STUDIES ON THE RELEASE OF EXOGENOUS SUBSTANCES FROM RAT LIVER LYSOSOMES

by

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ABSTRACT

The permeability of the lysosome membrane to substances related to cystine was examined in Chapter 3. The results of this study indicate that cysteine and cysteamine can permeate the lysosomal membrane, whilst cystamine shows pH-dependent permeability properties.

In Chapter 4, reports that ATP could stimulate the uptake of proteins by intact lysosomes in vitro were examined. There was no evidence that ATP influenced uptake of ^{125}I-labelled albumin by intact lysosomes. ATP disrupted intact lysosome-enriched suspensions by lowering the pH of the medium.

^{125}I-labelled PVP, [U-^{14}C]sucrose and [^{35}S]cystine were entrapped within negatively charged (DPPC/DPPA) multilamellar liposomes (Chapter 6). Both ^{125}I-labelled PVP and [U-^{14}C]sucrose were retained by these liposomes at 37^{o}C, but [^{35}S]cystine was not. At 37^{o}C ^{125}I-labelled PVP leaked from positively charged (DPPC/stearylamine) liposomes.

In Chapters 7 and 8, the distributions of injected ^{125}I-labelled PVP, [U-^{14}C]sucrose and [^{35}S]cystine in the rat were investigated. For all three substances, entrapment within negatively charged liposomes led to an increased hepatic uptake compared with non-entrapped substance. ^{125}I-labelled PVP remained within the liver after its initial uptake, at least over the 48h after injection. However, both liposome-entrapped [U-^{14}C]sucrose and liposome-entrapped [^{35}S]cystine had largely been removed from the liver 48h after injection.

The subcellular location of injected (DPPC/DPPA) liposome entrapped ^{125}I-labelled PVP suggests that these liposomes entered the liver cells by phagocytosis. If one assumes that liposomes of identical composition but with different contents are treated identically by cells (the evidence is discussed in Chapter 8), then the results suggest that [U-^{14}C]sucrose and [^{35}S]cystine are able to escape from the lysosomes.

Further evidence for the entry of DPPC/DPPA liposomes containing entrapped ^{125}I-labelled PVP into liver cells by phagocytosis was obtained in Chapter 9, in which the setting up of a method for exploring the permeability of the lysosome membrane to small molecules is described. The results of the first experiments using ^{125}I-labelled PVP indicate that it is unable to escape from intact lysosomes *in vitro*.

ABBREVIATIONS

Acid DNAase	:	acid deoxyribonuclease
Aryl-S.ase	:	(in Figure and Tables) arylsulphatase
DMPC	:	dimyristoylphosphatidylcholine
DOPC	:	dioleoylphospatidylcholine
DPPA	:	dipalmitoylphosphatidic acid
DPPC	:	dipalmitoylphosphatidylcholine
DSPC	:	distearoylphosphatidylcholine
EDTA	:	ethylenediamine tetraacetic acid
egg PC	:	egg phosphatidylcholine
EGTA	:	ethyleneglycol-bis-(β-aminoethyl ether) N,N'-tetraacetic acid
GIT	:	glutathione-insulin transhydrogenase
^{125}I-Labelled dBSA	:	formalin denatured, ^{125}I-labelled bovine serum albumin
^{125}I-Labelled PVP	:	^{125}I-labelled poly(vinylpyrrolidone)
^{125}I-PVP	:	(in Figure and Tables) ^{125}I-labelled poly(vinyl-pyrrolidone)
LUV	:	large unilamellar vesicles
MLV	:	multilamellar vesicles
MOPS	:	morpholinopropane sulphonic acid
N-Ac-β-G.ase)	:	(in Figures and Tables)
N-Ac-β-Gluc.ase)		N-acetyl-β-D-glucosaminidase
n.d.	:	(in Tables) not determined
PC	:	phosphatidylcholine
PVP	:	poly(vinylpyrrolidone)
S.E.M.	:	standard error of the mean
SUV	:	small unilamellar vesicles

CONTENTS

CHAPTER 1

General Introduction

1.1 Cystinosis : An unresolved problem

Cystinosis is an inherited disease in which vast amounts of cystine accumulate within cells, particularly those of the reticuloendothelial system (see Schulman, 1973; Seegmiller, 1973). Children with the disease appear normal for the first six months of life, but then gradually show polyuria and polydypsia, signs of a renal tubular defect of water reabsorption, which in turn leads to recurrent fevers (usually the presenting symptom). As the tubular dysfunction increases, other signs are presented, e.g. increased renal excretion of glucose, amino acids, phosphate and potassium. Careful examination at this stage may reveal the presence of glomerular damage, and it is the rate at which this progresses that determines the course of the disease. Cystinotic children remain below the third percentile in both height and weight throughout their lives, and as the disease progresses, the growth failure becomes more apparent. The glomerular damage progresses so that, unless treated, death usually occurs before puberty.

The disease is inherited as a simple autosomal recessive which, together with its rarity, means that it is often not identified until the more overt signs of glomerular damage occur. Diagnosis is confirmed by the presence of crystalline opacities in the conjunctiva and peripheral cornea, using a simple test performed with a slit lamp.

The blood of cystinotic children contains normal levels of cystine (Schneider, et al., 1967; Seegmiller et al., 1968), which indicate that the intracellular cystine crystals do not enter the cells from the extracellular fluid. The crystals are found primarily within the reticuloendothelial cells of bone marrow, liver, spleen and lymphatic system. Although there is an increased concentration of cystine within cystinotic cells, a comprehensive survey of all the known enzymes of cystine metabolism revealed no defect (Patrick, 1962; Tietze et al., 1972).

Schneider et al. (1967) found that the cystine in cystinotic leucocytes was largely contained in a granular fraction which co-sedimented with acid phosphatase, and Schulman et al. (1969), using similar cells, localised the cystine to a fraction rich in lysosomal enzymes. His group later published electron micrographs (Schulman et al., 1970) showing that in vitro, macrophages took up ferritin to the same cellular compartment as the cystine, while other workers (Patrick and Lake, 1968) have shown electron micrographs of lymph node cells containing cystine crystals surrounded by a membrane. This demonstration that excess cystine is within the lysosomes is compatible with cystine being derived from extralysosomal cystine which is abnormally concentrated by the lysosomes, or has its origin within the lysosomes. Recent work (Oshima et al., 1976) has shown that the cystine accumulated within the lysosomes is derived from protein material, possibly from intracellular protein (Thoene et al., 1977).

Although there has been much study of the ability of lysosomes to degrade proteins (see Barrett and Dingle, 1971), comparatively little is known of the fate of protein disulphides within the lysosomes. In order to speculate on the possible treatment of cystine residues by the lysosomes, and to examine the possible molecular basis of cystinosis, it is first necessary to discuss aspects of the lysosomal system (Section 1.2). However, even without this fundamental information, various forms of therapy have been attempted. Such therapy is of four types:

(i) Dietary therapy.

Cystine is not an essential amino acid, and several attempts have been made to limit the dietary intake of cystine in an effort to diminish the cellular levels (see Bickel et al., 1973). Although such diets succeed in lowering the plasma cystine concentrations they have little, if any, effect on the intracellular levels of the amino acid.

(ii) Therapy using reducing agents.

Clearly, if the cystine can be reduced to the soluble cysteine, then it may be possible to remove the deposits within the cells. Both dithiothreitol and ascorbic acid have been used in this way following reports than these substances lowered intracellular cystine levels of cystinotic fibroblasts *in vitro*. Unfortunately neither substance proved to be clinically effective (Seegmiller, 1973; Schneider and Schulman, personal communication).

(iii) Renal transplantation.

Since the cause of death in cystinosis is usually attributed to renal failure, a renal transplant may alleviate the symptoms of the disease. Additionally, since the allograft will be of a different genotype the glomerular damage might not be expected to recur. Since the first transplants in 1969, the evidence suggests that renal allografts do extend the life-expectancy of cystinotic children considerably (see Goodman *et al.*, 1973).

(iv) Diagnosis *in utero*.

There is now a reliable test for the identification of cystinosis in the foetus (Schneider et al., 1974). The test is only carried out when a family already has a cystinotic child, but does give the parents the chance of avoiding further cystinotic offspring, should the test prove positive, by opting for an abortion of the affected foetus.

1.2 The digestive organelle : The lysosome

1.2.1 The digestive capacity of lysosomes

The lysosome is a subcellular particle which consists of an array of digestive enzymes bounded by a semi-permeable membrane. The lysosomal enzymes are capable of degrading a wide range of biopolymers to small molecules (Holtzman, 1976). In general the enzymes are specific only in the type of bond they are capable of breaking. For example, there is a whole series of lysosomal glycosidases that is responsible for the sequential removal of specific sugar residues from gangliosides, each enzyme specific for one type of glycosidic bond.

The degradation of proteins is also carried out by a collection of enzymes known as the cathepsins (see Barrett and Dingle, 1971; Barrett, 1975). Of the major enzymes, cathepsins B1 and D are endopeptidases, and cathepsins A and B2 are carboxypeptidases. In addition cathepsin C (dipeptidyl aminopeptidase I) and dipeptidyl aminopeptidase II are present within lysosomes. Clearly, the lysosome is capable of degrading proteins to the level of amino acids.

1.2.2 The lysosomal membrane

The chief feature of lysosomes is the membrane which separates the enzymes from the surrounding cytosol. It is the permeability of this membrane which determines many of the properties of the lysosomes. The membrane effectively separates the enzymes within intact lysosomes from substrates outside, and it was this feature that led to their discovery by de Duve in 1949 (see de Duve, 1969) through the phenomenon of latency. The barrier preventing full expression of enzymic activity can be breached by the use of osmotic shock, ultrasonic irradiation, detergents and freeze/thaw cycles.

1.2.2.1. The permeability of the lysosomal mambrane

The lysosomal membrane is not freely permeable to all substances, e.g. the lysosomal enzymes and those substrates that confer the property

of latency upon lysosomes. When macromolecular substrates are digested within lysosomes many low molecular weight products result. If all of these small molecules were unable to escape from the lysosomes, an increase in the internal osmolarity of the lysosomes would occur. This would cause an influx of water which could lead to a rupture of the lysosomal membrane. Also, if the digestion products were to remain sequestered within the lysosomes, there would be no advantage in carrying out the breakdown of the initially entrapped substrates. It seems inevitable, therefore, that the digestion products must be capable of traversing the lysosomal membrane.

