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STUDIES ON THE LATENCY OF SOME GLYCOSIDASES IN RAT LIVER LYSOSOMES

by

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ABSTRACT

This thesis records results obtained between October, 1972 and September, 1975 in the Biochemistry Research Unit, University of Keele.

The latency of three glycosidases in intact rat liver lysosomes was examined with *p*-nitrophenyl substrates. The latency of the three enzymes observed with these substrates was unusually low, suggesting that *p*-nitrophenyl substrates can penetrate the membrane of intact lysosomes. A model, based on the heterogeneity of lysosomes with respect to their permeability properties, was proposed to explain these results. The validity of this model was examined by measuring the latency of α-glucosidase with substrates of differing size (Chapter 3).

The latency of two exogenous lysosomal glycosidases with *p*-nitrophenyl substrates was found to be much higher than that observed for the endogenous enzymes. The differing subcellular distribution of exogenous and endogenous enzymes suggests that the latency difference is connected with the vacuolar location of the exogenous enzymes: exogenous enzymes are located in vacuoles whose membrane is impermeable to *p*-nitrophenyl substrates (Chapter 4).

Lysosomes possess the enzymes which are capable of degrading nucleic acids to the level of nucleosides. In spite of their relatively high molecular weights, nucleosides were shown to be able to penetrate the membrane of intact lysosomes by demonstrating their inefficiency at providing prolonged osmotic protection to lysosomes. The relevance of these findings to the *in vivo* situation is discussed (Chapter 5).

Cysteine and ascorbate have both been previously reported to affect the stability of the lysosomal membrane. Neither substance had any measurable effect on the stability of rat liver lysosomes, as shown by the latency and sedimentibility of α-glucosidase.(Chapters 6 and 7). The validity of the use of leucyl β-naphthylamidase as a lysosomal marker enzyme is discussed (Chapter 7).

CONTENTS

		Page No.
	Acknowledgements	
	Abstract	
Chapter 1	General Introduction	1 - 13
Chapter 2	Materials and Methods	14 - 31
Chapter 3	Latency of some endogenous glycosidases in rat liver lysosomes	32 - 66
Chapter 4	Latency of some exogenous glycosidases in rat liver lysosomes	67 - 88
Chapter 5	Permeability of rat liver lysosomes to nucleic acid derivatives	89 - 99
Chapter 6	The effects of cysteine on the permeability and stability of rat liver lysosomes	100 - 114
Chapter 7	The effects of ascorbate on the permeability and stability of rat liver lysosomes	115 - 127
Chapter 8	General Discussion	128 - 130
	References	131 - 137

CHAPTER 1

GENERAL INTRODUCTION

As with many scientific discoveries, the initial observations that led to the discovery of the lysosome were purely accidental. De Duve and his colleagues were working on the effects of insulin on isolated liver tissue, and had resorted to the newly-developed technique of subcellular fractionation as a means of purifying glucose-6-phosphatase. The distribution of acid phosphatase activity in liver homogenates was done as a matter of course, largely as a control. In an experiment performed on December 16th, 1949, the livers were homogenized in 0.25M-sucrose and, although everything appeared to go well for glucose-6-phosphatase, there seemed to have been something wrong with the acid phosphatase assay, since the activity obtained in the homogenate was only about one-tenth of the value usually obtained in water extracts, whereas the activities of individual fractions added up to twice the value in the homogenate. The assays were repeated on the same fractions five days later, with the same reagents, and the results were more satisfactory: recovery was 85%, homogenate activity was of the right order of magnitude and displayed a distinct peak in the mitochondrial fraction. The entire experiment was then repeated and the effect was shown to be a real one; acid phosphatase did not initially display its full activity in homogenates prepared in 0.25M-sucrose. The full activity of the enzyme was only demonstrable after allowing the fractions to stand for several hours, or after subjecting them to various disruptive techniques. So it was that the lysosome first introduced itself in the form of latent acid phosphatase activity.

De Duve could not have envisaged the enormous consequences of his discovery, and probably regarded the investigations into the newly-discovered phenomenon of latency as a temporary diversion from the main course of his research. Lysosomes were not immediately recognized as distinct particles; indeed in the beginning it was taken for granted

that acid phosphatase was located in the mitochondrion, and the latency of the enzyme was initially reported as evidence for the existence of the mitochondrial membrane (de Duve et al., 1951).

In the same year, without committing themselves to the identity of acid phosphate-containing particles, Berthet et al. proposed a theoretical model of these particles. According to the model, the particles were considered to be sac-like structures surrounded by membrane; the enzyme was believed to be present inside in perfectly active and soluble form, and its apparent lack of activity was attributed to the impermeability of the particle membrane to β-glycerophosphate, the substrate used in the enzyme assay. This model was later supported by two key facts. β-glycerophosphate was found to afford osmotic protection equally well as sucrose, a role it could fulfil only if it were unable to penetrate within the particle (see below). It was also found that no relative increase in activity could be brought about by raising the concentration of β-glycerophosphate in the incubation medium, an observation which suggested an almost complete lack of permeability of the membrane to this compound (Appelmans and de Duve, 1955).

A number of other enzymes were investigated and shown to display latency and subcellular distributions very similar to acid phosphatase. By 1955 five enzymes had been difinitely shown to possess these characteristics: acid phosphatase; ribonuclease; deoxyribonuclease; cathepsin; acid β-glucuronidase. Careful examination of their subcellular distributions showed that they were all slightly different from mitochondrial enzymes, and de Duve et al. (1955) postulated that these enzymes were located in a hitherto unrecognized particle. The word lysosome, meaning lytic body, was proposed, along with an updated biochemical model, to describe these new organelles. It must indeed have been most

heartening for de Duve when later microscopic examination of partially purified fractions confirmed the existence of lysosomes as the morphologically-distinct pericanalicular dense bodies which had been noted by electronmicroscopists previously (Novikoff et al., 1956).

The biological function of lysosomes at that time was not understood but, as more enzymes were investigated and found to be located within the lysosome, a pattern seemed to emerge. All the enzymes were hydrolytic in nature, with an acid pH optimum, and together possessed the ability to degrade a whole range of cell constituents, not only phosphate esters, but also proteins, nucleic acids and glycosides. Purely from a safety point of view, it would seem reasonable that the cell would need to segregate such potentially destructive weapons, and the expression 'suicide bags' was coined to highlight the possible role of lysosomes in cellular death and autolysis.

Largely due to the pioneering work of Straus (see Straus, 1967 for review), lysosomes were realized to play a much more active role in the cell, and were not merely dormant killers waiting to destroy the host cell. Straus used horseradish peroxidase as a marker whose presence could be conveniently displayed morphologically in tissue sections, and also biochemically in tissue homogenates. By using a double-staining technique for peroxidase and lysosomal acid phosphatase, Straus was able to demonstrate the endocytic uptake of injected horseradish peroxidase by the kidney cortex cells of rats. The injected enzyme was shown to initially become entrapped within micropinocytic vesicles which were formed by invaginations of the plasma membrane. These pinocytic vacuoles were then found to fuse with the acid phosphatase-staining vacuoles to form the heterolysosomes within which digestion of the injected enzyme occurred.

The processes of endocytosis and intralysosomal digestion are now

more fully understood. It is now known that the lysosome is but part of the complex dynamic system which is responsible for intracellular digestion (de Duve, 1969). Lysosomes are now known to possess the enzymes which together are capable of the digestion of almost any type of naturally occurring macromolecule (Tappel, 1969; Barrett, 1972).

The properties of the lysosomal membrane are an integral part of the concept of the lysosome and they feature prominently in both the original biochemical definition (de Duve, 1955), and the subsequent cytochemical definition (Novikoff, 1961). Although de Duve's original biochemical model has been challenged by Koenig (1962), who envisages lysosomes as solid complexes in which the various enzymes are retained by ionic conjugation with acidic glycolipids, the bag-like model is still generally accepted as being the one most consistent with experimental observations. The importance of the lysosomal membrane has become increasingly apparent as more enzymes were attributed to lysosomes and their biological function established. If lysosomes are the organelles of intracellular digestion, the lysosomal membrane must satisfy three major requirements. Firstly, it must have permeability properties which allow digestion products to diffuse out of the lysosome but retain the enzymes and incompletely digested fragments. Secondly, it must be capable of fusing with other vacuoles. Thirdly, it must be able to maintain the acid environment within the lysosome which the enzymes require.

The experiments described later in this thesis have been designed to obtain more information about the membrane of the lysosome, particularly its permeability properties. Many experimental approaches have been used in the past to examine this aspect of lysosomal function, and no attempt will be made to list them all here. The methods which will be listed and briefly discussed are those which have been employed, or have directly

influenced the reasoning of, the majority of the work reported in this thesis.

Latency

Measurement of the latency of lysosomal enzymes has been perhaps the most frequently employed method of investigating the permeability properties of the lysosomal membrane, probably because it is the method by which lysosomes were first discovered. The degree to which lysosomal enzymes are latent has customarily been used as an index of the integrity of the lysosomal membrane. In freshly-prepared lysosome-rich fractions, resuspended in 0.25M-sucrose, one usually observes that only about 10-15% of the activity of lysosomal enzymes is initially demonstrable. Only after disruptive treatments, such as acid preincubation, freeze thawing, osmotic shock or detergent, do the latent enzymes manifest themselves.

The low value which one usually observes for the proportion of the total enzyme activity which is initially freely available (percent free activity) can be explained in two ways:

(1) The value of percent free activity for a lysosomal enzyme, initially about 10-15%, is a reflection of the number of damaged lysosomes in the preparation, since the substrate used in the detection of the enzyme is unable to penetrate the membrane of intact lysosomes. If the lysosomes are subjected to some injurious treatment, more are broken, their enzymes become fully accessible to the substrate, and hence an increase in the percent free activity is observed.

(2) The low value which one observes initially for percent free activity is a result of the limitations which diffusion of substrates across the lysosomal membrane to their respective enzymes imposes on the overall rate of reaction. Intact lysosomes are partially permeable to the substrate, but the rate at which substrates are able to penetrate is such

that the substrate concentration inside the lysosome is very much lower than that outside, and consequently the observed rate of hydrolysis is lower than that for disrupted lysosomes.

The method which has been frequently used in many investigations in this thesis to distinguish these two possibilities is the approach adopted by Appelmans and de Duve (1955), the full justification for which was provided later by de Duve (1965). If one measures the percent free activity of a lysosomal enzyme at concentrations above the Km of the enzyme, one can distinguish these two effects. If the percent free activity represents the proportion of broken lysosomes, then both free and total activities will be unaffected if the substrate is increased beyond enzyme-saturating concentrations, i.e. the percent free activity will remain constant. If, however, latency is the result of partial permeability of substrate, increasing the substrate concentration beyond enzyme-saturation will result in an increase in percent free activity, since raising the extralysosomal concentration, although not affecting the total activity, causes an increase in free activity by effectively raising the intralysosomal substrate concentration. Using this approach Appelmans and de Duve (1955) and Lloyd (1969) have demonstrated the impermeability of the lysosomal membrane to β-glycerophosphate and maltose and shown that the latency of the lysosomal enzymes measured with these substrates is an index of the number of lysosomes in the preparation whose enzymes are freely accessible to their substrate.

Latency is, however, a property which is not entirely exclusive to lysosomes: given the correct conditions, almost any enzyme surrounded by a semipermeable membrane will display latency. The latency of some of the mitochondrial dehydrogenases (Bendall and de Duve, 1960; Verity and Brown, 1973) and of microbody catalase (Baudhuin, 1964) are typical

examples. The latency of microbody catalase is an interesting case because it would seem very unlikely that its substrate, hydrogen peroxide, would be unable to permeate the microbody membrane. Here was a situation where the enzyme was so active that the diffusion of substrate to the enzyme became the rate-limiting step. It was not possible to confirm this suggestion by the method described earlier because catalase happens to display first-order kinetics over the entire concentration range and the diffusion barrier would exert the same dampening effect on the reaction rate at all substrate concentrations. Baudhuin (1964) was able to resolve this problem by progressively inhibiting catalase with increasing concentrations of cyanide. As the activity of catalase fell, membrane diffusion became relatively less rate-limiting and the apparent latency of the enzyme fell.

Clearly latency is not simply a property which can be attributed solely to the presence of a semi-permeable membrane and an impermeable substrate. Latency may also be observed with permeable substrates. Latency is better envisaged as the result of a number of interacting variables, such as: substrate permeability; membrane area; substrate concentration; enzyme activity; and Michaeli constant. This means that different enzymes surrounded by the same membrane might display different latency properties, or else, vacuoles of different size with the same enzyme concentration need not necessarily display the same latency. These points are particularly pertinent to a number of investigations described in Chapters 3 and 4, where the latency of endogenous and exogenous glycosidases is examined with a variety of substrates.

Osmotic Protection Studies

The ability of a molecule to afford osmotic protection to lysosomes is often used in conjunction with latency measurements to evaluate the

permeability properties of a substrate. The theory surrounding osmotic protection investigations has been fully discussed by de Duve (1965) and in the introduction to Chapter 5, and rests on the basic assumption that lysosomes are stable if resuspended in an isotonic solution of non-permeant solute. Immediately after resuspension in an isotonic solution, lysosomal enzymes display a typically low percent free activity of 10-15%, even if the solute is a permeable molecule such as glucose. In the presence of a non-permeant solute, such as sucrose, lysosomes are able to maintain this degree of latency for at least an hour at 25°C, whilst in the presence of a permeant solute, latency is quickly lost due to lysosome rupture. The speed at which the stabilising effectiveness of a solute wears off is an index of the permeability properties of the solute molecule. Using this approach, Lloyd (1969a; 1971) and Lee (1970;1971) have examined the permeability properties of a number of carbohydrates and peptides. The method has been used almost exclusively in Chapter 5 to evaluate the permeability properties of nucleosides.

Vacuolation Studies

Another approach is that employed by Cohn for the observation of the vacuolation of mouse peritoneal macrophages when exposed to indigestible non-permeable solutes, such as sucrose. Ehrenreich and Cohn (1969) and Cohn and Ehrenreich (1969) have observed by phase-contrast microscopy the appearance of large phase-lucent vacuoles in the cytoplasm of cells exposed to a variety of carbohydrates and peptides. It was found that monosaccharides with a molecular weight less than 220 failed to produce vacuolation, whilst a number of di-, tri-, and tetrasaccharides with higher molecular weights produced vacuolation similar to that of sucrose. The disaccharides which failed to produce vacuolation, despite their relatively high molecular weight, were found to

be susceptible to digestion by lysosomal enzymes. The vacuolation produced by sucrose could be abolished if the cells were exposed to invertase. This was accompanied by the intracellular hydrolysis of sucrose to fructose and glucose, which were promptly excreted into the medium. The uptake of invertase, as indicated by shrinkage of sucrose-laden vacuoles, was blocked by inhibitors of pinocytosis. No effect was noted when invertase was added to macrophages laden with Ficoll, a polysucrose which is not hydrolyzed by the enzyme. The results obtained with peptides support the concept of a molecular weight threshold of 220: non digestible molecules with a molecular weight above 220 cause vacuolation by their failure to permeate the lysosomal membrane and their subsequent accummulation within lysosomes.

This approach has proved a useful comparison to osmotic protection studies since it measures the outward movement of molecules rather than their inward movement. The results obtained from vacuolation experiments are generally in good agreement with the results obtained from osmotic protection experiments and suggest that the lysosomal membrane does not have vectorial permeability properties.

Introduction of Foreign Compounds into Lysosomes

Ever since the early work of Straus (1958), with horseradish peroxidase, the introduction of foreign compounds into lysosomes has proved a useful tool for cell biologists and biochemists. There are two major categories of foreign compound which have been introduced into lysosomes in order to examine their permeability properties: enzymes and radioactively-labelled marker substances.

By careful selection of an enzyme with known kinetic properties, it is possible to gain useful information about the fate of such enzymes and the properties of lysosomes. As already mentioned, Cohn and Ehrenreich (1969) demonstrated the uptake of invertase by mouse peritoneal

macrophages by observing the shrinkage of sucrose-laden vacuoles. Jacques (1968) followed up the earlier work of Straus (1967) on horse-radish peroxidase and demonstrated the latency of this enzyme in homogenates of livers from rats injected with the enzyme. Lloyd has also demonstrated the latency of two injected glycosidases in rat liver lysosomes. The introduction of foreign enzymes into lysosomes is a particularly useful tool for studying the permeability properties of the lysosomal membrane, and has been exploited in the experiments described in Chapter 5. It offers the possibility of examining the permeability properties of the lysosomal membrane with substrates that could not be used for the endogenous lysosomal enzymes.

The introduction of radioactively-labelled proteins into lysosomes has been extensively used since the classical studies of Mego and McQueen (1965) to study the properties of heterolysosomes and the fate of exogenous proteins. Maunsbach (1969) injected rats with ^{125}I-labelled albumin and observed the release of trichloroacetic acid-soluble radioactivity, presumably due to digestion, in the kidney cortex, and noted that the labelled protein was concentrated in the lysosomal fraction of kidney homogenates. Davies et al. (1969; 1971) have demonstrated the inhibition of intralysosomal digestion of albumin by trypan blue, seramin and aurothiomalate. These substances all exerted their effect without affecting endocytosis or lysosomal breakage.

This method, incidentally, does give some useful information about the permeability properties of lysosomes, if the digestion products of the foreign protein can be examined. This has only really been done for one type of molecule; ^{125}I-labelled albumin. Mego et al. (1967) and Williams et al. (1971) have both shown that, following digestion of the ^{125}I-labelled albumin within intact lysosomes, the release of trichloroacetic acid-soluble radioactivity was almost entirely in the form of monoiodotyrosine.

The digestive capacity of lysosomes has mostly been investigated *in vitro* with purified lysosomal extracts. Coffey and de Duve (1968) have studied the protein digestion by purified lysosomal extracts; Aronson and de Duve (1968) and Aronson and Davidson (1968) measured the capacity of lysosomal extracts to digest polysaccharides and mucopolysaccharides; Fowler and de Duve (1969) studied the digestion of lipids; and Arsenis et al. (1970) investigated the degradation of nucleic acids. These studies have established that normal lysosomes contain the capacity for digestion of almost every known macromolecular complex, and have provided some circumstancial evidence about the permeability properties of the lysosomal membrane. The final digestion products of most macromolecules have been shown by other methods to be able to permeate the lysosomal membrane. The ability of nucleosides to permeate the lysosomal membrane has been examined in Chapter 5, since they are thought to be the final digestion product of nucleic acids (Arsenis et al., 1970).

Lysosomal Storage Diseases

Lysosomes and storage diseases were brought together over ten years ago by Hers' discovery (Hers, 1963) of the lysosomal localization of the enzyme deficiency in Pompe's disease and the subsequent definition of the concept of 'inborn lysosomal error'. His notion of the inborn lysosomal disease now appears to be applicable to most congenital storage diseases. All lysosomal storage diseases are characterised by the deficiency, or lack, of a single lysosomal enzyme and the accumulation of incompletely-digested substances within membrane bound vacuoles. A third characteristic which often accompanies these two is the elevation of unaffected enzymes.

Not only have these observations helped provide a basis for the interpretations of the clinical manifestations of such diseases, but

the study of storage diseases has also influenced the understanding of normal lysosomal function. It has led to the identification of a number of specific hydrolases which would probably have escaped detection for a long time were it not for the experiments of the nature offered by storage diseases. The importance of cellular autophagy in normal functioning of cells has been realized since deposits found in lysosomes often must be of endogenous origin. One minor contribution which investigations into lysosomal storage diseases have made is to emphasise the permeability properties of the lysosomal membrane. Pompe's disease is the most striking in this respect; although the cells have quite normal cytoplasmic concentrations of glycogen, and the phosphorylase activity of the cells is normal, the accummulation of glycogen, due to the deficiency of lysosomal α-glucosidase, occurs inside the lysosome. Here, the glycogen is surrounded by a membrane which does not allow entry of phosphorylase, nor allow exit of the glycogen. A certain amount of the work described in the latter part of this thesis has been designed to shed more light on the storage disease cystinosis where one of the suggested causes of the disease is the lack of a membrane transport system. (Schulman and Bradley, 1970.)

Quantitative Cytochemistry

The technique which has been developed by Bitensky (Bitensky et al., 1973) for the investigation of experimentally induced alterations in lysosomal membrane function in tissue section, is that of microdensitometry. Based on a morphological approach, the stability and latency of lysosomes are examined by a quantitative cytochemical procedure which depends on the distribution of leucyl- β-naphthylamidase activity.

Using this approach, Bitensky et al. (1974a; b) have examined the effects a number of hormones and glucocorticoids on lysosomal permeability and stability, and Chayen et al. (1971; 1973) have obtained evidence for

the alteration in the permeability properties of the lysosomal membrane in lysosomes from the living cells of human rheumatoid joints.

Much of the criticism of this technique has been levelled at the use of leucyl β-naphthylamidase as a lysosomal marker enzyme. In Chapter 7 of this thesis this problem has been examined, along with the suggestion of Chayen et al. (1973) that the permeability of the lysosomal membrane can be influenced by redox reagents, such as ascorbate.

CHAPTER 2

MATERIALS AND METHODS

2.1 Preparation of a lysosome-enriched fraction of rat liver

There are now available a number of methods for the preparation of subcellular fractions by differential centrifugation. In these investigations, lysosome-enriched fractions of rat liver have been prepared by the method of Lloyd (1969a), which is based on the earlier schemes of Sawant et al. (1964) and de Duve et al. (1955).

The experiments were performed with male adult (4-6 months) Wistar rats, weighing 300-400g. The rats were starved overnight before use, but provided with a liberal supply of water. The rats were killed by a sharp blow to the back of the head, and their livers were quickly removed and placed in a beaker containing ice-cold 0.25*M*-mannitol. The liver was blotted dry to remove as much blood as possible, and weighed in a chilled beaker. The chilled liver (average weight 7-10g) was then forced through a stainless steel sieve (mesh $0.8cm^2$ approx.), in order to remove the major vascular and connective tissue and thus facilitate homogenization. The resulting liver pulp was weighed (average weight 5g approx.) and homogenized in 2.5 volumes (wet weight of liver pulp/ volume) of ice-cold 0.25*M*-mannitol. Homogenization was performed with a Potter-Elvejhem Teflon-on-glass-type homogenizer (Tri-R Instruments Inc., Rockville Centre, New York, N.Y. 11570, U.S.A., model K4I; diameter clearance 0.019 cm). Homogenization of the pulp was accomplished by forcing the tissue suspension past the pestle, which was rotating at approx. 3000 r.p.m. (speed setting 2.7), 3 times in 30 sec. Chilled 0.25*M*-mannitol was added to give a final volume of 10ml per g of liver pulp and the homogenate was then subjected to differential centrifugation at 4°C, using M.S.E. '4L', rotor no. 62303, and 'High Speed' '18', rotor no. 69181, refrigerated centrifuges.

A very short fractionation scheme was employed, in order that the mechanical disruption, which the repeated washings and resuspensions of

a fuller fractionation scheme might cause to subcellular particles, could be minimised, and the maximum possible latency of the lysosomal enzymes be observed. The fraction sedimenting between 1100g (10 min) and 22500g (10 min) was gently resuspended in ice-cold 0.25M-mannitol, usually 2.5ml per g of original liver pulp. Resuspension was achieved by gently forcing the pellet, still in the centrifuge tube, once only past a pestle rotating at approx. 1500 r.p.m. (Tri-R, speed setting 1.7).

This lysosome-enriched fraction was freshly prepared immediately prior to enzyme assays, and diluted further with ice-cold 0.25M-mannitol if necessary.

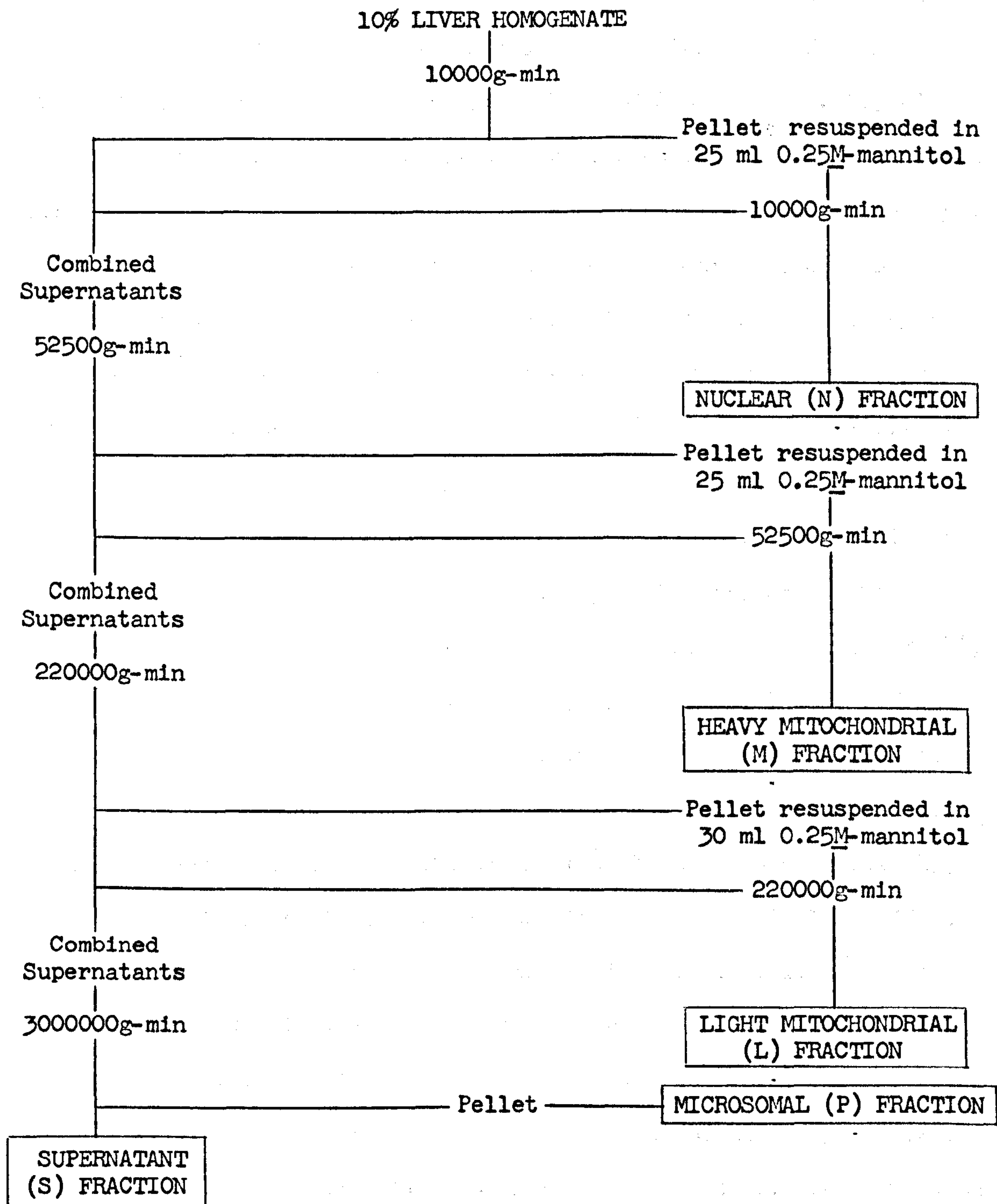


Fig. 2.1 Fractionation of tissue components by differential centrifugation

2.2 Fractionation of tissue components by differential centrifugation

The scheme adopted in the present investigations is shown diagramatically in Fig. 2.1 and is based on the earlier work of de Duve et al. (1955); de Duve (1965; 1967; 1971); and Sawant et al. (1964).

Male adult Wistar rats (300-400g) were used in these investigations. The rats had been starved overnight but provided with an adequate supply of water. Some of the rats used in these experiments had earlier received an intravenous injection of enzyme. The rats were killed by a blow to the back of the head, their livers quickly removed and a 10% homogenate in ice-cold 0.25M-mannitol prepared as described earlier in 2.1.

The tissue homogenate was separated at 4° into five subcellular fractions by centrifugation at successively higher speeds as outlined in Fig. 2.1, using M.S.E. '4 L', rotor No. 62303; M.S.E. 'High Speed 18', rotor No. 69181; and M.S.E. 'Superspeed 50', rotor No. 59590, refrigerated centrifuges. Some of the original 10% homogenate was also retained for enzymic and protein estimation. In this way, it was possible to obtain nuclear (N); heavy mitochondrial (M); light mitochondrial (L); and microsomal (P) pellets. The supernatant from the final 100,000g, 30min, centrifugation gave the soluble (S) fraction. Pellets were gently resuspended in ice-cold 0.25M-mannitol by forcing them once only past a Teflon pestle rotating at approx. 1500 r.p.m. (Tri-R, speed setting 1.7). All operations were performed as quickly as possible at 4°, the complete fractionation being 4-5h. in duration. The fractions were maintained overnight at -30° before assay, it having been ascertained that such storage resulted in no loss of any of the enzymic activities under investigation.

