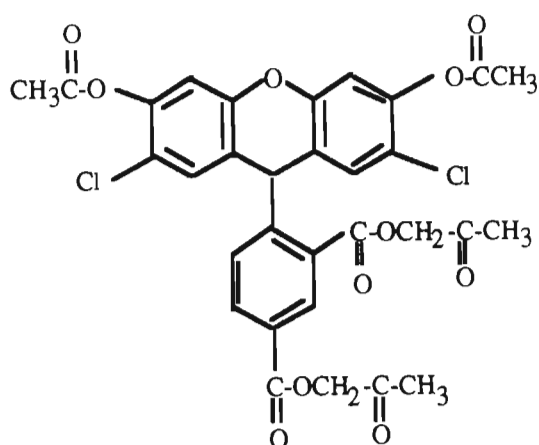
I = C-400 bis-acetoxymethyl ester
(Molecular Probes Catalog No. C-2938)
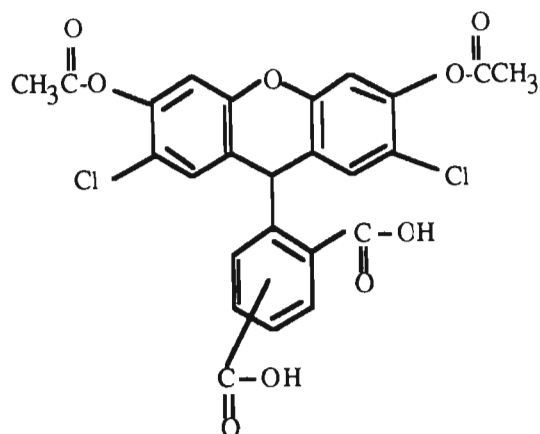
II = 5- (and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate
(Molecular Probes Catalog No. C-400)
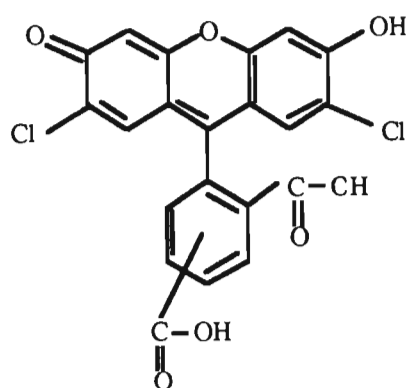
III = 5-(and-6)-carboxy-2',7'-dichlorofluorescein
(Molecular Probes Catalog No. C-368)
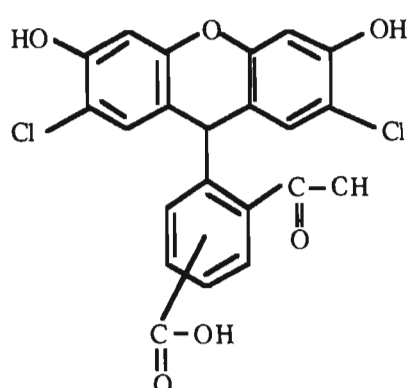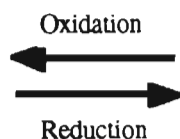
IV = 5-(and-6)-carboxy-2',7'-dichlorofluorescin

Figure 14. Molecular Probes Inc. C-2938 dye for detecting intralysosomal oxidising conditions.
C-2938 (I) is a bis-acetoxymethyl ester of C-400 (II). Unlike C-400, it is therefore uncharged and is thus membrane permeable. Intralysosomal esterases will hydrolyse the esters thereby trapping the dye in the organelle and concurrently generating the redox sensitive fluorophores (III) and (IV).

4.4.1 Emission profiles of LysoTracker and C-2938

The emission profiles of LysoTracker and C-2938 dyes were examined, firstly to see if the excitation wavelengths of the epifluorescent microscope would generate a suitable emission profile, as they were not the precise excitation wavelengths given for these dyes. Ideally LysoTracker should be excited at 577 nm and C-2938 at 495 nm, but the two excitation wavelengths available in the epifluorescent microscope were 560 nm and 490 nm. Secondly, the experiment involves loading the cells with both dyes simultaneously and therefore it was necessary to establish if there was interference between the spectra of the two dyes.

4.4.1.1 Reagents

MCF growth medium. The DMEM:Hams F-12 medium (1:1) was supplemented with horse serum [5% (v/v), 52.6 ml], EGF (20 µg, 0.43 ml), insulin (10 mg, 4.2 ml), antibiotics (100 mg Streptomycin and 100 000 units Penicillin G, 10 ml), Fungizone (0.25 mg, 1.1 ml) and hydrocortisone (0.5 mg, 2.14 ml).

1 mM LysoTracker Red stock solution. LysoTracker dye was obtained as a 1 mM stock solution and was stored at -4°C.

7.4 mM C-2938 oxidative probe stock solution. C-2938 (5 mg) was dissolved in DMSO (1 ml), aliquoted (50 µl) and stored in the dark at -20°C.

4.4.1.2 Procedure

The individual emission profiles of LysoTracker and C-2938 were examined by exciting each dye at the wavelength of the appropriate epifluorescent microscope filter. LysoTracker (2 µl) and C-2938 (2 µl) stock solutions were separately diluted in MCF growth medium (1 ml) and the emission profiles were determined in a Hitachi F-2000 spectrofluorimeter with excitation at 560 nm for LysoTracker and 490 nm for C-2938. Alternatively the two dyes were made up in the same solution and their emission profiles re-examined. LysoTracker (2 µl) and C-2938 (2 µl)

were mixed together in MCF growth medium (1 ml) and the emission profiles determined with excitation at 560 nm and at 490 nm.

4.4.2 Apparatus

Fluorescent studies in living cells have provided new insights into cell function and the availability of fluorescent probes directed at specific cellular functions has paved the way for these studies. In many cases well defined, stable culture conditions must be maintained during intermittent or continuous observations. In addition, free access to cells may be required for experimental procedures. Versatile closed micro-chambers have been designed for fluorescent as well as light microscopy. Ince *et al.* (1983) developed a micro-CO_2-incubator for use on a microscope to study phagocytosis, cell division, cell locomotion and differentiation, but the chamber did not allow for easy exchange of culture medium which was essential for this study. Ince *et al.* (1990) later developed a micro-perfusion chamber for single-cell fluorescent measurements. This chamber was designed to accommodate glass coverslips, it was made of non-toxic materials in easy to assemble parts, and allowed for the rapid exchange of fluids. The micro-chamber developed in this study was based on that of Ince *et al.* (1990) design, but was simplified, as a number of the chambers components were not required.