Cohn and Ehrenreich (1969) showed that when mouse peritoneal macrophages were cultured in the presence of sucrose, vacuolation of the cells occurred. They attributed this to the inability of sucrose to escape from the lysosomes of the macrophages either as sucrose or in a digested form. As well as sucrose, trisaccharides and some other disaccharides caused vacuolation, and so it was concluded that these substances could not escape from the lysosomes. In a similar survey Ehrenreich and Cohn (1969) found that of a series of amino acids and peptides tested, only D-alanyl-D-alanyl-D-alanine and D-glutamyl-D-glutamic acid caused vacuolation, suggesting that they could not cross the lysosomal membrane. The authors proposed (Cohn and Ehrenreich, 1969) that non-digestible substances having a molecular weight in excess of 220 could not permeate the lysosomal membrane.

A lysosome-enriched suspension will remain osmotically stable in an isotonic solution of an impermeant solute, and thus the latency of lysosomal enzymes is maintained in such circumstances. In similar solutions of permeant solutes, the increase in internal osmolarity occurring as the solute permeates will lead to an influx of water which will cause lysis of the organelles, and a progressive loss of latency. Utilizing this property of lysosomes, Lloyd (1969) found that the lysosomal membrane was permeable to most monosaccharides at 25°C, two notable

exceptions being potassium D-gluconate and D-sorbitol, which are probably too polar and too large (sorbitol cannot form a ring structure) respectively, so that they cannot penetrate the membrane of the lysosomes. All the disaccharides gave osmotic protection, and therefore could not have penetrated the lysosomal membrane. Lloyd (1971) found that amino acids and tripeptides penetrated only slowly, whereas dipeptides quickly caused disruption of the lysosomes. He attributed the inability of amino acids to cross the lysosomal membrane to their charged nature. Lloyd (1971) endorsed the hypothesis of Cohn and Ehrenreich (1969) but suggested that the conformation and charge of molecules might be as important as molecular weight in determining their ability to cross the lysosomal membrane. Subsequent work (Burton et al., 1975) has reinforced this view, since several nucleosides can apparently traverse the lysosomal membrane at 25°C. Lysosomal extracts were incapable of digesting the nucleosides and the authors concluded that the nucleosides were able to traverse the lysosomal membrane because of their hydrophobic aglycone.

A survey of the lysosomal membrane permeability to small ions was undertaken by Henning (1975) who found that the order of permeability was $H^+ >> Cs^+ > Rb^+ > K^+ > Na^+ > Li^+ >> Mg^{2+}$, Ca^{2+} in Tritosomes (lysosomes loaded with Triton WR-1339) at 4°C. At 37°C Casey et al. (1978) found a similar order for the cations Cs^{2+}, K^+ and Na^+, but found H^+ to be least permeable and $SCN^- > I^- > CH_3COO^- > Cl^- \approx HCO_3^- \approx P_i > SO_4^{2-}$ for anions. These results are qualitative, and it is therefore difficult to relate them to the observation that at 25°C sodium chloride solutions > 0.1M provide osmotic protection (Lloyd, 1971) to lysosomes. It has been suggested (Davidson and Song, 1975) that there is a thermally-induced alteration of lysosomal permeability to ions, and a recent report by Ruth and Weglicki (1978) provides evidence for the temperature-dependent loss of latency of lysosomal enzymes, involving a transition of the lysosomal membrane at approx. 15°C.

The lysosomal membrane is, as would have been expected, permeable to most small molecules, but it appears that the ability of substances to traverse the membrane is not determined solely by their molecular weight.

1.2.3 The method of entry of substrates into the lysosomes

The ability of the lysosomal membrane to exclude biopolymers has been discussed, and it is this property that necessitates a degree of complexity in the functioning of lysosomes. Since neither the lysosomal enzymes nor potential macromolecular substrates can traverse the membrane, there must be special mechanisms to present the enzymes with substrates.

1.2.3.1. Extracellular substrates

Endocytosis is the process by which cells internalize extracellular substances and present them to the lysosomal system. The substances are taken into an infolding of the plasma membrane which forms a vesicle. The vesicle subsequently fuses with a lysosome to initiate digestion of the contents of the vesicle. The involvement of lysosomes in the digestion of extracellular substrates was first shown by the histochemical work of Strauss (1964; 1967) on kidney tubule epithelia and Kupffer cells. Endocytosis has been classified according to the nature of the internalized material.

Particulate matter, often with a diameter in excess of 1μm, is captured by phagocytosis and is contained within a phagosome. This process requires the adsorption of the particle to the surface of the cell prior to uptake. The mechanism is thought to involve the microtubular system, and is energy dependent.

Smaller particles and soluble material are taken up by pinocytosis, a process which has been subclassified by Allison and Davies (1974) into macro- and micropinocytosis. They have proposed that uptake into small vesicles (pinosomes) of 70-100nm diameter is by micropinocytosis, an energy independent process. Pinosomes of a larger size are formed by macropinocytosis, a mechanism that is energy dependent.

Pinocytosed proteins can be taken up either in the fluid phase or adsorbed to the surface of the cell. The high rate of selective internalization of some proteins is due to their adsorption to the cell surface prior to uptake (Moore et al., 1977). This adsorption may be of a non-specific kind, such as that reported by Moore et al. (1977) to hydrophobic regions of ^{125}I-labelled denatured bovine serum albumin. Alternatively, the adsorption may be quite specific, due to recognition of specific chemical groups within a molecule. Several specific receptors are now known, e.g. the galactose recognition site of hepatocytes (Morrell et al., 1971) which binds to desialylated glycoproteins, and the phosphohexose recognition system of fibroblasts (Kaplan, 1977a; 1977b) involved in the binding of β-glucuronidase and other enzymes.

Using a lysosome-enriched fraction of mouse liver containing exogenous radiolabelled albumin, Mego and McQueen (1965) were able to show the digestion of the sedimentable protein to an acid-soluble form, and identified the majority of this product as iodotyrosine. Subsequent studies using rat yolk sac (Williams et al., 1971) and renal cells (Davidson, 1973; 1975b) have also shown the degradation of exogenous radiolabelled proteins within lysosomes, and the release into the medium of acid-soluble radioactivity.

1.2.3.2. Endogenous material

The incorporation of cytosol within a membrane-delimited vacuole (or autophagosome) has been observed in some cell types (see Ericsson, 1969). Autophagosomes so formed then fuse with lysosomes, although some may contain lysosomes (and thus lysosomal enzymes) at their conception. Autophagy seems to be more prevalent in tissues undergoing pathological or physiological regression (Novikoff and Essner, 1962), but autophagosomes are probably present in most cell types (Pfeifer and Scheller, 1975).

The involvement of the lysosomal system in the degradation and turnover of intracellular proteins has been suggested by several authors (Schimke,

1975; Segal and Dunaway, 1975; Natori, 1975; Segal et al., 1976; Lloyd, 1976). There is some experimental evidence (Dean, 1975a and 1975b; Natori, 1975) for the degradation of intracellular proteins within lysosomes, but there is no proof that protein turnover occurs chiefly within lysosomes.

1.2.4 The digestive system of lysosomes

1.2.4.1. The intralysosomal pH.

The majority of the lysosomal enzymes are most active at a slightly acid pH, and so it seems reasonable to suppose that the interior of the lysosomes has a pH below neutrality. Using an indicator dye attached to killed yeast cells which were endocytosed, Mandell (1970) estimated the intraphagosomal pH of leucocytes to be between 5.9 and 6.7.

By measuring the distribution of a radiolabelled weak acid or base across the lysosomal membrane of lysosomes suspended in different media it is possible to estimate the intralysosomal pH. Using [^{14}C]methylamine in this way, the pH inside lysosomes was found to be between 6.4 and 7.2, approx. 1 unit below the external medium (Henning, 1975). This agrees closely with other data obtained using this method (Reijngoud and Tager, 1973; Reijngoud et al., 1976). The general consensus is that the intralysosomal pH is of the order of 1.0 - 1.5 pH units lower than the surrounding medium. This being the case, a pH gradient exists across the lysosomal membrane.

1.2.4.2. The maintenance of intralysosomal pH.

Two alternative mechanisms have been proposed for the maintenance of the intralysosomal pH, one energy-dependent and the other not.

The existence of "an ATPase mechano-enzyme complex important in the governance of lysosomal integrity" was first postulated by Duncan (1966), although the major experimental evidence for the existence of a proton pump in lysosomes came from Mego et al. (1972). They showed that ATP activated the intralysosomal proteolysis of radiolabelled albumin at pH 8.

Furthermore, these workers showed that the activation was diminished as the pH was lowered so that there was no effect at pH 5. The activation of proteolysis by ATP was stimulated by Mg^{2+} and Mn^{2+} ions, but diminished by Ca^{2+} ions (Mego et al., 1972), and was inhibited by an ionophore and also by an uncoupler (Mego, 1975). Other workers (Huisman et al., 1974) have shown that ATP merely stabilises lysosomes at high pH, while Henning (1975) could find no effect of ATP upon the lysosomal pH gradient.

An energy-independent Donnan equilibrium operating across the lysosomal membrane was first postulated by Coffey and de Duve (1968). Reijngoud and Tager (1973 and 1975) carried out work using Tritosomes (lysosomes containing entrapped Triton WR-1339), and by measuring the distribution of [^{14}C]methylamine across the lysosomal membrane, found that the results were compatible with the existence of a Donnan equilibrium across the membrane. The variation of pH in the presence of various cations at 4°C is consistent with the existence of a Donnan equilibrium (Henning, 1975).

More recently, the observation of the low proton permeability of the lysosomal membrane at 37°C (Casey et al., 1978) has led to the proposal that the equilibrium maintaining the pH gradient is not a true Donnan equilibrium, in that the gradient is not wholly maintained by intralysosomal non-diffusible anions.