Particular care was taken in the separation of the heavy mitochondrial (M) and light mitochondrial (L) fractions. The heavy mitochondrial

sediment consists of a well-packed bottom layer, covered by a paler, loosely-packed 'fluffy layer'. In separating off the supernatant fluid, care was taken to avoid removing any of either layer. The light mitochondrial sediment is capped by a pinkish 'fluffy layer', which Appelmans et al. (1955) have shown to be poor in acid phosphatase activity, but rich in glucose 6-phosphatase activity. This pinkish 'fluffy layer' was therefore decanted with the rest of the supernatant, and finally recovered in the microsomal (P) fraction.

2.3 Administration of intravenous injections

All operations were performed whilst the rats were under light ether anaesthesia. Injections, usually 0.5ml total volume, were administered into the femoral vein. The anaesthetised rat was placed on its back, a small incision, about one inch in length, made in the groin, and the vein exposed by gently teasing away the overlying connective tissue. Best results were obtained with 25-gauge needles which had been bent to an angle of about 30° to allow easy puncture of the vein. After injection a small swab was held in place at the injection site, in order to quench any bleeding. The incisions were sutured and the animals allowed to recover.

2.4 Collection and analysis of blood from injected rats

All experiments were performed whilst the rats were under light ether anaesthesia. Rats were injected in the femoral vein as described in 2.3 and were maintained under ether anaesthesia for the duration of the experiment. Immediately before the injection a small incision was made to one of the hind feet, a sample of blood collected in a 20μl heparinized capillary tube (Hawksley & Son Ltd., Lancing, Sussex, Cat. No. A803), and diluted for assay. Bleeding was quenched by the application of a small tourniquet. Immediately after injection, and at appropriate time intervals later, the tourniquet was released and a further blood sample taken.

2.5 Analysis of liver concentrations of exogenous enzymes

Animals were injected as described in 2.3 and after the appropriate time interval were killed by a blow to the back of the head. The liver was quickly exposed and perfused *in situ* with 20ml chilled 0.25*M*-mannitol, by inserting a plastic cannula into the hepatic portal vein and forcing the buffer into the liver by means of a syringe. After removal, the liver was gently massaged, blotted dry and weighed. After such treatment livers became very pale in colour.

The livers were then finely minced with a pair of scissors and homogenized vigorously in 2.5 volumes (2.5ml buffer per g tissue) of ice-cold 0.25*M*-mannitol. Homogenization was performed on a Virtis '45' Homogenizer (Virtis Research Equipment, Gardiner, New York, U.S.A.), speed-setting 'Medium' for 5min. The homogenate was diluted with more ice-cold 0.25*M*-mannitol, to give a concentration of 10ml homogenate per g of original liver. The homogenate was examined immediately for enzymic activity.

2.6 Estimation of Glucose by Glucose Oxidase

The method employed for the estimation of glucose was that described by Lloyd and Whelan (1969), which permits the estimation of glucose in the presence of maltose. The method is essentially a refinement of the earlier work of Fleming and Pegler (1963), and utilises the inhibitory properties of Tris on α,1,4-glucosidase, an activity that is always a major contaminant of commercial preparations of glucose oxidase.

2.6.1 Preparation of Glucose Oxidase Reagent

The reagent was prepared by dissolving the following in 100ml of Tris-Glycerol Phosphate Buffer, pH 7.0: glucose oxidase (Boehringer, cat. No. 15424 EGAC), 30mg; horseradish peroxide (Boehringer, cat. No. 15302 EPAB), 3mg; o-dianisidine dihydrochloride (Sigma Chemical Co. Ltd., London) 10mg. It was found that o-dianisidine dihydrochloride dissolved only very slowly in the buffer at room temperature and a convenient method to prepare the reagent quickly was to prepare a stock solution of o-dianisidine dihydrochloride and add the appropriate volume along with the enzymes as required. After mixing, the reagent was filtered, if necessary, and stored at 4° under which conditions it gave satisfactory results for 2-3 weeks.

2.6.2 Procedure

To 1ml of test solution, containing 0-75μg glucose, 2ml glucose oxidase reagent were added, mixed well, and incubated for 30min at 37°. The reaction was stopped, and colour development achieved by the addition of 4ml 5N-Hydrochloric acid. The solution was mixed well and its extinction at 525nm measured using glass cells of 1cm path-length in a Cecil 'CE 373' spectrophotometer (Cecil Instruments Ltd., Cambridge).

A standard curve was constructed for glucose concentrations 0-75μg per ml, over which range the concentration showed a linear relationship with the extinction at 525nm.

2.7 Estimation of ^{125}I-radioactivity

Estimations of ^{125}I-radioactivity were performed on a Packard Selectronic Gamma Counter (Packard Instrument C6.,Inc.,Illinois,USA), samples being presented to the machine in disposable 3ml plastic tubes. Estimations were performed with a counting-time in the range 60-300sec, using adiscriminator level of 66, a channel width of 210, a high voltage of 1160V, and a gain setting of X 10.

2.8 Enzyme Assays

The following general assay methods have been employed for the enzymes listed below.

2.8.1 Determination of α-glucosidase activity by the hydrolysis of maltose

The activity of α-glucosidase (E.C.3.2.1.20) towards maltose can be conveniently monitored by the rate of production of the hydrolysis product, glucose. Glucose can be measured specifically in the presence of maltose by the use of the glucose oxidase reagent (Lloyd and Whelan, 1969). The method employed is that of Lloyd (1969a).

The method involves the incubation of the enzyme preparation (0.25ml), in an appropriate buffer (0.50ml) with maltose (Sigma Chemical Co. Ltd., London, Grade 2), at 37° for varying lengths of time. The most commonly employed buffer was 0.1M-acetic acid -sodium acetate, pH 5.0, in 0.25M-mannitol, which will be subsequently referred to as 'acetate-mannitol'. The reaction was stopped by the addition of 0.32M-sodium hydroxide (0.3ml), with mixing, followed immediately by the addition of 0.16M-zinc sulphate (0.3ml). The gelatinous precipitate thus obtained was removed by centrifugation at approx. 1500g, 5-10min (M.S.E. 'Super Minor' centrifuge). Aliquots of the clear supernatant were removed, assayed for glucose using the glucose oxidase assay described in 2.6, and their glucose concentration determined from a standard graph.

Suitable controls were always performed in which the enzyme preparation and maltose were incubated separately, and mixed only after the addition of 0.32M-sodium hydroxide.

2.8.2 Determination of α-glucosidase activity by the hydrolysis of glucose

It is well known that glycogen is a substrate for α-glucosidase

and it has been shown that the enzyme possesses the ability to hydrolyse both α(1.4) and α(1.6) linkages of glycogen, along with some trans-glycosylation reactions (Bruni et al., 1969, 1970; Jeffrey et al., 1970). The involvement of the enzyme in type 2 glycogenosis, the human storage disease, has also been implicated (Hers and de Barsy, 1973).

The assay technique is essentially that described in 2.8.1 for the hydrolysis of maltose, the hydrolysis of glycogen being estimated by the rate of production of glucose. The reaction is stopped and the glucose concentration measured in an identical manner. Suitable controls were again always performed where enzyme and substrate are incubated separately and mixed only after the addition of 0.32M-sodium hydroxide.

2.8.3 Assay method for α-fluoroglucosidase activity

Barnett et al. (1967a and b), have shown some glycosyl fluorides to be extremely good substrates, in some cases many times better than the corresponding p-nitrophenyl substrates, for several glycosidases. Barnett (1971) has shown α-fluoroglucoside to be an extremely good substrate for the α-glucosidase obtained from rat intestinal mucosa. The reaction has been followed by a number of techniques (Barnett, 1967a), but in the present investigation it was found most convenient to monitor the reaction by following the rate of glucose production.

The conditions of the assay were identical to those described for the estimation of maltose activity in 2.8.1. The reaction was stopped, and the product, glucose, estimated in an identical manner, using the glucose oxidase reagent. Appropriate controls were again always performed.

2.8.4 Estimation of cellobiase activity

The estimations of the activity of β-glucosidase (E.C.3.2.1.21) towards cellobiose were performed in an identical manner to that described for the estimation of maltase activity in 2.8.1, using a cellobiose concentration of 80mM. The reaction was stopped, and the hydrolysis

product, glucose, estimated in an identical manner, using the glucose oxidase reagent. Appropriate controls were again always performed.

2.8.5 General assay method for *p*-nitrophenyl glycosidases

Nitrophenyl substrates have been widely used in the past in the study of lysosomal enzymes (Conchie and Levvy 1957, 1959; Conchie and Hay, 1963; Gatt and Rapport, 1966), largely due to the ease of detection of the liberated aglycone, whose extinction at about 420nm at pH 10-11 can easily be measured. The same general method was adopted for the detection of α-glucosidase; β-glucosidase; β-galactosidase; and β-xylosidase activities, using the corresponding *p*-nitrophenyl glycosidic substrates (all obtained from Sigma Chemical Co. Ltd., London). Minor refinements, such as pH or substrate concentration have been made to the general assay method, depending upon the nature of the individual experiment.

The method adopted was essentially that of Barrett (1972), but 0.2*M*-sodium carbonate was used to develop the yellow colour of the liberated *p*-nitrophenol, since sodium hydroxide has been found to cause rapid hydrolysis of some substrates (Furth and Robinson, 1965).

The assay method involved the incubation of enzyme preparation (0.25ml) in acetate-mannitol, pH 5.0 (0.5ml), with the corresponding *p*-nitrophenyl glycoside (0.25ml) at 37° for a period of time, usually 10 min. After incubation the reaction was stopped and colour development achieved by the addition of 0.20*M*-sodium carbonate. Such treatment was found to elevate the pH of the solution to a value of 10-11 but caused little or no hydrolysis of any of the substrates employed. At this pH liberated *p*-nitrophenol can be estimated spectrophotometrically at 420nm. All measurements of extinction were performed in a Cecil '373' linear-readout spectrophotometer (Cecil Instruments Ltd., Cambridge), using glass cells of 1cm path-length.

When lysosome-enriched liver fractions were used as the enzyme-source it was often found that turbidity became troublesome and the yellow colour of liberated *p*-nitrophenol was masked. In such cases, the reaction was stopped and deproteination achieved by the addition of 0.5ml of 3.3% trichloroacetic acid (TCA). The precipitate was removed by centrifugation at 1500g for 10 min, 1.0ml supernatant added to 2.0ml 0.2*M*-sodium carbonate, and liberated *p*-nitrophenol estimated spectrophotometrically as described above. Suitable control experiments were always performed in which substrate and enzyme solutions were incubated separately and mixed only after the addition of TCA.

A calibration curve was constructed at *p*-nitrophenol concentrations of 0-0.200μmoles per ml. A linear relationship was observed between the concentration of *p*-nitrophenol and the extinction at 420nm.

2.8.6 Assay method adopted for the detection of aryl sulphatase activity (E.C.3.1.6.1.)

The group of enzymes known collectively as arylsulphatases still remain rather mysterious, although there are known to be three distinct arylsulphatases: A, B and C. The two former activities are known to be lysosomal in origin, whilst the latter has been found to be a microsomal enzyme (Roy, 1960). Although arylsulphatases A and B have been shown to be quite distinct enzymes (Roy, 1958; Wynn, 1966), they are indistinguishable by the usual assay employed. The usual assay involves the detection of liberated *p*-nitrocatechol after the hydrolysis of the ester-sulphate bond of *p*-nitrocatechol sulphate. Although the kinetics of the reaction are somewhat complicated (Roy, 1953b; Dodgson and Spencer, 1956), the reaction can be conveniently followed by the spectrophotometric estimation of *p*-nitrocatechol, after stopping the reaction with alkali (Roy, 1953a).

The method of assay is essentially that of Lloyd (1969a). The

incubation mixture comprised: 0.5ml acetate-mannitol, pH 5.0; 0.3ml p-nitrocatechol sulphate (Sigma Chemical Co. Ltd., London); and 0.2ml enzyme preparation. Incubation was for 10 min at 37° and was terminated by the addition of 6ml alkaline quinol reagent (Roy, 1953a). Liberated nitrocatechol was determined by measuring the extinction at 540nm. Control experiments were again performed in which substrate and enzyme solutions were incubated separately and mixed only after the addition of alkaline quinol reagent. Except where otherwise specified the substrate concentration in the enzyme assay was 5mM.

A calibration curve of nitrocatechol showed a linear relationship between the extinction at 540nm and concentration, in the range 0-0.8 μmoles per ml.

2.8.7 Assay method for L-Leucyl-β-naphthylamidase activity

L-Leucyl-β-naphthylamidase activity was used in a histochemical procedure for the observation of lysosomes in tissue sections (Bitensky, 1973; Bitensky et al. 1974a, b; Butcher et al. 1973; Chayen et al. 1971, 1973). The techniques have been employed to demonstrate changes in stability of the lysosomal membrane. The hydrolysis of L-Leucyl-β-naphthylamide in these investigations has been assumed to be caused by cathepsin B1 activity, lysosomal in origin. Although there is evidence to support this, (Snellman, 1969; Mahadevan and Tappel, 1967), there is also some evidence suggesting that cathepsin B1 is not responsible for the activity, but other enzymes, aminopeptidases, non-lysosomal in origin, are responsible (Barrett and Poole, 1969; McDonald et al., 1970). Indeed it does seem rather anomalous that a lysosomal enzyme should display a pH optimum near neutrality, the conditions usually employed in naphthylamidase assays.

Two assay methods have been employed in the present investigations: the first, Method a, based on the histochemical method of Felgenhauer

and Glenner (1966); the second, Method b, based on the method of Peters et al. (1972). Both techniques are essentially the same, differing only in the method of estimation of the product β-naphthylamine.

Method a

The incubation comprised: 0.2ml enzyme preparation, in 0.25M-mannitol; 1.7ml acetate-mannitol buffer, pH 5.0; and 0.1 30mM-L-leucyl-β-naphthylamide (Sigma Chemical Co. Ltd., London). After incubation at 37° the reaction was stopped and liberated β-naphthylamine estimated by the addition of 1ml of a solution of 1M-sodium acetate, pH 4.2, containing 10% 'Tween 20' (Polyoxyethylene Sorbitan Monolaurate, Sigma) and 0.1% Fast Garnet G.B.C. (o-Amino Azotoliene, Diazonium salt, Sigma). After allowing to stand 15 min at room temperature the extinction at 525nm was measured. Appropriate control experiments were always performed in which enzyme and substrate solutions were incubated separately and mixed only after having stopped the reaction. A calibration curve of β-naphthylamine constructed for concentrations 0-0.08μmoles per ml showed a linear relationship with extinction at 525nm.

Method b

The incubation mixture comprised: 0.25ml enzyme preparation, in 0.25M-mannitol; 0.5ml acetate-mannitol buffer, pH 5.0; and 0.25ml 1mM-L-leucyl - β-naphthylamide (Sigma). After incubation at 37° the reaction was stopped by the addition of 2ml 100mM-sodium hydroxide-glycine buffer, pH 10.4. Liberated β-naphthylamine was estimated fluorimetrically in a Perkin Elmer Fluorescence Spectrophotometer, Model 204, with Perkin Elmer 150 Xenon power-supply, using an extinction wavelength of 340nm and an emission wavelength of 410nm. The fluorescence was compared to a standard solution of β-naphthylamine containing 0.007μmoles per ml. Appropriate controls were again performed in which enzyme and substrate solutions were incubated separately and mixed only after the addition of 100mM-sodium hydroxide-glycine buffer.

2.8.8 Assay method employed to estimate the hydrolysis of ^{125}I-labelled albumin

The method employed to estimate the hydrolysis of ^{125}I-labelled albumin in lysosome-enriched fractions is based on the work of Mego (1971) and Mego et al.(1965, 1967). The release of $[^{125}I]$-iodotyrosine as trichloroacetic acid (TCA) - soluble radioactivity - is used as an index of the rate of the reaction.

The incubation mixture comprised: 0.25ml enzyme preparation; 0.5ml acetate-mannitol buffer, pH 5.0; and 0.25ml ^{125}I-labelled Bovine Serum Albumin (BSA) (Koch-Light Laboratories Ltd., Bucks.) Cat. No. 0142T, 100g per ml. The BSA had been radio-labelled with $[^{125}I]$ iodide (Radiochemical Centre, Amersham, Bucks.) by the method of Williams et al. (1971) and had been denatured in the presence of formalin, pH 10, for 3 days at 4°. Unreacted $[^{125}I]$ iodide was dialysed off against 201 1% sodium chloride.

After incubation at 37° the reaction was stopped by the addition of 0.5ml 20% TCA. The precipitate was centrifuged at 2,000g (M.S.E. '4L' refrigerated centrifuge) for 45 min in order to pack the precipitate in the bottom of the tube and thus eliminate any geometric factors which a dispersed precipitate might cause. The 'Total' radioactivity of the tube and its contents were then estimated as described in 2.7. After estimation of the 'Total' radioactivity the clear supernatant was carefully decanted into a clean tube and the 'TCA-soluble' radioactivity estimated in a similar manner.

Suitable control experiments were performed in which enzyme and substrate solutions were incubated separately and mixed only after addition of TCA.

2.9 Estimation of protein

The estimation of protein was performed by the method of Lowry et al. (1951). The general method adopted was as follows: to 0.5ml diluted protein sample were added 0.5ml N-sodium hydroxide, and allowed to stand at room temperature for 30 min, in order to solubulise all the protein present. 5ml of alkaline-copper reagent were then added with mixing and allowed to stand a further 10 min at room temperature. 0.5ml Foln Ciocalteu's Reagent (diluted 1:1 with distilled water) were then added with mixing. Colour development was complete after 30 min at room temperature, and was measured spectrophotometrically at 750nm (Cecil 'CE373' Spectrophotometer) using glass cells of 1cm path-length.

Bovine Serum Albumin (Sigma, crystallized and lyophilized) was used as the reference. A standard curve was constructed for concentrations of BSA 0-400μg per ml.

2.10 Estimation of cysteine

The estimation of cysteine was performed by the method of Zahler and Cleland (1968), based on the reaction of monothiols with 5,5'-dithiobis (2-nitrobenzoic acid), (DTNB), to form complexes which can be estimated spectrophotometrically.

The reaction mixture comprised: 0.2ml 1.0M-Tris buffer, pH 8.1; 0.1ml 3mM-DTNB in 50mM-acetate buffer, pH 5.0; and distilled water to a total volume of 2.9ml. After addition of 0.1ml cysteine solution, colour development was complete within 10min at room temperature, and was examined spectrophotometrically, using glass cells of 1cm path-length. Appropriate control experiments were always performed. The concentration of cysteine showed a linear relationship with the extinction at 412nm.

CHAPTER 3

LATENCY OF SOME ENDOGENOUS GLYCOSIDASES IN RAT LIVER LYSOSOMES

3.1 Introduction

Latency, perhaps the most striking property of lysosomes, has been attributed to the presence of a limiting membrane which surrounds a matrix of lysosomal hydrolases and acts as a permeability barrier to the substrates that are usually employed for the detection of such enzymes. Several experimental approaches have reinforced the concept of structure-linked latency and a number of theoretical models have been proposed to explain the phenomenon (Romeo and de Bernard, 1966; Weissman et al., 1971; Koenig, 1962). The simplest model compatible with most experimental evidence is de Duve's bag-like model of the lysosome (de Duve, 1965).

Appelmans and de Duve (1955) attributed the latency of acid phosphatase to the impermeability of the lysosomal membrane to β-glycerophosphate. From what is known about the permeability properties of the lysosomal membrane (Lloyd, 1969a, 1971; Ehrenreich and Cohn, 1969; Cohn and Ehrenreich, 1969), the latency of most lysosomal enzymes can also be explained by the inability of their substrates to penetrate the membrane of intact lysosomes. It has, however, been suggested over the years that some substrates are able to permeate the lysosomal membrane, reducing the apparent latency of the enzymes which they are employed to detect (Conchie and Hay, 1963; Furth and Robinson, 1965; Robinson and Wilcox, 1969; Koenig, 1969; Rosenburg and Janoff, 1968; Rosenburg, 1970). It is true that one never observes complete latency in lysosome-enriched fractions; a fact which has been attributed to the mechanical damage of a small proportion of the lysosomes during the isolation technique (Lloyd, 1969a; Bowers et al., 1967). It has been repeatedly suggested, however, that rather than an increase in the number of broken lysosomes, a graded permeabilization of the lysosomal membrane to substrates underlies the unmasking of lysosomal hydrolases

caused by various treatments (Shibko et al., 1965; Shibko and Tappel, 1965; Misch and Misch, 1969). Shibko et al. (1965) observed the loss of latency as a two phase event: first the increase in availability of enzymic activity, without a release of enzyme, followed by the release of enzyme from the lysosome.

Most of the substrates which are usually employed in the detection of lysosomal enzymes are polar molecules with molecular weights above the threshold of 220 proposed by Ehrenreich and Cohn (1969). Such molecules are unlikely to possess the ability to permeate the membrane of intact lysosomes, and subsequently yield high values for the latency of the enzymes they are used to detect.

Relatively little work has been performed with substrates which are either small enough, or hydrophobic enough, to permeate the membrane of intact lysosomes. We considered that by investigating the latency of enzymes with permeable substrates, one might gain useful information about the rates of entry and hydrolysis of such compounds. The apparent latency of microbody catalase activity, resulting from the rate-limitation imposed by substrate entry (Baudhuin, 1964), is the classical example of such a system. Much of the work in this chapter has been devoted to the discovery, and examination of, such compounds.

A criterion customarily employed as an index of the permeability properties of a molecule is its ability to afford osmotic protection to lysosomes (de Duve, 1965) (see General Introduction). Lloyd (1969a) demonstrated that methyl α and β-glucosides did not provide sustained protection to rat liver lysosomes, indicating their ability to penetrate the membrane of intact lysosomes. Initial studies were therefore aimed at using the methyl glucosides as substrates for the corresponding lysosomal glucosidases, and investigating the latency of these activities. This approach was however abandoned when preliminary experiments showed

both methyl glucosides to be extremely poor substrates for the lysosomal enzymes. No detectable hydrolysis of either substrate was observed, even after prolonged incubation at 37°. These substrates were therefore useless in investigations of latency which demand a very short assay time, usually 10 min or less, in order to minimise the latency loss during the actual assay (de Duve, 1963).

Another possible experimental approach for examining the latency of permeable substrates, and one which has been employed by Jacques (1968) and Lloyd (1969b), is the introduction of exogenous enzymes of known kinetic properties into lysosomes, and measuring the latency of these activities. Both methyl glucosides were found to be very poor substrates for two appropriate non-lysosomal enzymes: amyloglucosidase from *Aspergillus niger* (Qureshi, 1967), and sweet-almonds, emulsin β-glucosidase (Sigma) (Veibel, 1950; Pazur and Klepp, 1962). This approach was therefore abandoned.

Another class of substrates frequently employed for the detection of lysosomal enzymes, and have from time to time been suggested to be able to permeate the lysosomal membrane, are the *p*-nitrophenyl compounds (Conchie and Hay, 1963; Furth and Robinson, 1965; Robinson and Wilcox, 1969; Baccino and Zuretti, 1975). Robinson and Wilcox (1969) measured the apparent latency of lysosomal acid phosphatase activity with a number of different substrates and found its latency was much lower with nitrophenyl phosphate than with either β-glycerophosphate or -methyl-lumbelliferyl phosphate. Furth and Robinson (1965) and Baccino and Zuretti (1975) have suggested that *p*-nitrophenyl β-galactoside can permeate the lysosomal membrane of intact lysosomes. In both laboratories the free β-galactosidase activity, measured with this substrate, was observed to rise at concentrations of substrate which were inhibitory to total activity. Such results suggest that the relatively high percent

free activity of β-galactosidase observed with *p*-nitrophenyl β-galactoside arises from the penetration of the substrate into intact lysosomes. In view of these suggestions, the latency of several lysosomal enzymes have been examined with a number of *p*-nitrophenyl substrates.

3.2 Latency of α and β-glucosidase activities

3.2.1 Measurement of initial latency

The general scheme adopted for the measurement of initial latency involved the use of the nonionic detergent Triton X-100 as a membrane-disrupting agent (Wattiaux and de Duve, 1956), and closely resembles the methods employed by Barret (1972) and Lloyd (1969a). The latency of several lysosomal glucosidases has been estimated by comparing the 'free' activity with the measurable 'total' activity obtained after treatment with Triton X-100.

A lysosome-enriched fraction of rat liver was prepared as described in 2.1, and resuspended in ice-cold 0.25M-mannitol. 0.25ml of this suspension were incubated at 37° for 10 min in 0.5ml acetate-mannitol buffer, pH 5.0, in the presence of the appropriate glycosidic substrate, 0.25ml, in 0.25M-mannitol. The reaction was stopped and products estimated as described for individual enzyme activities in 2.8. 'Total' activities were estimated in a similar manner, but the acetate-mannitol buffer, pH 5.0 contained 0.2% Triton X-100 (B.D.H. Chemical Co. Ltd.). In all cases suitable controls were performed in which enzyme preparation (lysosome-enriched fraction) and substrate solution were incubated separately and mixed only after stopping the reaction.

3.2.2 Enzyme Assays

General methods for enzyme assay are outlined in 2.8.

3.2.2.1 α-glucosidase (EC.3.2.1.20 and EC 3.2.1.11)

The lysosomal origin of acid α-glucosidase has been demonstrated, both in rat liver (Lejeune et al., 1963; Jeffrey et al., 1970a), and in canine liver (Torres and Olivarría, 1964). Torres and Olivarría (1964) were able to distinguish the lysosomal enzyme from another α-glucosidase, active at a higher pH, present in microsomal or soluble fractions of canine liver. In addition to its α-glucosidase activity

(EC 3.2.1.20), the lysosomal enzyme has been shown to possess isomaltase (EC 3.2.1.11) (Bruni et al., 1969) and transglycosylase activity (Bruni et al., 1970). The enzyme, or rather an absence or defect of the enzyme, is responsible for Type 2 glycogenosis (Hers and de Barsy, 1973).

The latency of α-glucosidase activity in lysosome-enriched fractions has been estimated with a variety of substrates; maltose; _p_-nitrophenyl α-glucoside; glycogen; and α-fluoroglucoside. Assay techniques are fully described in 2.8.

3.2.2.2 β-glucosidase (EC 3.2.1.21)

Evidence that β-glucosidase is located in the lysosomes of rat liver has been provided by Beck and Tappel (1968). The enzyme was found to be distinct from β-galactosidase and β-acetylglucosaminidase activities by column chromotography. β-glucosidase activity was not however separable from β-xylosidase activity, suggesting that the same enzyme is responsible for both activities. A different situation was found to exist in rat kidney, where Price and Dance (1967) found β-glucosidase activity predominantly in the soluble phase, with little evidence of any lysosomal enzyme. Gatt and Rapport (1966b) have prepared an enzyme from beef-brain, separable from β-galactosidase, which is capable of hydrolysing ceramide glucosidase and nitrophenyl substrates. A similar enzyme, capable of hydrolysing ceramide glucosidase, has also been discovered in lysosome-enriched fractions of rat liver (Weinreb et al., 1968). The enzyme has also long been known to be involved in Gaucher's disease, the human lipid-storage disease (see Brady and King, 1973).

The latency of β-glucosidase activity has been investigated using _p_-nitrophenyl β-glucosidase and cellobiose as substrates. Details of assays are given in 2.8.5 and 2.8.4.

3.2.2.3 β-galactosidase (EC 3.2.1.23)

The lysosomal origin of β-galactosidase has been very well established, both in rat (Sellinger et al., 1960; Furth and Robinson, 1965), and in mouse (Conchie and Hay, 1959). The enzyme shows considerable tissue variations, and has been shown to exist in multiple forms (Furth and Robinson, 1965). The activity of the enzyme has been measured, mainly with synthetic substrates; nitrophenyl (Furth and Robinson, 1965; Conchie and Hay, 1959) and umbelliferyl (Robinson and Wilcox, 1969). There are a number of naturally occuring substrates of β-galactosidase, amongst which are the ceramide lactoside and glycolipids which Gatt and Rapport (1966a, b) found to be susceptible to a rat brain enzyme. The enzyme is involved in the degradation of galactose-containing glycoproteins and its deficiency is involved in G_{M1}-Gangliosidosis (Van Hoof, 1973).

In these investigations the latency of β-galactosidase in lysosome-enriched fractions has been examined with p-nitrophenyl β-galactoside as substrate - details of the assay are given in 2.8.5.

3.2.3 Determination of Michaelis Constants (Km app.)

The Michaelis Constants (Km app.) of maltase, p-nitrophenyl α and β-glucosidase and p-nitrophenyl β-galactosidase activities were estimated from double-reciprocal plots of reaction velocity against substrate concentration (Lineweaver and Burk, 1934; Dixon, 1953). From the straight lines obtained the values of Km app. and Vmax. were calculated for each activity. The values thus obtained are shown in Table 3.1.