The micro-chamber was constructed in the Mechanical Instrument Workshop of the University of Natal, Pietermaritzburg. It consists of a perspex™ block, 70 x 30 x 8 mm, with a circular chamber turned out of the middle of one of the large faces. This chamber was 4 mm deep and had stepped sides: from the face down to 2 mm the diameter was 27 mm and from 2 to 4 mm the diameter was 22.2 mm. A 0.5 mm pitch thread was cut into the face from 0 to 2 mm. Opposite this chamber the face was relieved to a depth of 0.5 mm to provide an optically clear surface, protected from surface abrasion. The chamber was sealed with a removable 22 mm diameter coverslip, sandwiched between two R2075 O-rings (1/16" section x 3/4" i.d.), and held in place by an annular perspex nut. The perspex nut was 1.5 mm thick: its outer edge was threaded with a 0.5 mm pitch thread, complementary to that in the chamber, and its inner edge (18 mm diameter) was chamfered at the same angle as the microscope objective lens. Fluid access to the chamber was provided by to stainless steel tubes, 1.5 mm in diameter, press-fitted into holes drilled in the block which intercepted 1.5 mm diameter holes drilled vertically into the floor of the chamber (Fig. 15).
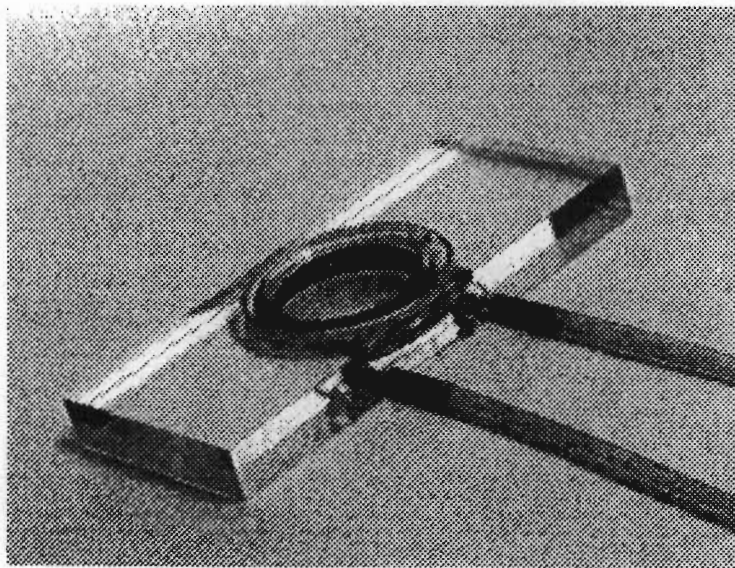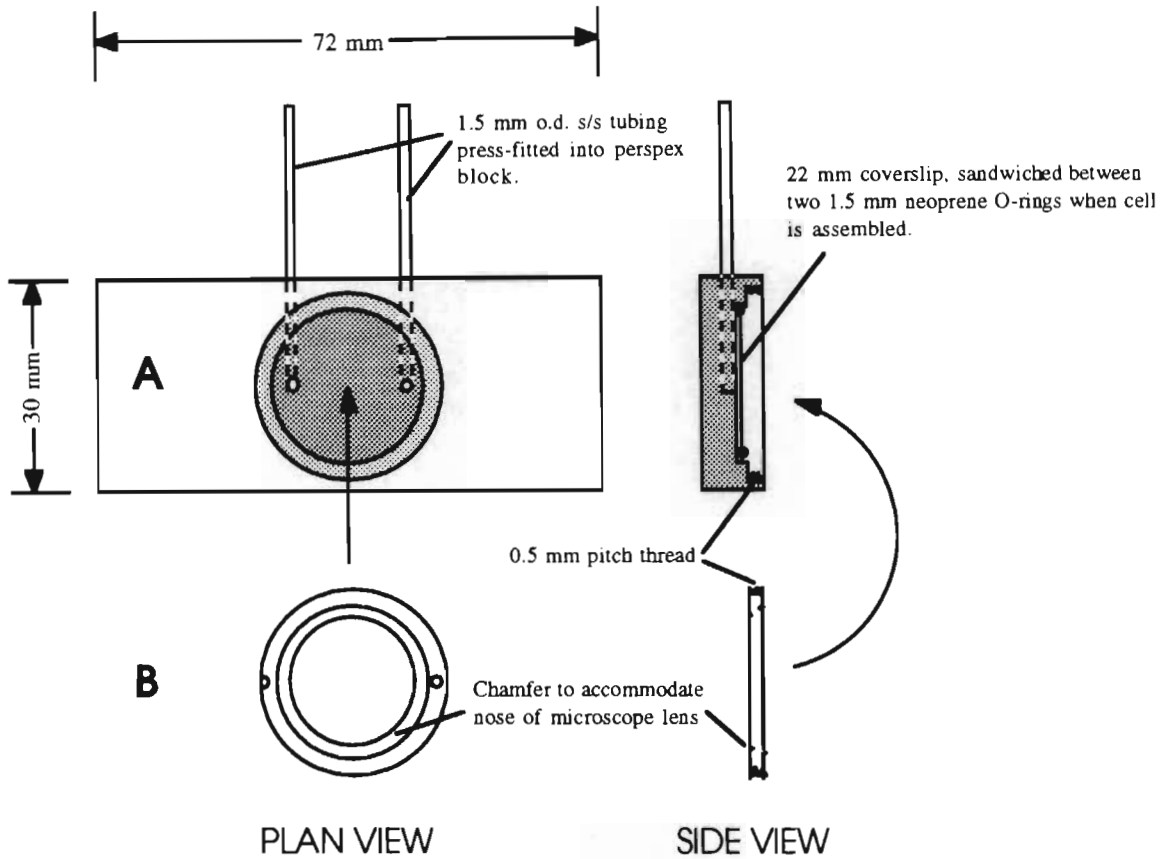
Figure 15. Live cell micro-chamber for fluorescent microscopy.

4.4.3 The MCF-10A and MCF-10A-Neo-T breast epithelial cell lines

The MCF-10A and MCF-10A-Neo-T-c-Ha-*ras* human cell lines are a unique pair of cell lines, comprised of an adherent, immortal, 'normal' breast epithelial cell line (designated MCF-10A) and a transformed cell line equivalent (designated MCF-10A-Neo-T, or simply Neo-T), derived from the MCF-10A cell line, by transfection with the *ras* oncogene. These cell lines were developed at the Michigan Cancer Foundation (hence, 'MCF'), and were supplied to this laboratory by Dr Bonnie Sloane, of Wayne State University, Detroit USA.