It can be seen that the combination of the permeability properties of the lysosomal membrane, together with the intralysosomal pH and the lysosomal enzymes, produce a system which is capable of the digestion of a wide variety of biopolymers to small molecules. The small digestion products can then escape from the lysosomes to be reused elsewhere within the cell.

1.2.5 Lysosome storage diseases

1.2.5.1. The fate of normal lysosomes.

As digestion proceeds in normal lysosomes, the products of digestion

diffuse from the organelle, so that eventually all the substrates will have been removed. During the digestion, therefore, the internal osmolarity of the lysosomes is decreasing, so that water will pass from the organelles by osmosis, leading to a loss of turgidity of the membrane of the lysosomes. It has been proposed (Lloyd, 1976; Dean, 1977; Duncan and Pratten, 1977) that the regression of lysosomes may occur by the budding off of surplus lysosomal membrane, either inwards or outwards. Lysosomal involution may afford one means by which cytosol may be taken into the lysosomes. The addition of cytosol proteins to the external face of the lysosomal membrane might then be the point at which the selection of proteins for turnover could take place (Lloyd, 1976; Dean, 1977). Lysosomes that have finished digestion of substrates, but that still contain some indigestible or non-permeant material, are termed residual bodies. Some cell types may be able to expel the contents of such vacuoles by the mechanism of exocytosis (also termed defaecation), e.g. liver cells (de Duve and Wattiaux, 1966), although undigestible dextran (Wiesmann, 1974) or sucrose (Jacques, 1968) do accumulate within fibroblasts and liver cells respectively for several days, suggesting that, in these cells at least, the residual bodies have a long lifetime.

1.2.5.2. The concept of lysosomal storage diseases.

The lysosomal storage diseases have an important place in any description of lysosomes. The theoretical basis of this group of inherited disorders was first explained by Hers (1963). Since the degradation of all substrates by lysosomes is dependent on the existence of the lysosomal enzymes, the absence of any enzyme must mean the inability of the lysosomes to digest one specific linkage of some substrate(s). This will result in the accumulation of such undigested molecules within the lysosomes, so that storage of indigestible non-permeable "substrates" eventually disrupts the lysosomal system. It should be noticed that, since several substrates

may be digested by the same enzyme, the stored substances may, as in the mucopolysaccharidoses, not be homogeneous. Many lysosomal storage diseases have now been identified, and there is an extensive literature on the subject (see e.g. Hers and van Hoof, 1973; Desnick et al., 1976). All known lysosomal storage diseases are inherited as a Mendelian recessive character, and are usually due to the absence of a single enzyme, but in multiple sulphatase deficiency, a variant of metachromatic leukodystrophy, three similar sulphatases are all missing. In general, patients suffering from lysosomal storage diseases appear normal at birth. The development of the symptoms of the disease is progressive, the rate depending on the levels of stored materials and the major sites of storage. In several storage diseases, the nervous system is severely affected, although this is not a universal symptom.

1.2.5.3. Enzyme therapy of lysosomal storage diseases.

Extracellular proteins are internalized by endocytosis, and enter the lysosomal system. In cases where a lysosomal enzyme is missing, it may therefore be possible to supply a substitute from an extracellular source. Several methods of administering missing enzymes have been tried in different storage diseases, such experiments falling into three groups:

i) Injection of replacement enzyme.

A summary of the results of human trials of direct enzyme-replacement therapy in various lysosomal storage diseases is contained in the review by Desnick et al. (1976). The success of this approach appears to be somewhat limited, depending largely on the resistance to proteolysis of the injected enzyme within the affected tissues.

ii) Entrapment of replacement enzyme within liposomes.

The enzymes are administered within a vesicle so that the targetting to specific cell types can be accomplished without the modification of the enzyme itself. Experiments by Gregoriadis and Buckland (1973) using a

model system have shown that liposomes containing β-fructofuranosidase (invertase) are capable of removing the vacuoles of stored sucrose in mouse peritoneal macrophages and human embryo lung fibroblasts. Tyrrell et al. (1976a) attempted to treat a patient with type II glycogenosis (Pompe's disease) by the administration of liposomes containing amyloglucosidase. The patient died on the eighth day of therapy, but even in this time the liver glycogen levels had been reduced (compared to levels in Pompe controls) and trace amounts of the enzyme could be detected in the liver and spleen. The main problem still to be overcome is that of the targetting of the liposomes, so that at the present time this mode of therapy is of limited use.

iii) Enzyme replacement from allografts.

Histocompatible grafts of normal cells may be able to provide a continuous supply of the missing enzyme over a long period of time, and thus remove the need for regular treatment at shorter intervals. Dean et al. (1976) implanted histocompatible fibroblasts into a patient with Hunter syndrome (absence of lysosomal sulpho-L-iduronate sulphatase). The excretion of Hunter corrective factor rose from 0.0 - 1.04 units per day before treatment to approx.3 units per day (6.7% of normal) and the pattern of excretion of uronic acids and glycosaminoglycans were consistent with correction of the defect. A more recent paper (Dean et al., 1978) has extended this study to include three Hunter patients. All three patients had increased plasma levels of Hunter corrective factor compared with Hunter controls, three years after the implantation of the fibroblasts.

1.3 Lysosomal metabolism of cystine residues

Lysosomes possess a system capable of digesting proteins to the level of amino acids, but the fate of the cystine residues in such proteins is unclear. A theoretical consideration of the problem supports several possible routes for the fate of cystine residues in lysosomes; these are shown in Figure 1.1.

Three of the routes depicted in Figure 1.1 assume that intact cystine cannot cross the lysosomal membrane and require the reduction of cystine (molecular weight 240) to the sulphydryl form, cysteine (molecular weight 121) prior to its escape from the lysosomes. The differences between these routes are concerned solely with the site of the reduction.

Route A:

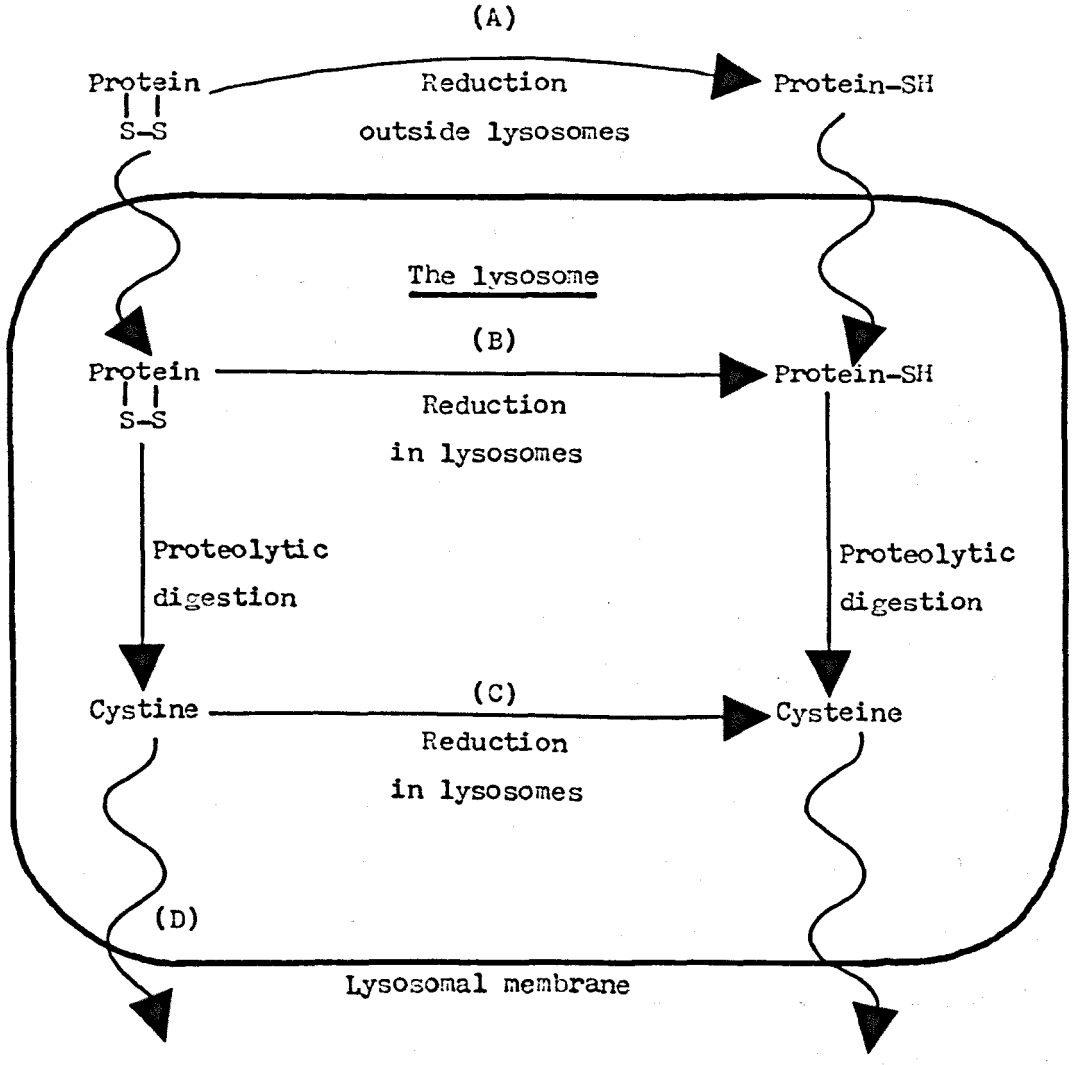
In this scheme, the reduction occurs before the proteins enter the lysosomes, so that the subsequent intralysosomal proteolysis produces cysteine, which is presumably able to escape from the lysosomes. An enzyme capable of reducing the cystine residues in insulin by means of reduced glutathione (glutathione insulin transhydrogenase: GIT) has been found in the microsomal fraction of rat liver (Ansorge et al., 1973). It may be that these small particles are pinosomes, and that the enzyme originates from the plasma membrane. The enzyme may possess the ability to reduce proteins other than insulin, so that their reduction is complete prior to any contact with lysosomal enzymes.