Kinetic studies were always performed with lysosome-enriched liver fractions which had been pre-treated to destroy latency prior to their examination. Experimental conditions were identical to those described in 2.8, substrate concentrations of 1 to 10 mM being employed in every case except that of p-nitrophenyl β-galactosidase activity, which displayed substrate-inhibition above a concentration of 4 mM.

Activity	Km app. (mM)	Relative rate of hydrolysis per unit of preparation
Maltase	2.1 ± 0.1	10.6
p-Nitrophenyl α-glucosidase	1.8 ± 0.3	1.0
p-Nitrophenyl β-glucosidase	0.9 ± 0.2	2.5
p-Nitrophenyl β-galactosidase	1.1 ± 0.2	3.1

Table 3.1 Michaelis constant (Km app.) of some glycosidases in lysosome-enriched liver fractions

3.2.4 Latency of p-nitrophenyl α and β-glucosidase activities in lysosome-enriched liver fractions

The latency of p-nitrophenyl α and β-glucosidases in lysosome-enriched liver fractions were examined by the method outlined in 3.2.1, employing an assay time of 10 min at 37°. Both activities were measured simultaneously in the same lysosome preparation in order to eliminate the discrepancies that individual preparations might show. p-Nitrophenyl α and β-glucosidase activities were assayed by the general methods outlined in 2.8, employing substrate concentrations of 10, 8, 6, 5 and 4 mM.

The results obtained in such experiments are summarized in Table 3.2, the 'free' activity being expressed as a percentage of the total activity for each substrate. Results are the mean of four separate experiments.

The per cent free maltase activity is in good agreement with Lloyd (1969a), and the value obtained (11.9 ± 4.0%) is probably an index of the integrity of the lysosome preparation, i.e. the percentage of lysosomes which have been broken in the isolation technique. Lloyd (1969a) showed there to be no increase in the proportion of total maltase activity which was freely available in intact lysosomes even if the substrate concentration was raised to 20mM, about 10 times the Km of the enzyme. Such a result is consistent with the impermeability of the lysosomal membrane to maltose, which is confirmed by its ability to afford sustained osmotic protection to lysosomes.

The most striking feature of the results illustrated in Table 3.2 is the difference in the availability of α-glucosidase, as displayed by the percent free activities of maltase and p-nitrophenyl α-glucosidase. If the same enzyme is responsible for the hydrolysis of both maltose and p-nitrophenyl α-glucoside, one must conclude that the enzyme

Substrate	Substrate concentration (m$\underline{M}$)				
	10	8	6	5	4
$\underline{p}$-Nitrophenyl α-glucoside	68 ± 15.9	61 ± 14.1	66 ± 14.7	72 ± 5.3	74 ± 14.0
$\underline{p}$-Nitrophenyl β-glucoside	115 ± 19.0	114 ± 21.0	121 ± 34.0	108 ± 36.0	112 ± 43.0
Maltose				11.4 ± 4.0	

Table 3.2 Initial percent free activity of α and β-glucosidases in lysosome-enriched liver fractions at 37°

can be simultaneously inaccessible to maltose and accessible to p-nitrophenyl α-glucoside. The simplest explanation for such a result is that much of the p-nitrophenyl α-glucoside is hydrolysed inside lysosomes, following its penetration through the intact membrane.

As was discussed in Chapter 1, the usual method of demonstrating such penetration is to observe the rise in percent free activity as the substrate is increased beyond enzyme-saturating concentrations (de Duve, 1965; Bowers et al., 1967). Although the substrate concentration has been raised to a value of approximately five times the Km of the enzyme, no such increase in percent free activity was observed. This question is taken up in later sections of this chapter.

Total p-nitrophenyl β-glucosidase activities showed a high degree of variability and were often found to be lower than free activities. The most plausible explanation for such a result is an inhibitory effect of Triton X-100 on p-nitrophenyl β-glucosidase activity. In order to investigate this question further, the rate of hydrolysis of pnitrophenyl β-glucoside was examined in the absence and presence of 0.1% Triton X-100, and compared to that observed with other glycosidic substrates.

3.3 Time-course of hydrolysis of some glycosides

The time-course of hydrolysis of maltose, p-nitrophenyl α and β-glucoside, and p-nitrophenyl β-galactoside have been examined in freshly prepared lysosome-enriched liver fractions, employing incubation periods from 10 to 60 min at 37°, and a substrate concentration of 5mM. Experimental conditions were identical to those described in 3.2, individual activities being assayed by the methods outlined in 2.8.

Typical results obtained in such experiments are illustrated in Fig. 3.1. The rate of hydrolysis of all the substrates examined in the presence of 0.1% Triton X-100 was found to be constant from the beginning of the experiment, indicating that all these enzymes were fully accessible to their corresponding substrates and that the enzymes were all stable under these conditions. In the absence of Triton X-100, the initial rate of hydrolysis was relatively low and rose until it reached a constant value. Except in the case of p-nitrophenyl β-glucosidase activity, this final rate of hydrolysis was equal to that seen in the presence of Triton X-100.

For all incubation periods longer than 10 min the rate of hydrolysis of pnitrophenyl β-glucosidase in the absence of Triton X-100, exceeded that in its presence. This inhibitory effect of Triton X-100 explains how the free activity of p-nitrophenyl β-glucosidase could exceed its total activity (Table 3.2). The variability of total p-nitrophenyl β-glucosidase activities previously encountered may be explained by the fact that the inhibition of this enzyme by Triton X-100 was found to be time-dependent, maximal inhibition being obtained after a pre-incubation period of about 5 min.

An interesting aspect of the time-course curves (Fig. 3.1) is the time taken for the free activities of different enzymes to reach a

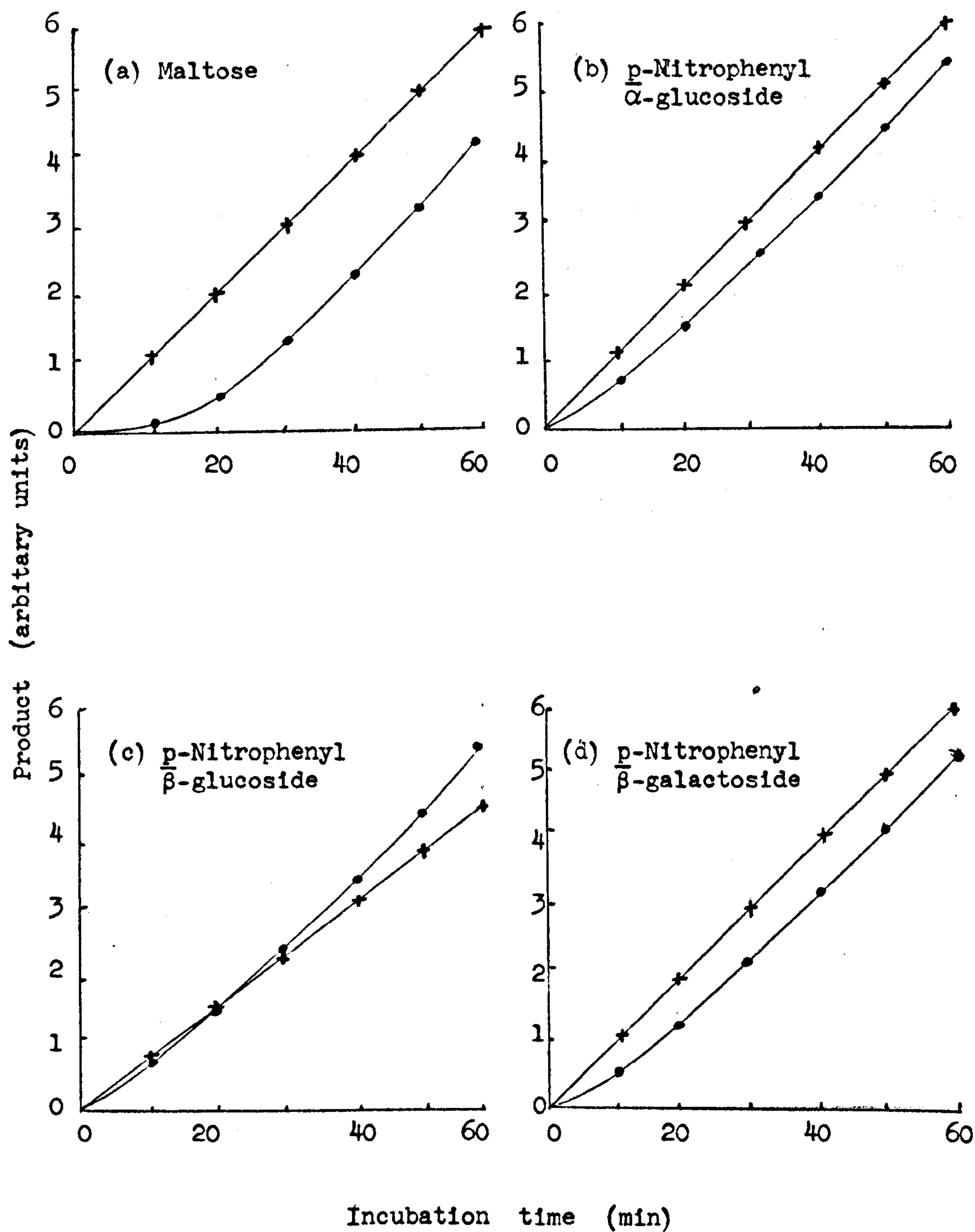


Fig.3.1 Time-course of hydrolysis of some glycosides at 37° in lysosome-enriched liver fractions, in the absence (•) and presence (+) of 0.1% Triton X-100

constant value. This is particularly striking in the case of α-glucosidase activity where the time taken to reach this constant value depended on the substrate used. With maltose, a constant value was not obtained until an incubation period of about 40 min, compared to a time of about 20 min for p-nitrophenyl α-glucoside. The free activity of the other three enzymes behaved very similarly to p-nitrophenyl α-glucosidase. The question whether this effect is conneeted with the penetration of p-nitrophenyl substrates into intact lysosomes is taken up later in this thesis.

3.4 <u>The inhibitory effect of Triton X-100 on p-nitrophenyl β-glucosidase aetivity</u>

Triton X-100 has been very widely used as a means of disrupting lysosomal membranes in the estimation of 'total' enzyme activity. At the concentration usually employed, 0.1%, Wattiaux and de Duve (1956) found no inhibition of any of the lysosomal enzymes they were investigating. Its main advantage over other methods of membrane-disruption, such as, freeze-thawing or sonication, are its cost, speed and ease in application.

From Fig. 3.1 it is apparent that p-nitrophenyl β-glucosidase activity becomes fully available within 40 min, experiments have therefore been performed in which lysosome-enriched liver fraction, pre-incubated at 37° for 40 min in acetate-mannitol to destroy latency, was assayed for p-nitrophenyl β-glucosidase activity at varying concentrations of substrate and in the presence of varying concentrations of Triton X-100. Since it has been suggested that lysosomal β-xylosidase and β-glucosidase activities may be due to a single enzyme (Robinson and Abrahams, 1967; Beck and Tappel, 1968), the effect of Triton X-100 on the hydrolysis of p-nitrophenyl β-xyloside by the lysosome-enriched liver fraction was investigated. It was found that at a substrate concentration of 5mM β-xylosidase activity was inhibited approximately 25% by 0.1% Triton X-100 and approximately 50% by 0.2% or 0.3%.

Attempts were made in Fig. 3.2 to examine the kinetics of inhibition by the methods of Dixon and Webb (1964) and Dixon (1953). Although Lineweaver-Burk plots were linear (Fig. 3.2), they did not conform to any single inhibition type. Dixon plots were not,therefore, linear and it was impossible to calculate a meaningful K_I. The results in Table 3.2 are therefore expressed as the percentage loss of β-glucosidase activity in the presence of varying concentrations of Triton X-100.

Beck and Tappel (1968) demonstrated that the β-glucosidase activity

Concentration of Triton X-100	Concentration of p-nitrophenyl β-glucoside (m$\underline{M}$)				
	1	2	3	4	5
0.1	34	39	39	31	21
0.2	59	60	45	41	80
0.3	61	57	47	43	41

Each result is the mean of 2 experiments. Inhibition is expressed as a percentage loss of the activity found in the absence of Triton X-100

Table 3.2 Inhibitory effects of Triton X-100 on p-nitrophenyl β-glucosidase activity in lysosome-enriched liver fractions

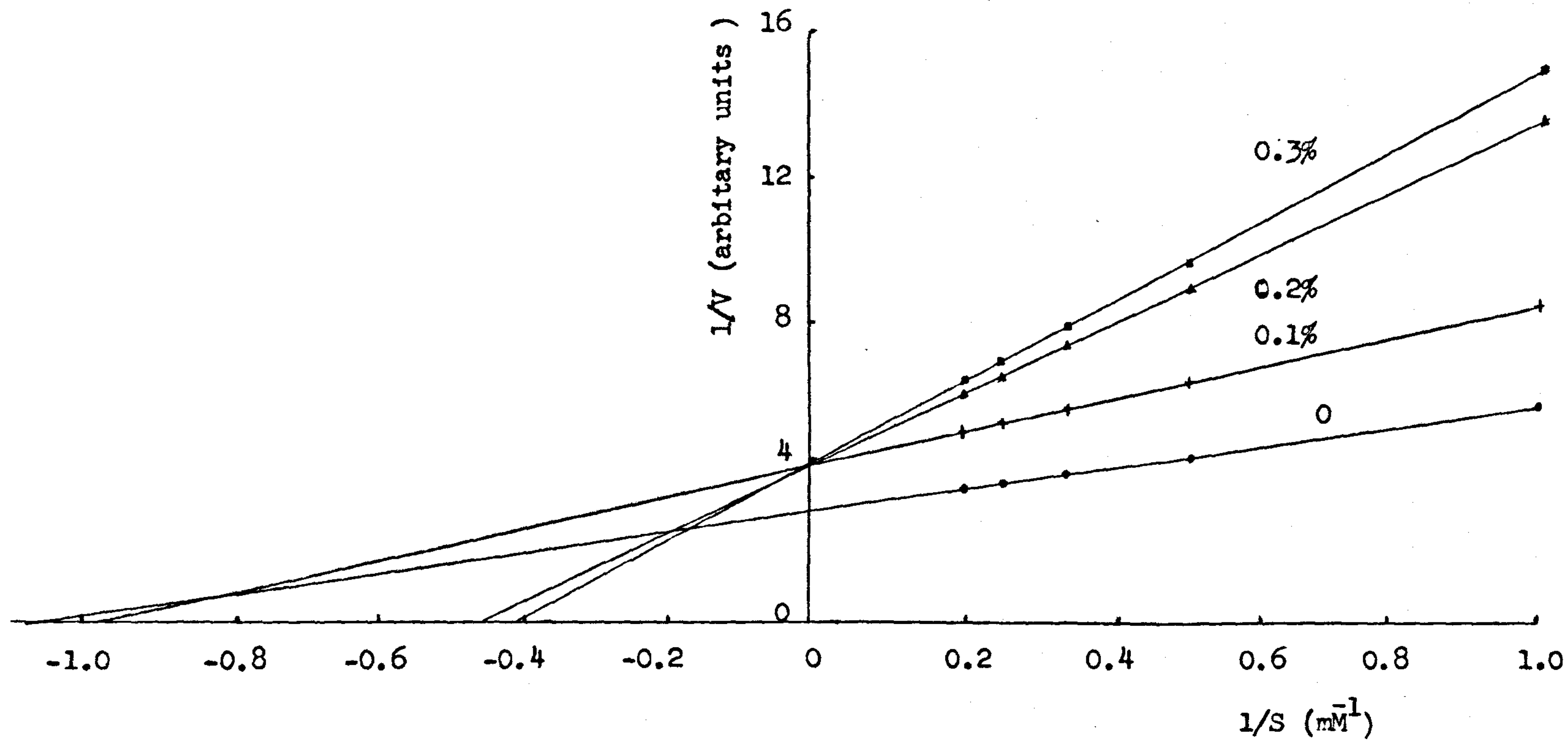


Fig.3.2 Lineweaver-Burk plot of p-nitrophenyl β-glucosidase activity in lysosome-enriched liver fractions at 37°-effect of Triton X-100

of rat liver lysosomes was strongly bound to the lysosomal membrane. It is possible that the enzyme is to some extent dependent on the integrity of the membrane for its activity, and conditions that destroy this integrity cause a reduction in enzyme activity. To examine this suggestion the effects of freeze-thawing on β-glucosidase activity have also been investigated. Lysosome-enriched fractions which had been freeze-thawed in a carbon dioxide-acetone mixture through 10 cycles displayed an activity loss of approximately 30%, at a substrate concentration of 5mM.

Clearly freeze-thawing and Triton X-100 are both of limited value for the estimation of total β-glucosidase activities, and in this work pre-incubation at 37° has always been employed to estimate total activities of lysosomal β-glucosidase, the justification for which is provided in 3.5.

3.5 Effects of substrate concentration upon the latency of some glycosidases

The time-course of hydrolysis of several glycosides in the absence and presence of 0.1% Triton X-100 was examined in 3.3. The method is now used to investigate the latency of maltase, *p*-nitrophenyl α and β-glucosidase and *p*-nitrophenyl β-galactosidase at varying substrate concentration, in an effort to resolve the question posed by the difference in the latency of α-glucosidase as displayed by its maltase and *p*-nitrophenyl α-glucosidase activities (Table 3.2). The estimation of latency is based on the argument that, when the graph of 'free' activity against time becomes linear (see Fig. 3.1), all the enzyme is freely accessible to the substrate and measurement of the slope of the graph after this point is reached gives a measure of the total activity. The method is more useful than a straightforward 10 min estimation of latency (3.2), firstly, because the progressive loss of latency on incubation can be observed, and, secondly, because it avoids the use of Triton X-100, which is undesirable with *p*-nitrophenyl β-glucosidase activity.

Latency investigations have been performed with freshly prepared lysosome-enriched liver fractions, employing incubation periods from 10 to 60 min, and substrate concentrations from 1 to 10mM. Experimental conditions have been outlined earlier in 3.2 and 2.8. Table 3.4 shows the free activity for each 10 min time interval expressed as a percentage of total activity, for each substrate at a range of substrate concentrations.

The main feature of the results shown in Table 3.4 is the difference in the latency of maltase and *p*-nitrophenyl α-glucosidase activities. The initial percent free activity of *p*-nitrophenyl α-glucosidase was about five times higher than the value for maltase activity and, in

Substrate	Concentration (mM)	Time interval (min)						Number of experiments
		(0-10)	(10-20)	(20-30)	(30-40)	(40-50)	(50-60)	
p-Nitrophenyl α-glucoside	1	66 ± 4	91 ± 15	93 ± 10	94 ± 22	92 ± 13	113 ± 21	4
	2	77 ± 8	78 ± 16	100 ± 15	92 ± 6	99 ± 9	96 ± 17	
	4	70 ± 7	105 ± 10	101 ± 15	105 ± 16	93 ± 16	111 ± 20	
	5	69 ± 11	72 ± 19	105 ± 8	90 ± 13	114 ± 11	85 ± 24	
	10	66 ± 7	70 ± 18	89 ± 19	109 ± 9	99 ± 15	111 ± 16	
p-Nitrophenyl β-glucoside	1	67 ± 6	106 ± 23	117 ± 14	94 ± 3	89 ± 12	87 ± 17	4
	2	70 ± 11	75 ± 9	105 ± 14	108 ± 19	98 ± 7	95 ± 17	
	4	66 ± 3	95 ± 13	101 ± 10	110 ± 9	91 ± 23	92 ± 17	
	5	72 ± 5	74 ± 23	100 ± 14	96 ± 3	102 ± 12	97 ± 20	
	10	74 ± 7	80 ± 5	100 ± 11	113 ± 15	91 ± 18	104 ± 24	
p-Nitrophenyl β-galactoside	1	52 ± 7	59 ± 12	94 ± 8	83 ± 15	106 ± 10	95 ± 22	4
	2	52 ± 1	65 ± 16	89 ± 9	88 ± 13	118 ± 18	107 ± 19	
	4	59 ± 3	64 ± 11	93 ± 7	94 ± 7	112 ± 23	85 ± 24	
	5	57 ± 5	62 ± 13	91 ± 13	105 ± 10	100 ± 22	108 ± 24	
	10	51 ± 5	59 ± 5	110 ± 21	108 ± 9	108 ± 10	85 ± 22	
Maltose	1	16 ± 4	56 ± 14	82 ± 12	91 ± 21	101 ± 19	127 ± 23	4
	2	9 ± 5	38 ± 12	66 ± 11	84 ± 9	88 ± 22	119 ± 14	
	4	16 ± 3	65 ± 12	79 ± 18	85 ± 21	100 ± 18	109 ± 19	
	5	12 ± 4	35 ± 15	98 ± 19	76 ± 20	99 ± 21	104 ± 22	
	10	14 ± 5	46 ± 14	72 ± 10	74 ± 19	87 ± 13	114 ± 18	

Table 3.4 Percent free activities of some glycosidases in lysosome-enriched liver fractions at 37°

agreement with previous observations, (Table 3.2), was independent of substrate concentration in the range employed (1-10mM). The rate at which maltase and p-nitrophenyl α-glucosidase lost their latency is also interesting. With p-nitrophenyl α-glucosidase activity, the graph of free activity against time (Fig. 3.1) became linear after an incubation period of about 20 min, compared to a value of about 40 min for maltose. As was suggested in 3.2.4, if the same enzyme is responsible for the hydrolysis of maltose and p-nitrophenyl α-glucoside, this difference may reflect a difference in the permeability of the lysosomal membrane to maltose and p-nitrophenyl α-glucoside. Maltose, being the larger, less lipophilic molecule might logically be expected to be the least permeable. It is interesting to note that even after a higher proportion of maltase activity became available, after an incubation period of 20 to 30 min, the percent free activity of this enzyme still remained independent of substrate concentration. The effect of incubation at 37° at pH 5.0 on free maltase activity seems to be an 'all or none' type of effect, not an increase in the permeability of the lysosomal membrane to maltose, but rather an increase in the number of lysosomes whose enzyme is fully accessible to maltose. Enzyme appears to be either fully available or totally unavailable to maltose. The ways how an enzyme could be simultaneously inaccessible to one substrate and accessible to another are discussed below.

The initial percent free activities of p-nitrophenyl β-glucosidase and p-nitrophenyl β-galactosidase were also about five times higher than the value observed for maltase, and independent of substrate concentration in the range employed (1-10 mM). This result is particularly interesting in the case of p-nitrophenyl β-galactosidase, which happens to display substrate inhibition beyond a substrate concentration of 4mM. Free and total activities of this enzyme, nevertheless, showed similar

levels of inhibition to subsequent increases in substrate concentration. Furth and Robinson (1965) and Baccino and Zuretti (1975) have, however, shown the percent free activity of p-nitrophenyl β-galactosidase to rise as the substrate concentration was increased. The discrepancy in these observations may reside in the different ionic strength and/or pH of the buffers used in the assay. It is interesting to note that the rate at which p-nitrophenyl β-glucosidase and p-nitrophenyl β-galactosidase lost their latency was very similar to that observed with p-nitrophenyl α-glucosidase; little if any, latency of these activities remained after an incubation period of about 20 min. The high initial percent free activity and rapid loss of latency of all three p-nitrophenyl glycosidase compared to maltase may reflect the penetration of p-nitrophenyl glycosides into intact lysosomes.

If p-nitrophenyl glycosides are able to penetrate the membrane of all intact lysosomes, how can one explain the value of about 60% which one observes for the initial percent free activity of lysosomal enzymes with such substrates? One explanation is that their rate of penetration into the lysosome is not sufficient to maintain an intra-lysosomal concentration equal to that in the bulk assay medium (de Duve, 1965). This explanation seems extremely unlikely as no increase in the percent free activity of any of the enzymes was observed when the substrate concentration was increased to values of several times the Km of the enzymes.

A second, and more plausible, explanation is the heterogeneity of lysosomes in the rat liver preparation. Lysosomes have graded permeability properties; the percent free activity of a lysosomal enzyme represents the proportion of lysosomes in the preparation whose enzymes are freely accessible to the substrates used in the detection of the enzymes. Such differing permeability properties could arise, either as

a secondary effect of the isolation technique, or as an inherent difference in the properties of lysosomes from different cell types (hepatocytes, Kupffer cells) or of different intracellular status (primary, secondary lysosomes). As lysosomes are incubated in buffer at 37° , the membrane's permeability increases, either by membrane-disintegration, or by lysosomal swelling, and the proportion of lysosomes that allow the penetration of substrates, increases. The penetration of substrates occurs at a rate that is fast enough to avoid becoming rate-limiting. By this hypothesis one would predict that initially all lysosomes are impermeable to maltose, whilst only about 50% are initally impermeable to p-nitrophenyl glycosides.

There are, however, a number of other possible explanations for the high availability of p-nitrophenyl glycosidases:

(1) The high apparent availability of p-nitrophenyl glycosidases is caused by enzymes, present in the lysosome-enriched liver fraction, but outside intact lysosomes, which are active against p-nitrophenyl α-glucoside, but not active against maltose. Such enzymes could be either genuinely cytoplasmic enzymes adsorbed on the membrane of lysosomes or else lysosomal enzymes which are situated on the citoplasmic face of the lysosomal membrane, such as has been suggested for esterase (Shibko and Tappel, 1964).

(2) p-Nitrophenyl glycosides or their hydrolysis product, p-nitrophenol, cause instability of the lysosomal membrane.

(3) p-Nitrophenyl glycosides are able to penetrate the membrane of all rat liver lysosomes, whilst maltose is initially unable to penetrate any. One never observes an initial 100% availability of p-nitrophenyl glycosidases because in a 10 min assay of initial free activity, a significant time is taken before equilibrium is reached between intra- and extra-lysosomal concentrations of substrate.

(4) *p*-Nitrophenyl glycosides and maltose are initially unable to penetrate any lysosomes. After a few minutes incubation in buffer at 37°, the enzymes become freely accessible to *p*-nitrophenyl substrate, but remain totally inaccessible to maltose. Only after further incubation do the enzymes become freely accessible to maltose.

The first explanation is easily examined by measuring the non-sedimentable activity of the enzymes. If the non-sedimentable activity of *p*-nitrophenyl α-glucosidase is initially low and of a similar value to that of maltase activity, penetration of *p*-nitrophenyl α-glucoside into intact lysosomes is suggested.

The second alternative explanation is quite easily examined by measuring the initial latency of another lysosomal enzyme in the absence and in the presence of either *p*nitrophenol or *p*-nitrophenyl glycosides.

The third and fourth possibilities are both 'beginning of experiment' effects which can be investigated in a number of ways. Firstly at 25° lysosomes are much more stable than at 37°. By examining the latency of these glycosidases at 25° one can help to eliminate the possibility of an equilibration effect, such as is suggested in (3). Secondly, by measuring the initial latency after a period of pre-incubation at 37° one can also help to eliminate the equilibration effect. Thirdly, and most crucially, if the initial percent free activity is the same in both 5 and 10 min estimations of initial latency, the third and fourth possibilities are both eliminated.

3.6 Sedimentability of glycosidases

The rates of release of maltase, p-nitrophenyl α and β-glucosidase, and p-nitrophenyl β-galactosidase activities have been examined by measuring the rate at which these activities became non-sedimentable. 0.5ml of lysosome-enriched liver fraction, freshly prepared and resuspended in 0.25M-mannitol, was mixed with 1.5ml acetate-mannitol buffer, pH 5.0, incubated at 37° for a period of time, and then subjected to centrifugation at 4° at 25000g for 15min (M.S.E. High Speed '18' refrigerated centrifuge). Incubation periods varied from 0 to 60 min. Enzyme determinations on aliquots of the 25000g, 15 min supernatant yielded the non-sedimentable activity. Total activities were estimated on a similarly treated sample, incubated in acetate-mannitol for 50 min, but omitting the final centrifugation. Individual enzymes were assayed by the methods outlined in 2.8, a substrate concentration of 5mM being employed, except in the case of p-nitrophenyl β-galactosidase, where a concentration of 4mM was employed.

Table 3.5 shows the non-sedimentable activity of maltase, p-nitrophenyl α and β-glucosidase, and p-nitrophenyl β-galactosidase activities, expressed as a percentage of the total activity, following pre-incubation of the lysosome-enriched fraction in acetate-mannitol for periods of time up to 60 min.