The MCF-10 cell line is an oestrogen receptor-negative, spontaneously immortalised breast epithelial cell line, derived from mastectomy tissue from a 36-year-old parous, premenopausal woman with fibrocystic disease (Tait *et al.*, 1990; Soule *et al.*, 1990). This cell line is unique as it is the first spontaneously immortalised line of breast epithelium, for which there is direct evidence that the normal diploid chromosome pattern of the original explanted tissue was retained for over a year in culture. Transformation to aneuploid or near diploid karyotype, characteristic of an immortal cell line, moreover, took place spontaneously, with minimal chromosome rearrangement, whereas in other currently available breast epithelial cell lines, immortality was induced by treatment with carcinogenic drugs, or the cell lines were transformed using the SV40 virus and still harbour some transforming SV40 genetic information (Soule *et al.*, 1990)

The parent MCF-10A cell line, therefore, represents the nearest to 'normal' breast epithelial cell line available, allowing the effect of a single oncogene transfection to be more easily discerned. Transformation with the *ras* oncogene is especially interesting. *ras*-Transformation of human and murine fibroblasts has been shown to cause slight alkalisation of the lysosomes (Jiang *et al.*, 1990). Alkalisation of acidic compartments, such as endosomes and lysosomes, in turn, causes disturbances in protease processing and secretion (Nishimura *et al.*, 1988). The use of the MCF-10A and Neo-T cell lines provides a unique opportunity to study the possible effect of the transforming c-Ha-*ras* protooncogene on the alkalisation and the redox status of the intralysosomal milieu.

4.4.4 Reagents

MCF growth medium. See Section 4.4.1.1.

1 mM LysoTracker Red stock solution. See Section 4.4.1.1. When required, it was diluted to a working strength of 75 nM in MCF growth medium.

7.4 mM C-2938 oxidative probe stock solution. See Section 4.4.1.1. When required, it was diluted to a working strength of 5 μM in MCF growth medium.

4.4.5 Procedure

MCF-10A and Neo-T cells were grown to near confluence in MCF growth medium, in a 95% humidified atmosphere, containing 5% CO_2, at 37°C. Cells were washed with HBSS, trypsinised with 1X trypsin-EDTA solution, and seeded (at approx. 1×10^4 cells/well) into duplicate 12-well Nunc Multiwell plates, one plate containing a 22 mm diameter sterile glass coverslip in each well. When cells were near 60% confluence the coverslips were inverted and placed in the micro-chamber described in Section 4.4.2. The cells were double labelled, first with the C-2938 oxidative probe for 60 min at 37°C, followed by incubation in LysoTracker for 40 min at 37°C. The micro-chamber was flushed with MCF growth medium and the cells viewed with an epifluorescent microscope using the appropriate filters. The results were captured on XP2 400 black and white film and analysed using National Institute of Health (NIH) image analysis software (NIH-image), which is available without cost on the internet.

4.5 Results

4.5.1 The effect of cysteine concentration on three lysosomal enzymes

The effect of cysteine concentration on the activity of cathepsins S and B and acid α-glucosidase is shown in Fig. 16. The activity of cathepsin L is also shown (Dennison *et al.*, 1992). The activities of cathepsins S and B showed a similar increase in activity with increase in cysteine concentration. However, cathepsin S showed a 20% residual activity before activation with cysteine whereas cathepsin B showed zero activity, the same as cathepsin L. Acid α-glucosidase showed only a slight fluctuation of activity over the range of cysteine concentrations. The three cysteine cathepsins all showed the greatest increase in activation up to 30 mM cysteine and thereafter their activity increased less and in the case of cathepsin S and B the activity decreased slightly after a cysteine concentration in excess of 100 mM.
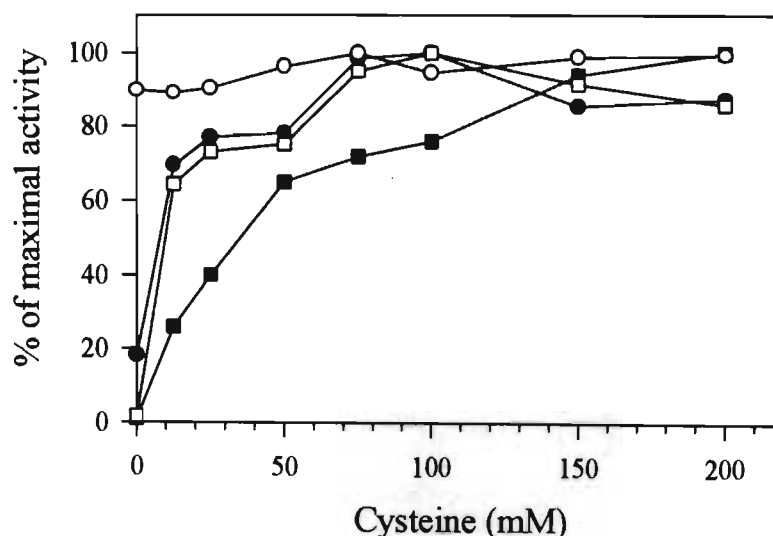
Figure 16. The effect of cysteine concentration on the activity of cathepsins S, B and L and acid α-glucosidase. Artificial substrate hydrolysis by equimolar amounts of the enzymes was measured in standard 10 min assays using constant pH buffers containing a range of cysteine concentrations, as described in Section 4.3. Cathepsin S (●), cathepsin B (□), acid α-glucosidase (O), cathepsin L (■) (Dennison *et al.*, 1992). Each point represents the mean of three determinations.

4.5.2 Emission profiles of LysoTracker and C-2938

The emission profiles of LysoTracker and C-2938 are illustrated in Fig. 17. LysoTracker, excited at 560 nm, emitted at 595 nm, while C-2938, excited at 490 nm, emitted at 525 nm. A mixture of the two dyes excited at their respective wavelengths gave identical emission profiles.
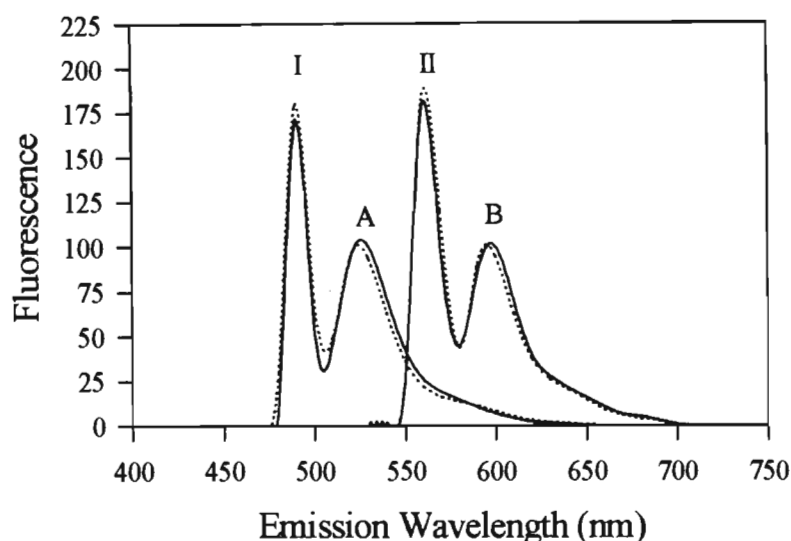
Figure 17. Emission spectra of C-2938 and LysoTracker.