Routes B and C:

In these schemes, the reduction occurs after the proteins enter the lysosomes, either at the protein level or after digestion, at the free cystine level. It might be expected that any enzyme acting at this stage would be a "typical" lysosomal enzyme, i.e. possess slightly acid pH optimum. A recent paper (Grisolia and Wallace, 1976) reports the existence of a GIT in rat liver Tritosomes. Any intralysosomal reductase

Figure 1.1. The Possible Theoretical Fates of the Cystine Residues of Proteins Entering Lysosomes.

The routes A, B, C and C are described in Section 1.3.


Cytosol
(A)
Protein
S–S
Reduction
outside lysosomes
Protein-SH
The lysosome
(B)
Protein
S–S
Reduction
in lysosomes
Protein-SH
Proteolytic
digestion
Proteolytic
digestion
Cystine
(C)
Reduction
in lysosomes
Cysteine
(D)
Lysosomal membrane

must have a supply of oxidizable cofactor in order to maintain its operation. Such cofactors must be obtained either from outside the lysosome, or be already within the organelle. An extralysosomal reducing agent must either be able to traverse the lysosomal membrane in the reduced form, or be transported in some way. A reducing agent arising in situ must either be present when proteins are selected for lysosomal entrapment, or arise from the digestion of the lysosomal substrates.

Route D:

This route supposes that cystine itself can cross the lysosomal membrane, either via a transport mechanism, or because the normal lysosomal membrane is freely permeable to cystine.

1.3.1 The possible defect in cystinosis

Clearly cystinosis is a defect in the normal route of elimination of cystine residues from the lysosomes. If the cystine residues are reduced prior to lysosomal entrapment (Route A), then presumably cytinosis is the result of the absence of a non-lysosomal enzyme, perhaps one that is associated with the plasma membrane. This would explain why no lysosomal enzyme defect could be found by Tietze et al. (1972).

If lysosomal reduction of cystine occurs, (Routes B and C) then cytinosis might be due to the absence of a "typical" lysosomal enzyme. The report of a GIT in lysosomes (Grisolia and Wallace, 1976) poses the problem of the means of entry of the reducing agent. On the basis of present evidence (Cohn and Ehrenreich, 1969; Lloyd, 1971), it would seem doubtful that glutathione could cross the lysosomal membrane, either in the reduced or oxidised form, although it is quite possible that glutathione may not be the physiological substrate. It may be that the penetration of the reducing agent is controlled by a transport mechanism. This would satisfy the requirement for the involvement of a protein, so that in this case cystinosis would be the result of the absence of the transport mechanism for the reducing agent.

If cystine can escape from normal lysosomes, then cystinosis is either due to the absence of a transport mechanism for cystine, or else the result of the absence of a membrane protein which raises the permeability of the membrane so that cystine cannot diffuse from the lysosomes as it would normally. It seems likely that cystine may be just too large to escape from normal lysosomes (Cohn and Ehrenreich, 1969; Lloyd, 1971), although cystine itself is far too insoluble to be tested by the techniques at present available.

1.4 Aims of this thesis

This thesis describes an investigation into the fate of cystine residues in lysosomes. In Chapter 3 the permeability of the lysosomal membrane to some disulphide and sulphydryl-containing compounds was examined. Chapter 4 describes experiments with formalin-denatured ^{125}I-labelled bovine serum albumin, which were carried out in order to evaluate a technique claimed to achieve the uptake of substances into lysosomes *in vitro*. The subsequent Chapters describe the setting up and use of a system to achieve uptake of liposome-entrapped substances into rat liver lysosomes, so as to observe the rate of escape of substances from intact lysosome-enriched suspensions *in vitro*. Such a system would enable the selective introduction of relatively small molecules into the lysosomal system. Previously it has only been possible to follow the fate of macromolecules after their localization within secondary lysosomes by endocytosis.

CHAPTER 2

Materials and Methods

2.1 Fractionation Procedures

2.1.1 Preparation of a lysosome-enriched fraction of rat liver

In these investigations, lysosome-enriched fractions of rat liver have been prepared by the method of Lloyd (1969). The fraction so prepared contains other cell particles (particularly mitochondria and peroxisomes) apart from lysosomes, but the preparation time (approx. 1h) is shorter than that of a more rigorous purification. The lysosomes are thus more likely to retain their functional integrity.

Male Wistar rats weighing 250 - 350g were starved for 18h before use, but allowed water ad libitum. The rats were killed by cervical dislocation, and the liver quickly perfused with 20 - 30ml ice-cold 0.25M sucrose, and then excised, blotted dry and weighed. The liver was then forced through a stainless steel sieve (mesh size 1.0mm^2), in order to remove the major vascular and connective tissues. This liver pulp was weighed and then 2.5ml/g pulp of ice-cold 0.25M sucrose were added. The resulting suspension was homogenised in a Potter-Elvejhem Teflon/glass homogeniser (Tri-R Instruments Inc., New York; 0.019cm clearance) at 3,000 rpm (speed setting 2.7), forcing the tissue past the pestle three times in 30 sec. Ice-cold 0.25M sucrose was then added to give a 10% (pulp wt/volume) suspension of the liver homogenate.

A two stage centrifugation scheme was employed in order to minimize the mechanical disruption of the lysosomes during the preparation. The homogenate was centrifuged at 3,300g x 10min (MSE 18, Rotor no. 43114-106) and this supernatant centrifuged at 21,000g x 10 min in the same centrifuge. During the whole of the isolation procedure the homogenate and fractions were kept on ice, and the centrifugations were at 4°C. The fraction that sedimented at the higher speed was resuspended in a 0.25M solution by gently forcing the pellet (still in the centrifuge tube) once past a pestle rotating at 1,500rpm (Tri-R, speed setting 1.7).

This lysosome-enriched fraction was always used immediately after preparation. Some slight variations to this general method were introduced in some of the experiments. In the experiments of Chapter 3, 0.25M mannitol replaced sucrose as the homogenising medium, and in Chapter 9 the preparation was carried out using 0.3M sucrose containing 5mM MOPS, pH7.4, and 5mM EGTA as the homogenising medium.

2.1.2 Subcellular fractionation of rat liver

The differential subcellular fractionation scheme employed is described fully in Section 5.2.1.

2.2 The preparation of large multilamellar liposomes

Following experiments in which the quantities and concentrations of various substances were adjusted (Chapter 6), the following method was adopted for the preparation of negatively charged liposomes.

Dipalmitoylphosphatidylcholine (DPPC, 9mg) and dipalmitoylphosphatidic acid (DPPA, 1mg), supplied by Sigma, Poole, Dorset, were suspended in 5ml "Aristar" grade chloroform (BDH Ltd., Poole, Dorset) in a 100ml pear-shaped "Quickfit" flask under nitrogen. (These lipids gave single spots on thin layer chromatograms when 100µg was applied, solvent $CHCl_3$: CH_3OH : NH_3 : H_2O, 65 : 30 : 4 : 1). The suspension was evaporated *in vacuo* at 61°C using a Buchi rotary evaporator, to yield a thin film of lipid on the wall of the flask. The liposomes were formed by adding 1ml 5mM phosphate-buffered saline (see below) and resuspending the film by intermittent vortex mixing, maintaining the flask at 61°C. Substances to be entrapped were dissolved in the saline. The liposomes were left at room temperature (21°C) for 1h and then purified by centrifugation.

Three centrifugation steps were employed, each 50,000g x 10min (MSE 50 centrifuge, rotor no. 59113, 21°C). Following each centrifugation, the supernatant was decanted, and the pellet of liposomes resuspended in 2ml 5mM phosphate-buffered saline, except that in some cases the final pellet was resuspended to a smaller final volume.

Positively charged liposomes were prepared by replacing DPPA by octadecylamine (stearylamine) (Koch-Light, Colnbrook, Bucks.) in the method.

Phosphate-buffered saline, pH7.4, (5mM) was prepared from 16g NaCl, 1.15g $Na_2PO_4.12H_2O$, 0.2g KH_2PO_4 and 0.2g KCl in water, adjusted to pH7.4 with NaOH and made up to 2,000ml. The substances to be entrapped were used at the following concentrations; ^{125}I-labelled PVP, 500µg/ml; [U-^{14}C] sucrose, 500µg/ml; [^{35}S] cystine dihydrochloride, 100µg/ml in 67mM NaCl, or 150µg/ml in 0.1M HCl. The [^{35}S] cystine dihydrochloride

at 150 μg/ml was used only in Chapter 6.

This method of preparation was employed for the liposomes used in Chapters 7-9, although the quantities used were increased by two to four times. The methods used for ascertaining the stability and other properties of the liposomes are described in Chapter 6.

2.3 Enzyme assays

2.3.1 p-Nitrophenyl-N-acetyl-β-D-glucosaminidase

The assay method used was essentially that of Barrett and Heath (1977) in which the ability of N-acetyl-β-D-glucosaminidase to liberate p-nitrophenol from the p-nitrophenyl glycoside is measured spectrophotometrically. The substrate (p-nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside, Koch-Light, Colnbrook, Bucks.) was dissolved in 150mM acetate buffer, pH5.0, to a concentration of 15mM. To 0.3ml of this substrate solution in a centrifuge tube was added 0.3ml 0.5M sucrose containing 0.3% v/v Triton X-100. To initiate the assay, 0.3ml of enzyme preparation was added, and the mixture incubated at 37°C for 5min. In experiments where the enzyme preparation was not suspended in sucrose, 0.75M sucrose containing 0.3% v/v Triton X-100 replaced the second solution.

To terminate the assay, 1ml 8% v/v trichloroacetic acid was added, and the tube was then centrifuged in an MSE Minor bench centrifuge for 10 min at 3,000rpm. The supernatant (1ml) was added to 2ml 0.3M glycine, buffered to pH10.5 with NaOH. The resulting yellow colour of p-nitrophenol was measured using a Cecil CE 373 Spectrophotometer (Cecil Instruments Ltd., Cambridge) set to 400nm. The free activity of the enzyme was measured by omitting the Triton X-100 from the assay. Appropriate blanks were always measured.

Some modifications to this assay were made during the course of the work described in this thesis. In Chapter 3, the assay was carried out at 25°C, necessitating the use of an incubation time of 15min. Throughout these experiments mannitol replaced sucrose. In Chapter 4, the assay was carried out as described, but, in the later experiments (Section 4.3.2 onwards) the incubation time was increased to 10min at 37°C due to the alteration in the pH of the assay (noted in Section 4.2.2).