Maltase and p-nitrophenyl α-glucosidase displayed almost identical non-sedimentable activities at all incubation times, suggesting that the same enzyme is responsible for both activities. The percent non-sedimentable activity of maltase and p-nitrophenyl α-glucosidase, initially about 5%, rose gradually until a maximum value of 70-75% was reached after an incubation period of about 50 min. Although p-nitrophenyl β-galactosidase was released to a lesser extent than

Substrate	Concentration (mM)	37° Pre-incubation time (min)						
		0	10	20	30	40	50	60
p-Nitrophenyl α-glucoside	5	6.1 ± 1.6	11.2 ± 6.4	45.3 ± 22.2	53.9 ± 14.4	67.2 ± 12.9	70.5 ± 13.5	68.8 ± 6.9
p-Nitrophenyl β-glucoside	5	2.3 ± 0.7	2.0 ± 0.6	2.8 ± 1.7	3.3 ± 1.3	7.7 ± 2.1	28.5 ± 18.6	7.4 ± 6.7
p-Nitrophenyl β-galactoside	4	5.4 ± 1.9	8.5 ± 2.6	33.5 ± 15.4	47.9 ± 9.6	56.5 ± 6.9	63.9 ± 8.7	56.7 ± 7.5
Maltose	5	5.5 ± 0.5	12.4 ± 3.9	44.2 ± 18.2	55.3 ± 13.6	66.3 ± 88.7	73.9 ± 12.6	71.5 ± 9.0

Each result is mean ± S.D. for four experiments

Table 3.5 Non-sedimentable activity of some glycosidases in lysosome-enriched liver fractions

α-glucosidase activity, it showed a very similar trend of release, maximal non-sedimentable activity being attained after an incubation period of about 50 min. The non-sedimentable activity of p-nitrophenyl β-glucosidase remained very low throughout the experiment, in agreement with Beck and Tappel (1968), who found the β-glucosidase activity in rat liver homogenates to be very strongly membrane bound. The difference in the maximum percent non-sedimentable activity of β-galactosidase and β-glucosidase is undoubtedly a reflection of the strength with which these enzymes are bound to or associated with the lysosomal membrane.

The results with p-nitrophenyl β-glucosidase emphasize the danger of equating the increase in free activity of an enzyme with its release from the lysosome. Enzyme readsorption is also a problem which complicates the interpretation of sedimentability data (Shibko and Tappel, 1965). Although it is not possible to compare in absolute terms the percent free activity and percent non-sedimentability of an enzyme, the comparative rates of increase of free and non-sedimentable activity may be compared.

The maximal non-sedimentability of maltase was achieved after an incubation period of about 50 min, an almost identical time to that required for the enzyme to become freely available to maltose (3.5). After an incubation period of 10 min the percent non-sedimentability of maltase is very similar to its percent free activity, and is further evidence that the initial latency of maltase activity is an index of the number of damaged and broken lysosomes in the preparation. Although p-nitrophenyl α-glucosidase was fully available after an incubation period of about 20 min, it did not display its maximum sedimentability until an incubation period of about 50 min. These findings are consistent with suggestions that p-nitrophenyl α-glucoside is able to penetrate

the membrane of intact lysosomes. p-Nitrophenyl β-glucosidase activity, like p-nitrophenyl β-galactosidase, is fully available before the enzyme is fully released from the lysosome. p-Nitrophenyl β-glucosidase is fully available in spite of the fact that the enzyme is tightly bound to the lysosomal membrane.

3.7 Latency of glycosidases at 25°

The latency of maltase, p-nitrophenyl α and β-glucosidase and p-nitrophenyl β-galactosidase have been examined in lysosome-enriched liver fractions which had been pre-incubated at 25° in acetate-mannitol, pH 5.0. The latency of each activity following this pre-incubation period was estimated by a further 10 min assay at 25°, similar to that described in 3.2. A substrate concentration of 5mM was employed, except in the case of p-nitrophenyl β-galactosidase activity, where a concentration of 4mM was employed.

Table 3.6 shows the initial free activity (first 10 min of incubation) of glycosidases, expressed as a percentage of the total activity, measured at 25° following pre-incubation of the lysosome-enriched fraction in acetate-mannitol for periods of time up to 120 min.

Using maltase activity as an index of lysosome integrity, the lysosome-enriched fractions appear to be much more stable at 25° than at 37°. The initial percent free maltase activity is of a similar value to that obtained at 37°, but the rate of increase in free activity was much less rapid. The p-nitrophenyl-glycosidases all displayed a much higher initial percent free activity than maltase activity, but in all cases, the value was found to be lower than that obtained at 37°. The increase of free activity was much less dramatic than at 37°, where latency appeared to be lost within 30 min.

Substrate	Concentration (mM)	25° Pre-incubation time (min)					Number of experiments
		0	30	60	90	120	
p-Nitrophenyl α-glucoside	5	57.5 ± 5.7	47.0 ± 2.7	56.5 ± 4.4	64.0 ± 6.3	75.0 ± 9.6	4
p-Nitrophenyl β-glucoside	5	47.5 ± 3.3	54.5 ± 3.3	72.3 ± 9.8	96.2 ± 18.7	107.3 ± 17.6	4
p-Nitrophenyl β-galactoside	4	38.7 ± 5.4	61.1 ± 8.3	73.5 ± 11.7	81.3 ± 9.7	89.0 ± 10.8	4
Maltose	5	15.5 ± 1.9	25.7 ± 5.5	47.3 ± 10.6	69.0 ± 13.8	72.2 ± 12.2	4

Table 3.6 Initial percent free activities of some glycosidases in lysosome-enriched fractions at 25°

3.8 Latency of glycosidases in a 5 min assay

The initial free activity of the four glycosidases has been investigated using assay times of 5 min and 10 min at 37°. The method is identical to that outlined in 3.2, employing substrate concentrations of 5mM except in the case of p-nitrophenyl β-galactosidase activity, where a concentration of 4mM was used. Total p-nitrophenyl β-galactosidase activity was estimated in lysosome-enriched fractions which had been pre-incubated in acetate-mannitol, pH 5.0 for 40 min in order to destroy latency.

Table 3.7 shows the initial free activity of maltase, p-nitrophenyl α and β-glucosidases, and p-nitrophenyl β-galactosidase in 5 and 10 min assays, expressed as a percentage of the total activity. The values for 5 and 10 min assays are almost identical, suggesting that the observed initial free activity is a real effect and not some 'beginning of experiment' phenomenon suggested in 3 and 4 previously.

Substrate	Concentration (mM)	Assay-time (min)		Number of experiments
		5	10	
p-Nitrophenyl α-glucoside	5	54.8 ± 4.0	60.3 ± 1.8	4
p-Nitrophenyl β-glucoside	5	50.8 ± 1.6	52.5 ± 5.7	4
p-Nitrophenyl β-galactoside	4	51.6 ± 2.5	49.5 ± 1.6	4
Maltose	5	14.7 ± 2.5	14.0 ± 1.6	4

Table 3.7 Effect of assay-time upon the initial percent free activities of glycosidases in lysosome-enriched liver fractions at 37°

3.9 Effects of pre-incubation on latency

The initial free activity of the four glycosidase activities has been examined in lysosome-enriched fractions which have been pre-incubated at 37° in acetate-mannitol for periods of time from 0 to 40 min. Initial free activities were determined by the method described in 3.10.2, using an assay time of 10 min.

Table 3.8 shows the initial percent free activity of maltase, *p*-nitrophenyl α and β-glucosidase, and *p*-nitrophenyl β-galactosidase in lysosome-enriched fractions that had been pre-incubated in acetate-mannitol at 37° . The initial free activities all show a similar trend to that observed earlier (3.8), activities apparently becoming fully accessible to their respective substrates after a pre-incubation period of about 30 min.

Substrate	Concentration (m$\underline{M}$)	37° Pre-incubation time (min)					Number of Experiments
		0	10	20	30	40	
$\underline{p}$-Nitrophenyl α-glucoside	5	60.3 ± 1.8	63.9 ± 12.2	73.6 ± 8.5	94.7 ± 11.1	98.4 ± 10.0	4
$\underline{p}$-Nitrophenyl β-glucoside	5	52.5 ± 5.7	64.2 ± 5.4	79.5 ± 14.1	93.9 ± 10.0	100.0 ± 0.0	4
$\underline{p}$-Nitrophenyl β-galactoside	4	49.5 ± 1.6	52.8 ± 7.2	65.7 ± 5.4	98.3 ± 12.8	110.1 ± 11.7	4
Maltose	5	14.0 ± 1.6	24.6 ± 7.2	71.4 ± 5.4	95.4 ± 12.8	102.8 ± 11.7	4

Table 3.8 Effects of pre-incubation upon initial percent free activity of glycosidase in lysosome-enriched liver fraction

3.10 Effects of p-nitrophenol and p-nitrophenyl glycosides on latency

The effect of p-nitrophenol on the stability of rat liver lysosomes was examined, using the initial free activity (first 10 min of hydrolysis) of maltase activity as an index of the integrity of the lysosome preparation. The method employed was identical to that described in 3.2, employing a substrate concentration of 5mM, and encorporating p-nitrophenol into the acetate-mannitol buffer. No loss of latency was observed with concentrations of p-nitrophenol up to 0.2μmoles per ml.

The effect of p-nitrophenyl glycosides on the stability of rat liver lysosomes was examined in lysosome-enriched liver fractions using the initial percent free p-nitrocatechol sulphatase activity as an index of the integrity of the preparation (Lloyd, 1969a). The substrate of this enzyme, p-nitrocatechol sulphate, unlike nitrophenyl glycosides, is unable to penetrate the lysosomal membrane because of the presence of a sulphate group in the molecule. Latency was investigated by the method outlined in 2.8, employing a substrate concentration of 5mM.

p-Nitrocatechol sulphatase activity showed an initial percent free activity of 14.1 ± 1.6%. The percent free activity was found to be unaffected by the incorporation of a 5mM solution of either of the p-nitrophenyl glucosides or of p-nitrophenyl β-galactoside into the incubation mixture.

* * * * *

The results presented in sections 3.6 to 3.10 tend to support the idea that the high initial percent free activities of p-nitrophenyl glycosidases is a real effect, connected with the permeability properties of such substrates, and not some beginning of experiment phenomenon. It has also been demonstrated (3.10) that the effect is not caused by the direct influence of p-nitrophenyl glycosides or of p-nitrophenol upon lysosomal stability or enzyme activity. It seems reasonable to envisage lysosomes as a heterogeneous collection of vacuoles with graded permeability

properties. The differing latency which one observes for a single enzyme with different substrates is probably a reflection of this heterogeneity.

In order to examine this hypothesis it would be extremely useful if one could measure the latency of a lysosomal enzyme with a variety of substrates, ranging from a small, permeable substrate to an impermeable macromolecular substrate. Maltose and *p*-nitrophenyl α-glucosidase have both been employed as substrates for lysosomal α-glucosidase, and, in order to provide a wider variety of substrate size, the latency of α-glucosidase has also been examined with glycogen and α-fluoroglucoside as substrates.

3.11 Latency of Glycogen-hydrolase activity

Glycogen is known to be a substrate for lysosomal α-glucosidase (Jeffrey et al., 1970). The hydrolysis of glycogen in lysosome-enriched liver fractions was monitored by the assay method outlined in 2.8, employing glucose oxidase in the estimation of the product, glucose. A substrate concentration of 20mg per ml was used (rabbit liver glycogen, type 3, Sigma, London). Latency and sedimentability experiments were always performed on freshly-prepared lysosome-enriched liver fractions by the methods outlined in 3.2 and 3.6. The free activity and sedimentability of maltase activity were always performed on the same preparation and used as an index of integrity.

Table 3.9 shows the percent free activity of glycogen-hydrolase activity for each 10 min interval at 37°, pH 5.0.

Table 3.10 shows the non-sedimentable glycogen-hydrolase activity, expressed as a percentage of the total activity, following pre-incubation of the lysosome-enriched fraction in acetate-mannitol for periods of time up to 60 min.

The initial percent free activity (first 10 min of incubation) of glycogen-hydrolase is similar to that obtained with maltose as substrate, suggesting that this figure also represents the proportion of broken and damaged lysosomes initially present in the preparation. The free activity of glycogen hydrolase rose slowly, but did not reach the constant value observed with previous substrates. Even after an incubation period of 60 min, free glycogen-hydrolase activity was less than that observed in the presence of 0.1% Triton X-100. The percent non-sedimentable activity of glycogen-hydrolase was very similar to those observed with maltose or p-nitrophenyl α-glucoside (Table 3.5), reaching a maximum value of about 70% after 50 min. The remaining 30% of the activity which is still sedimentable is presumably adsorbed or

Substrate Concentration	Time interval (min)						Number of Experiments
	(0-10)	(10-20)	(20-30)	(30-40)	(40-50)	(50-60)	
20mg/ml	12.5 ± 6.7	21.7 ± 14.7	25.1 ± 13.1	62.3 ± 16.4	67.7 ± 11.7	79.0 ± 3.4	4

Table 3.9 Percent free activity of glycogen-hydrolase in lysosome-enriched fractions at 37°

37° Pre-incubation time (min)							Number of Experiments
0	10	20	30	40	50	60	
1.6 ± 1.4	5.8 ± 2.2	20.5 ± 13.4	50.7 ± 8.4	68.1 ± 8.6	70.1 ± 3.6	67.5 ± 5.5	4

Table 3.10 Non-sedimentability of glycogen-hydrolase activity in lysosome-enriched fractions

tightly bound to the lysosomal membrane and is not freely released. This may explain why glycogen hydrolase never became fully available. With a substrate as large as glycogen, the enzymes could well become non-sedimentable before they become freely accessible. A smaller substrate might be freely accessible to the enzymes before they become non-sedimentable.

Such results are not inconsistent with the hypothesis postulated earlier. It seems likely that *p*-nitrophenyl substrates would pass more easily through the lysosomal membrane than either maltose or the enzyme. It seems probable from the results shown in Tables 3.9 and 3.10 that for enzymes to pass through the membrane, a similar degree of lysosomal damage (most likely to be complete membrane-rupture) is necessary as for glycogen to enter, since the values of free and non-sedimentable activities with this substrate are very similar.

3.12 <u>Latency of α-fluoroglucosidase activity</u>

Glycosyl fluorides have been shown to be extremely good substrates for a number of non-lysosomal glycosidases (Barnett et al., 1967a, b). In particular, α-fluoroglucoside has been shown to be rapidly hydrolysed by a rat intestinal α-glucosidase (Barnett, 1971). α-Fluoroglucoside has the same molecular weight as glucose and is therefore likely to be able to permeate the lysosomal membrane equally well as glucose. Unfortunately, the limited supply of this substrate did not permit the confirmation of this fact by the technique of osmotic protection which requires the use of large amounts of solute in order to prepare isotonic solutions. It is also probable that if α-fluoroglucoside is hydrolysed by lysosome-enriched fractions, the same enzyme, whose latency properties have already been examined with glycogen, maltose and *p*-nitrophenyl α-glucoside, will be responsible.

The hydrolysis of fluoroglucosides can be assayed by the number of techniques (Barnett et al., 1967a), and preliminary experiments with lysosome-enriched fractions showed that the reaction could be conveniently followed by estimating glucose production, using the method outlined earlier (2.8) for maltase activity. The reaction was shown to proceed linearly with time and display typical enzymic behaviour to variations in enzyme or substrate concentrations. Kinetic examination of α-fluoroglucosidase activity by the methods of Lineweaver and Burk (1934) and Dixon and Webb (1964) confirmed the usefulness of α-fluoroglucoside as a substrate for the lysosomal enzyme. α-Fluoroglucoside was found to be hydrolysed at a rate of 4-5 times that of maltose, with a Michaelis constant (Km app.) of 2.9-3.3m*M*. The activity of α-fluoroglucosidase was found to be unaffected by the encorporation of 0.1% Triton X-100 into the incubation mixture.

The latency and sedimentability of α-fluoroglucosidase has been examined in freshly prepared lysosome-enriched liver fractions, as

Substrate Concentration (mM)	Time-interval (min) (0-10)	(10-20)	(20-30)	(30-40)	(40-50)	(50-60)	Number of Experiments
1	28 ± 3.0	43 ± 3.0	47 ± 6.5	77 ± 3.5	100 ± 10.0	107 ± 7.5	
2	24 ± 2.0	41 ± 4.0	60 ± 3.5	71 ± 8.5	100 ± 1.0	94 ± 8.5	
4	30 ± 1.0	42 ± 1.5	64 ± 12.0	89 ± 2.0	100 ± 1.0	97 ± 19.0	2
5	28 ± 1.0	43 ± 1.0	62 ± 1.5	78 ± 8.0	101 ± 1.5	106 ± 4.0	
10	37 ± 1.5	48 ± 6.5	62 ± 1.0	90 ± 3.0	103 ± 3.5	107 ± 7.0	

Table 3.11 Percent free activity of α-fluoroglucosidase in lysosome-enriched liver fractions at 37°

37° Pre-incubation time (min) 0	10	20	30	40	50	60	Number of Experiments
2.6 ± 3.6	6.8 ± 14.1	42.2 ± 3.5	68.1 ± 6.8	69.0 ± 4.7	80.1 ± 5.3	77.6 ± 8.8	4

Table 3.12 Non-sedimentability of α-fluoroglucosidase activity in lysosome-enriched liver fractions

described in 3.8 and 3.9. The free activity of maltase was also examined in the same lysosomal preparation, and used as an index of the integrity of the same preparation.

The time-course of hydrolysis of α-fluoroglucoside at 37°, pH 5.0, was examined at substrate concentrations of 1, 2, 3, 4, 5 and 10mM, both in the absence, and in the presence of 0.1% Triton X-100. The assay of non-sedimentable activity was performed at a substrate concentration of 5mM.

The results in Table 3.11 show the free activity of α-fluoroglucosidase for each 10 min interval, expressed as a percentage of the total activity, at each substrate concentration.

Table 3.12 shows the non-sedimentable activity of α-fluoroglucosidase, expressed as a percentage of the total activity, following pre-incubation of the lysosome-enriched preparation in acetate-mannitol for periods of time up to 60 min.

The percent non-sedimentable activity of α-fluoroglucosidase was found initially to be low (2.6%), rising to a maximum value of about 80% after an incubation period of 50 min. The release of α-fluoroglucosidase was almost identical to that observed with glycogen, maltose and *p*-nitrophenyl α-glucoside as the substrate, suggesting that the same enzyme is responsible for the hydrolysis of all four substrates.

In spite of this low sedimentability, α-fluoroglucosidase displayed an initial percent free activity of about 30% in preparations which showed typically low initial percent free maltase activity. Such a result suggests that α-fluoroglucoside is able to penetrate the membrane of intact lysosomes.

One would have predicted, however, that α-fluoroglucoside entry into the lysosome would have been very rapid and that α-glucosidase would have displayed little, or no, latency. The unexpectedly low

initial percent free activity of α-fluoroglucosidase may be a consequence of the high activity of α-glucosidase towards this substrate. Just as Baudhuin (1964) has shown the apparent latency of microbody catalase to be a result of the rate-limitations imposed by the entry of hydrogen peroxide, so the observed latency of α-fluoroglucosidase may be a result of the relatively slow rate of penetration of α-fluoroglucosidase into intact lysosomes. This is supported by the fact that the Km app. for free α-fluoroglucosidase activity was slightly higher than the value observed for total activity (Table 3.13), and that the initial percent free activity of α-fluoroglucosidase rose at the highest substrate concentration (10mM). Unfortunately, the limited supply of substrate did not permit further investigation into this effect.

If the initial percent free activity of α-fluoroglucosidase had displayed no such concentration dependence, one would have needed to postulate the unlikely explanations of the differing permeability properties of α-fluoroglucoside and glucose, or the existence of some lysosomes, possibly primary lysosomes, which neither glucose nor α-fluoroglucoside could initially penetrate.

3.13 Discussion

The work reported in this chapter was motivated by an interest in lysosomal latency, in particular, the possibility of altering the apparent latency of an enzyme by using substrates with differing permeability properties. The theory surrounding this type of experiment has been discussed at length by de Duve (1965) and in Chapter 1. The fundamental concept underlying many of the experiments described in this chapter is that if the latency of an enzyme represents the partial permeability of the lysosomal membrane to the substrate used in the enzyme's detection, then the observed latency should be dependent on the concentration of substrate.

The latency of maltase and p-nitrophenyl α-glucosidase have been shown to differ, although the evidence of sedimentability experiments suggests that the same enzyme, α-glucosidase, is responsible for the hydrolysis of both substrates. The initial percent free activity of p-nitrophenyl α-glucosidase, although about five times higher than the value observed with maltose as substrate, showed no concentration dependence. In agreement with this finding the values of Km app. for free and total activities of p-nitrophenyl α-glucosidase were identical (Table 3.13). Similar high initial percent free activities were observed with p-nitrophenyl β-glucosidase and p-nitrophenyl β-galactosidase.

In order to explain how an enzyme can be simultaneously accessible to one substrate and inaccessible to another, a simple model has been proposed. The model is based on the heterogeneity of the lysosome preparation with regard to the permeability of the lysosomal membrane. According to this model, the high initial availability of p-nitrophenyl glycosidases can be explained by the ability of p-nitrophenyl glycosides to penetrate the intact membrane of some lysosomes. Penetration of such substrates is assumed to occur at a rate which is sufficient to

	Km app. (mM)	
	Free activity	Total Activity
Maltase	2.2	2.2
p-Nitrophenyl α-glucosidase	2.0	2.0
α-Fluoroglucosidase	3.2	3.0
p-Nitrophenyl β-glucosidase	1.0	1.0
p-Nitrophenyl β-galactosidase	1.1	1.1

Table 3.13 Michaelis constants (Km app.) of free and total glycosidase activities in lysosome-enriched liver fractions

maintain the intralysosomal substrate concentration equal to that in the bulk assay medium. Conversely, the low initial availability of maltase is explained by its inability to penetrate any lysosomes.

This model has been put to the test in a number of subsequent investigations with substrates of differing size. Glycogen has been used as a macromolecular substrate for lysosomal α-glucosidase, and α-fluoroglucoside has been employed as a permeant substrate molecule for the same enzyme. The observed latency of α-glucosidase was different for each substrate. In keeping with a substrate molecule as large as glycogen, the availability of α-glucosidase with glycogen as the substrate was found to be concomitant with its release from the lysosome. α-Fluoroglucoside was shown to possess the ability to penetrate the membrane of intact lysosomes, and thus yield a high value for the initial percent free activity of α-glucosidase. Its rate of penetration was not, however, sufficient to maintain an intralysosomal substrate concentration equal to that in the bulk assay medium. The behaviour of both glycogen and α-fluoroglucoside could be explained by the heterogeneity of the lysosomal preparation with respect to their permeability properties of the lysosomal membrane.

The loss of latency which is observed when lysosomes are incubated at 37° in an acidic buffer could also be explained by this model. Rather than the increase in the permeability of the lysosomal membrane that has been suggested by several authors (Shibko et al., 1965; Shibko and Tappel, 1965; Misch and Misch, 1969; Badenoch-Jones and Baum, 1973), such latency loss is suggested to accompany the increase in the number of lysosomes whose enzymes are freely available to substrate. In agreement with this suggestion, Hainsworth and Wynn (1966) have observed that rat liver lysosomes become permeable to glycerol, sorbose and *p*-nitrophenol after a 30 min incubation at room temperature.

Clearly, the latency of lysosomal enzymes is not simply a question of the molecular weight of the substrate employed; other factors, noteably the hydrophobicity and charge of the molecule, and the rates of penetration and hydrolysis are also important parameters to consider (Lee, 1970; Robinson and Wilcox, 1969; de Duve, 1965). The very high initial availability which p-nitrophenyl glycosidase exhibit is probably connected with the hydrophobicity of the aromatic ring in the p-nitrophenyl moiety of such compounds. The 'molecular weight barrier', which is often referred to in connection with lysosome permeability, is not an absolute value, applicable to all types of compound, but rather an empirical figure, only applicable to a specific homologous series of molecules, such as oligopeptides or carbohydrates. It has also been suggested that the latency of lysosomal enzymes may depend on the properties of individual vacuoles, e.g. natüre, size, origin. The lysosomes obtained from rat spleen have been shown to have quite different fragility properties to those obtained from rat liver (Bowers et al., 1964).

Although seemingly a relatively homogenous tissue, liver is in fact composed of a significant proportion of cells other than hepatocytes. Dauost and Cantero (1959) showed that only about 60% of the cells of normal rat liver were hepatocytes, and that the proportion of these cells to other cell types can change drastically during experimental procedures such as those which involve the experimental induction of liver cancers. In later investigations where the latency of exogenous enzymes are measured, these considerations will be particularly pertinent.

CHAPTER 4

LATENCY OF SOME EXOGENOUS GLYCOSIDASES IN RAT LIVER LYSOSOMES

4.1 Introduction

The term 'lysosomotropic' has been recently coined to describe substances that are taken up selectively into lysosomes, irrespective of their chemical nature or mechanism of uptake (de Duve et al., 1974). It is now known that some observations made more than a century ago, for instance the early studies on vital staining, were merely a manifestation of this phenomenon. The recognition of the importance of lysosomotropism has led to the proposal of new types of drug-therapy. Almost any foreign substance could in theory be directed into the lysosomal system if it were attached to an appropriate carrier.

There are two major modes of entry of exogenous substances into lysosomes: endocytosis and permeation. The main, and probably only, route of entry for large polar molecules, such as proteins, is by endocytosis, which depends on their enclosure within vacuoles derived from invaginations of the plasma membrane. In most cases, these pinocytic vacuoles subsequently fuse with lysosomes, either primary or secondary, to form heterolysosomes in which enzymic degradation of the endocytized material occurs.

A number of authors have demonstrated uptake of exogenous enzymes into lysosomes. Straus (1958, 1962, 1964, 1967, 1971), using a double staining technique for exogenous horseradish peroxidase and lysosomal acid phosphatase, has demonstrated cytochemically the fusion of peroxidase-laden pinocytic vacuoles with lysosomes to form large heterolysosomes. Jacques (1968) has demonstrated that, after intravenous injection of yeast invertase into rats, the enzyme was taken up largely by the liver, where it decays slowly, with a half-life of 5.4 days, residing within particles identifiable as lysosomes. The lysosomal location of yeast invertase was also confirmed by its ability to digest the sucrose which had accumulated within lysosomes following its

injection into rats. The recent advances with liposome-entrapped enzymes (Gregoriadis and Ryman, 1972; Gregoriadis et al., 1971, 1974) have also proved useful in examining the fate of endocytized enzymes.

The latency of endocytized enzymes has been demonstrated by both Jacques (1968) and Lloyd (1969b). The latency of yeast invertase, observed by Jacques (1968), is not altogether surprising in view of the impermeability of the lysosomal membrane to sucrose, the substrate used in the assay of invertase. Jacques (1968), in similar work with horseradish peroxidase, found the endocytized enzyme to be non-latent in intact lysosomes. The non-latency of horseradish peroxidase was attributed to the high permeability of the lysosomal membrane to N,N-dimethyl-p-phenylenediamine (NNPD). Lloyd (unpublished) has confirmed the penetration of NNPD into intact lysosomes by demonstrating its inability to afford prolonged osmotic protection to rat liver lysosomes. In contrast with the results of Jacques, John et al. (1967) found the myeloperoxidase of human neutrophil lysosomes to be latent. Whether this result is a consequence of the use of guaicol as the hydrogen donor, or a reflection of the differing permeability properties of lysosomes of different origin remains unresolved.

Lloyd (1969b) demonstrated the latency of amyloglucosidase and emulsin in lysosome-enriched liver fractions from rats injected with these enzymes. The latency of amyloglucosidase was again not surprising in view of the use of maltose as the substrate in its detection. Emulsin was also shown to be almost wholely latent when cellobiose or p-nitrophenyl β-glucoside were employed as substrates. The endogenous β-glucosidase activity, measured with p-nitrophenyl β-glucoside, was, however, found to be fully available in intact lysosomes, and was suggested to demonstrate the impermeability of p-nitrophenyl β-glucoside, and the location of endogenous β-glucosidase on the cytoplasmic face of the lysosomal membrane. As was discussed at length in the previous

chapter, the latter result was almost certainly attributable to the use of Triton X-100 for estimations of total β-glucosidase activity. If, as is suggested in the previous chapter, p-nitrophenyl β-glucoside is able to penetrate the membrane of intact lysosomes, it is difficult to explain how the exogenous β-glucosidase activity could be completely latent. In order to take this question further, the latency of amylo-glucosidase and emulsin have been re-examined.

4.2 Administration of exogenous enzymes

Male Wistar rats (300-400g) were starved overnight and enzyme solutions in isotonic saline (0.5ml) injected into the femoral vein under light ether anaesthesia as described in 2.3. The enzyme injected was *Aspergillus niger* amyloglucosidase (Qureshi, 1967) at 250mg/Kg body weight, or almond emulsin β-glucosidase (Sigma) at 125mg/Kg body weight. Control animals received an injection of isotonic saline.

4.3 Assay of amyloglucosidase and emulsin β-glucosidase

The activity of amyloglucosidase was assayed with both maltose and *p*-nitrophenyl β-glucoside as substrate. Emulsin β-glucosidase activity was estimated by its activity towards cellobiose and *p*-nitrophenyl β-glucoside. The general assay methods for all enzymes are provided in Chapter 2.