C-2938 was excited at 490 nm and LysoTracker was excited at 560 nm, (–) A and (–) B represent the respective emission profiles, while (–) I and (–) II represent the emission generated by reflection of the excitation beam. (···), represents the emission profiles when a mixture of the two dyes was excited at their respective wavelengths.

4.5.3 Qualitative measurement of the redox potential of acidic compartments *in vivo*

MCF-10A and MCF-10A-Neo-T breast epithelial cell lines were labelled with C-2938 and LysoTraker, as described in Section 4.4.5, and the resulting fluorescent patterns (Fig. 18 and 19) were photographed and analysed on NIH-image analysis software to ascertain where the two fluorescent images overlapped. The overlap between fluorescent patterns indicates which of the acidic organelles correspond with organelles which are oxidising. The fluorescent intensity of C-2938 gives an indication of the redox potential in a particular acidic organelle.

(A)   (B)

(C)
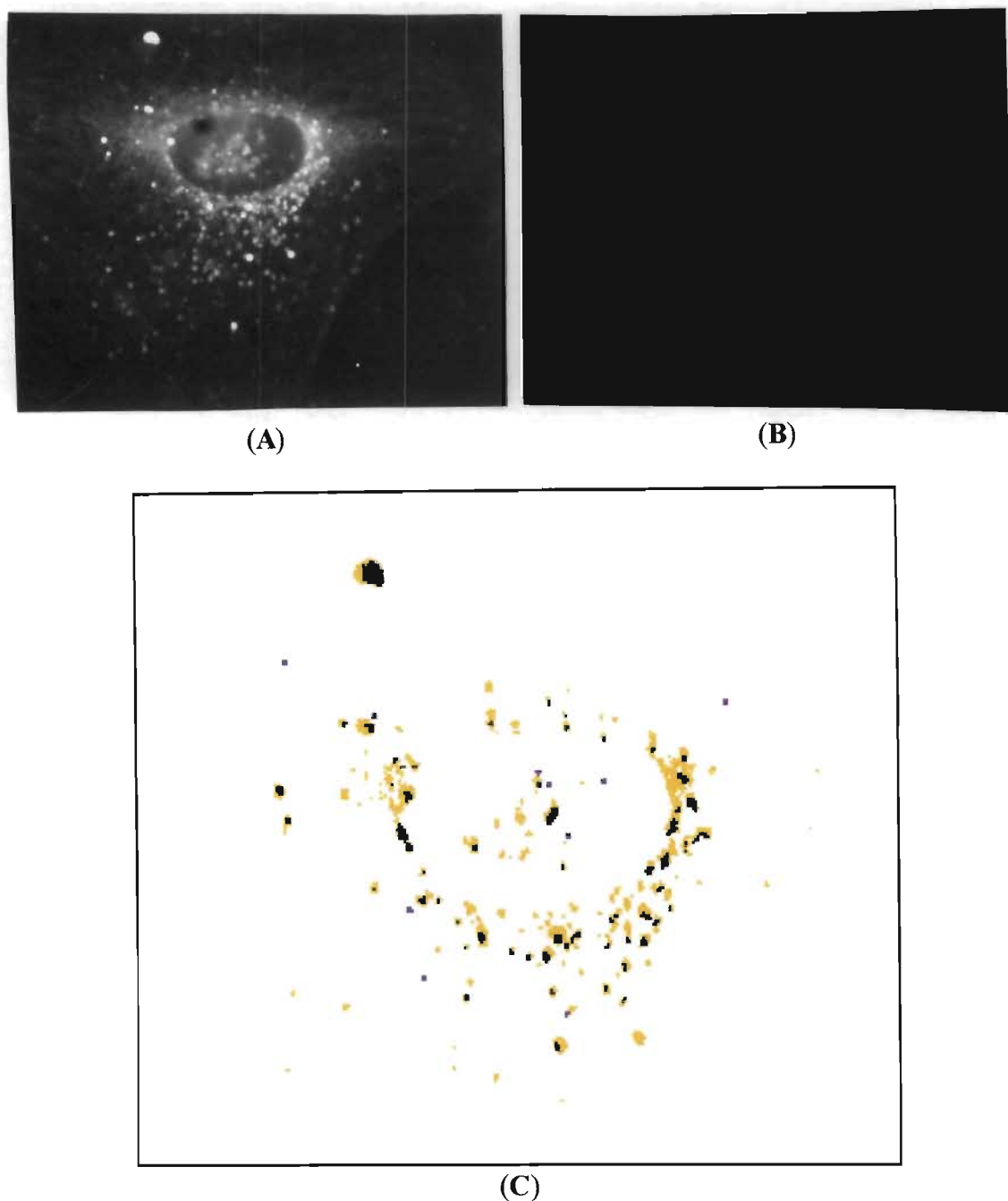
Figure 18. MCF-10A breast epithelial cell labelled with C-2938 and LysoTracker.

Cells were incubated, in culture medium, with C-2938 for 1 h at 37°C, followed by LysoTracker for 40 min at 37°C (Section 4.2.1). The results were viewed and photographed through an epifluorescent microscope with excitation at 560 nm for LysoTracker (**A**) and 490 nm for C-2938 (**B**). The photographs were analysed using the programme NIH-image (**C**). Yellow = LysoTracker, Purple = C-2938, Black = overlap between the two dyes. The result shown is representative of (approximately) 5 replicate experiments.

(A)   (B)

(C)

Figure 19. MCF-10A-Neo-T breast epithelial cell labelled with C-2938 and LysoTracker.