2.3.2 4-Methylumbelliferyl-N-acetyl-β-D-glucosaminidase

The fluorimetric assay of N-acetyl-β-D-glucosaminidase is similar in principle to the spectrophotometric assay of 2.3.1. In this case, the product of the hydrolysis is 4-methylumbelliferone, which fluoresces strongly at 450nm when excited by light of 350nm wavelength. The substrate (4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside, Koch-Light Ltd., Colnbrook, Bucks.), 9.5mg, was dissolved in 5ml 150mM acetate buffer, pH5.0. To 25µl of the substrate solution was added 25µl 0.5M sucrose containing 0.3% v/v Triton X-100 and 25µl of the enzyme preparation. After 5min incubation at 25°C, the reaction was stopped by the addition of 2ml 0.3M glycine, buffered to pH10.5 with NaOH. The fluorescence of this solution was measured on a Perkin-Elmer 1000 Fluorescence Spectrophotometer using an excitation wavelength of 350nm and an emission wavelength of 450nm.

Where the enzyme preparation was not suspended in sucrose, the 0.5M sucrose was replaced by 0.75M sucrose. The free enzyme activity was measured by omitting the Triton X-100. Preliminary experiments indicated that the Triton X-100 did not interfere with the assay, but appropriate blanks were always measured.

2.3.3 Arylsulphatase

Arylsulphatases A and B are both located within the lysosomes, whereas arylsulphatase C is a microsomal enzyme (Austin, 1973). The assay used measures both the A and B form of the enzyme, but there is probably little interference by arylsulphatase C because it is most active at a higher pH.

A solution of 15mM nitrocatechol sulphate (Koch-Light, Colnbrook, Bucks.) in 150mM acetate buffer, pH5.0 was prepared, and 0.3ml was added to 0.3ml 0.5M sucrose containing 0.3% v/v Triton X-100. This mixture was incubated with 0.3ml enzyme preparation at 37°C for 5 min. To terminate the assay, 2.7ml 0.2M NaOH was added. The absorbance of the nitrocatechol in this solution was then measured at 540nm using a Cecil

CE 373 Spectrophotometer. The free enzyme activity was measured by omitting the Triton X-100 from the assay.

In Chapter 9, the assay was modified so that the incubation was 15min at 25°C. Where the enzyme preparations were not suspended in sucrose, the 0.5M sucrose containing 0.3% v/v Triton X-100 was replaced by 0.75M sucrose containing 0.3% v/v Triton X-100.

2.4 Estimation of protein

Protein was estimated by the method of Lowry et al. (1951). To 0.5ml diluted protein sample was added 0.5ml 1M NaOH, and the resulting mixture allowed to stand at room temperature for 30min to enable solubilization of the protein to take place. Folin A solution (5.0ml) was then added with mixing, and after another 20min, 0.5ml 50% v/v Folin Ciocalteau reagent (B.D.H. Ltd., Poole, Dorset.) was added and immediately mixed. The colour was allowed to develop for 45min at room temperature, and was then measured spectrophotometrically at 750nm using a Cecil CE 373. Bovine serum albumin (Sigma, Poole, Dorset.) was used as a reference protein to construct a standard curve of 0-500µg/ml each time the assay was used.

Folin A solution was prepared from 2% w/v anhydrous sodium carbonate (100ml), 1% w/v copper (II) sulphate pentahydrate (1ml) and 2% w/v sodium tartrate (1ml), freshly made up from stock solutions before use.

In Chapter 5, reference is made to a series of experiments in which the protein material of the samples is precipitated with acid in order to remove traces of EDTA. In these experiments 0.1ml protein-containing sample was mixed with 0.1ml of 10% w/v sodium tungstate solution and 0.1ml 1.3M H_2SO_4. After centrifugation at 3,000rpm for 5min in a MSE Minor bench centrifuge, the supernatant was removed, and the pellet resuspended in 5ml Folin A solution. After 10min at room temperature, 0.5ml Folin Ciocalteau reagent was added and immediately mixed. The colour was allowed to develop over the next 25min and measured spectrophotometrically at 750nm as described above.

2.5 Estimation of cysteine

This was performed using the method of Zahler and Cleland (1968), based on the spectrophotometric estimation of complexes formed by the reaction of monothiols with 5,5'-dithiobis(2-nitrobenzoic acid), (DTNB).

The solution under examination (0.1ml) was added to a mixture of 0.2ml 1.0M tris buffer, pH8.1, 0.1ml 3mM DTNB, in 50mM acetate buffer, pH5.0, and 2.6ml water. Development of the colour was complete after 10min incubation at room temperature, and the absorbance of the solution at 412nm was measured using a Cecil CE 373 Spectrophotometer. The relationship of cysteine concentration to absorbance at 412nm was linear over the range 0-4mM.

2.6 Estimation of radioisotopes

2.6.1 ^{125}I-Radioactivity

The activity of samples containing ^{125}I-radioactivity was measured using a Packard Selektronic Gamma Scintillation Counter (Packard Instrument Co. Inc., Illinois, USA). Counting times were in the range 30-300sec, using a discriminator level of 66, channel width of 210, high voltage of 1160V and a gain of x10. The samples were contained in disposable 3ml plastic tubes, usually in a volume of 1ml. If the volume of the sample was not 1ml, a correction factor was calculated and applied to take into account the alteration of geometry.

2.6.2 ^{14}C-Radioactivity

^{14}C-Radioactive samples were measured using a Packard 2425 Liquid Scintillation Counter utilising the preset facility of this machine. Samples (volume 1ml) were counted in a scintillant of 6.7ml toluene (scintillation grade, B.D.H. Ltd., Poole, Dorset.) and 3.3ml Triton X-100 (Bohm and Haas, U.K. Ltd.), containing 6g/l t-butyl PBD (Koch-Light Ltd., Colnbrook, Bucks). Following the first duplicate counting of between 5 and 20min, the samples were spiked with 50µl [U-^{14}C]sucrose and recounted for 2min. In this way a correction for the degree of quenching could be applied.

2.6.3 ^{35}S-Radioactivity

Samples containing ^{35}S-radioactivity were counted on the Packard 2425 in the above scintillation cocktail. In a preliminary experiment the optimum settings for the machine were found to be 50 for the lower discriminator, 1000 for the upper discriminator with a gain setting of 19.0%. Quenching was corrected as for ^{14}C-radioactivity (above).

2.7 Intravenous injections

Male rats weighing 250 - 350g were used in these experiments. Under light ether anaesthesia the rat was placed on its back and a small incision approx. 1.5cm in length was made in its left groin. The left femoral vein was carefully exposed by removal of the surrounding tissue and the area swabbed gently to remove any blood. The substance (usually in a volume of less than 0.5ml) was taken into a disposable syringe fitted with a 25 gauge needle, and injected into the vein. A swab was held over the area to staunch any bleeding. The incision was sutured and the animal allowed to recover.

2.8 Collection and analysis of blood from injected rats

Rats injected as described in Section 2.7 were held under ether anaesthesia when the collection of blood samples was undertaken. A small incision was made in the right foot pad and a sample of blood collected in a 50µl heparinised tube (Hawkesley and Son Ltd., Lancing, Sussex.). Bleeding was quenched by a small tourniquet. Further samples were taken by temporarily loosening the tourniquet.

2.8.1 Blood containing ^{125}I-radioactivity

The 50µl samples were placed in 2ml 1M NaOH and left at room temperature for 2h. This solution was divided into two equal portions and counted as described in Section 2.6.1.

2.8.2 Blood containing ^{14}C-radioactivity

The 50µl samples were suspended in 1ml 0.25M NaOH and sonicated for 2h in a Kerry bath-type sonicator (Kerry Ultrasonics Ltd.). After this, 1ml 0.25M HNO_3 was added, and two equal portions were counted for ^{14}C-radioactivity as described in Section 2.6.2.

2.8.3 Blood containing ^{35}S-radioactivity

After taking the 50µl samples, they were placed in 2ml water. This suspension was divided into two equal quantities and counted as described in Section 2.6.3. The estimated radioactivity was multiplied by a correction factor of 2.48. Several methods of counting were tried, but this method gave the best measurable radioactivity.

2.9 Counting of radioactive tissue samples

2.9.1 Tissues containing ^{125}I-radioactivity

Tissues containing ^{125}I-radioactivity were weighed and a portion of known weight was added to a quantity of 1M NaOH in a flask. After solubilization of the protein (2 - 3h at 37°C) the volume of liquid was measured, and duplicate 1ml samples taken for estimation of ^{125}I-radioactivity as described in Section 2.6.1. The ^{125}I-radioactivity of liver fractions was estimated by counting 1ml of the resuspended subcellular fraction.

2.9.2 Tissues containing ^{14}C-radioactivity

Tissue ^{14}C-radioactivity was estimated by taking 0.5g from the weighed tissue and adding 2ml 0.25M NaOH. After 2h sonication (see 2.8.2), 2ml 0.25M HNO_3 was added, and the volume was made up to 5ml with water. Duplicate 1ml samples were then counted (see 2.6.2). Liver fractions were counted in a similar way, using 1ml of suspension in place of 0.5g tissue, and omitting the water.

2.9.3 Tissues containing ^{35}S-radioactivity

Tissue levels of ^{35}S-radioactivity were estimated using the method described in Section 2.9.2, and counting as in 2.6.3.

2.10 Source of radioisotopes

All substances containing radioisotopes were purchased from The Radiochemical Centre, Amersham, U.K., except for formalin-denatured ^{125}I-labelled bovine serum albumin (see below), which was prepared using iodine-125. The specifications for the products were as follows:

[^{125}I]Iodinated poly(vinylpyrrolidone), product number IM.33P. Sterile aqueous solution containing 1% benzyl alcohol. 125μCi/ml; 20 - 60μCi/mg PVP.

[U-^{14}C]Sucrose, product number CFB.146. Sterile aqueous solution containing 3% ethanol. 350mCi/mmol.

L-[^{35}S]Cystine hydrochloride, product number SJ.138. Sterile solution in 0.1M HCl. 206mCi/mmol; 897mCi/ml.