4.4 Serum and liver concentrations of emulsin and amyloglucosidase

Male Wistar rats (300-400g) were starved overnight, injected as described in 4.2, and allowed to revive. After the appropriate time interval, the rats were re-anaesthetised with ether, and blood was collected from the descending aorta. The blood was allowed to clot at 4° and serum separated by centrifugation at 1500g for 10min. Immediately after collection of blood, the liver was perfused *in situ* with 20ml isotonic saline, dissected free, blotted dry, weighed and homogenized vigorously as described in 2.5. Sera and liver homogenates were maintained at 0° and assayed within 1 hour of preparation.

Table 4.1 shows the maltase activity in the blood and liver of rats that had received an injection of amyloglucosidase. The activity is expressed as a percent of the injected dose, after allowance for the innate maltase activity of the serum and liver. In the case of serum, innate maltase activity was very low, but in liver it was quite significant.

Table 4.2 shows the β-glucosidase activity, assayed by its activity towards *p*-nitrophenyl β-glucoside, in the blood and liver of rats that had received an injection emulsin. The serum of control rats contained negligible β-glucosidase activity, so that only the values for liver had to be corrected for endogenous activity.

Immediately after injection, the serum concentrations of emulsin and amyloglucosidase were consistent with the doses of the injected enzymes. The serum concentrations of both enzymes fell markedly within 4 hours of injection. Emulsin was almost totally cleared from the bloodstream within 4 hours, whereas about 50% of the original dose of amyloglucosidase remained in the blood after the same time-interval. It is impossible to say from these preliminary investigations whether this difference is a consequence of the higher dosage of amyloglucosidase, or whether it is connected with the chemical properties of the two enzymes.

Time after injection (h)	Maltase Content			
	Serum		Liver	
	% of injected enzyme, per ml	Exogenous enzyme activity (Maltase activity of control rats = 1)	% of injected enzyme, per liver	Exogenous enzyme activity (Maltase activity of control rats = 1)
1	15.8	57.7	3.1	1.9
2	13.0	44.2	3.6	2.2
3	12.0	43.5	7.2	3.5
4	8.5	31.6	10.0	4.6

Table 4.1 Serum and liver concentrations of maltase after intravenous injection of *Aspergillus niger* amyloglucosidase into rats.

Time after injection (h)	β-glucosidase Content		
	Serum	Liver	
	% of injected enzyme, per ml	% of injected enzyme, per liver	Exogenous enzyme activity (β-glucosidase activity of control rats = 1)
0.5	9.8	14.8	68.4
1	8.4	15.6	76.4
2	1.7	27.9	135.4
3	0.2	22.6	110.0
4	0.007	21.4	103.8

Table 4.2 Serum and liver concentrations of β-glucosidase after intravenous injection of emulsin β-glucosidase into rats.

The liver concentrations of the exogenous enzymes may provide some answers to this question. It is evident from the results in tables 4.1 and 4.2 that the liver is one of the major organs concerned with the removal of emulsin and amyloglucosidase from the blood of injected rats. Amyloglucosidase concentration in the liver of injected rats showed a gradual increase, compatible with the rate of clearance of amyloglucosidase from the blood. Four hours after injection with the enzyme, the liver concentration was still rising and had reached a value of 10% of the original dose. The appearance of emulsin activity in the liver of rats injected with the enzyme, was more rapid, and to a higher degree, than that shown by amyloglucosidase. The liver concentration of emulsin had reached a peak after 2h, in agreement with its rapid removal from the blood. After this peak of activity a gradual decline in the liver concentration of emulsin was noted, presumably due to the digestion of the enzyme by lysosomal proteinases.

The liver concentration of the enzymes is very difficult to interpret because it is the result of two opposing effects: uptake and digestion. Nevertheless, both injected enzymes have been shown to be cleared from the bloodstream by the liver, where they presumably enter the lysosomal system and are digested within hetero-lysosomes. The experiments described here, however, were performed on individual rats whose blood volumes and liver function might vary. In order to avoid this problem, and to obtain a more realistic estimate for the rates at which emulsin and amyloglucosidase are cleared from the blood, experiments were performed in which serial blood samples were taken from injected rats, rather than a single sample after a given time interval from a number of rats.

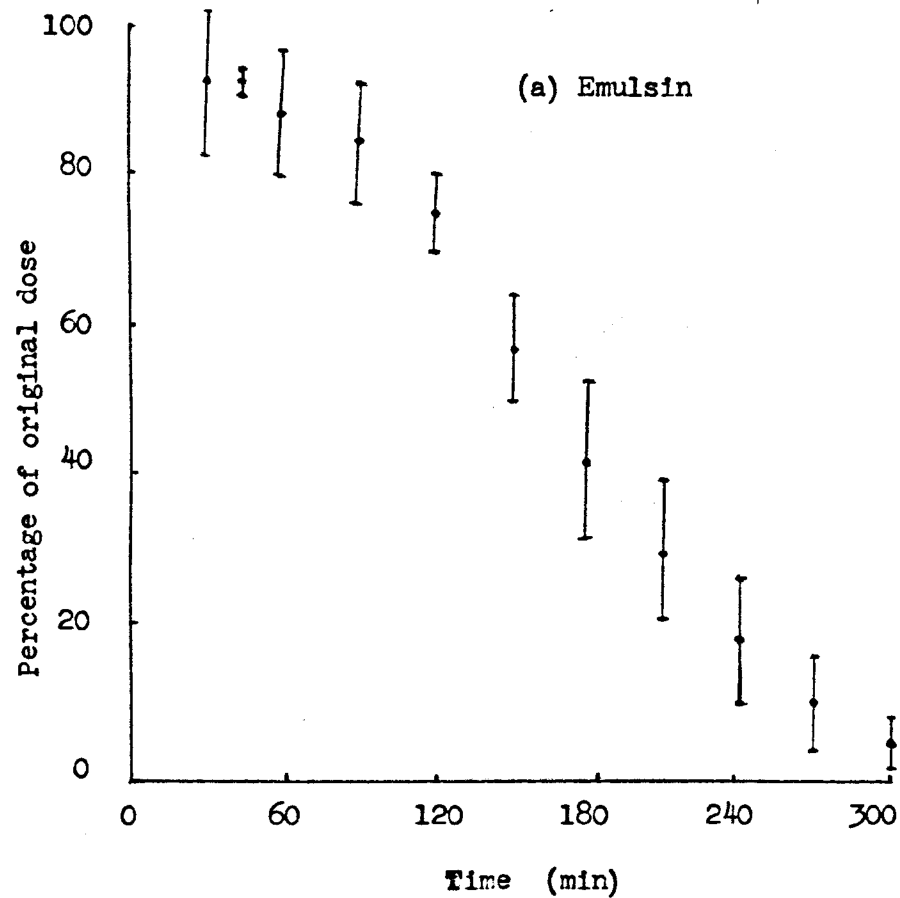
In these experiments 20μl blood samples were taken at intervals from rats, maintained under light anaesthesia with an ether:air mixture

of 1:10, as described in 2.4. Samples, resuspended in 1ml isotonic saline containing 1 unit per ml Heparin, were stored at 4°, and assayed for maltase and β-glucosidase activity as described in 2.8. For each rat a blood sample was taken immediately before injection and used to estimate the endogenous enzyme activity. A sample taken immediately after injection of enzyme was used as the zero time blood concentration of the exogenous enzyme, and was used to estimate the blood volume of individual rats (mean blood volume 26.5ml). At each time interval the blood concentration of emulsin or amyloglucosidase was calculated as a percentage of the original dose, after suitable correction for innate β-glucosidase or maltase activity.

Fig. 4.1 shows the blood concentrations of emulsin and amyloglucosidase, expressed as a percentage of the original dose. Results are the mean of 4 experiments.

The blood concentration of emulsin showed a gradual decline after injection, until, after 5h, only about 5% of the injected dose remained. This decline proceeded almost linearly with time, suggesting that the uptake system for emulsin had been saturated. The method of blood-sampling proved somewhat unsatisfactory in the case of amyloglucosidase. Within the first 90 min of amyloglucosidase injection, the blood appeared to be extremely viscous and clotted very readily, making accurate sampling almost impossible. This probably explains the inaccuracy in the early part of the amyloglucosidase curve. Nevertheless, amyloglucosidase was shown to be cleared slowly from the blood of injected rats, over 50% of the original dose remaining after 5h had elapsed. This slow rate of removal may be connected with the high dose employed. Huijing et al. (1973) found the recovery of amyloglucosidase in the livers of injected rats to be dependent on the amount administered.

If one compares the results in Fig. 4.1 to those in Tables 4.1



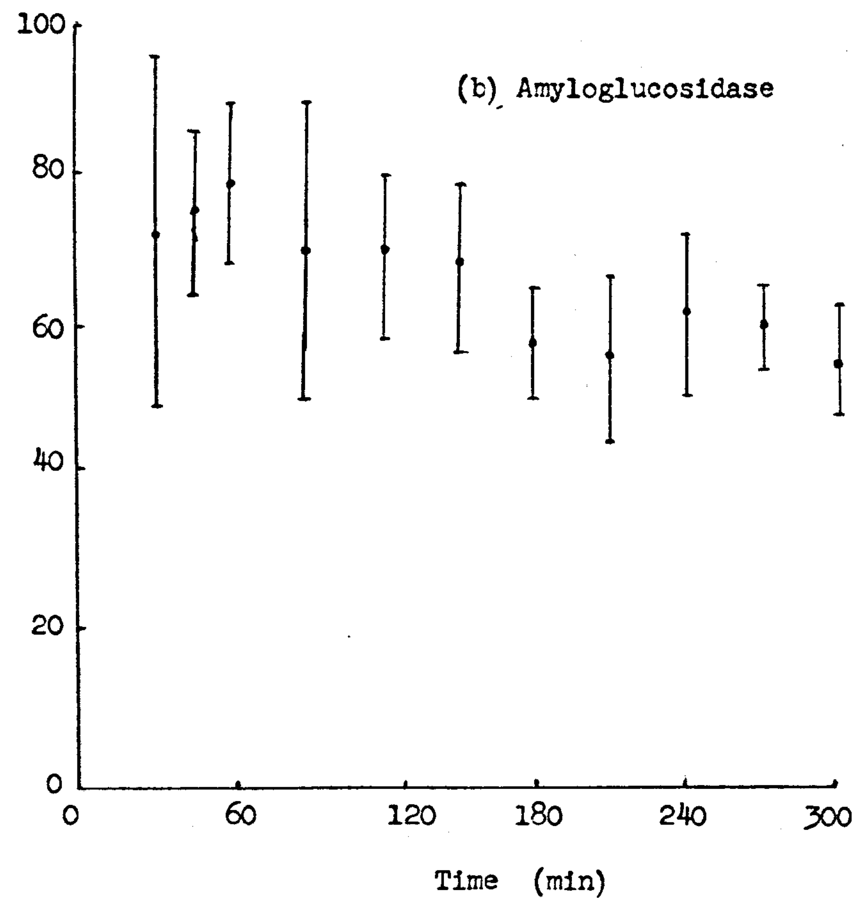


Fig. 4.1 Blood concentrations of emulsin (a) and amyloglucosidase (b) after intravenous injection of these enzymes into rats

and 4.2, there are some minor discrepancies. In previous experiments, in which rats were allowed to revive after injection of the enzyme, emulsin appeared to be cleared much more rapidly from the blood; after 3h very little enzyme remained. This effect may have been the result of prolonged ether anaesthesia causing some impairment, or loss of efficiency, of liver function.

4.5 Subcellular distribution of exogenous enzymes

Male Wistar rats (300-400g) were starved overnight and injected intravenously, under light anaesthesia, with purified *Aspergillus niger* amyloglucosidase (Qureshi, 1967) (250mg/Kg body weight), with almond emulsin β-glucosidase (Sigma) (125mg/Kg body weight), or with isotonic saline only. Enzymes were dissolved in isotonic saline and administered in a total volume of 0.5ml. Rats were killed 4.0-4.5h after injection and their livers quickly removed and subjected to differential centrifugation at 4° as described in 2.2. Nuclear (N), heavy mitochondrial (M), light mitochondrial (L), microsomal (P) and final supernatant (S) fractions were prepared. Pellets, gently resuspended in ice-cold 0.25M-mannitol, and the final supernatant fraction were maintained overnight at -30°, it having been ascertained that such storage resulted in no loss of enzyme activity. After storage, the fractions were thawed, diluted if necessary, and assayed for enzyme and protein concentration. Fractions were examined for p-nitrophenyl α and β-glucosidase, p-nitrophenyl β-galactosidase, p-nitrocatechol sulphatase and maltase activities, by the assay methods described in 2.8. A substrate concentration of 5mM was employed in all cases except p-nitrophenyl β-galactosidase, where a substrate concentration of 4mM was employed. The protein concentration of the original liver homogenate and the various subcellular fractions was estimated by the method of Lowry et al. (1951).

The enzyme activity in each fraction was calculated as a percentage of the total recovered activity, the same calculation being performed for the protein concentration of each fraction. From these findings the Relative Specific Activity of each enzyme was calculated for all the fractions. In the case of the endogenous activities, the Relative Specific Activity is the ratio of the percentage of the total activity

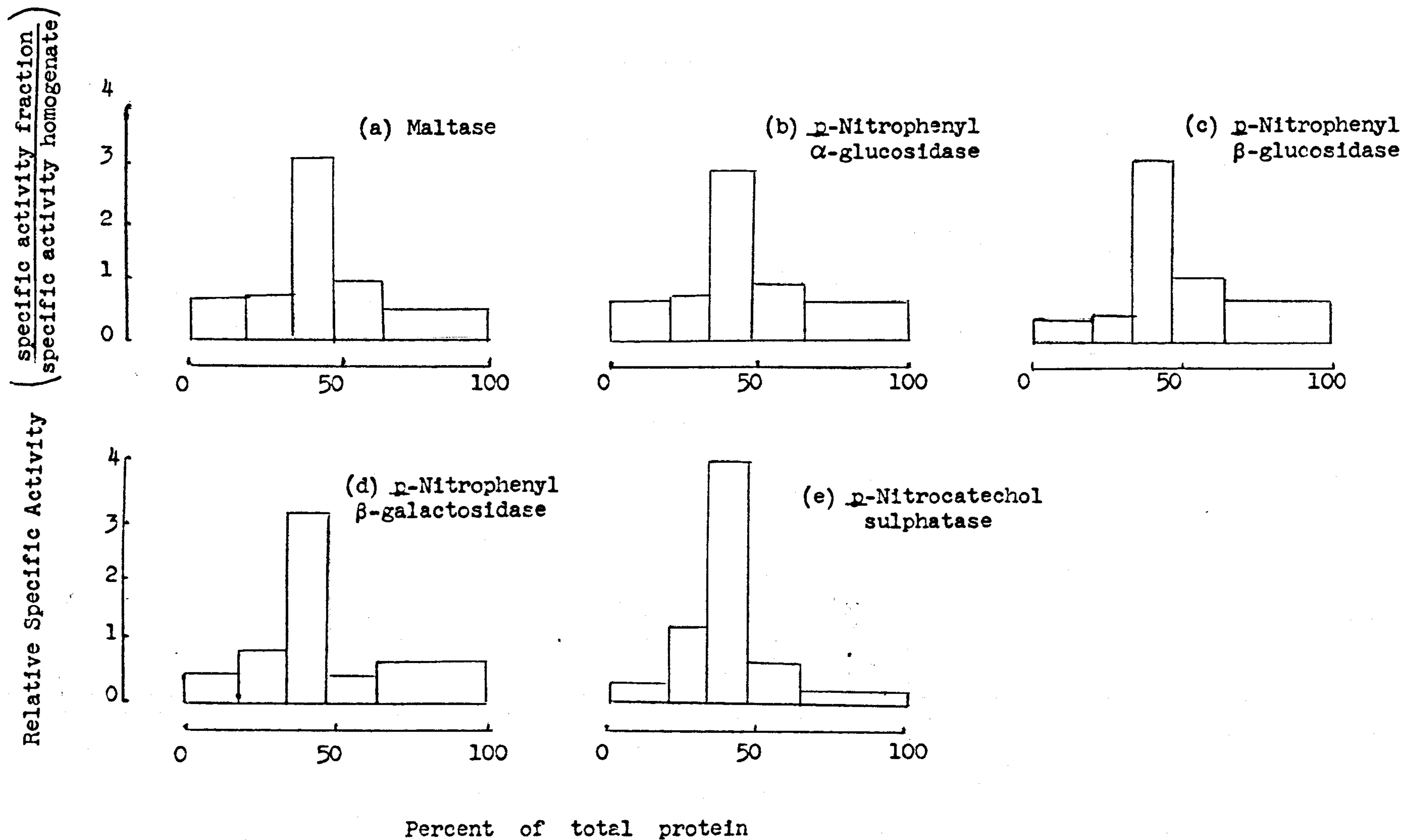


Fig. 4.2 Subcellular distribution of some endogenous glycosidases in rat liver

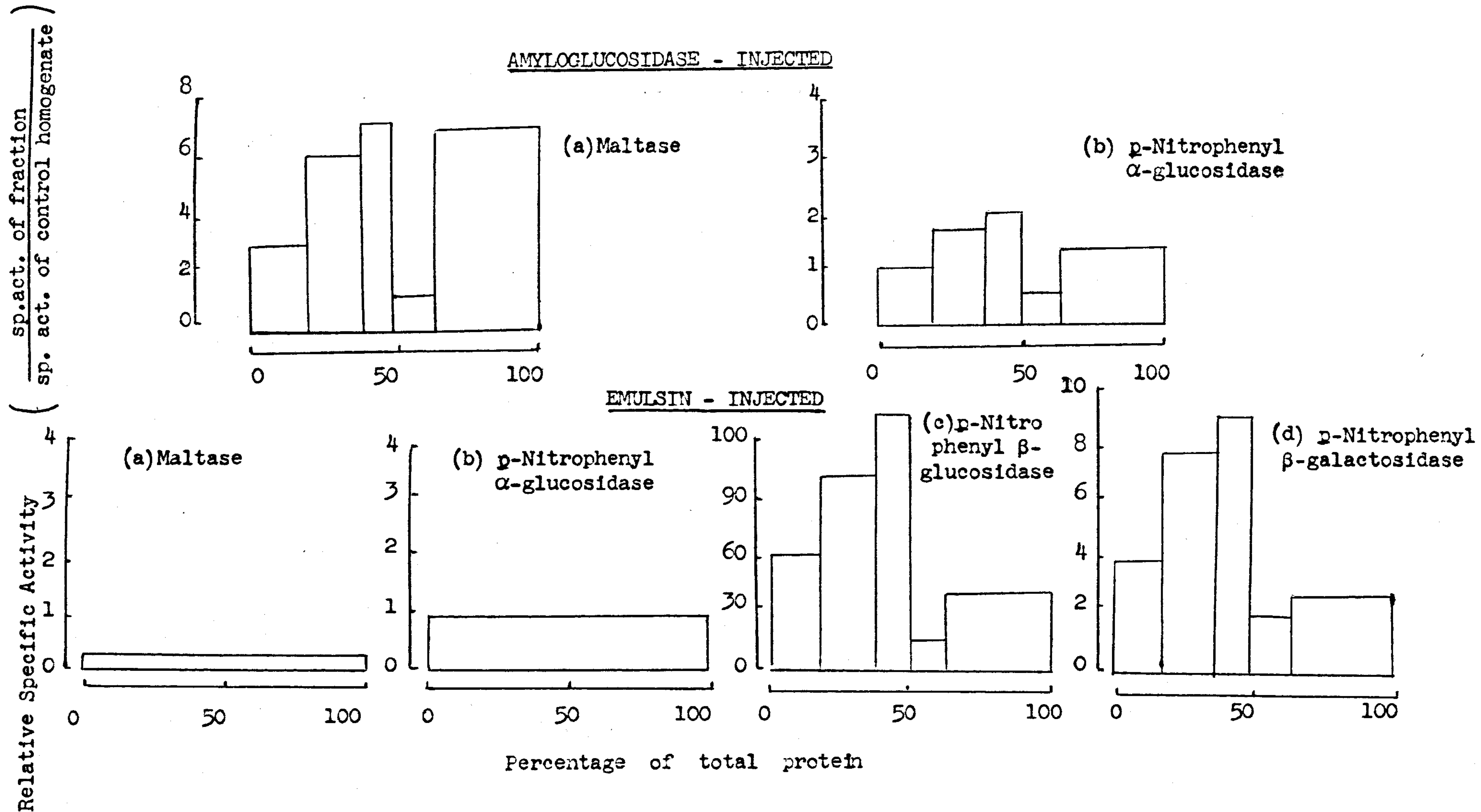


Fig. 4.3 Subcellular distribution of additional enzyme activities after injection of emulsin and amyloglucosidase into rats

in the fraction to the percentage of the total protein in the fraction. Block diagrams were prepared according to de Duve et al. (1955), fractions appearing from left to right in the order of their isolation (Fig. 4.2).

All the enzyme activities examined in control rats have been previously shown to be lysosomal in origin, and all typically displayed their highest specific activity in the light mitochondrial fraction. The most interesting aspect of these results is the distribution of maltase and *p*-nitrophenyl α-glucosidase activities. These activities showed almost identical distribution, further evidence to support the suggestion in the previous chapter that the same enzyme is responsible for both activities.

Similar block diagrams (Fig. 4.3) were constructed for the additional enzyme activities which resulted from the injection of emulsin or amyloglucosidase. In this case, however, the relative specific activity of each additional activity was calculated by comparing its specific activity with that of control homogenates. In some cases, additional activities were too low for individual fractions to be assayed with accuracy; relative specific activities of the additional activity in the whole homogenate are therefore given.

Following injection of amyloglucosidase into rats, maltase activity was elevated to a value of 6-7 times that of control animals, whilst *p*-nitrophenyl α-glucosidase activity only displayed approximately a third of this rise. These results are in keeping with the affinity of amyloglucosidase for maltose and *p*-nitrophenyl α-glucoside (Pazur and Klepp, 1962). *p*-Nitrophenyl β-glucosidase, *p*-nitrophenyl β-galactosidase and *p*-nitrocatechol sulphatase activities remained the same in magnitude and distribution to controls.

The additional activities of maltase and *p*-nitrophenyl α-glucosidase,

attributable to amyloglucosidase, displayed almost identical distributions; amyloglucosidase was present in significant amounts in all the subcellular fractions. Although such a distribution is probably consistent with the vacuolar location of an endocytized enzyme, it is possible that such a diffuse distribution may have resulted from the presence of blood in the liver. Previous experiments (Table 4.1) have shown that about 50% of the injected dose of amyloglucosidase remains in the blood after 4h. Experiments were therefore performed on amyloglucosidase-injected rats whose livers had been perfused with 20ml isotonic saline before fractionation. Such livers yielded almost identical enzyme distributions, both for endogenous and exogenous activities, to non-perfused liver.

The distribution of amyloglucosidase is therefore a true reflection of the number and type of vacuole in which the endocytized enzyme may be found 4h after injection. Although the present fractionation scheme differed from that of Huijing et al. (1973), the distribution of amyloglucosidase was found to be very similar; the highest specific activity of the enzyme being observed in the heavy and light mitochondrial fractions. Such a distribution is consistent with the ability of amyloglucosidase to deplete liver glycogen levels in patients with Pompe's disease (Hug and Schubert, 1966). The ability of amyloglucosidase to deplete the liver glycogen levels in conditions where non-lysosomal enzymes are deficient (Huijing et al., 1973; Cuthbertson et al., 1966), and accumulation of glycogen is cytoplasmic, is somewhat surprising, although the present investigations have shown a significant amount of amyloglucosidase to be present in the soluble fraction. The most likely explanations for this soluble activity are that it derives from the breakage of large phagosomes or heterolysosomes which are too fragile to withstand homogenization, or that the endocytized enzyme is located

in extremely small vacuoles which do not sediment at the speeds employed. It seems doubtful that the endocytized enzyme would be able to diffuse out of lysosomes into cytoplasm.

Following injection of emulsin into rats, all the control activities except _p_-nitrocatechol sulphatase were elevated. The most dramatic effect of emulsin was the increase in _p_-nitrophenyl β-glucosidase activity to a value of about 60-70 times that of controls. A somewhat unexpected result was the elevation of _p_-nitrophenyl β-galactosidase to a value of approximately 10 times that of controls. Conchie et al. (1967) and Hayworth and Walker (1962) have shown that emulsin possesses both β-glucosidase and β-galactosidase activities; the elevation of _p_-nitrophenyl β-galactosidase activity in emulsin-injected rats is undoubtedly a manifestation of this fact. Another unexpected result was the increase in maltase and _p_-nitrophenyl α-glucosidase activities. The doubling of maltase activity and the 50% increase in _p_-nitrophenyl α-glucosidase activity were caused by the multiple specificity of emulsin itself, or by the presence of a contaminant α-glucosidase in the preparation. Substrate contamination was eliminated as a possible cause.

The distribution of the additional activities, all of which can be attributed to emulsin, were calculated and are depicted in Fig. 4.3. Additional activities of maltase and _p_-nitrophenyl α-glucosidase were too low for individual fractions to be assayed with accuracy; relative specific activities of the additional activity in the whole homogenate are therefore given. Like amyloglucosidase, emulsin was located predominantly in the mitochondrial fractions, with relatively less activity than amyloglucosidase in the nuclear and supernatant fractions. This slight difference in distribution of amyloglucosidase and emulsin may be connected with the rates at which the enzymes are cleared from the bloodstream (Fig. 4.1) or may simply be a reflection of the different doses at which the enzymes were administered.

The distribution of amyloglucosidase and emulsin have both been shown to differ considerably from the endogenous lysosomal enzymes, both exogenous enzymes being widely distributed in significant amounts throughout all subcellular fractions. Clearly, if such distributions are a reflection of the number and variety of vacuoles in which the endocytized enzymes are present, the latency of emulsin and amyloglucosidase might well differ from that of endogenous enzymes.

4.6 Latency of emulsin and amyloglucosidase in lysosome-enriched liver fractions of injected rats

Animals, treated 4h previously with either the enzymes or isotonic saline, were killed by a blow to the back of the head, and lysosome-enriched liver fractions prepared as described in 2.1. The initial latency of *p*-nitrophenyl α and β-glucosidase, maltase, *p*-nitrocatechol sulphatase, and cellobiase activities were estimated in freshly-prepared lysosome-enriched fractions as described in 3.2, employing an assay time of 10 min at 37°.

Except in the case of *p*-nitrophenyl β-glucosidase activity, total activities were estimated by the incorporation of 0.1% Triton X-100 into the incubation mixture. Because rat liver lysosomal β-glucosidase is inhibited by 0.1% Triton X-100 (3.4), total activities of *p*-nitrophenyl β-glucosidase in saline and amyloglucosidase-injected rats were measured in lysosome-enriched fractions which had been pre-incubated at 37° in acetate-mannitol for 40min, prior to the 10min assay. With rats injected with emulsin, however, all the total activities were measured in the presence of 0.1% Triton X-100. This was permissible because emulsin β-glucosidase was not inhibited by Triton X-100, and was present in amounts that effectively swamped the endogenous enzyme (see Table 4.2). This method was considered preferable to pre-incubation as a means of estimating total activities because an incubation of 40 min at 37° was found to cause a loss of β-glucosidase activity, probably by the degradation of exogenous enzyme by lysosomal proteinases within intact lysosomes. A substrate concentration of 5m*M* was employed for *p*-nitrocatechol sulphatase and maltase activities. Cellobiase activity was assayed at a concentration of 80m*M*. *p*-Nitrophenyl α and β-glucosidase were assayed at substrate concentrations of 10, 5, 4, 2 and 1m*M*.

Substrate	Concentration (mM)	Injection received: Isotonic Saline	Injection received: Amyloglucosidase (250mg/Kg b.w.)	Injection received: Emulsin β-glucosidase (125mg/Kg b.w.)	Number of Experiments
Nitrocatechol sulphate	5	14.1 ± 1.6	15.7 ± 2.9	15.1 ± 1.0	4
Maltose	5	11.9 ± 2.7	9.0 ± 2.4	10.1 ± 4.5	4
Cellobiose	80	*	*	13.2 ± 4.4	4
p-Nitrophenyl α-glucoside	1	67.0 ± 4.4	47.4 ± 3.4		
	2	68.4 ± 7.7	52.1 ± 5.6		
	4	64.5 ± 1.4	50.0 ± 4.4		4
	5	69.1 ± 4.1	50.0 ± 3.8	41.7 ± 1.8	
	10	66.7 ± 5.1	54.3 ± 3.7		
p-Nitrophenyl β-glucoside	1	57.4 ± 4.2		13.2 ± 3.2	
	2	59.8 ± 3.9		12.0 ± 2.0	
	4	64.3 ± 2.9		11.0 ± 1.0	4
	5	66.3 ± 3.8	62.0 ± 5.8	11.6 ± 1.6	
	10	65.8 ± 5.5		10.5 ± 0.8	

* Cellobiase activity was detectable only in livers from rats injected with emulsin β-glucosidase.