Cells were labelled as described in Fig. 18. LysoTracker (**A**), C-2938 (**B**) and the composite NIH-image (**C**). Yellow = LysoTracker, Purple = C-2938, Black = overlap between the two dyes. The result shown is representative of (approximately) 5 replicate experiments.
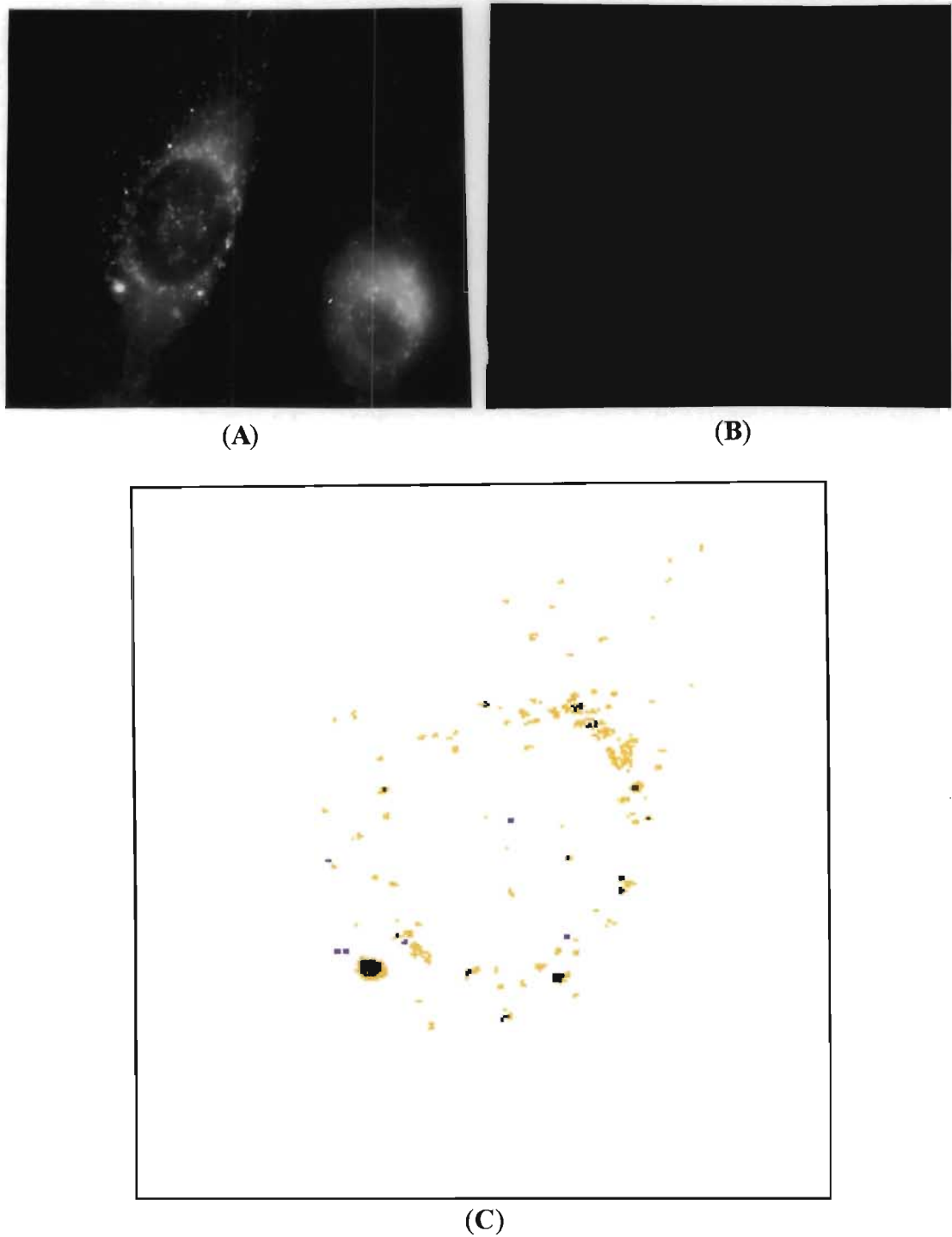
4.6 Discussion

The role of redox potential in the regulation of lysosomal function has been approached from two different angles. The first approach involved assessing the effect of increasing concentrations of the reducing agent cysteine on the three lysosomal enzymes isolated as described in Chapter 3. The effect of reducing agents on lysosomal enzyme activity was directly linked to the following *in vivo* experiment as it showed a possible way in which the redox potential could affect or even regulate certain lysosomal enzymes. This second, more direct, approach assessed the *in vivo* intralysosomal redox potential and whether this potential varied among the lysosomes of a single cell. This particular problem has not been previously addressed, so the experimental procedure had to be devised, optimised, and the validity of the results assessed.

The activation profiles of the two lysosomal cysteine proteinases, cathepsins S and B (Fig. 16), with the reducing agent cysteine, were very similar. Both enzymes showed a sharp increase in activity up to 30 mM cysteine followed by a more gradual increase to maximal activity at 100 mM cysteine. This sharp activation gradient between 0 to 30 mM cysteine means that a small change in the cysteine concentration would significantly alter the enzymes' activity. Therefore, varying the reducing potential of the medium could constitute a regulatory mechanism for these enzymes. The response of a third lysosomal cysteine proteinase, cathepsin L (Dennison *et al.*, 1992), was included for comparison. Its activation was more uniform over the given range of cysteine concentrations and did not show the same rapid activation at lower cysteine concentrations. However, it still supports the idea that the reducing potential of the medium could affect its activity. An interesting difference between the activity of cathepsin S and that of cathepsins B and L is that cathepsin S shows residual activity in the absence of a reducing agent, whereas cathepsins B and L show no activity until they are activated by a reducing agent. The reason for this is unclear but it does mean that cathepsin S could remain partially active at all times. Cathepsin S, however, apparently has a limited tissue distribution, unlike cathepsins B and L which are ubiquitous.

These results do not give any direct insight into the *in vivo* response of these enzymes to varying redox potentials. The need for a reducing agent for activation of these enzymes may be a result of oxidation of the active site cysteine during isolation. However, the results discussed here indicate a possible mechanism for regulating lysosomal cysteine cathepsin function by varying redox potential.

The activity of acid α-glucosidase did not significantly alter in the presence of the reducing agent, cysteine (Fig. 16). Its activity increased about 5% at 100 mM cysteine and decreased again, it was therefore concluded that a reducing environment had no direct effect on the activity of this enzyme. This was not unexpected as it does not have a cysteine residue in its active site, therefore it should not require activation by a reducing agent. However, as discussed in Chapter 1, acid α-glucosidase digests the smaller β-glycogen particles faster than the larger protein-rich, disulfide linked, α-glycogen particles. This is thought to be due to steric hindrance, as the α-particles hinder hydrolysis by not allowing the enzyme, once bound, to attack the glucosidic bonds (Calder and Geddes, 1989a). Reduction of the disulfide bridges of the α-particles by a reducing agent such as cysteine, and digestion of the protein core by the activated cysteine cathepsins, would liberate the smaller β-particles which are more susceptible to enzymatic attack by acid α-glucosidase. Therefore, a reducing environment may indirectly form part of a regulatory mechanism of acid α-glucosidase on lysosomal glycogen, by making its substrate more susceptible to enzymatic attack, rather than by directly activating the enzyme.