Iodine-125, product number IMS.30. Supplied as iodide in sterile NaOH solution, pH8 - 11. 100mCi/ml. This preparation was used to iodinate bovine serum albumin (Sigma, Poole, Dorset. Lyophilized and crystallized) by the method of Williams *et al.* (1971).

CHAPTER 3

The permeability of rat liver lysosomes to some sulphydryl compounds

3.1 Introduction

As explained in Section 1.2.2.1, the permeability properties of the lysosomal membrane can be inferred from studies on the ability of substances to afford osmotic protection to isolated intact lysosomes. It would obviously be of interest to carry out experiments of this type using cystine, to find out whether the lysosomal membrane was permeable to this amino acid. However, cystine itself is too insoluble to be used in this way, and so the ability of some similar substances to give osmotic protection to lysosome-enriched suspensions has been examined, in an attempt to gather information which might aid in determining the fate of cystine residues in lysosomes.

There are two ways in which the stability of lysosomes in suspension can be followed by measuring the activities of lysosomal enzymes. In both the non-latent enzyme activity is compared with the total lysosomal enzyme activity, which is usually measured by assaying the enzyme in the presence of the non-ionic detergent, Triton X-100.

The first method measures the "free activity" of a lysosomal enzyme, that is the proportion of the enzyme that has access to the substrate. The enzyme is measured under conditions that maintain the integrity of the lysosomes in the suspension, usually by assaying in 0.25M sucrose. If the lysosomes were wholly intact, there would be no interaction between substrate and enzyme and, conversely, if all the lysosomes were broken, the free activity should equal the total activity, (the activity measured in the presence of Triton X-100). The "non-sedimentable activity", on the other hand is measured quite differently. To determine the degree of lysosome breakage, a sample of the suspension is centrifuged at high speed, and the enzyme activity of the supernatant is compared to the total enzyme activity measured in the presence of Triton X-100.

The non-sedimentable activity may not equal the free activity of the

enzyme, for several reasons. First, enzymes inside partially disrupted lysosomes may contribute to the free activity, but would not be in the supernatant, and therefore not be included in the non-sedimentable activity. Secondly, any binding of released enzyme to the lysosomal membrane would decrease the measured non-sedimentable activity. For instance, the non-sedimentable activity of arylsulphatase is similar to its free activity (see Figure 9.1) under conditions where the non-sedimentable activity of N-acetyl-β-D-glucosaminidase is barely measureable (results not shown) even though the percentage free activities are the same.

Finally, the speed of the centrifugation will affect the non-sedimentable activity, as, for example, occurred in the experiments described in Chapter 9 (c.f. Figures 9.1 and 9.3).

The percentage free lysosomal enzyme activity is probably the more appropriate as an estimate of the degree of lysosome damage, while the percentage non-sedimentable activity is better envisaged as a measure of the degree of dissociation of the enzyme from the lysosomes.

3.2 Experimental procedures

The experiments were performed using a lysosome-enriched fraction of rat liver, prepared as previously described in Section 2.1.1. The final pellet, derived from one quarter of the rat liver, was resuspended in an ice-cold 0.25M (isotonic) solution of the substance to be tested (5ml/g of initial tissue pulp). The suspension was adjusted to pH5.0, 6.0 or 7.4, using either NaOH or HCl. This avoided the use of a buffer solution, which would have increased the osmolarity of the suspension, and thus increased the osmotic protection given to the lysosomes. These suspensions were then incubated in a water bath at 25°C for 2h.

The first series of experiments utilized 10 - 15ml lysosome-enriched suspension for each experiment at any one pH. At various time intervals the activity of p-nitrophenyl-N-acetyl-β-D-glucosaminidase was determined in the absence (free activity) and presence (total activity) of Triton X-100, as described in 2.3.1. The duration of these assays was 15min, and the assay temperature 25°C.

In order to carry out experiments with more expensive solutes (cysteamine, cystamine), the assays were scaled down so that less material was required. In preparing the lysosome-enriched fraction, suspension equivalent to only 1g initial liver pulp was placed in each tube for the second centrifugation. One pellet was used for each experiment, and after it was resuspended in 5.0ml of the test substance, the suspensions were divided into three 1.5ml lots in separate tubes, and adjusted to pH as described above. A fluorometric substrate, 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside (molecular weight 379) was used to assay N-acetyl-β-D-glucosaminidase, as described in Section 2.3.2. The 0.25M solutions of cysteamine hydrochloride were kept under nitrogen to minimise the possible oxidation to cystamine. No significant oxidation of any of the other substances was envisaged.

3.3 Permeability of rat liver lysosomes to the tested compounds

The percentage of free activities of N-acetyl-β-D-glucosaminidase observed in 0.25M solutions of the tested compounds over 2h incubation at 25°C are shown in Tables 3.1 and 3.2.

Clearly, suspension in mannitol leads to relatively little increase in the percentage free enzyme activity during the course of the incubations, confirming the observations of Lloyd (1969) that it is unable to penetrate the lysosomal membrane, despite its low molecular weight. This has been attributed to the inability of mannitol to adopt a pyranose form (Lloyd, 1969). In contrast with the statement of Lloyd (1971), the degree of osmotic protection afforded by mannitol at 25°C was affected by the pH of the suspension.

Glucose and L-glycyl-L-glycine did not afford osmotic protection to the lysosome-enriched suspension, implying that both could rapidly penetrate the membrane, in agreement with earlier reports (Lloyd, 1969, 1971; Cohn and Ehrenreich, 1969). It has been postulated (Lloyd, 1971) that glycylglycine is able to cross the lysosomal membrane when the positive and negatively charged regions of the molecule are adjacent. This cis rotamer is less polar than other possible configurations.

Cysteine and serine have nearly identical structures, the only difference being that the hydroxyl group in serine is replaced by a sulphydryl group in cysteine. The difference causes the properties of the two to differ in two ways. First, the pK_a of an aliphatic hydroxyl group is approx. 16, which is 5-7 units greater than that of a sulphydryl group. This means that, although the proton of the hydroxyl of serine is essentially immovable, the sulphydryl proton of cysteine plays an important part in the ionization of the molecule, particularly at high pH values. Secondly, unlike the serine hydroxyl group, the sulphydryl of cysteine is capable of oxidation to a disulphide, forming insoluble cystine.

Table 3.1 Percentage free p-nitrophenyl-N-acetyl-β-D-glucosaminidase activity of lysosome-enriched suspensions in 0.25M solutions of some compounds following incubation at 25°C

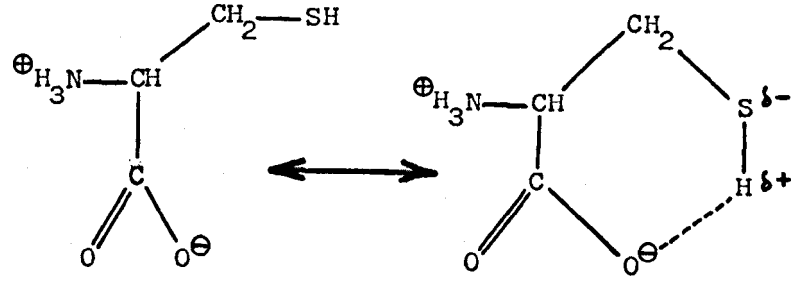
The lysosome-enriched pellet (see Section 2.1.1) derived from one quarter of the rat liver pulp was resuspended in an ice-cold 0.25M solution of the test substance (5ml/g initial liver pulp). The suspension was adjusted to the pH shown with NaOH or HCl, and then incubated at 25°C. At the times shown, the p-nitrophenyl-N-acetyl-β-D-glucosaminidase activity was measured in the absence (free activity) and presence (total activity) of 0.1% v/v Triton X-100, as described in Section 2.3.1, and the percentage free activity calculated. The data shown are the mean ± S.E.M.

Medium	pH	No. expts.	Incubation time at 25°C. (min.)				
			0	30	60	90	120
Mannitol	5.0	3	9.4 ± 0.8	13.1 ± 1.1	17.4 ± 0.5	25.0 ± 3.8	35.2 ± 4.9
	6.0	4	9.3 ± 1.0	11.5 ± 1.3	14.1 ± 1.4	16.8 ± 1.0	21.1 ± 1.6
	7.4	4	8.4 ± 0.5	11.2 ± 0.9	18.2 ± 2.4	22.1 ± 1.3	28.9 ± 2.3
D-Glucose	5.0	3	10.2 ± 2.0	34.8 ± 5.1	62.1 ± 8.6	78.2 ± 4.0	79.0 ± 6.2
	6.0	3	10.1 ± 1.9	39.4 ± 6.2	61.2 ± 5.5	72.4 ± 4.0	81.9 ± 6.5
	7.4	3	9.1 ± 0.6	48.5 ± 6.6	67.9 ± 5.3	75.8 ± 2.2	81.4 ± 5.2
L-Glycyl-L-glycine	5.0	4	8.4 ± 0.8	30.5 ± 2.6	58.7 ± 6.9	88.9 ± 4.7	93.6 ± 3.5
	6.0	4	11.1 ± 1.7	44.0 ± 5.6	70.2 ± 5.3	90.2 ± 1.9	95.5 ± 2.9
	7.4	4	10.9 ± 1.6	69.5 ± 10.3	90.2 ± 8.7	101.0 ± 7.1	98.4 ± 5.4
L-Cysteine	5.0	6	11.9 ± 1.2	17.0 ± 2.0	48.1 ± 7.6	79.9 ± 7.9	93.6 ± 4.5
	6.0	5	11.1 ± 1.5	15.7 ± 1.4	37.4 ± 8.5	54.7 ± 12.2	75.1 ± 11.3
	7.4	7	11.1 ± 1.3	16.0 ± 2.4	29.5 ± 6.6	53.3 ± 10.8	84.1 ± 8.3
L-Serine	5.0	3	10.5 ± 1.3	12.3 ± 2.4	24.1 ± 9.4	34.6 ± 10.0	58.5 ± 16.2
	6.0	3	11.1 ± 2.2	13.1 ± 2.4	20.2 ± 3.4	28.0 ± 6.4	43.2 ± 10.2
	7.4	3	9.4 ± 0.6	13.0 ± 1.5	28.8 ± 2.1	40.0 ± 2.1	54.7 ± 3.7
Mercapto-ethanol	5.0	2	101.9	100.0			
	6.0	2	99.1	102.8			
	7.4	2	99.1	96.3			

After 120 min. incubation at 25°C, serine caused 40-60% release of free activity, lower than any other permeant solute tested. These figures are in line with those for glycine, alanine and valine (Lloyd, 1971). At all three pH values, cysteine penetrates the lysosomal membrane more rapidly than serine. The most rapid increase in the free activity occurs at pH5.0.