Table 4.3 Percent free activities of enzymes in lysosome-enriched liver fractions from rats receiving injections of saline, amyloglucosidase or emulsin 4h before death

Table 4.3 shows the initial free activities (first 10 min of hydrolysis), expressed as a percentage of the total activity, of p-nitrophenyl α and β-glucosidase, maltase, cellobiase and p-nitrocatechol sulphatase in lysosome-enriched fractions of rat liver.

The latency of p-nitrocatechol sulphatase activity was performed on all preparations and used as an index of the integrity of the lysosomes in the preparation. p-Nitrocatechol sulphatase activity, both free and total, was found to be unaffected by any of the injections, and the low percent free activity observed was in good agreement with previous observations on untreated rats.

In saline-injected rats p-nitrophenyl α and β-glucosidase and maltase activities all displayed similar percent free activities to untreated animals. The initial percent free activity of p-nitrophenyl α and β-glucosidase, although very high, showed no substrate concentration dependence. Cellobiase activity was not detectable in a 10 min assay in fractions from saline-injected rats.

The effect of emulsin injections upon p-nitrophenyl β-glucosidase activity was most dramatic, total activity being increased to 30-45 times the value observed in control (saline-injected) experiments. p-Nitrophenyl β-glucosidase activity now appeared to be 85-90% latent, in agreement with Lloyd (1969b). By correction for endogenous β-glucosidase activity, it was possible to calculate the additional p-nitrophenyl β-glucosidase activity, attributable to emulsin. Calculations showed that this additional activity was 89-91% latent. The initial percent free activity of emulsin β-glucosidase activity (Kmapp. 3.9-4.5m$\underline{M}$) showed no dependence on the substrate concentration in the range employed (1 to 10m$\underline{M}$).

The rise in p-nitrophenyl α-glucosidase activity following emulsin injections, which had been noted previously (see Fig. 4.3) and attributed

to enzyme contamination or multiple specificity, was also noted in these investigations. As a consequence of this additional activity, the apparent percent free activity of *p*-nitrophenyl α-glucosidase activity fell by about 27% from the value observed in saline-injected rats. Such a decrease in percent free activity is compatible with the observed increase in total activity of about 80%, if the additional activity is assumed to be 90% latent, i.e.

	p-nitrophenyl α-glucosidase activity		
	Free	Total	Percent Free Activity
Control	65	100	65.0
Emulsin-injected	73	180	40.6

Cellobiase activity, which was only measurable in emulsin-injected rats, displayed an initial percent free activity of 13.2 ± 4.4%. This result is not surprising in view of the osmotic protection studies of Lloyd (1969a), who showed that cellobiose affords extremely good osmotic protection to rat liver lysosomes.

The effects of amyloglucosidase-treatment are not nearly as dramatic as those observed after injection with emulsin. *p*- Nitrocatechol sulphatase and *p*-nitrophenyl β-glucosidase activities remained unaffected whilst cellobiase activity was not measurable in homogenates from amyloglucosidase-injected animals. In keeping with the substrate specificity of amyloglucosidase (Pazur and Klepp, 1962), the rise in total maltase activity was approximately three or four times that observed for *p*-nitrophenyl α-glucosidase activity. Additional maltase activity, attributable to amyloglucosidase, was estimated to be 90% latent. The observed decrease of 15-20% in percent free *p*-nitrophenyl α-glucosidase is again explicable by a 50% increase in total activity, assuming the additional activity to be 90% latent.

Although it was 15-20% lower than in control experiments, the percent free activity of *p*-nitrophenyl α-glucosidase in amyloglucosidase-injected rats (Km app. amyloglucosidase 4-5m*M*) did not show any substrate concentration dependence in the range employed (1-10m*M*).

4.7 Discussion

The experiments described in this chapter were designed to provide further information about the latency of exogenous enzymes, and are based on the earlier work of Lloyd (1969b), who demonstrated the latency of emulsin β-glucosidase and amyloglucosidase in lysosomes obtained from the livers of rats injected with these enzymes. The latency of emulsin when p-nitrophenyl β-glucosidase was used as the substrate was somewhat surprising in view of the evidence presented in the previous chapter for the ability of the substrate to penetrate the membrane of intact lysosomes.

Distribution and recovery experiments have revealed that, following intravenous injection, both enzymes were removed fairly rapidly from the blood stream. Emulsin was cleared from the bloodstream of injected rats more quickly than amyloglucosidase, little, if any, enzyme remaining in the blood 4h after injection. About 50% of the injected dose of amyloglucosidase remained in the blood after the same time-interval.

After removal from the blood, both enzymes were found in considerable amounts in the liver (Tables 4.1 and 4.2), where they displayed very diffuse subcellular distributions, significant amounts of enzyme being found in all subcellular fractions. The distribution of emulsin differed from that of amyloglucosidase, possibly as a result of their differing doses and rates of clearance. The distribution of both exogenous enzymes was, however, quite different from the endogenous lysosomal enzymes, and is undoubtedly a reflection of the number and type of vacuoles in which the endocytized enzymes are located 4h after their injection into rats.

Besides their dose and relative rate of uptake, the susceptibility of such molecules to hydrolytic attack by the lysosomal enzymes is also an important consideration. It ought to be noted that in all these

investigations enzyme activity has been used as an index of the amount of enzyme present in subcellular fractions. Enzymes, being protein in nature, are susceptible to catheptic digestion, and it would therefore seem probable that one would always underestimate the amount of exogenous enzyme present in secondary lysosomes.

Amyloglucosidase and emulsin have both been shown to be latent in livers of injected rats, in agreement with Jacques (1968) and Lloyd (1969b), who have also shown endocytized enzymes to be almost wholely latent. Partial apparent increases in latency, such as were observed with *p*-nitrophenyl α-glucosidase following injection of either emulsin or amyloglucosidase, could be explained if the additional activity, attributable to the exogenous enzyme, was wholely latent. In these investigations, however, the low initial percent free activities of emulsin and amyloglucosidase were observed with the *p*-nitrophenyl substrates which have been previously demonstrated to yield very high values for the corresponding endogenous enzymes in intact lysosome-enriched liver fractions. The high accessibility of such substrates to the enzymes of intact lysosomes was previously explained by the heterogeneity of lysosomes, and the existence of graded permeability properties. The latency which an enzyme displays towards a substrate has been envisaged as an index of the number of lysosomes in the preparation in which the substrate is freely accessible to the enzymes. The results obtained in these investigations support such a concept, if the injected enzymes are located in a class of vacuole that does not allow penetration of *p*-nitrophenyl substrates. This contention is supported by the fact that the percent free activity of both enzymes was found to be independent of substrate concentration. If the observed low percent free activity was a result of the increased enzyme activity making membrane-diffusion of the substrate relatively more rate-limiting,

then one would have anticipated that the percent free activity of the exogenous enzymes would be concentration dependent (de Duve, 1965; Baudhuin, 1964).

It is tentatively suggested that the latency difference which one observes for endogenous and exogenous enzymes with _p_-nitrophenyl substrates is one of vacuole-type, emulsin and amyloglucosidase being sequestered into vacuoles that are initially impermeable to _p_-nitrophenyl α and β-glucoside. It is well known that lysosomes of different origin or type often have differing fragility and stability properties (Bowers et al., 1967; Jacques, 1968). Lysosomes of different intracellular status (primary, secondary) might well differ in their permeability properties. Since it is likely that these exogenous enzymes are predominantly endocytized by Kupffer cells in the liver (Straus, 1962; Goldfischer et al., 1970), the latency difference could also be connected with the permeability properties of lysosomes from Kupffer cells and hepatocytes.

How the nature or origin of a vacuole could determine its permeability properties is a matter for debate. It is possible to envisage that phagosomes, secondary lysosomes and primary lysosomes might possess inherent differences in their permeability properties, or that apparent differences might arise as a result of their isolation. Relatively little work has been done on the ability of digestible substances, such as proteins, to bring about such changes. The only real evidence in this field has been gained from the protein catabolism studies of Mego (1964) and Mego et al. (1965; 1967), where monoiodotyrosine was shown to possess the ability to diffuse out of intact heterolysosomes.

It would be very interesting to examine the subcellular distribution and latency of these exogenous enzymes at differing times after injection, and see how this correlated with their rates of clearance. In this way,

one might be able to estimate the amount of enzyme present in each vacuole-type and examine the latency of individual vacuole-types, rather than that of a heterogeneous mixture.

CHAPTER 5

PERMEABILITY OF RAT LIVER LYSOSOMES TO NUCLEIC ACID DERIVATIVES

5.1 Introduction

The latency of lysosomal enzymes in lysosome-enriched fractions, freshly prepared and resuspended in an osmotically buffered medium, such as 0.25M-mannitol or sucrose, results from the permeability barrier which the lysosomal membrane presents to the substrates employed in their detection (de Duve, 1963, 1965). The latency of lysosomal enzymes in such preparations is, however, never found to be complete, a value of about 10-15% usually being observed, for the initial percent free activity. The initial percent free activity observed with impermeable substrates, such as maltose, has been attributed to the mechanical disruption of a number of lysosomes during the isolation technique (Lloyd, 1969a).

If lysosomes are resuspended in an isotonic solution of non-permeant solute, such as sucrose, and incubated at 25° very little latency loss, if any, is observed within 60 min. When lysosomes are resuspended in an isotonic solution of permeant solute, such as glucose, protection is only transient, and the percent free activity of a lysosomal marker enzyme rises as the lysosomal suspension is incubated. The question whether this increase in percent free activity is a result of lysosomal breakage or an increase in the permeability of the lysosomal membrane is examined in this chapter. The speed at which the stabilising effectiveness of a solute wears off is used as an index of the ease with which a molecule is able to penetrate the lysosomal membrane.

Using this approach, Lloyd (1969a, 1971) and Lee (1970, 1971a, 1971b, 1972) have examined the permeability properties of the lysosomal membrane with a series of carbohydrates and small oligopeptides. The results from such an approach support the suggestion by Cohn and Ehrenreich (1969) and Ehrenreich and Cohn (1969) of a molecular weight ceiling around 220, beyond which water-soluble substances such as

carbohydrates and oligopeptides apparently cannot penetrate the lysosomal membrane.

As was discussed in Chapter 1, circumstancial evidence from the identification of the digestion products of macromolecules in lysosomal extracts suggests that the permeability properties of the lysosomal membrane are ideally suited to the digestive capacity of lysosomes. The digestion of proteins (Coffey and de Duve, 1968), carbohydrates (Aronson and Davidson, 1968), and mucopolysaccharides (Fowler and de Duve, 1969) by lysosomal extracts have all been studied in some detail, and appear to follow a distinct pattern. The final digestion products of all these macromolecules are small molecules which could easily penetrate the membrane of intact lysosomes and pass into the cytosol, where anabolic or further catabolic reactions could take place.

Lysosomes contain ribonuclease and deoxyribonuclease activities which are doubtless employed in the intralysosomal degradation of RNA and DNA. The products of such digestion are the mononucleotides, which could be further degraded by an enzyme or enzymes of the acid phosphatase complex to yield the nucleosides (Arsenis et al. 1970). The fate of nucleosides arising within lysosomes in this way is not known. All the common nucleosides have molecular weights in excess of the threshold proposed by Ehrenreich and Cohn (1969), and it would, at first sight, seem doubtful that they would be able to penetrate the membrane of intact lysosomes. There have been no reports, however, of any enzymes within lysosomes which are capable of attacking the N-glycosidic bond of nucleosides to yield the base and pentose sugar.

In order to examine whether nucleosides possess the ability to penetrate the membrane of intact lysosomes, their efficiency at affording osmotic protection to rat liver lysosomes has been measured.

5.2 Permeability of rat liver lysosomes to nucleosides

The format for these experiments is essentially that of Lloyd (1969a, 1971), and involves the measurement of free and total *p*-nitrocatechol sulphatase activities in lysosome-enriched fractions after incubation in 0.25*M* solutions of different test compounds. A lysosome-enriched fraction of rat liver was prepared as described in 2.1 and gently resuspended in the centrifuge tube, by forcing the suspension once only past a teflon pestle rotating at approx. 1500 r.p.m., in a 0.25*M* solution of test compound at 4°. After incubation for varying time intervals at 25°, aliquots of the suspension were withdrawn and assayed at 25° for free and total *p*-nitrocatechol sulphatase activity, by the general methods outlined in Chapter 2. A substrate concentration of 5m*M* was always employed, and enzyme assays were always of 10 min duration at 25° in order to minimise latency loss within the actual assay. Total enzyme activities were estimated in the presence of 0.1% Triton X-100. Enzyme reactions were terminated and colour development of liberated *p*-nitrocatechol achieved by the addition of 6.0ml alkaline quinol reagent (Roy, 1960).

Uridine, Thymidine, Purine and Glucose were obtained from Sigma, Cytidine was obtained from Koch-Light, and Mannitol was obtained from Hopkins and Williams.

Unfortunately, the survey was not nearly as complete as was originally hoped. Of the nucleosides, only the pyrimidine derivatives, thymidine, uridine and cytidine, were found to be soluble enough in water to prepare the 0.25*M* solutions which this technique required. None of the free bases, whether purine or pyrimidine derivatives, were found to be soluble enough to prepare 0.25*M* solutions. However, the parent base purine, which is very soluble in water, was examined along with the three nucleosides as it might provide some insight into the

Test Compound	25° Preincubation Time (min)			Number of Experiments
	0	30	60	
Mannitol	14.9 ± 2.9	15.2 ± 4.5	20.0 ± 4.0	4
Glucose	16.3 ± 6.0	35.1 ± 6.3	55.1 ± 5.3	4
Uridine	18.6 ± 5.1	29.2 ± 5.2	48.8 ± 4.5	4
Thymidine	20.2 ± 5.6	77.1 ± 15.7	89.7 ± 5.4	4
Cytidine	17.8 ± 7.4	22.8 ± 5.2	35.9 ± 5.3	4
Purine	84.5 ± 12.7	91.2 ± 14.3	96.7 ± 5.4	4

Table 5.1 Initial percent free activity of p-nitrocatechol sulphatase in lysosome-enriched liver fractions at 25°

Test Compound	25° Preincubation Time (min)			Number of Experiments
	0	30	60	
Mannitol	13.9 ± 2.7	20.2 ± 5.1	17.0 ± 3.5	4
Uridine	18.5 ± 4.2	38.3 ± 5.1	42.1 ± 2.8	4
Thymidine	26.4 ± 8.2	71.4 ± 5.3	86.8 ± 2.9	4
Cytidine	23.2 ± 3.0	35.4 ± 4.6	41.7 ± 3.2	4
Purine	77.9 ± 9.5	67.9 ± 11.9	81.0 ± 12.2	4

Table 5.2 Initial percent free activity of maltase in lysosome-enriched liver fractions at 25°

behaviour of the free bases. Mannitol and glucose were employed as non-permeant and permeant reference compounds respectively, the percent free activity of p-nitrocatechol sulphatase being estimated in their presence for every lysosome-enriched fraction.

Table 5.1 shows the percent free activity, in a 10 min assay of p-nitrocatechol sulphatase of lysosome-enriched liver fractions incubated at 25° for 0, 30 or 60 min in 0.25M solutions of mannitol, glucose, uridine, cytidine, thymidine or purine.

In agreement with Lloyd (1969a) mannitol gave prolonged, but glucose only transient, osmotic protection. The percent free activity of p-nitrocatechol sulphatase in fractions resuspended in 0.25M-glucose rose by about 40% in 60 min. Purine failed even to give initial protection, indicating a very rapid rate of entry into lysosomes. This is consistent with its low molecular weight (120). The initial percent free activity of p-nitrocatechol sulphatase in lysosomes resuspended in 0.25M-mannitol was unaffected by the incorporation of 0.25M-purine into the buffer, indicating that the instability of lysosomes in purine solutions is an osmotic phenomenon and not a direct effect of purine on the lysosomal membrane. The nucleosides all afforded good initial osmotic protection, but this was lost on incubation for 60 min. The rate of loss of latency was relatively slow for lysosomes incubated in 0.25M-cytidine or uridine, indicating a slow rate of penetration into the lysosome. Lysosomes incubated in thymidine lost their latency more quickly, indicating a more rapid penetration into lysosomes than the other two nucleosides. Purely on the basis of their molecular weights, one would have predicted that thymidine would have penetrated the fastest, with cytidine and uridine somewhat slower. Stein (1967) has related the molecular weight and number of hydrogen bonds formed with water to the rate of diffusion of solutes across biological membranes. The fact that thymidine is a

nucleoside of deoxyribose, and hence contains one less potential hydrogen-bonding hydroxyl group than ribose-containing nucleosides, may also be an important consideration.

The possibility that the nucleosides exert their labilising effect on lysosomes by virtue of their pH has been examined. Although the initial pH of 0.25M solutions of individual nucleosides varied, the lysosome-enriched fractions were shown to possess sufficient buffering capacity to give a final pH in all cases of 6.7 ± 0.1, which was identical to that measured in fractions resuspended in either mannitol or glucose. The pH of fractions resuspended in purine were slightly lower than 6.7, but not sufficient to significantly change the activity of p-nitrocatechol sulphatase. The activity of p-nitrocatechol sulphatase was also unaffected by the three nucleosides under observation.

Table 5.2 shows similar data for a second lysosomal enzyme, maltase. The latency of maltase activity at 25° was estimated by the methods outlined in Chapters 2 and 3. The results obtained with maltase as the lysosomal marker enzyme were almost identical to those obtained with p-nitrocatechol sulphatase; thymidine's stabilising effectiveness wearing off much more quickly than either cytidine's or uridine's. Also in agreement with previous results, mannitol afforded sustained protection, whilst purine gave little, if any, protection.

It was suggested in the introduction to this chapter that there were two possible explanations for the increase in percent free activity which one observes when lysosomes are incubated in an isotonic solution of permeant solute. One explanation is that, as lysosomes swell, their membranes become progressively more leaky: substrates may enter, but not rapidly enough to achieve an intralysosomal concentration of substrate equal to that in the bulk assay medium. Another possible explanation is that the loss of latency reflects an increased proportion of ruptured

Test Compound	Concentration of Maltase (min)					Number of Experiments
	1	2	4	5	10	
Uridine	32.0 $\pm$ 3.2	29.1 $\pm$ 8.3	31.3 $\pm$ 6.7	33.7 $\pm$ 1.5	35.1 $\pm$ 3.0	3
Cytidine	35.0 $\pm$ 0.2	34.3 $\pm$ 0.1	35.4 $\pm$ 1.4	30.5 $\pm$ 2.1	36.0 $\pm$ 2.6	3

Table 5.3 Effect of substrate concentration on the percent free maltase activity of lysosome-enriched fractions pre-incubated at 25° for 30 min.

lysosomes in the population. As was discussed in Chapter 1, the plausibility of these two suggestions may be examined by measuring the percent free activity at a range of substrate concentrations.

If the first explanation is true, the percent free activity of a lysosomal enzyme will rise as the substrate concentration is increased beyond concentrations which saturate the enzyme. If the second explanation is true, the percent free activity will be constant at all substrate concentrations.

Table 5.3 shows the effect of substrate concentration on the percent free maltase activity, measured in a 10 min assay at 25°, of lysosome-enriched fractions which had been pre-incubated for 30 min at 25° in a 0.25M solution of either uridine or cytidine. The percent free activity of maltase (Km app. 2mM) was independent of substrate concentration in the range employed (1-10mM). The latency loss which accompanies the incubation of lysosomes in 0.25M-uridine or cytidine is therefore probably caused by the rupture of lysosomes.

That the latency loss in such conditions is progressive, rather than abrupt, deserves comment. The answer probably lies in the heterogeneity of lysosomes in the rat liver preparation. Lysosomes have a spectrum of sizes and respond at differing rates to an osmotic imbalance generated by a permeant solute. If one assumes that all lysosomes are approximately spherical in shape, and all enclosed by a membrane of uniform thickness and tensile strength, then a large vacuole would be more resistant to rupture than a small vacuole, if both were placed in a solution of permeant solute. Since the value of the surface area/ volume ratio of a sphere falls as the radius of a sphere increases, the relative increase in volume by inward diffusion of solute is smaller as the radius increases, i.e. smaller vacuoles will rupture first. Size is not the only possible source of a graded response; lysosomes

from different cell types (hepatocytes, Kupffer cells) or of different intracellular status (primary, secondary lysosomes) might well differ in their permeability properties .

5.3 Permeability of rat liver lysosomes to nucleotides

Before considering the question of the permeability of rat liver lysosomes to nucleotides, it is extremely important to ascertain the permeability properties of the lysosomal membrane with inorganic phosphate buffers alone. Nucleotides may be regarded as monoesterified derivatives of phosphoric acid, and as such, are strong dibasic acids with two ionizable hydroxyl groups in their phosphoric moiety. Although the disodium salt of nucleotides could be employed, it too is readily ionized in water to yield sodium ions which would exert an osmotic pressure in their own right, besides that of the nucleotide anions. It would be very difficult to quantitate the ionization and calculate the concentration of each osmotically-active species. The pH of nucleotide solutions also makes practical application very difficult; it is almost impossible to obtain the same pH as control non-ionizable compounds without the involvement of osmotically-active buffers. Eck and Lloyd (unpublished) have partially resolved this problem by titrating the disodium and acid forms of AMP to the required pH.

The ability of a series of inorganic phosphate buffers ($Na\ H_2\ PO_4\ 2H_2O$-$Na_2\ HPO_4$), of differing pH, to afford osmotic protection to rat liver lysosomes has been examined as previously described, employing maltase as the lysosomal marker enzyme. p-Nitrocatechol sulphatase was not employed in these investigations as it is inhibited by minute traces of inorganix phosphate (Roy, 1960), and was shown to be totally inhibited by 0.25M--AMP. Maltase did not suffer any of these effects.

Table 5.4 shows the percent free activity, in a 10 min assay, of maltase in lysosome-enriched liver fractions, incubated at 25° for 0, 30 or 60 min in phosphate buffers.

Phosphate buffers, both 0.125M and 0.25M, provided sustained osmotic protection, although, in all cases, the percent free maltase activity

Test phosphate buffer	25° Preincubation Time (min)			Number of Experiments
	0	30	60	
0.125M, pH 7.0	33.9 ± 11.9	25.8 ± 7.7	28.6 ± 6.5	4
0.25M, pH 7.0	33.9 ± 9.4	27.0 ± 12.4	25.3 ± 8.6	4
0.125M, pH 6.0	28.2 ± 10.5	22.9 ± 14.1	21.6 ± 6.6	4
0.25M, pH 6.0	23.5 ± 4.7	29.8 ± 2.8	25.7 ± 4.1	4
0.125M, pH 5.0	25.1 ± 4.1	29.5 ± 11.8	39.9 ± 9.7	4
0.25M, pH 5.0	18.4 ± 6.4	18.2 ± 9.3	22.6 ± 3.0	4
0.25M-Mannitol	13.9 ± 2.7	20.2 ± 5.1	17.0 ± 3.5	

Table 5.4 Initial percent free activity of maltase in lysosome-enriched liver fractions at 25°

immediately after resuspension was somewhat higher than the value observed in 0.25M-mannitol. The results at pH 7.0 are rather puzzling in this respect, the initial value being higher than that observed after incubation, although the total activity of maltase was unaffected. This is extremely unlikely and is probably an artifact produced by the inaccuracies inherent in this technique. Results do, however, indicate a very slow rate of penetration of phosphate and sodium ions into lysosomes at every pH examined.

5.4 Discussion

The experiments described in this chapter are in essence an extension of those carried out by de Duve and his colleagues some years ago (Appelmans and de Duve, 1955; Berthet et al., 1951), and more recently by Lloyd (1969a, 1971) and Lee (1970, 1971a,b, 1972). Lloyd (1969a) and Lee (1970) have both demonstrated the importance of charge in determining the permeability properties of carbohydrates to lysosomes. Gluconate, glucuronate and lactate anions were all observed to enter lysosomes much more slowly than uncharged carbohydrates of similar molecular weight. Although non ionic media such as 0.25M-sucrose or mannitol are usually preferred as osmotic buffers in the preparation of subcellular fractions, ionic solutions, such as β-glycerophosphate (Appelmans and de Duve, 1955) or potassium chloride (Bowers et al., 1967) have frequently been used for this purpose in the past. Lloyd (1971) has also demonstrated the effectiveness of sodium chloride solutions as osmotic protectors for rat liver lysosomes.

In view of these observations, the protection which phosphate buffers have been shown to afford to rat liver lysosomes is hardly surprising. It would seem very unlikely indeed that a large charged molecule such as a nucleotide would be able to enter lysosomes at a greater rate than either sodium or phosphate ions, which themselves only penetrate very slowly. In support of this argument, Lloyd and Eck (unpublished) have demonstrated the effectiveness of 0.125M-AMP as an osmotic protector for rat liver lysosomes. This property could, however, be argued to be entirely attributable to sodium ions, since the disodium salt of the nucleotide was employed. It is interesting to speculate the fate of inorganic phosphate in secondary lysosomes *in vivo*, if indeed it is one of the intralysosomal digestion products of nucleic acid degradation. The lysosomal membrane may possibly

possess vectorial permeability properties with respect to phosphate, allowing phosphate to permeate outward, but not inward. One could also suggest the existence of an active transport system, such as the ATPase which has been suggested by Mego et al. (1972).

Although only the pyrimidine-derived nucleosides were soluble enough for investigation, all those which were examined showed considerable ease of penetration into the lysosome, in spite of their relative high molecular weights. The explanation possibly lies in the considerably hydrophobic nature of the bases, particularly in the case of thymidine. In accordance with these findings, Lloyd and Eck (unpublished) have demonstrated the inability of lysosomal extracts to digest uridine or cytidine. Chromotographic examination of the digestion products revealed no traces of uracil or cytosine; unchanged nucleoside was the only detectable component. Similar experiments in which uridine 5'-phosphate was incubated with lysosomal extracts revealed the rapid production of uridine.

The ability of nucleosides to penetrate the lysosomal membrane is ideally suited to the digestive capacity of lysosomes. Degradation beyond the level of the nucleoside seems unnecessary, and it is unlikely that rat liver lysosomes possess any enzymes with this capability.

CHAPTER 6

THE EFFECTS OF CYSTEINE ON THE PERMEABILITY AND STABILITY OF RAT LIVER LYSOSOMES

6.1 Introduction

One of the most extensively utilized and most convenient non-microscopic methods for the study of uptake of macromolecules into cells is to expose cells or tissue to a suitable macromolecule labelled with a radioactive isotope. The molecule most frequently employed to study the uptake and fate of proteins is radioiodinated bovine serum albumin (BSA). Uptake may be quantitated by measuring the amount of radioactivity in the cell which cannot be removed by extensive washing.

Using this approach, Mego and McQueen (1965) have demonstrated that after formaldehyde-treated albumin was cleared from the bloodstream of injected mice, it appeared predominantly in the mitochondrial fractions of mouse liver, where it was rapidly digested. The digestion of radioiodinated BSA was followed by measuring the increase in trichloroacetic acid-soluble radioactivity when the partially-purified lysosomal fraction was incubated. Chromatographic examination of the digestion products showed that this radioactivity was almost entirely in the form of monoiodotyrosine. Mego et al. (1967) later demonstrated that the digestion of exogenous protein only occurred inside intact lysosomes, implying that monoiodotyrosine was able to cross the membrane of intact heterolysosomes. If the vacuoles were disrupted, e.g., by treatment with Triton X-100, digestion ceased almost completely, presumably due to the huge dilution which enzymes and foreign protein undergo when they are released from their intimate contact within the heterolysosome.

The involvement of cathepsins in the digestion of proteins, suggested by Mego and McQueen (1965) after demonstrating that the rate of digestion was affected by cysteine and iodoacetamide, is also supported by a number of more recent investigations (Huisman et al., 1973a, b; Kussendrager et al., 1972). Cysteine and iodoacetamide were

shown by Greenbaum and Fruton (1957) to behave respectively as activator and inhibitor of cathepsin B activity. If cysteine and iodoacetamide exert their respective effects on the intralysosomal digestion of injected protein by a direct influence on cathepsins, they both must be able to permeate the lysosomal membrane. This seems feasible in view of their relatively low molecular weights.

At concentrations of iodoacetamide which Greenbaum and Fruton (1957) had found to cause complete inhibition of catheptic activity, Mego and McQueen (1965) only observed a reduction of approximately 50% in digestive activity. Similarly, cysteine only activated digestive activity by about 50% at the concentration which Greenbaum and Fruton (1957) had observed to cause 5-6 fold activation. Although this degree of activation or inhibition could merely represent the partial permeability of the lysosomal membrane to cysteine and iodoacetamide, i.e. the amount of each present within heterolysosomes, or be indicative of the involvement of other enzymes in the digestion of injected protein, their effects may not necessarily be connected with an influence on enzymic activity. Their effects could equally well be explained if they affected the stability of lysosomes on incubation, or the rate at which the enzymes or substrate are released from broken lysosomes.