The effect of cysteine on the isolated lysosomal cysteine cathepsins was encouraging in the context of this study as it supports the hypothesis that intralysosomal redox potential may constitute part of a regulatory mechanism for lysosomal function. Therefore, the next step was to devise a method to assess the intralysosomal redox potential in living cells. This involved the development of some novel experimental approaches. The redox potential of a given system is governed by the relative amounts of reductant and oxidant, thus maintaining this system in its natural state during the assessment is crucial. Conventional labelling techniques involve permeablising, labelling and fixing the cells before viewing. This procedure was unsuitable for this experiment as permeablising and fixing the cells would destroy the natural intralysosomal redox potential, as this cannot be maintained without the reducing and oxidising molecules. To measure the potential within lysosomes, the intralysosomal redox potential must be maintained at, or as near to, its natural state as possible. Therefore, the labelling procedure was carried out on cultured cells, in the stable environment of a cell culture incubator.

The crucial problem centred around the development of a specific redox indicator system which would target the lysosome. Since no previous experimental procedure had been devised to assess the intralysosomal redox potential, a novel system was developed as described in Section 4.4. Using this system it was possible to qualitatively assess the redox status of the intralysosomal milieu. The oxidative probe (C-2938), which is not selectively loaded into lysosomes, was used in conjunction with a lysosomal marker dye, LysoTracker. The two probes fluoresce with different excitation wavelengths, making it possible to load both into the same cell and view the two different fluorescent patterns separately. This was achieved by using an epifluorescent microscope with specific excitation filters for the two dyes, making it possible to excite each dye separately.

The emission profiles of the two dyes were assessed (Fig. 17) before they were used in the experiment. These emission profiles were generated by exciting the dyes at their specific excitation wavelengths and scanning the emission spectra. Both emission spectra showed two peaks, one at the same point as the excitation wavelength and another smaller emission peak. The first peak was due to reflected excitation light which is recorded by the emission sensor. The second peak represented the emission wavelength of each dye, with LysoTracker and C-2938 showing emission peaks at 595 nm and 525 nm respectively. These values compare favourably with the reported emission values for these dyes, with LysoTracker reported to emit at 592 nm and C-2938 at 518 nm (Molecular Probes bulletin No. 21). A second experiment was carried out to see the effect that mixing the dyes would have on their emission profiles. This was relevant as it was imperative that a specific excitation wavelength only caused fluorescence of one dye, otherwise it would be impossible to distinguish whether the emission pattern was due to specific cellular conditions or just an interaction between the two dyes. The emission profiles showed no change when the dyes were mixed which indicated that they had no effect on each other, so it may be concluded that fluorescent patterns should not be influenced by a cross-reaction between the dyes.

Viewing the labelled cells posed some problems of its own. If the cells were stressed in any way during viewing it would alter the results, as the labelling system developed in this study is dynamic and would indicate any change in redox status caused by stress. Conventional viewing involved mounting the cells on a microscope slide in order to view them under an epifluorescent microscope. In this system the cells would no longer be immersed in culture medium, and would soon become stressed through lack of nutrients and oxygen. Moreover, it was found that cells on

a slide coved by a coverslip were distorted by the weight of the coverslip. This led to the development of a special purpose micro-chamber which allowed for viewing of live cells while still immersed in culture medium. This effectively reduced the possibility of artefacts being produced by abnormal cellular responses to external factors which could not be easily controlled. However, the mounting slide was not temperature controlled so all the procedures prior to viewing were done in the temperature controlled environment of the cell culture incubator. Viewing times, therefore, were limited so that the temperature of the cultured cells environment would not vary too much. An obvious modification to this micro-chamber would be the addition of a mechanism to control the temperature. For the immediate purpose, however, this was not considered necessary.

In order to evaluate the fluorescent patterns generated by the two dyes, the images had to be accurately compared. To achieve this, photographs were taken of the fluorescent patterns, and the amount of overlap between the two images was assessed. Initially colour photographs of the fluorescent patterns were attempted, in order to show the different fluorescent colours of the dyes, but colour film was not sensitive enough to capture the images, as so little light was emitted. For this reason, also, it was not possible to capture the images directly by video camera. The problem was compounded by the fact that the dyes were rapidly quenched, i.e. their fluorescence decreased rapidly with time of exposure to the excitation wavelength. This complicated determination of optimal photographic exposure times. To solve this problem in future studies, a computer controlled automatic exposure system with spot metering was acquired but this equipment arrived too late to be useful in the present study. With advice from the Centre for Electron Microscopy, University of Natal,, a sensitive black and white film (XP2-400) was used to capture the fluorescent images. However, this makes the images look similar, as the colour difference was no longer apparent. Following the successful capturing of the images, a suitable method for comparing the images was required. The most accurate method proved to be an image analysis software package developed by the NIH. The photographic images were transferred, via a video camera link-up, into the image analysis program, where a graphic representation of the fluorescent patterns was made. The two different patterns were then overlaid and rendered in artificial colour to highlight where the images overlapped.

Using the black and white photographs and the composite image it was possible, firstly, to see which of the acidic organelles showed fluorescence of the oxidative probe, and secondly, to see the fluorescent intensity of the oxidative probe in the acidic organelles, which would indicate the level of oxidation. Using these criteria the fluorescent and composite images obtained from the MCF-10A and MCF-10A-Neo-T cell lines were analyzed to see if the oxidation status of the lysosomes varied within a single cell and also if the general intralysosomal oxidation/reduction levels in the two different cell lines differed.

A significant result is that there was <u>any</u> fluorescence due to C-2938, as this throws into question the view that lysosomes are invariably reducing. The fluorescent patterns obtained from the 10A and Neo-T cells were not obviously different (Figs. 18 and 19 are representative of several replicate experiments conducted). From a simple visual inspection it could not be concluded that there was any difference in the number or distribution of acidic organelles, or that the number and intensity of oxidative organelles differed between the two cell lines. The difference was such that a conclusion could only be drawn if a large number of both cell types were analyzed which was not done in this preliminary study. The only obvious difference between the two was the shape of the cells, with the Neo-T cells being more elongated, while the 10A cells maintained a more symmetrical shape. An interesting feature which can be seen in both cells is the presence of a single large acidic vesicle which also shows labelling with the oxidative probe. Similar large acidic vesicles have been studied by Montcourrier et al. (1990; 1994) but they reported that these large acidic vesicles were 2 to 20 fold more abundant in breast cancer cells than in normal mammary epithelial cells. However, in this study no difference in the number of large acid vesicles was seen between the transformed and non-transformed cell lines. The large acidic vesicles might be involved in digestion of insoluble extracellular matrix constituents. The present cells were grown on liquid media and this might have suppressed development of the large acid vesicles. As no conclusive difference could be seen between the two cell lines the results discussed will concentrate on the 10A cell (Fig. 18) as this represents the closest to the normal condition.