If the cysteine was oxidised during the course of the experiments, then the external osmolarity would drop, and the compensating influx of water might cause rupture of the lysosomes. However, at 25°C, 0.25M cysteine appears to be stable (95 -98%) at all three pH values for two hours, measured by assaying for sulphydryl by the method of Zahler and Cleland (1968) which is described in Section 2.5. It is therefore unlikely that oxidation of cysteine to cystine is the major cause of the observed rise in free enzyme activity.

At all three pH values used the major form of cysteine is the zwitterion (Friedman, 1973), below left, which is the most abundant form at



pH5.0, and is less abundant at pH6.0 and 7.4. It can be stabilised in solution by adopting a ring structure, as shown above right, which is likely to be more stable than the corresponding structure in serine, considering the high pK_a of the hydroxyl group. It is possible that this smaller, less polar structure is able to penetrate the lysosomal membrane more easily.

Mercaptoethanol causes immediate and complete loss of lysosomal enzyme latency, implying a rapid penetration and disruption of the lysosomal membrane. Since mercaptoethanol is a potent reducing agent, it seems

likely that chemical breakdown of the membrane may occur. The substrate blanks of the enzyme assay were normal, showing that there was no significant action of mercaptoethanol upon the enzyme assay. A similar effect may have been observed by Davidson (1975a), who stated that mercaptoethanol 0.05M inhibited the digestion of ^{125}I-labelled ribonuclease at 37°C by mouse kidney lysosomes *in vitro*.

In the second series of experiments, cystamine ($NH_3^+CH_2CH_2SSCH_2CH_2NH_3^+$), as its dihydrochloride, exhibited strongly pH-dependent behaviour in its ability to preserve lysosomal integrity (Table 3.2). At pH5.0 and pH6.0 cystamine did not permeate the membrane of the lysosomes, and was in fact better than mannitol for preserving lysosomal integrity, as judged by these studies. At pH7.4 the percentage free activity rose quickly, in a similar manner to that occurring in glucose solutions. The two positively charged amino groups probably play an important role in the pH-dependent permeability of cystamine. The amino groups probably lose their protons at around pH8, and the lower polarity of the molecule at higher pH values may therefore account for the faster permeability of cystamine through the lysosomal membrane at pH7.4. Lloyd (1971) has shown that chloride ions above 0.1M act as osmotic protectors in this experimental system, and thus the rise in percentage free enzyme activity obtained in with cystamine is unlikely to be due to chloride ion penetration.

Cysteamine, the reduced form of cystamine, used as its hydrochloride, caused as increase in the free enzyme activity at all three pH values, although at pH6.0 the increase plateaued after 60-90min. incubation. Since the molecular weight of cysteamine is 88, it is probably small enough to enter the lysosomes. It is difficult to find a cause for the slower rate of penetration at pH6.0.

Table 3.2 Percentage free 4-methylumbelliferyl-N-acetyl-β-D-glucosaminidase activity of lysosome-enriched suspensions in 0.25M solutions of cystamine dihydrochloride and cysteamine hydrochloride at 25°C

The lysosome-enriched pellet (see Section 2.1.1) derived from 1g initial liver pulp was resuspended in 5.0ml ice-cold 0.25M cystamine dihydrochloride or cysteamine hydrochloride. This suspension was divided into three 1.5ml lots, which were separately adjusted to the required pH using NaOH or HCl, and then incubated at 25°C. At the times shown, the 4-methylumbelliferyl-N-acetyl-β-D-glucosaminidase activity was measured in the absence (free activity) and presence (total activity) of 0.1% v/v Triton X-100 (see 2.3.2), and the percentage free activity calculated. The data shown are the mean ± S.E.M.

Medium	pH	No. Expts.	Incubation time at 25°C. (min.)				
			0	30	60	90	120
Cystamine di HCl	5.0	3	12.1 ± 1.2	15.6 ± 1.4	17.0 ± 2.4	19.4 ± 1.5	20.9 ± 1.8
	6.0	3	11.2 ± 1.4	14.3 ± 0.9	15.7 ± 1.3	18.8 ± 0.4	21.0 ± 2.5
	7.4	3	11.6 ± 1.0	26.2 ± 4.4	47.8 ± 5.8	64.1 ± 3.3	74.9 ± 1.1
Cysteamine HCl	5.0	3	15.4 ± 4.2	48.8 ± 7.9	66.4 ± 2.3	76.1 ± 3.8	77.8 ± 0.6
	6.0	3	15.5 ± 1.7	22.4 ± 0.6	43.4 ± 3.4	52.9 ± 3.3	48.4 ± 4.2
	7.4	3	20.9 ± 4.5	54.0 ± 7.6	51.2 ± 1.4	60.9 ± 5.0	86.7 ± 10.7

3.4 Discussion

Cystine itself is unfortunately much too insoluble to be tested by the method used in this Chapter, but it would be a useful exercise to extend the present study to include other disulphides of a similar molecular weight. Schulman and Bradley (1970) examined a series of disulphides in experiments similar to those of Cohn and coworkers (e.g. Ehrenreich and Cohn, 1969) using cystinotic and normal fibroblasts. The rationale of this technique has already been discussed in Section 1.2.2.1. Schulman and Bradley (1970) found that all the isomeric penicillamine-cysteine mixed disulphides (molecular weight 268) and penicillamine disulphides caused vacuolation in cystinotic cells, but that only the D- and L-penicillamine disulphides (molecular wieght 296) vacuolated the normal fibroblasts. The lysosomes of normal fibroblasts were presumed to be capable of either the transport or the digestion of the mixed disulphides, but not of penicillamine disulphide.

A similar study using the osmotic protection technique would provide valuable data. D-penicillamine disulphide was obtained from Aldrich Chemical Co., Milwaukee, Wis. (product P110-1), but was found not to be soluble to 0.25M, so that experiments could not be carried out using this compound. It may however, be possible to carry out these experiments using, say, 0.1M sucrose with 0.15M D-penicillamine to obtain useful data, providing adequate control experiments are performed.

CHAPTER 4

Investigation of a method for the introduction of a protein into intact lysosomes *in vitro*

4.1 Introduction

In 1973, Natori and coworkers published a paper (Hayashi et al., 1973), in which they showed an apparent enhancement by ATP of the rate of appearance of degradation products when proteins were incubated in the presence of intact rat liver lysosomes. They claimed that this effect was due to an activation of the transport of the protein substrate into the lysosomes. It occurred to us that, if this were the case, the property might be usefully exploited to prepare lysosomes loaded with a particular substance, thus permitting an investigation of the release of substances from intact lysosomes. After "loading" of the organelles, excess substrate could be removed by centrifugation, and the release of the substrate or degradation products back into fresh medium could be observed.

The technique used by Hayashi et al. (1973) has been investigated in order to ascertain the validity of the claims (Hayashi et al., 1973; Natori, 1975) that ATP can promote the transport of a substrate into lysosomes. In this study, ^{125}I-labelled, formalin-denatured bovine serum albumin (Williams et al., 1971) was used as substrate, since it was readily available in the laboratory. If the ^{125}I-labelled dBSA was taken up by intact lysosomes, then the radiolabel would initially become sedimentable with the lysosomes. Subsequent digestion of the ^{125}I-labelled dBSA by the lysosomal proteases would lead to the appearance of acid-soluble radioactivity.

4.2 Experimental procedures

4.2.1 Measurement of lysosomal stability at various pH values

A series of experiments were carried out in order to establish the optimum pH for lysosome stability. A lysosome-enriched fraction from approx. 7.5g liver pulp, prepared as in Section 2.1.1, was resuspended in 20ml ice-cold 0.25M sucrose or mannitol. A sample of this suspension (2ml) was diluted with a mixture of 2ml 0.5M sucrose (or mannitol as appropriate) and 2ml 0.06M tris (adjusted to various pH values with citric acid), to give a final concentration of 0.25M sucrose (or mannitol) with 0.02M tris. The suspensions were incubated for 60min at 37°C, and then assayed for free and total N-acetyl-β-D-glucosaminidase activity for 5min at 37°C (see Section 2.3.1).

4.2.2 Measurement of the effect of ATP and $MgCl_2$ on the degradation of ^{125}I-labelled dBSA added to lysosome-enriched suspensions

A lysosome-enriched fraction of rat liver was prepared as described in Section 2.1.1 and the pellet derived from approx. 7.5g liver pulp was resuspended in 20ml ice-cold 0.25M sucrose. Aliquots of this suspension were diluted to one-third concentration with other ice-cold solutions to yield the final suspensions (see below for details) to which ^{125}I-labelled dBSA (2μg/ml) was added.