The effect of cysteine on the latency and sedimentability of a number of lysosomal enzymes has therefore been examined in order to investigate these possibilities.

6.2 Estimation of cysteine

Since cysteine is known to undergo rapid oxidation to cystine when placed in aqueous solution, the stability of cysteine was examined in order to assess the feasibility of its use in aqueous systems. Cysteine was assayed by the method of Zahler and Cleland (1968), exploiting its reaction with 5.5'-dithiobis (2-nitrobenzoic acid) (DTNB). Experimental details are fully described in Chapter 2.

Cysteine hydrochloride has been frequently used in the past as an alternative to the free amino acid, mainly because of its higher stability, particularly in acidic conditions. The hydrochloride was not employed in these experiments, however, because of the relative high acidity of aqueous solutions. The stability of cysteine was examined by its reaction with DTNB following pre-incubation in a variety of conditions. Preliminary investigations had shown that 1m*M* solutions of cysteine were less stable at pH 5.0 than at pH 7.4. Little significant loss of -SH content was observed in 40m*M* solutions which had been pre-incubated at either pH for 6h at room temperature. Cysteine was therefore used at a concentration of 40m*M* without the fear of significant oxidation within the duration of any of the subsequent experiments.

6.3 Effect of cysteine on the stability of rat liver lysosomes

6.3.1 Time-course of hydrolysis of p-nitrophenyl α-glucoside

The effect of cysteine on the stability of rat liver lysosomes was investigated by examining its effect upon the latency and sedimentability of α-glucosidase activity in rat liver lysosomes at 37°. The latency of α-glucosidase was examined by following the time-course of hydrolysis of *p*-nitrophenyl α-glucoside in lysosome-enriched liver fractions at 37°. Maltase activity was not measured because preliminary experiments had shown that 40m*M*-cysteine caused almost total inhibition of the glucose oxidase assay for glucose.

The time-course of hydrolysis of *p*-nitrophenyl α-glucoside was examined at pH 5.0, both in the absence and in the presence of 0.1% Triton X-100, for incubation periods of 0 to 60 min, employing a substrate concentration of 5m*M*. Full experimental details of latency investigations and individual enzyme assays are provided in Chapters 3 and 2 respectively. Cysteine (Sigma), dissolved in acetate-mannitol buffer, pH 5.0 immediately prior to use, was incorporated in the incubation mixture to yield a final concentration of 40m*M*.

Table 6.1 shows the effect of 40m*M*-cysteine on the latency of *p*-nitrophenyl α-glucosidase in lysosome-enriched liver fractions at 37°. Results show the free activity for each 10 min time interval, expressed as a percentage of the total activity (activity observed in the presence of 0.1% Triton X-100), in the absence and in the presence of 40m*M*-cysteine.

In agreement with previous observations, the initial percent free activity (first 10 min of incubation) of *p*-nitrophenyl α-glucosidase was found to be extremely high in preparations which displayed a typically low (about 15%) value for *p*-nitrocatehcol sulphatase activity. Cysteine was found to have no measurable effect on either free or total *p*-nitrophenyl α-glucosidase activity.

Buffer	Time interval (min)						Number of Experiments
	(0-10)	(10-20)	(20-30)	(30-40)	(40-50)	(50-60)	
Acetate-Mannitol, pH 5.0	62.6 ± 3.2	88.8 ± 2.2	95.9 ± 8.8	97.4 ± 8.5	95.1 ± 10.8	98.3 ± 9.1	4
Acetate-Mannitol, pH 5.0 + 40mM Cysteine	63.1 ± 4.6	87.8 ± 8.6	102.5 ± 4.9	93.1 ± 5.0	106.1 ± 11.4	100.5 ± 3.8	4

Table 6.1 Effect of Cysteine on the latency of p-nitrophenyl α-glucosidase activity in lysosome-enriched liver fractions at 37°

6.3.2 Sedimentibility of p-nitrophenyl α-glucosidase

As has been previously demonstrated, the initial percent free activity of p-nitrophenyl α-glucosidase activity is very high, even though the enzyme is almost entirely sedimentible. If cysteine affects the rate at which enzymes are released from the lysosome and become non-sedimentible, this effect would not necessarily be detectible by measurements of p-nitrophenyl α-glucoside latency. In an effort to resolve this problem, the sedimentibility of p-nitrophenyl α-glucosidase activity has also been examined.

The technique employed was identical to that described in 3.9. 0.5ml of lysosome-enriched liver fraction was mixed with 1.5ml acetate-mannitol buffer, pH 5.0, with or without 40mM-cysteine, incubated at 37°, and centrifuged at 4°at 25000g for 15 min. Enzyme determinations on the supernatant yielded non-sedimentible enzyme activity. Total enzyme activity was estimated in a similar manner, ommitting the final centrifugation.

Table 6.2 shows the non-sedimentible activity of p-nitrophenyl α-glucosidase, expressed as a percentage of the total activity, following pre-incubation of the lysosome-enriched liver fraction in acetate-mannitol, with or without 40mM-cysteine, for periods of time up to 60 min.

The rate of release of p-nitrophenyl α-glucosidase activity at 37° was found to be unaffected by the presence of 40mM-cysteine, the activity reaching a peak in non-sedimentible activity after an incubation period of 30-40 min.

6.3.3 Effect of cysteine pre-incubation on the initial free activity of p-nitrocatechol sulphatase

In order to examine if cysteine's effect is time-dependent, the initial free activity of p-nitrocatechol sulphatase was examined in lysosome-enriched liver fractions which had been pre-incubated at 25°

Buffer	37° Pre-incubation time (min)							Number of Experiments
	0	10	20	30	40	50	60	
Acetate-Mannitol, pH 5.0	2.2 ± 0.3	5.8 ± 2.9	44.4 ± 12.0	81.4 ± 7.2	82.6 ± 1.7	84.0 ± 1.1	80.0 ± 3.2	4
Acetate-Mannitol, pH 5.0 + 40m*M* Cysteine	3.3 ± 0.5	8.1 ± 0.3	45.1 ± 3.5	84.6 ± 2.8	80.6 ± 8.6	83.9 ± 8.6	79.0 ± 10.0	4

Table 6.2 Non-sedimentible activity of p-nitrophenyl α-glucosidase activity in lysosome-enriched liver fractions at 37°

in the presence of 40m*M*-cysteine. Lysosome-enriched liver fractions were prepared as in previous investigations and resuspended, either in 0.25*M*-mannitol, or in freshly-prepared 0.25*M*-mannitol containing 40m*M*-cysteine. After pre-incubation for varying time-intervals at 25°, aliquots of the suspension were withdrawn and assayed for *p*-nitrocatechol sulphatase activity at pH 5.0 in acetate-mannitol buffer, pH 5.0. *p*-Nitrocatechol sulphatase activity was assayed as described in Chapter 2, employing a substrate concentration of 5m*M*. The initial free activity of *p*-nitrocatechol sulphatase was examined in short, 10 min, assays and compared to the activity in the presence of 0.1% Triton X-100.

Table 6.3 shows the initial free *p*-nitrocatechol sulphatase activity, expressed as a percentage of the total activity, measured at 25°, following pre-incubation of the lysosome-enriched fraction in either 0.25*M*-mannitol or in 0.25*M*-mannitol containing 40m*M*-cysteine at 25°.

40m*M*-cysteine was found to have no measurable effect on the initial free activity of *p*-nitrocatechol sulphatase. Even after a pre-incubation period of 90 min, the free and total *p*-nitrocatechol sulphatase activities were found to be almost identical to those observed in the absence of cysteine. In both cases, a latency-loss of about 20% was observed after 90 min.

Buffer	25° Pre-incubation time (min)				Number of Experiments
	0	30	60	90	
Acetate-Mannitol, pH 5.0	13.9 ± 1.4	16.0 ± 7.0	22.2 ± 2.1	33.1 ± 7.2	4
Acetate-Mannitol, pH 5.0 + 40m*M* Cysteine	13.8 ± 3.1	17.6 ± 1.5	27.4 ± 2.6	34.7 ± 5.3	4

Table 6.3 Effect of pre-incubation in the presence of cysteine on the initial free p-nitrocatechol sulphatase activity in lysosome-enriched liver fractions at 25°

6.4 Effects of cysteine on the digestion of ^{125}I-labelled BSA

The lysosomal digestion of protein is known to be enhanced by cysteine (Mego and McQueen, 1965; Huisman et al., 1973), most probably by the activation of cathepsin B1 and other thiol proteinases (Barret, 1972). The hydrolysis of ^{125}I-labelled BSA (formalin-denatured) was assayed by the measuring of the increase in TCA-soluble radioactivity, as described in Chapter 2.

Lysosome-enriched liver fractions were prepared as previously, and resuspended in water to destroy any latency which might complicate the kinetics of the reaction. The time-course of hydrolysis of formalin-denatured ^{125}I-labelled BSA at 37° was followed, in the absence and in the presence of 40m*M*-cysteine, for incubation periods up to 100 min. The reaction was stopped by the addition of 20% TCA, and the resulting solution examined for 'total' and TCA-soluble radioactivity as previously described in Chapter 2.

Fig. 6.1 shows a typical product/time graph which one obtains in such experiments. Product is expressed in terms of the increase in the percentage of TCA-soluble radioactivity from zero time.

Very little TCA-soluble radioactivity was observed, either in the absence or in the presence of cysteine, within the first 20 min of incubation. This is not due to residual latency of the lysosomal fraction but rather due to the fact that hydrolysis is due to the concerted effect of a number of enzymes, and their products, peptides, need to reach a certain size before they become TCA-soluble. Mego et al. (1967) and Williams et al. (1971) have demonstrated that when exogenous proteins are digested in heterolysosomes, radioactivity is released mainly in the form of monoiodotyrosine. After this initial lag-period, TCA-soluble radioactivity increased linearly with time. In the presence of 40m*M*-cysteine, the reaction proceeded about 2-3 times faster than in its

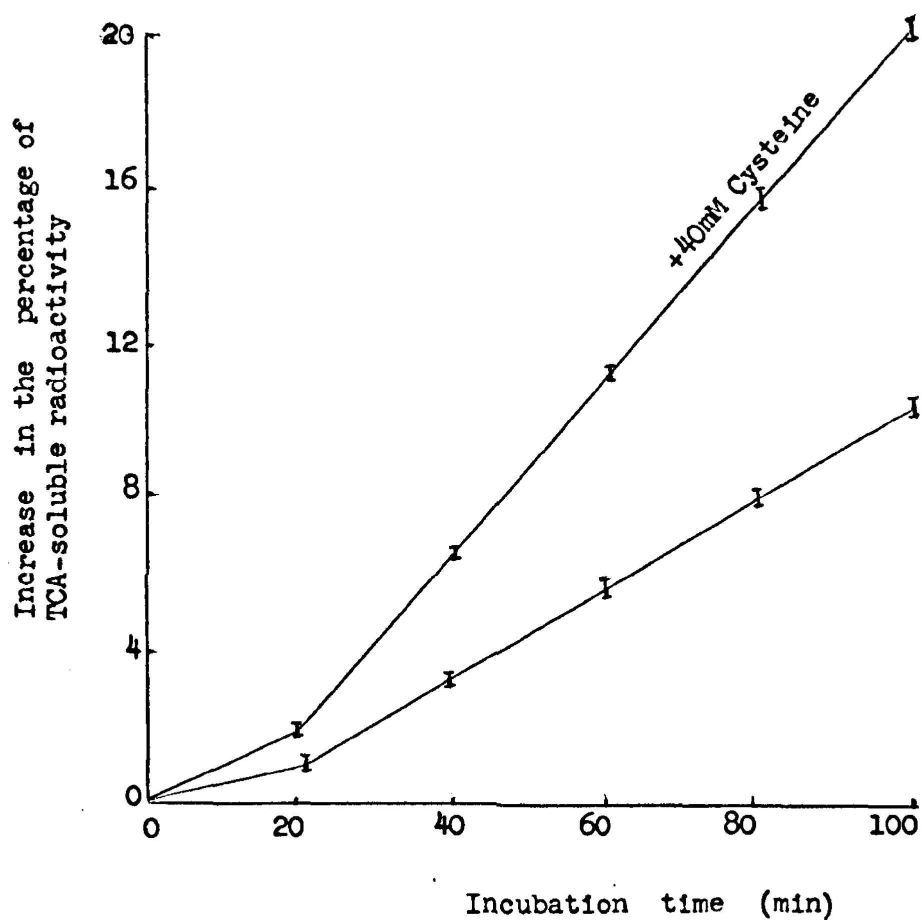


Fig. 6.1 Effect of Cysteine on the digestion of ^{125}I-labelled BSA by lysosome- enriched liver fractions at 37°

absence. The increase in digestion of albumin observed by Mego and McQueen in isolated heterolysosomes in the presence of 40m*M*-cysteine may merely be a reflection of this activation.

6.5 Sedimentability of ^{125}I-labelled BSA-hydrolase activity

The digestion of exogenous protein within intact heterolysosomes ceases if the particles are broken (Mego et al., 1967), presumably by diluting the exogenous protein and proteolytic enzymes from their intimate contact within the heterolysosome into a relatively large volume of medium. One plausible explanation for the activating effect of cysteine is an effect upon the rate at which proteolytic enzymes and/or exogenous protein are released from lysosomes after they are broken during incubation at 37°. In order to examine this possibility, the rate at which BSA-hydrolase activity is released from the lysosome has been investigated by measuring the rate at which it becomes non-sedimentable.

The technique was identical to that described previously in Chapter 3. 0.5ml lysosome-enriched liver fraction was mixed with 1.5ml acetate-mannitol buffer, pH 5.0, with or without 40m*M*-cysteine, incubated at 37°, and centrifuged at 4° at 25000g for 15min. The supernatant was examined for non-sedimentible proteolytic activity with ^{125}I-labelled BSA (formalin-denatured) as previously described. Total enzyme activities were estimated in a similar manner in fractions which had been suspended in non-osmotically-buffered medium, omitting the final centrifugation.

Table 6.4 shows the non-sedimentible activity of BSA-hydrolase, expressed as a percentage of the total activity, following pre-incubation of the lysosome-enriched liver fractions in acetate-mannitol, with or without 40m*M*-cysteine, for periods of time up to 60 min.

The rate at which BSA-hydrolase activity is released from the lysosome is very similar to that observed for other lysosomal enzymes (see Chapter 3), maximal non-sedimentibility being attained after an incubation period of approximately 50 min. Cysteine had no significant effect upon the rate of enzyme-release. In view of these results, it would seem unlikely that cysteine's activating effects are due to its

Buffer	37° Preincubation Time							Number of Experiments
	0	10	20	30	40	50	60	
Acetate-Mannitol, pH 5.0	9.5 ± 9.1	21.0 ± 6.7	41.1 ± 15.6	83.3 ± 10.5	79.4 ± 16.0	79.6 ± 4.5	65.7 ± 24.1	4
Acetate-Mannitol, pH 5.0 + 40mM Cysteine	11.5 ± 0.9	13.7 ± 2.7	41.3 ± 4.5	87.2 ± 1.5	86.4 ± 7.3	88.6 ± 11.4	88.2 ± 12.1	4

Table 6.4 Non-sedimentible activity of ^{125}I-labelled BSA-hydrolase activity in lysosome-enriched liver fractions at 37°

influence on the binding of lysosomal enzymes.

6.6 Effect of cysteine on the stability of heterolysosomes

The effect of cysteine on the digestion of protein (Mego and McQueen, 1965) was observed in heterolysosomes, whose physical properties may well differ from primary lysosomes. Cysteine's stabilising effect, if indeed it has one, might only be manifested in the increased stability of heterolysosomes, which are generally accepted to be more susceptible to mechanical disruption than primary lysosomes. The rate of release of radio-labelled polyvinyl pyrrolidone ($[^{125}I]$-PVP) from isolated heterolysosomes was examined in order to investigate this possibility.

$[^{125}I]$-PVP is a most convenient marker for heterolysosomes, firstly, because it is easily detected by means of its gamma radiation, and secondly, and more importantly, it is not susceptible to lysosomal digestion, and therefore accumulates within the lysosome. Moore, Williams and Lloyd (unpublished) have demonstrated the ability of rats to concentrate $[^{125}I]$-PVP in their livers after intravenous injection.

Male adult rats (300-400g) were starved overnight and injected in the femoral vein, under light ether anaesthesia, with $[^{125}I]$-PVP (Radiochemical Centre, Amersham) approximately 2 x 10^6c.p.m., 130 g, in 0.5ml iso-osmotic saline. After 18h the rats were killed and their livers quickly removed and weighed, after perfusion *in situ* with 20ml 0.25*M*-mannitol. Heterolysosomes were prepared in the form of a lysosome-enriched liver fraction, isolated as previously described, and resuspended in 0.25*M*-mannitol at 4°.

The release of $[^{125}I]$-PVP from heterolysosomes was examined by measuring the rate at which it became non-sedimentible. The technique employed is identical to that described earlier in 3.9. 0.5ml of liver fraction suspension was mixed with 1.5ml of acetate-mannitol, pH 5.0, incubated at 37°, and centrifuged at 4° at 25000g for 15 min. Estimations

Buffer	37° Preincubation time (min)							Number of Experiments
	0	10	20	30	40	50	60	
Acetate-Mannitol, pH 5.0	6.9 ± 0.4	11.3 ± 5.4	16.4 ± 7.1	33.5 ± 3.7	39.8 ± 5.3	45.8 ± 7.0	50.2 ± 2.7	4
Acetate-Mannitol, pH 5.0 + 40mM Cysteine	6.9 ± 0.9	13.7 ± 4.8	17.9 ± 6.8	42.3 ± 8.2	46.7 ± 4.8	54.0 ± 2.3	50.9 ± 5.1	4

Table 6.5 Non-sedimentible [^{125}I]-PVP radioactivity in lysosome-enriched liver fractions at 37°

of $[^{125}I]$-radioactivity in the supernatant yielded non-sedimentible $[^{125}I]$-PVP. Total activities were estimated in a similar manner, omitting the final centrifugation.

Table 6.5 shows the non-sedimentible $[^{125}I]$-PVP radioactivity, expressed as a percentage of total radioactivity, following preincubation of the lysosome-enriched liver fraction in acetate-mannitol, with or without 40mM-cysteine, for periods of time up to 60 min.

40mM-cysteine was found to have little effect on the rate at which $[^{125}I]$-PVP radioactivity became non-sedimentible. Both in the absence, and in the presence, of 40mM-cysteine, $[^{125}I]$-PVP was approx. 93% sedimentible, good evidence for its lysosomal location. Although the level to which it was released from the lysosome was somewhat lower than that observed earlier for lysosomal enzymes, the rate at which $[^{125}I]$-PVP was released was very similar, maximal non-sedimentible activity being attained after about 50 min. Recovery experiments later showed that the comparatively low level of the maximal non-sedimentible activity of $[^{125}I]$-PVP was almost certainly caused by readsorption onto the broken membranes and protein which remained sedimentable.

6.7 Discussion

The effects of cysteine on the stability and permeability of rat liver lysosomes has been examined by measuring the latency and sedimentibility of a number of lysosomal enzymes. At no time has any stability change in lysosomes been observed. Desai et al. (1964) observed an increase in the fragility of rat liver lysosomes, as displayed by the increase in non-sedimentible aryl sulphatase activity, when they were exposed to cysteine. Autoxidizable compounds, such as cysteine, were suggested to exert their effect on lysosomes by initiating the peroxidation of the lysosomal membrane, leading to lysosomal disruption. The use of aryl sulphatase as an index of lysosomal integrity is, however, somewhat suspect; less that 30% of the total activity of aryl sulphatase was found to become non-sedimentable after an incubation period of 1h at 37° at pH 5.0, although latency is entirely lost. It seems probable therefore, that Desai et al. (1964) were studying the binding of aryl sulphatase rather than the stability of lysosomes.

The two alternative explanations which were previously proposed to explain the stimulatory effect of cysteine on the intralysosomal digestion of proteins have been examined and now seem somewhat unlikely in the light of the evidence presented in this chapter. In disrupted lysosome-enriched liver fractions, the digestion of formalin-denatured ^{125}I-labelled BSA was found to be increased 2-3 fold in the presence of 40m*M*-cysteine. The stimulatory effect of cysteine on intralysosomal protein digestion probably resides in its ability to penetrate the membrane of intact heterolysosomes and cause direct activation of the thiol-cathepsin which are responsible for digestion. The degree of activation observed is undoubtedly a reflection of the intralysosomal concentration of cysteine. The ability of cysteine to penetrate the lysosomal membrane of intact heterolysosomes seems reasonable in view

of its low molecular weight (121), which is well below the molecular weight threshold proposed by Ehrenreich and Cohn (1969).

Much of the work reported in this chapter was motivated by an interest in cytinosis, a recessively-inherited human storage disease. On a classical pathological basis alone, cytinosis bears a remarkable resemblance to enzymatically-defined storage diseases. Storage of cystine occurs in truly massive amounts, particularly in reticulo-endothelial cells, and other endocytically-active cells, such as those in the kidney tubule. Although the precise metabolic defect responsible for cystine storage remains an enigma, the classification of cytinosis as a lysosomal storage disease seems justified, particularly in view of the results of Schulman and Bradley (1970). In their investigations the penicillamine-cysteine disulphides were observed to cause vacuolation of cystinotic, but not of normal, fibroblasts. Their failure to cause vacuolation in normal cells, which vacuolized on exposure to smaller molecules, suggested that normal lysosomes possess either an enzymatic mechanism for converting the disulphide into a smaller more permeable molecule, or a transport system for facilitating efflux of the disulphide from the lysosome.

If such defects do exist, one could, in theory, lower the concentration of cystine in cystinotic cells by increasing the permeability of the lysosomal membrane to allow cystine to diffuse out. Chayen et al. (1973) and Desai et al. (1964) have suggested that ascorbate can cause an increase in the permeability of the lysosomal membrane. Interest has also grown in the possible use of ascorbate as a reducing agent. Ascorbate is small enough to penetrate the membrane of intact lysosomes and reduce cystine to cysteine. Hope for the possible therapeutic use of ascorbate has been provided by Kroll and Schneider (1974), who have observed that ascorbate causes a decrease in free cystine content of cultured cystinotic fibroblasts.

In the following chapter the effect of ascorbate on the stability and permeability of rat liver lysosomes has therefore been examined.

CHAPTER 7

THE EFFECTS OF ASCORBATE ON THE PERMEABILITY AND STABILITY OF RAT LIVER LYSOSOMES

7.1 Introduction

There are now known to be many reagents capable of causing stability changes in the lysosomal membrane. The labilizing effect of retinol and the stabilizing effect of cortisol on lysosomes are well documented phenomena (Roels, 1969; Weissmann, 1969). Despite the attention that such compounds have received, particularly the anti-rheumatic agents, the mechanism by which they exert their effects is by no means fully understood. Lucy (1969; 1970) has suggested that such reagents may be active by virtue of their effect on micelle-formation in the lysosomal membrane. Weissman (1969) suggested that labilisers may exert their effect by insertion into the lipid components of the lysosomal membrane, causing distention of the membrane and increasing its susceptibility to thermal disruption. The majority of these observations have been made on isolated lysosomes by noting the effect of reagents on the latency and sedimentibility of lysosomal enzymes, such as those observed by Ignarro (1973). Equating these results with the _in vivo_ action of the drug is complicated by the fact that the response may be dose-dependent (Roels, 1969), or may differ in lysosomes of different origin (Ignarro, 1971).

The effect of metal ions in such systems is also interesting. A number of metal ions have been observed to affect the stability of isolated lysosomes (Desai et al., 1964; Verity and Reith, 1967; Chvapil et al., 1972; Davidson, 1975). Davidson (1975) observed an increase in the digestion of injected $[^{125}I]$-ribonuclease in mouse kidney heterolysosomes in the presence of chelating agents, such as EDTA. Although this effect was attributed to the reduction in the inhibitory effects of metal ions on lysosomal enzymes, a more likely explanation is that the chelating agents counteract the labilizing effect of metal ions.

The evidence for the effect of ascorbate on the stability of

isolated lysosomes is contradictory. Verity and Reith (1967) observed that ascorbate provides approximately 50% protection to the labilizing effects of Hg^{2+}. Desai et al. (1964), however, noted a large increase in the fragility of rat liver lysosomes, manifested by the release of aryl sulphatase activity, in the presence of ascorbate.

A cytochemical technique has been developed by Bitensky and her colleagues (Bitensky et al., 1973) for measuring stability and permeability changes in the lysosomal membrane. The technique is based on the latency of Leucyl β-napthylamidase activity in tissue sections. Using this approach, Chayen et al. (1971) have obtained evidence for the increased permeability of the lysosomal membrane of the lysosomes from human rheumatoid synovial cells, and Bitensky et al. (1974a, b) have observed the effects of a number of glucocorticoids and hormones on such lysosomes. Butcher et al. (1973) suggested that the high permeability of lysosomes from rheumatoid cells is connected with a redox imbalance in diseased tissue. Diseased tissue had been shown to possess a more reducing potential than normal tissue, with a greater proportion of sulphydryl groups in the reduced form compared to normal cells. Chayen et al. (1973) examined this suggestion by measuring the latency of Leucyl β-naphthylamidase in the presence of hydrogen acceptors and donors. Dehydroascorbate and menadione were employed as hydrogen acceptors and ascorbate as a hydrogen donor. Ascorbate was found to cause a decrease in the latency of normal tissue, but did not effect the non-latent state of rheumatoid tissue. Conversely, acceptors caused an increase in the latency of Leucyl β-napthylamidase in lysosomes from rheumatoid tissue, interpreted as a diminution of the reducing potential of the membrane, and hence an increase in membrane stability.

In order to examine the conflicting evidence of the effects of ascorbate on lysosomal stability and permeability, the latency and

sedimentibility of a number of lysosomal enzymes have been examined in its presence.

7.2 The effect of ascorbate on the latency of rat liver lysosomes

7.2.1 Time-course of hydrolysis of maltose and p-nitrophenyl α-glucoside

The effect of ascorbate on the latency of rat liver lysosomes was investigated by examining its effect on the time-course of hydrolysis of maltase and p-nitrophenyl α-glucosidase in lysosome-enriched liver fractions at 37°. The rate of hydrolysis of both substrates was estimated at pH 5.0, both in the absence and in the presence of 0.1% Triton X-100, for incubation periods ranging from 0 to 60 min, employing a substrate concentration of 5mM. Full experimental details of latency investigations and individual enzyme assays are described in Chapters 3 and 2 respectively. Ascorbate (Sigma), dissolved in acetate-mannitol buffer, pH 5.0 immediately prior to use, was incorporated in the incubation mixture to yield a final concentration of 1mM, the highest concentration employed by Chayen et al. (1973).

Tables 7.1 and 7.2 show the effect of 1mM-ascorbate on the latency of maltase and p-nitrophenyl α-glucosidase in lysosome-enriched liver fractions at 37°. Results show the free activity for each 10 min time interval, expressed as a percentage of the total activity (activity observed in the presence of 0.1% Triton X-100), in the absence and in the presence of 1mM-ascorbate.

In agreement with previous observations, maltase displayed a typically low initial percent free activity of 15.0% in the absence of ascorbate. As in previous investigations, the free activity rose steadily unitl, after an incubation period of about 40-50 min, it was approximately equal to that observed in the presence of 0.1% Triton X-100. Ascorbate was found to have no measurable effect on either free or total maltase activity. The latency of maltase activity in lysosome-enriched liver fractions was previously shown (Chapter 3) to be an index of the proportion of broken and damaged lysosomes in the preparation. If ascorbate does indeed have an effect upon lysosomal permeability, such

Buffer	Time interval (min)						Number of Experiments
	(0-10)	(10-20)	(20-30)	(30-40)	(40-50)	(50-60)	
Control (Acetate-Mannitol) pH 5.0	67.3 ± 5.4	68.3 ± 8.0	88.2 ± 11.1	92.5 ± 17.6	101.0 ± 8.5	106.9 ± 5.4	4
Acetate-Mannitol, pH 5.0 + 1m*M* Ascorbate	76.4 ± 3.9	70.8 ± 2.2	92.2 ± 6.2	98.1 ± 7.1	100.7 ± 20.0	102.7 ± 12.7	4

Table 7.2 Effect of Ascorbate on latency of p-nitrophenyl α-glucosidase activity in lysosome-enriched liver fractions at 37°

Buffer	Time interval (min) (0-10)	(10-20)	(20-30)	(30-40)	(40-50)	(50-60)	Number of Experiments
Control (Acetate-Mannitol) pH 5.0	15.0 ± 6.6	15.9 ± 8.8	40.7 ± 7.7	55.3 ± 9.5	77.5 ± 17.8	96.2 ± 11.0	4
Acetate-Mannitol, pH 5.0 + 1mM Ascorbate	15.5 ± 5.8	17.0 ± 4.3	45.4 ± 7.7	52.8 ± 19.1	77.8 ± 12.6	102.2 ± 15.1	4

Table 7.1 Effect of Ascorbate on latency of maltase activity in lysosome-enriched liver fractions at 37°

as is suggested by Chayen et al. (1973), it possibly is not an irreversible effect, and as such, would not be detected by the latency of a large impermeable substrate such as maltose, whose accessibility to α-glucosidase is most probably concomitant with its release from the lysosome. One might be able to detect a more subtle change in permeability by examining the latency of a more permeable substrate. It was for this reason that *p*-nitrophenyl α-glucoside was chosen. Ascorbate was however found to have no detectible effect on either the free or total *p*-nitrophenyl α-glucosidase activity in lysosome-enriched fractions at 37° (Table 7.2).