The fact that the internal milieu of some of the acidic organelles (lysosomes) were able to oxidise the C-2938 probe, while others showed no fluorescence, suggests that there is a difference in the redox potential of the acid organelles. Also, the few organelles which lit up with C-2938, but not with LysoTracker, might correspond with the non-acidic lysosomes reported by Butor et al. (1995)

(Chapter 5). The photograph of the oxidative dye shows a range of fluorescent intensity which can be interpreted as a variable redox potential between the lysosomes of a single cell. This preliminary result supports the hypothesis that the redox potential might be a controlling influence upon lysosomal function.

The system devised in this study is novel but has not been perfected. The results suggest that a more extensive, and quantitative, analysis is required. Challenges that remain are, i) how to quantitatively measure the intralysosomal redox potential in living cells and ii) how to measure the level of cyst(e)ine in lysosomes and preferably, also, the cysteine/cystine ratio in individual lysosomes.

CHAPTER 5

GENERAL DISCUSSION

Lysosomes are traditionally defined as acidic terminal compartments of the endocytic pathway that are devoid of recycling receptor molecules and enriched in the lamp/lgp class of membrane glycoproteins (of unknown function), as well as in acid hydrolases (Griffiths *et al.*, 1988; Kornfeld and Mellman, 1989). However, these "markers" are also found in high concentrations in structures now referred to as late endosomes (which are kinetically earlier than lysosomes). Therefore, the interpretation of a "lysosome" is being redefined as more knowledge is accumulated on the endosomal-lysosomal pathway. In this dissertation the methods used were unable to distinguish between lysosomes and late endosomes and the term "lysosome" therefore refers to lysosomes and late endosomes unless otherwise stated.

The rapid development of more sensitive and specific techniques for studying endosomal-lysosomal biogenesis have made it possible to acquire information in ways which were previously not possible. Current observations on the function of the endosomal-lysosomal pathway suggest a far more dynamic system which may be able to respond to changes in the needs of a cell, thus making this far more than "just a system for continuous degradation of proteins and other molecules". The membranes of the endosomes and lysosomes may also be more than just cordons protecting the cytoplasm from the dangerous brew of hydrolytic enzymes. Their "permeability" properties increasingly appear to be tailor-made to regulate their function, as more specific membrane transporters are being discovered.

In this study the role of the redox potential on lysosomal function has been investigated using some novel techniques. For their role in proteolysis, lysosomes must be reducing, both for the function of the cysteine cathepsins and for the reduction of disulfide bonds in the substrate proteins. This raised the question of whether the intralysosomal milieu is invariably reducing and proteolytic or whether the intralysosomal redox status and thus proteolytic activity may be variable, which in effect would be a switch between "on" or "off" limits at different times.

The results obtained in this study gave further insight into this hypothesis. They show that the activity of the lysosomal cysteine cathepsins, *in vitro*, is sensitive to the concentration of reducing agent present in the assay. Therefore, the redox potential in the *in vivo* medium could similarly affect their activity, thus constituting part of a regulatory mechanism for proteolysis in the lysosome. This hypothesis is supported by the results of the *in vivo* experiments on the redox potential in lysosomes. These results were interesting: firstly the mere fact that there was any C-2938 labelling means that the conventional view, i.e. that lysosomes are invariably reducing, must be questioned. Secondly, the lysosomes showed variable oxidation of the dye which suggests that the intralysosomal redox status may not be uniform in the lysosomes of a single cell. Thirdly, a few organelles labelled with C-2938 but not with LysoTracker, which could be interpreted as non-acidic lysosomes, showing a certain amount of oxidation of C-2938. This interpretation is supported, to some extent, by the recent results of Butor *et al.* (1995) which suggest that lysosomes may not always be strongly acidic.

Some lysosomal hydrolases have shown substantial residual activity in a more alkaline pH range (Kirschke and Barrett, 1980; Dawson and Tsay, 1977). Furthermore, prior studies have suggested that sialic acid-specific *O*-acetylesterase (Higa *et al.*, 1989; Butor *et al.*, 1993a,b) and a glycosylasparaginase (Tollersrud and Aronson, 1992; Mononen *et al.*, 1993), which have neutral pH optima, were found in lysosomes (Butor *et al.*, 1995). Dehrmann *et al.* (1995;1996) have also shown that the lysosomal cysteine proteinase cathepsin B also has an alkaline pH optimum. These apparently paradoxical observations were explored by Butor *et al.* (1995). Factors modifying the pH/activity profiles of these enzymes could not be found in lysosomal extracts, therefore, Butor *et al.* (1995) postulated that the function of the enzymes with neutral pH optima must either depend upon their minimal residual activity at acid pH, or the possibility that lysosomes are not always strongly acidic. Since most methods used to identify lysosomes take advantage of their acid interior (Ohkuma and Poole, 1978; Yamashiro and Maxfield, 1987a,b; Anderson and Orci, 1988), a subset of lysosomes with neutral pH at the time of observation may have been previously missed. Therefore, to detect lysosomes in living cells without regard to internal pH, Butor *et al.* (1995) used the uptake of a fluorescently labelled mannose-6-phosphate protein as a lysosomal marker. They showed that the labelled organelles did not all rapidly accumulate Acridine Orange, a vital stain that is specific for acidic compartments. From this they suggested that lysosomal pH may fluctuate, allowing hydrolytic

enzymes with a wide range of pH optima to efficiently degrade macromolecules. Butor *et al.* (1995) concluded that the pH of the late endosomal and lysosomal compartments might be more dynamic than previously thought, fluctuating over a wide pH range, with a preference for a more acidic state.

The results reported by Butor *et al.* (1995) lend support to the idea that there are non-acidic lysosomes which show oxidation. However the C-2938 labelled organelles which do not correspond with LysoTracker labelling could be unrelated organelles as it is not conclusively known whether C-2938 is selectively loaded into lysosomes. Thus establishing how selectively C-2938 is loaded into lysosomes is necessary in further studies.

Another perspective linked to pH regulation is the effect pH has on the lysosomal redox status. The redox potential generated by a specific redox pair, in this case cystine and cysteine, varies not only according to the concentration of each species but also with the pH of the medium. The lower the pH, the easier it will be to reduce the oxidised molecules (cystine) of the redox pair, and the harder it will be to oxidise the reduced molecules (cysteine) (Kyte, 1995). This means that when the lysosome is more acidic it will have a lower reducing potential or higher oxidising potential for the same concentrations of a redox pair. Therefore, any future studies on either lysosomal pH or redox potential will have to take note of this relationship and establish what effect it has on the system under investigation. This link between redox potential and pH may give further insight into a very dynamic lysosomal regulation system.

The *in vivo* experiments carried out in this project were novel and considerable time was spent developing the techniques. The results constitute preliminary findings and a great deal can still be done to further perfect this technique, both in execution and interpretation of the results. In this study the results were qualitative as time constraints did not allow for the fluorescent intensity of the dye to be calibrated with respect to redox potential and pH. Therefore, the intensity of fluorescence could not be quantitatively correlated with a level of oxidation but rather the variance of fluorescence intensity could only be interpreted qualitatively. Quantitative measurement of intraorganelle fluorescence has recently been described. Overly *et al.* (1995) quantitatively measured the pH in the endosomal-lysosomal pathway in neurons using radiometric imaging with pyranine. This technique may prove to be difficult to transfer to the present study,

however, as the neurons studied by Overly *et al.* (1995) only contained a few relatively large acid organelles which could easily be distinguished from each other. In the present study the labelled organelles were not only small but also relatively clumped together, making it more difficult to differentiate the individually labelled organelles.

Further work could also be done to assess if the redox status of the lysosomes changes with time. A single cell could be continuously viewed and photographed at specific time intervals. This would give a dynamic picture of the intralysosomal redox status and the movement of the lysosomes within the cell. This could be done by modifying the micro-chamber so that the chamber could be temperature controlled, either in itself, say with Peltier couples, or by using a heated microscope stage. Using this system, further experiments could also be done to see the effect of cell stress such as anoxia on the intralysosomal redox potential. This type of experiment could give greater insight into the possible role of redox potential in lysosomal regulation.

A number of models have been proposed for the mechanism of the endosomal-lysosomal pathway and the principal ones have been briefly discussed in Chapter 1. However, further comment is required on a model very recently proposed by Griffiths (1996) as it provides some interesting insights to the present study. In this model Griffiths (1996) suggest that the membrane organelles involved in the endosomal-lysosomal pathway may be classified either as "vesicles" or "compartments" (see page 2 for definition). He argues that the lysosomes are "vesicles", namely of membrane cargo which bud off the late endosomal "compartment" and periodically fuse back with the same "compartment" in a regulated manner (Fig. 20). Thus the function of the lysosome, as defined by Griffiths (1996), is to store mature lysosomal enzymes in a form such that they can be readily injected, by fusion, into late endosomal compartments, when needed. In this model lysosomes are more akin to storage vesicles, while the late endosomes are collectively, visualised in Griffiths' (1996) words, as the cell "stomach". However, little is known about the relative *in vivo* degradative abilities of the two sets of organelles. If, as Griffiths (1996) suggests, the late endosome represents the major **compartment** where degradation occurs and the lysosomes are mostly storage **vesicles**, a prediction is that lysosomes should fuse *in vitro* with purified late endosomes but not with themselves. Yet despite the vast literature on lysosomes over the past thirty years the structural and functional relationship between the late endosomes and lysosomes requires more attention.
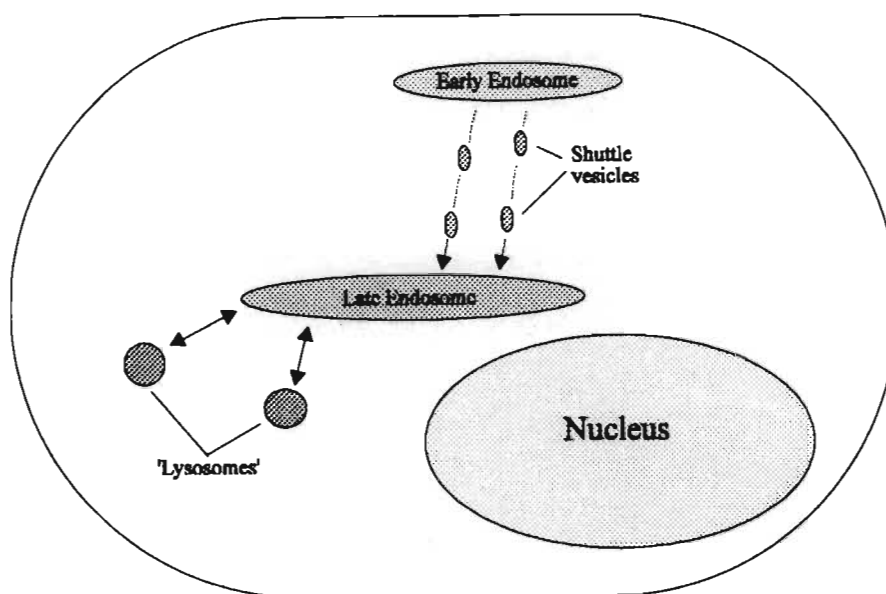
Figure 20. The endosomal-lysosomal pathway.
Materials are moved between the early and late endosomes by specialized shuttle vesicles which can not fuse with each other, but only with the early and late endosomal compartments. The lysosomes are also visualised as vesicles which bud off the late endosome and are able to again fuse with the late endosome but not with each other (adapted from Griffiths, 1996).

Griffiths' (1996) theory that lysosomes may play more of a storage role, while the late endosome is the site for active protein degradation, is interesting in the context of this study. The methods used to analyse lysosomes in this study could not distinguish between lysosomes and late endosomes as the marker dye labelled all acidic organelles. With this in mind, the results obtained are not inconsistent with Griffiths' hypothesis. If lysosomes perform a storage function, the intralysosomal environment should not be conducive to proteolytic action and, therefore, may be more oxidising. Thus, the acidic organelles which showed labelling with the oxidative probe C-2938 could be lysosomes, in terms of Griffiths (1996) theory, while the acidic organelles which showed no labelling with C-2938 could be late endosomes, having a reducing interior and consequently active thiol proteinases for protein digestion. The concept of lysosomes as redox-dependent storage vesicles is also consistent with the observations on lysosomal glycogen, which is stored in a protein-crosslinked form. In fact, this hypothesis provides a tentative answer to the question, "why are lysosomal proteinases cysteine proteinases?" The answer being that only these could be subject to redox regulation.

Our perception of lysosomes is subject to constant review, in the light of new evidence. The latest view, put forward in the review by Griffiths (1996), is of a complex and dynamic lysosome/late endosome system, in which lysosomes perform a storage role. The contribution of the studies reported in this dissertation has been to draw together evidence, both from the literature and from original experiments, on how lysosomes might perform this storage role, i.e. through redox-regulation of enzyme activity. The studies reported here represent a tentative beginning but may have opened a door to a new research field.

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