7.2.2 Effect of ascorbate-preincubation on the initial percent free maltase activity

In order to determine if ascorbate's effect is time-dependent, the initial percent free maltase activity was measured in lysosome-enriched fractions which had been pre-incubated at 25° in the presence of ascorbate. Lysosome-enriched liver fractions were prepared as previously described and resuspended either in 0.25*M*-mannitol or in freshly-prepared 0.25*M*-mannitol containing 1m*M*-ascorbate. After incubation at 25°, aliquots of the suspension were withdrawn and assayed for maltase activity at 25° in acetate-mannitol buffer, pH 5.0. Maltase activity was estimated as described in Chapter 2, employing a substrate concentration of 5m*M*. The initial percent free activity of maltase was examined in short, 10 min, assays and compared to the measurable total activity, in the presence of 0.1% Triton X-100.

Table 7.3 shows the initial free maltase activity, expressed as a percentage of the total activity, measured at 25°, following pre-incubation of the lysosome-enriched fraction in either 0.25*M*-mannitol or 0.25*M*-mannitol containing 1m*M*-ascorbate at 25°.

Ascorbate was found to have no measurable effect on the initial percent free maltase activity. Even after a pre-incubation period of

Buffer	25° Preincubation time (min)				Number of Experiments
	0	20	40	60	
Acetate-Mannitol pH 5.0	11.6 ± 6.9	14.8 ± 11.3	17.6 ± 10.0	17.8 ± 13.0	4
Acetate-Mannitol, pH 5.0 + 1mM Ascorbate	12.7 ± 6.8	8.0 ± 3.3	21.5 ± 10.0	11.1 ± 7.6	4

Table 7.3 Effect of preincubation in the presence of ascorbate on the initial percent free maltase activity in lysosome-enriched liver fractions at 25°

60 min, the free and total maltase activities were found to be identical to those observed in the absence of 1m$\underline{M}$-ascorbate. In both cases, very little latency-loss was observed within 60 min.

7.3 Latency and subcellular distribution of Leucyl β-naphthylamidase

Some doubt has been cast over the use of leucyl β-naphthylamidase as a lysosomal marker enzyme (Barret and Pode, 1969; McDonald et al., 1970). Nevertheless, leucyl β-naphthylamide is still frequently used as a chromogenic substrate for the detection of lysosomes (see Bitensky, 1969). In such studies it is presumed to be hydrolysed, either by lysosomal aminopeptidases (Mahadevan et al., 1967; Beck et al., 1969) or by cathepsin B (Snellman, 1969), of lysosomal origin. Since the observations by Chayen et al. (1973) were made in such a system, the latency of leucyl β-naphthylamidase has been investigated.

Firstly, an assay for leucyl β-naphthylamidase activity, similar to the cytochemical method of Bitensky et al. (1973), was sought in order to examine the latency characteristics of the enzyme. Unfortunately many of the cytochemical techniques involve the use of reagents, particularly metal ions, which themselves could affect lysosomal latency (Chvapil et al., 1972; Davidson, 1975). The two assay methods that have been developed are essentially the same, differing only in the method of estimation of the product, β-naphthylamine (see 2.8.7). In the first method, Method a, the product was estimated by spectrophotometric measurement of the diazo product with Fast Garnet. The method is essentially a refinement of the cytochemical technique of Felgenhauer and Glenner (1966), and closely resembles that of Chayen et al. (1973). In the second method, Method b, β-naphthylamine is measured directly by its fluorescence at alkaline pH (Peters et al., 1972).

7.3.1 Subcellular distribution and pH optimum of leucyl β-naphthylamidase

The hydrolysis of leucyl β-naphthylamide by lysosome-enriched liver fractions at 37° was shown by both methods to proceed linearly with time, and display typical enzymic responses to changes in enzyme or substrate concentration. The pH profile of the reaction was very

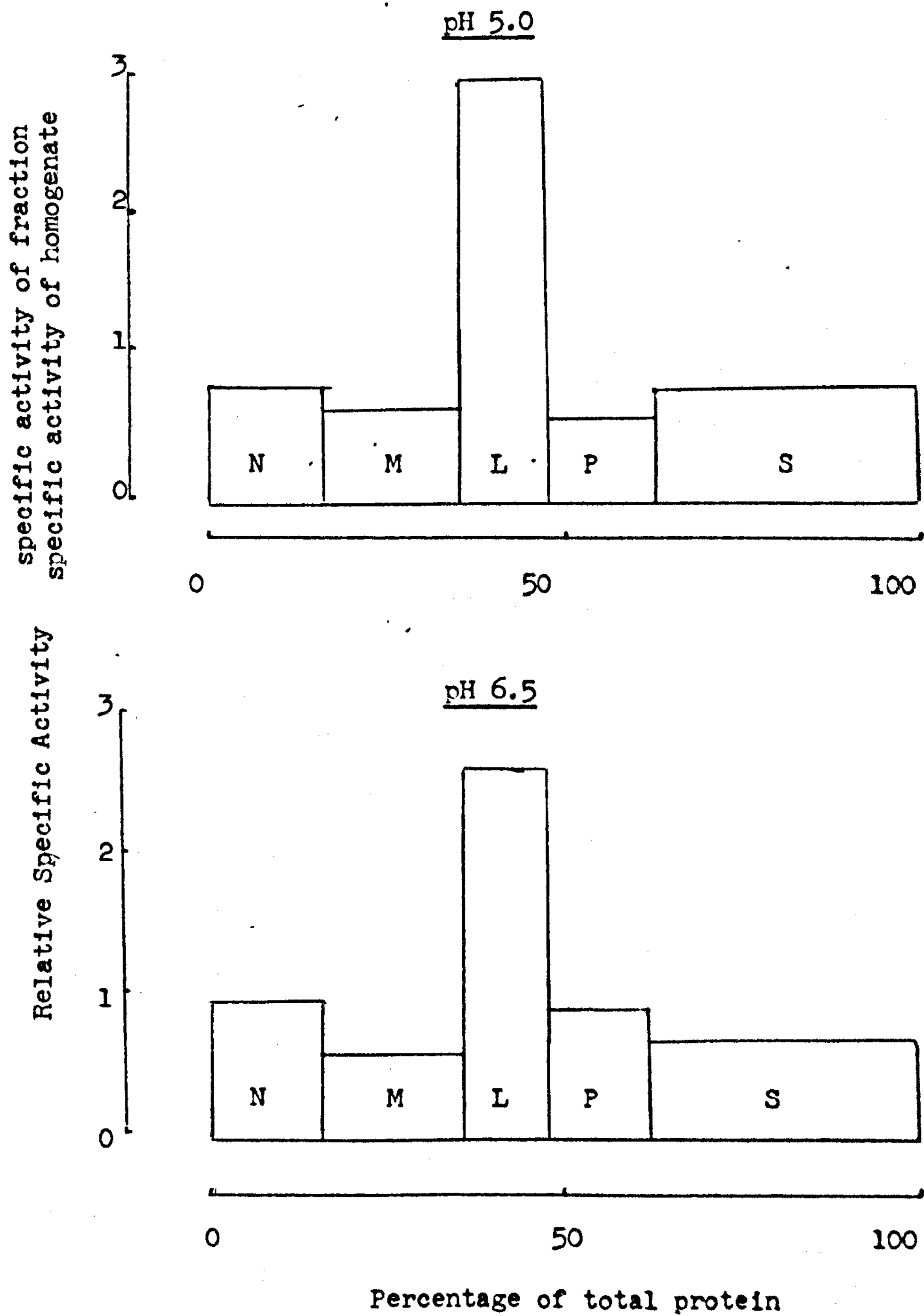


Fig. 7.1 Subcellular distribution of Leucyl β-naphthylamidase in rat liver

interesting and suggested the probable involvement of two or more enzymes. The reaction showed a small broad peak of activity at pH 4.0-5.5, as well as a larger more pronounced peak of activity at pH 6.5-7.0. The activity at pH 6.5 was found to be 2-3 times greater than that observed at pH 5.0. The Michaelis constants (Km app.) for the activity were estimated by both assay methods, and showed good correlation: at pH 5.0 the Km app. was 0.5-1.0m*M*, at pH 6.5 the Km app. was 0.15-0.20m*M*.

Subcellular fractions of rat liver were prepared as previously described in 2.2, resuspended in chilled 0.25*M*-mannitol, and examined for protein concentration and leucyl β-naphthylamidase activity. Since the pH profile and Michaelis constants were indicative of the involvement of at least two enzymes, leucyl β-naphthylamidase was examined both at pH 5.0 and at pH 6.5 in all fractions, employing assay method a.

Fig. 7.1 shows the subcellular distribution of leucyl β-naphthylamidase activity in rat liver. Results are presented graphically as described by de Duve et al. (1955), fractions appearing from right to left in the order of their isolation, i.e. N, M, L, P, S.

The activity of leucyl β-naphthylamidase, both at pH 6.5 and pH 5.0, displayed a subcellular distribution very similar to that observed for other known lysosomal enzymes (Fig. 4.1). Although such evidence suggests that leucyl β-naphthylamidase is a lysosomal enzyme, similar distributions may have been observed if the enzyme were cytoplasmic in origin but adsorbed onto the outer face of the lysosomal membrane. The latency characteristics of the enzyme were therefore examined in an effort to establish its true location.

7.3.2. Latency of leucyl β-naphthylamidase in lysosome-enriched liver fractions - Method a.

The latency of leucyl β-naphthylamidase activity has been examined at pH 6.5 and pH 5.0. The time-course of hydrolysis of leucyl

β-naphthylamine at 37°, in lysosome-enriched liver fractions, has been examined in the absence and in the presence of 0.1% Triton X-100, by the method outlined in 3.5. A substrate concentration of 1.5m<u>M</u> was always employed. The latency of maltase activity was estimated in all lysosome preparations and used as an index of the integrity of the preparation.

Triton X-100 was found to cause inhibition of leucyl β-naphthylamidase activity. At pH 5.0 activity was almost entirely abolished by 0.1% Triton X-100, whilst the activity at pH 6.5 showed a decrease of approximately 50%. In lysosome-enriched fractions that displayed typically high (85-90%) initial maltase latency, no, or very little, latency of leucyl β-naphthylamidase was observed. The free leucyl β-naphthylamidase activity was constant from the beginning of the reaction, and found to be equal to that observed in lysosomes resuspended in water.

Encorporation of 1m<u>M</u>-ascorbate into the incubation mixture lead to a high degree of variability in the measurable leucyl β-naphthylamidase activity. Freshly-prepared ascorbate was found to cause a drastic reduction in activity, whilst ascorbate that had been dissolved earlier and left at room temperature for 4h showed little inhibitory effect. Although this effect was shown to be dependent on the concentration of ascorbate, kinetic investigations proved fruitless. It was, however, shown that ascorbate exerts its effect, not on the enzyme reaction, but on the diazotization reaction of β-naphthylamine and Fast Garnet. Fig. 7.2 shows the effect of ascorbate on the calibration curve for β-naphthylamine. 10^{-3}<u>M</u>-ascorbate showed an inhibitory effect of about 80%, whilst 10^{-4} and 10^{-5}<u>M</u>-ascorbate had no detectible effect. Using this method, it is therefore impossible to examine the effect of ascorbate on the latency of leucyl β-naphthylamine.

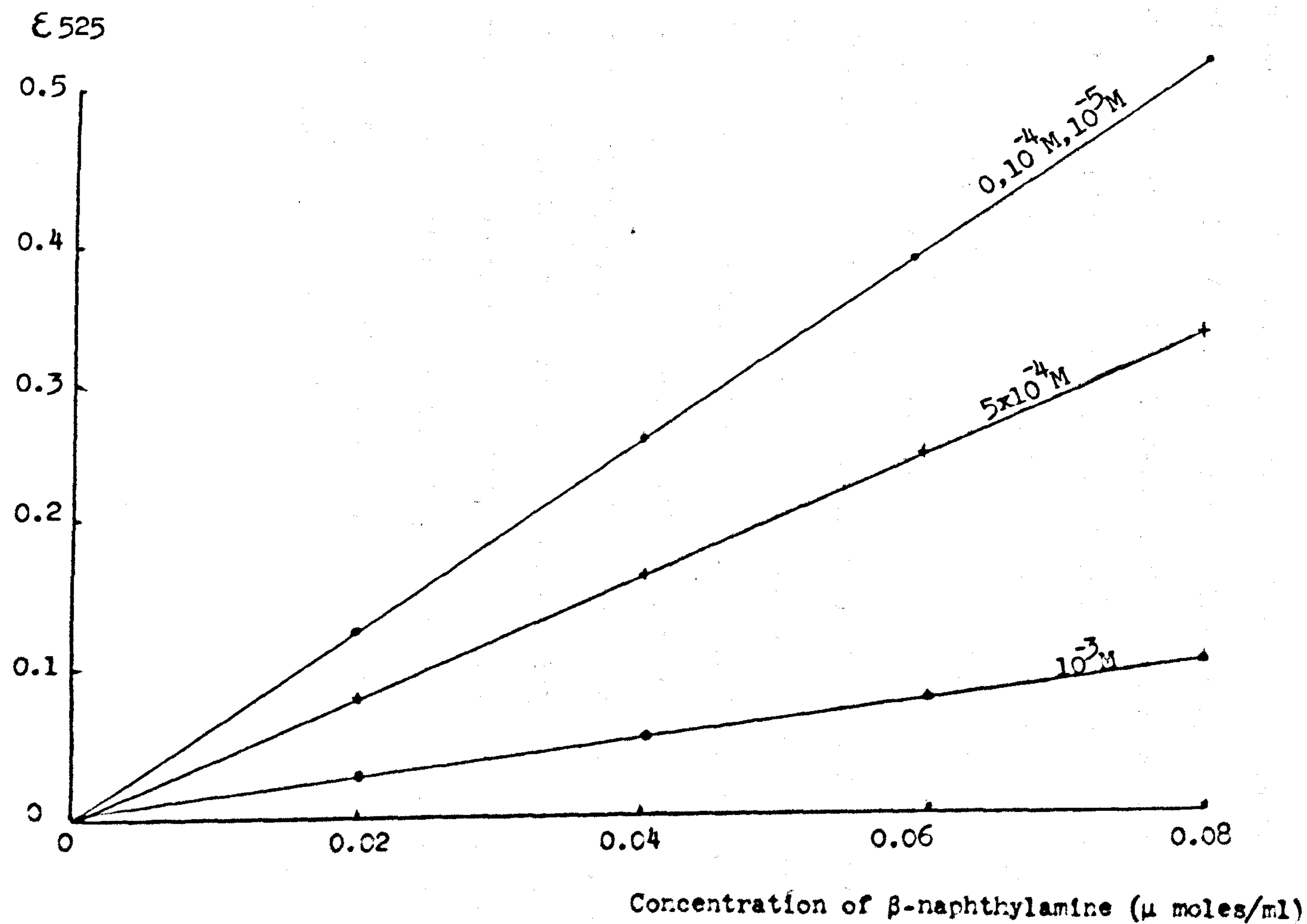


Fig. 7.2 Effect of ascorbate on the calibration-curve for β-naphthylamine

7.3.3 Latency of leucyl β-naphthylamine in lysosome-enriched liver fractions - Method b.

The time-course of hydrolysis of leucyl β-naphthylamine at 37° has been examined in a similar manner to that described earlier, the product, β-naphthylamine,being estimated directly by its fluorescence in alkaline solution (see 2.8). Triton X-100 was again shown to have an inhibitory effect on leucyl β-naphthylamidase of similar proportions to that observed with Method a. Ascorbate was found to have no inhibitory effect upon the enzyme reaction, confirmation that the inhibition observed in Method a is due to some effect on the trapping reaction of β-naphthylamine, and not on the enzyme itself.

The free activity of leucyl β-naphthylamidase was found to be constant from the beginning of the experiment, and equal to that displayed in non-osmotically buffered media. Table 7.4 shows the initial free leucyl β-naphthylamidase activity, expressed as a percentage of the total activity (activity observed by suspensions in water), at pH 5.0 and pH 6.5, in the absence and presence of 1m*M*-ascorbate, in lysosome-enriched liver fractions at 37°. Ascorbate was found to have no effect on the availability of leucyl β-naphthylamidase activity at 37°, which was found to be fully available, both in the presence and absence of 1m*M*-ascorbate, at both pH's.

	pH of Acetate-Mannitol buffer 6.5	5.0	Number of Experiments
No Ascorbate	99.7 ± 9.3	102.7 ± 18.6	4
+ 1mM Ascorbate	104.5 ± 18.2	99.0 ± 20.9	4

Table 7.4 Initial percent free activity of leucyl β-naphthylamidase in lysosome-enriched liver fractions at 37°

7.4 Effects of ascorbate on the sedimentibility of leucyl β-naphthylamidase

The technique of scanning microdensitometry which has been developed by Bitensky and her colleagues (Bitensky et al., 1973) for investigating experimentally-induced changes in the permeability properties of lysosomes, is essentially a cytochemical method, and as such, involves the use of tissue sections that have been frozen and thawed. It seems probable that this treatment itself could prove injurious to lysosomes, particularly to the lysosomal membrane. It seems likely that in this technique it is the rate of release of enzyme from the lysosome that is being monitored, rather than an increase in the permeability of the lysosomal membrane. As discussed in Chapter 3, if a permeable substrate is used in the detection of the enzyme, these two phenomena will not be manifested concomitantly, as they would be if a non-permeable substrate were employed.

In order to investigate if ascorbate has an effect upon the rate at which enzymes are released from the lysosome, the sedimentibility of α-glucosidase and leucyl β-naphthylamidase have been examined. The technique employed is identical to that described earlier in 3.9. 0.5ml of liver fraction suspension was mixed with 1.5ml of acetate-mannitol buffer, either pH 5.0 or pH 6.5, incubated at 37°, and centrifuged at 4° at 25000g for 15 min. Enzyme determinations on the supernatant yielded the non-sedimentible activity. Total activity was estimated in non-osmotically buffered media, omitting the final centrifugation.

Tables 7.5 and 7.6 show respectively the non-sedimentible activity of leucyl β-naphthylamidase and p-nitrophenyl α-glucosidase, expressed as a percentage of the total activity, following preincubation of the lysosome-enriched fraction in acetate-mannitol, with or without 1m*M*-ascorbate, for periods of time up to 60 min.

The rate of release of both p-nitrophenyl α-glucosidase and leucyl

Buffer	37° Preincubation time (min)							Number of Experiments
	0	10	20	30	40	50	60	
Acetate-Mannitol, pH 5.0	1.3 ± 1.1	0.5 ± 0.2	5.8 ± 3.8	13.0 ± 3.8	18.7 ± 4.5	14.7 ± 8.5	17.0 ± 7.1	4
Acetate-Mannitol, pH 5.0 + 1mM Ascorbate	2.9 ± 5.0	2.9 ± 5.0	9.8 ± 4.1	11.0 ± 8.3	16.6 ± 7.9	11.9 ± 0.5	21.6 ± 15.0	4
Acetate-Mannitol, pH 6.5	5.9 ± 1.5	7.0 ± 1.8	5.3 ± 4.1	5.5 ± 2.5	7.7 ± 3.6	7.6 ± 3.0	7.1 ± 3.7	4
Acetate-Mannitol, pH 6.5 + 1mM Ascorbate	6.3 ± 1.8	5.2 ± 1.5	5.3 ± 0.5	5.8 ± 2.3	6.6 ± 1.3	8.3 ± 1.9	10.0 ± 4.5	4

Table 7.5 Non-sedimentible activity of leucyl β-naphthylamidase in lysosome-enriched liver fractions at 37°

Buffer	37° Preincubation time (min)							Number of Experiments
	0	10	20	30	40	50	60	
Acetate-Mannitol, pH 5.0	4.5 ± 3.6	9.6 ± 2.6	31.4 ± 14.6	53.3 ± 18.7	76.3 ± 13.5	74.8 ± 3.9	81.5 ± 8.1	4
Acetate-Mannitol, pH 5.0 + 1mM Ascorbate	3.5 ± 3.6	5.1 ± 2.9	27.4 ± 2.9	40.9 ± 9.6	74.4 ± 1.1	70.4 ± 4.5	78.3 ± 8.4	4
Acetate-Mannitol, pH 6.5	5.6 ± 3.7	4.2 ± 2.9	7.3 ± 3.0	6.6 ± 0.4	14.1 ± 2.5	10.7 ± 2.6	12.1 ± 1.8	4
Acetate-Mannitol, pH 6.5 + 1mM Ascorbate	2.6 ± 2.1	2.2 ± 1.4	6.7 ± 2.8	3.9 ± 2.1	12.1 ± 3.6	13.9 ± 5.0	19.6 ± 1.3	4

Table 7.6 Non-sedimentible activity of p-nitrophenyl α-glucosidase activity in lysosome-enriched liver fractions at 37°

β-naphthylamidase activities, at both pH's seems to be unaffected by the presence of 1m*M*-ascorbate. From the rate of increase in non-sedimentible *p*-nitrophenyl α-glucosidase activity, lysosomes appear to be much more stable at pH 6.5 than at pH 5.0. Even at the lower pH, leucyl β-naphthylamidase activity was released very slowly from the lysosome, approximately 80% of the activity remaining sedimentible after a pre-incubation period of 60 min. At the higher pH very little increase was observed in the non-sedimentible activity of leucyl β-naphthylamine.

7.5 Discussion

As in the previous chapter, much of the work reported here was motivated by an interest in cytinosis, in particular, the possible use of ascorbate as a means of reducing the accumulation of cystine in the disease. Ascorbate, it was suggested, might accomplish this in two ways; either by the chemical reduction of cystine to cysteine, or by increasing the permeability of the lysosomal membrane.

The latter suggestion was made largely in response to observations made by Chayen et al. (1973). Efforts were made to parallel Chayen's work by biochemical methods, using the latency and sedimentibility of a number of enzymes in isolated rat liver lysosomes as the criteria of permeability and stability. The validity of the use of leucyl β-naphthylamidase as a lysosomal marker has also been examined. Although leucyl β-naphthylamidase activity was distributed throughout subcellular fractions of rat liver in a very similar manner to other known lysosomal enzymes, it was not found possible to demonstrate any latency of the enzyme in intact lysosomes. Such non-latency could be explained if the substrate used in the detection of the enzyme could penetrate the lysosomal membrane (see Chapter 3), or if the enzyme were located either in another organelle or on the cytoplasmic face of the lysosomal membrane. Leucyl β-naphthylamidase activity did, in fact, only slowly become non-sedimentible when lysosome-enriched fractions were incubated at 37°, indicating that it was very tightly bound. An observation that directly affects the interpretation of the results of Chayen et al. (1973) was the inhibitory effect of ascorbate on the diazotization reaction which is employed to estimate the enzyme's hydrolysis product. This inhibition was found to be dependent on the concentration and age of the ascorbate solution, and is undoubtedly connected with the autoxidation that ascorbate readily undergoes in aqueous solution. Latency measurements by an

independent method not involving this diazotization reaction confirmed, however, that leucyl β-naphthylamidase was fully available in intact lysosomes and was unaffected by the presence of ascorbate. Its use as an index of lysosomal stability and permeability therefore appears somewhat suspect.

A much more reliable enzyme for this purpose is α-glucosidase, whose lysosomal origin is well-documented. Even with this enzyme, however, ascorbate was found to have no detectable effect on the permeability or stability of rat liver lysosomes. In discussing the inability of many non-steroidal anti-inflammatory agents to stabilize membranes of isolated lysosomes, Weissman (1969) considered it was possible that they might act as stabilizers only if administered to intact cells. This suggestion might be particularly relevant to ascorbate whose effects were noted in tissue sections. The fact that lysosomes of different origin may vary in their response is also a possible explanation (Ignarro, 1971). The main hope for the therapeutic use of ascorbate in cytinosis, however, probably resides in its reductive capacity and not its ability to labilize the lysosomal membrane.

CHAPTER 8

GENERAL

DISCUSSION

In the Introduction to this thesis, the methods that have generally been employed in the past to evaluate the permeability properties of lysosomes were listed, along with an historical account of how these approaches have helped in our understanding of lysosomes and their function. The experimental approach which has been most frequently employed in this thesis to measure the permeability characteristics of the lysosomal membrane is that of latency. The latency of lysosomal glycosidases were examined as these enzymes offered the possiblity of using a wide variety of substrates.

Glycosidases were shown to possess similar latency properties to other classes of lysosomal enzymes, and have proved a useful tool for investigating the effect of substrate size and exogenous enzymes on the latency of lysosomal enzymes. The latency of glycosidases has also proved helpful in evaluating the permeability of the lysosomal membrane to nucleic acid derivatives and in examining the effects of ascorbate and cysteine on lysosomal permeability and stability.

It is evident from the results presented in this thesis, particularly those in Chapters 3, 4 and 5, that the latency of a lysosomal enzyme cannot simply be attributed to the presence of a semi-permeable membrane surrounding a matrix of lysosomal enzymes. The observed latency of a lysosomal enzyme depends largely on the chemical nature of its substrate and on the rate at which the substrate enters the lysosome and is hydrolysed. The permeability of the lysosomal membrane is, therefore, better visualized as a kinetic property than as a fixed value. Sucrose, and other such classical 'non-permeable' compounds, are better envisaged as permeating the lysosomal membrane very slowly, rather than not at all: how else could one explain the slow regression of Triton WR-1339 (Wattiaux et al., 1963) or sucrose (Cohn and Ehrenreich, 1969) in heterolysosomes laden with these undegradable substances?

It has been suggested (Segal et al., 1969, 1974) that protein turnover in the cell is closely connected with the permeability properties of the lysosomal membrane and the susceptibility of proteins to digestion by lysosomal proteinases. Although it seems doubtful that the penetration of proteins into intact lysosomes would be sufficiently rapid to maintain turnover, the involvement of lysosomes in protein turnover is still an attractive concept. Perhaps some proteins enter lysosomes by an hitherto unrecognized process similar to autophagy. Future research may reveal how such a process, if it exists, could achieve the required specificity.

The ability of nucleosides to penetrate the membrane of intact lysosomes, slightly unexpected in view of their relatively high molecular weights, is a good illustration of how the chemical properties of a molecule can determine its membrane permeability. The ability of lysosomes to digest nucleic acids to the level of the nucleoside raises the interesting question about the fate of phosphate when it is produced intralysosomally. The lysosomal membrane could possess vectorial permeability properties, allowing large ions, such as phosphate and sulphate, to diffuse out of, but not into the lysosome, or else, such ions may exit by some unrecognized active transport process.

The accumulation of basic dyes within lysosomes is a well-documented phenomenon, but the concentration mechanism remains a matter for conjecture. It has been suggested that such dyes penetrate the membrane in their unprotonated form, (Reijngoud and Tager, 1973; de Duve et al., 1974) and are trapped by protonation, with a proton pump in the lysosomal membrane providing the driving force for continuing uptake. Evidence suggests that Chloroquine enters lysosomes by this mechanism, and exerts its biological effects by inhibiting proteolysis (Wibo and Poole, 1974). The presence of a proton pump in the lysosomal membrane is clearly

important, not only in the maintenance of intralysosomal pH, but also in the entry of certain substances into lysosomes. More work is required to establish the nature of the proton pump.

Investigations on the permeability of the lysosomal membrane such as have been described in this thesis, have direct bearing on our understanding of the mechanisms that ensure osmotic protection of lysosomes within the cell. Rather than a static phenomenon, this should be conceived as a dynamic system (Lucy, 1969), the equilibrium of which depends on many variables, such as: the entry into lysosomes by endocytosis of osmotically-active molecules; the production of osmotically-active fragments in the degradation of macromolecules; and the possible presence of energy-dependent transport systems (Mego et al., 1972). Obviously, passive permeability is not the only means by which a molecule may be translocated across the lysosomal membrane, but there seems no doubt that it is one of the fundamental properties which enables lysosomes to fulfil their intended role.

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