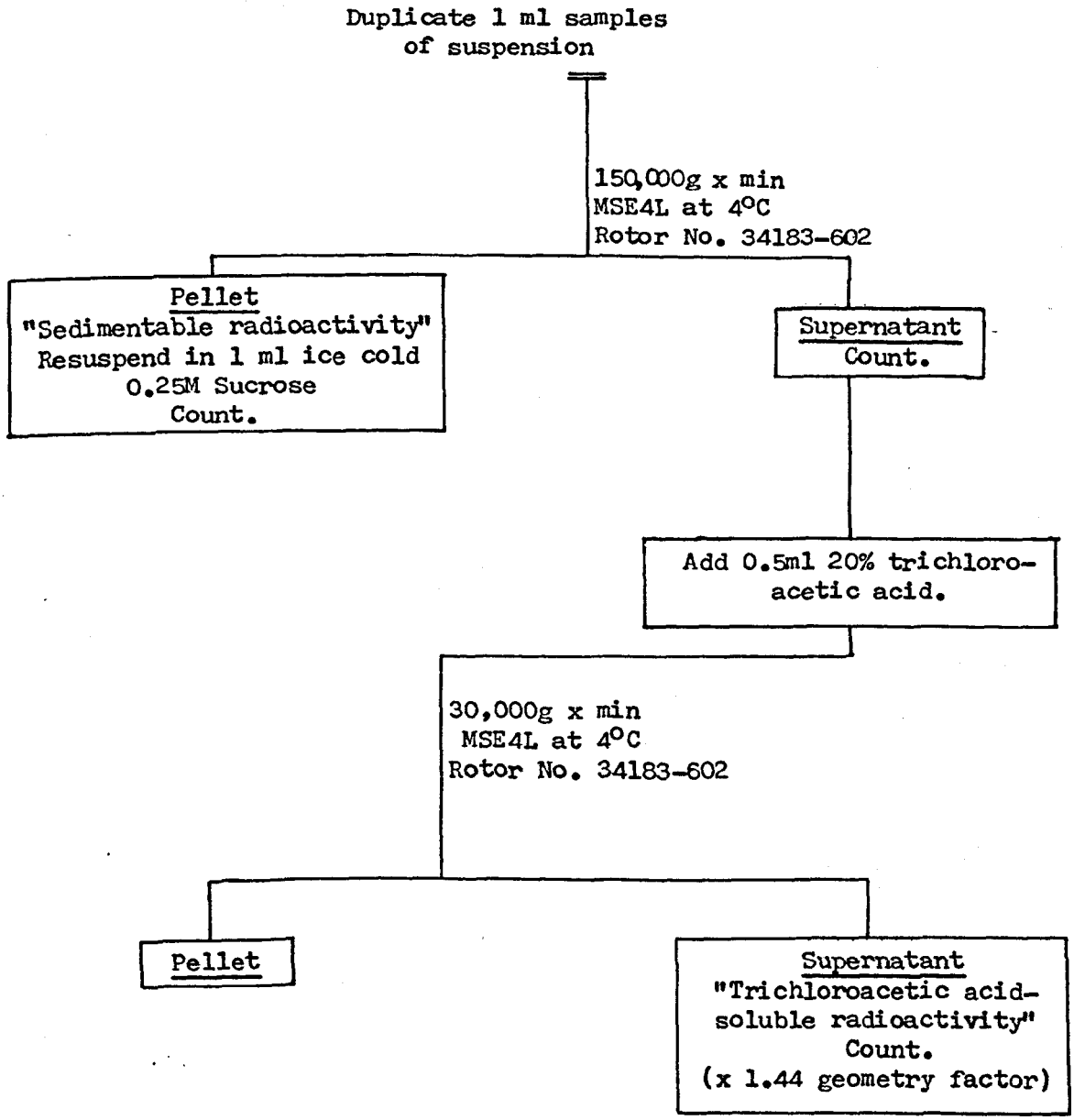
The lysosome-enriched suspensions with various additions were incubated at 37°C unless otherwise stated. The fate of ^{125}I-labelled dBSA was followed by withdrawing duplicate 1ml samples 0, 30 and 60min after the start of the incubation. These were assayed for sedimentable and trichloroacetic acid-soluble radioactivity as described below. After 60min incubation, i.e. at the end of the experiment, the free and total N-acetyl-β-D-glucosaminidase activities were measured. The percentage free enzyme activity was estimated at the beginning of every experiment to ensure that the lysosomes in the preparations were initially intact. The free enzyme activity was measured in preference to the non-sedimentable activity, since this gave a more realistic

estimate of the degree of lysosomal disruption (see Section 3.1).

The duplicate samples taken for radioactivity analysis were treated as shown in Figure 4.1, and counted as described in Section 2.6.1. An appropriate correction for geometry (shown in Figure 4.1) was made where the volume counted was not 1ml. If no binding of ^{125}I-labelled dBSA to the tubes occurred, the sedimentable radioactivity would represent material that was either within the lysosomes or bound to lysosomal membranes. The trichloroacetic acid-soluble radioactivity recorded is due to any degradation of ^{125}I-labelled dBSA that occurs, although a small amount of acid-soluble radioactivity (1-2% of the total radioactivity) is due to residual free [^{125}I]iodide.

N-Acetyl-β-D-glucosaminidase was assayed essentially as described in Section 2.3.1 with the exception of the 0.05M acetate buffer, pH 5.0, which was replaced by either 0.02M tris, adjusted to pH 6.0 with citric acid, or 0.02M phosphate buffer, pH 6.0, as appropriate. The enzyme and substrate were incubated for 10min at 37^{o}C.

Figure 4.1 Centrifugation and radioactivity counting scheme employed, using duplicate 1 ml samples of the lysosome-enriched suspension following incubation at 37°C with 2µg/ml ^{125}I-labelled dBSA


Duplicate 1 ml samples of suspension
150,000g x min
MSE4L at 4°C
Rotor No. 34183-602
Pellet
"Sedimentable radioactivity"
Resuspend in 1 ml ice cold
0.25M Sucrose
Count.
Supernatant
Count.
Add 0.5ml 20% trichloro-
acetic acid.
30,000g x min
MSE4L at 4°C
Rotor No. 34183-602
Pellet
Supernatant
"Trichloroacetic acid-
soluble radioactivity"
Count.
(x 1.44 geometry factor)

4.3 Results

4.3.1 The pH-dependence of lysosome stability at 37°C

Figure 4.2 shows the results of the experiments in which lysosome-enriched fractions were incubated at 37°C for 1h at a range of pH values. At the end of the incubation, the free and total N-acetyl-β-D-glucosaminidase activities were measured. Incubation in 0.25M sucrose and in 0.25M mannitol gave rise to similarly shaped curves of percentage free enzyme activity against pH. The minimum percentage free activities occurred after incubation at pH values between 5.5 and 6.5, presumably indicating that the lysosomes were most stable at these pH values. The recorded percentage free activities were always much lower when the incubation was in 0.25M sucrose, compared with 0.25M mannitol. In order to afford greater protection to the lysosome-enriched suspensions in future studies, subsequent experiments were carried out at pH 6.0 in 0.25M sucrose.

4.3.2 The effect of ATP and $MgCl_2$ on the degradation of ^{125}I-labelled dBSA added to lysosome-enriched suspensions.

(i) tris buffers

In the first series of experiments, the lysosome-enriched suspensions were incubated in a medium containing 0.25M sucrose and 0.02M tris, adjusted with citric acid to pH 6.0. In this solution there is no significant conversion of ^{125}I-labelled dBSA (2μg/ml) into either a sedimentable or an acid-soluble form, at 37°C or at 4°C (see Table 4.1). Also, there appears to be no disruption of lysosome stability, as judged by percentage free enzyme activity. When 3.3mM ATP and 3.3mM $MgCl_2$ are present in the solution, there is a steady increase in the sedimentable radioactivity, but no evidence of protein digestion. The rise in sedimentable radioactivity is unfortunately coupled with a (very variable) increase in the percentage free enzyme activity, indicating widespread

Figure 4.2 The percentage free N-acetyl-β-D-glucosaminidase activity found following the incubation of lysosome-enriched fractions in solutions of various pH at 37°C for 1h. Points are mean ± SEM of three experiments

KEY:

o . . 0.25M sucrose, 0.02M tris, buffered with citric acid to pH shown.

● . . 0.25M mannitol, 0.02M tris, buffered with citric acid to pH shown.

% Free N-Ac-β-Gase
0
50
100
pH of incubation
5.0
6.0
7.0
8.0

Table 4.1 Acid-soluble radioactivities and sedimentable radioactivities measured after incubation of lysosome-enriched fractions with ^{125}I-labelled dBSA for the times shown

Each figure is the mean $\pm$ SEM of three experiments

All of the lysosome-enriched fractions were suspended in 0.25M sucrose, 0.02M tris, buffered with citric acid to pH6.0, with 2μg/ml ^{125}I-labelled dBSA. $MgCl_2$ and ATP were both 3.3mM. In the experiment blanks, the radiolabelled albumin was added directly to the tubes used for counting the radioactivity (see Figure 4.1).

Conditions	Temp. °C.	Min. incubn.	% free N-Ac-β-D-glucosaminidase	Sedimentable radioactivity, % of total	Acid-soluble radioactivity, % of total
Control	37	0	7.6 ± 0.9	6.2 ± 1.4	2.0 ± 0.1
		30	n.d	5.2 ± 1.1	1.6 ± 0.2
		60	9.4 ± 0.7	3.8 ± 0.2	1.5 ± 0.1
Control	4	0	7.6 ± 0.9	6.5 ± 1.2	2.1 ± 0.1
		30	n.d	5.2 ± 0.4	1.8 ± 0.1
		60	9.0 ± 1.7	4.9 ± 0.2	2.0 ± 0.1
+ATP +$MgCl_2$	37	0	10.8 ± 3.9	13.2 ± 4.5	2.1 ± 0.2
		30	n.d	34.7 ± 9.2	1.5 ± 0.1
		60	58.0 ±21.6	44.3 ± 8.6	2.3 ± 1.0
Blank (+ATP, +$MgCl_2$)	37	0	7.7 ± 1.0	26.3 ± 7.4	1.8 ± 0.1
		30	n.d	25.3 ± 5.9	1.4 ± 0.1
		60	57.9 ±29.0	31.5 ± 9.8	2.7 ± 1.4

damage to the lysosomal membrane. Furthermore, the rise in sedimentable radioactivity and free enzyme activity was detected in the blank experiments using 3.3mM ATP and 3.3mM $MgCl_2$, where the ^{125}I-labelled dBSA was added directly to the tubes in which the duplicate samples of medium were taken for counting. These experiments were all repeated using 0.02M phosphate buffer in place of tris/citrate buffer in order to eliminate the possibility that the use of tris was affecting the properties of the lysosomal membrane.

4.3.3 The effect of ATP and $MgCl_2$ on the degradation of ^{125}I-labelled dBSA added to lysosome-enriched suspensions

(ii) phosphate buffers

The conditions used for these experiments were identical to those of Section 4.3.2 (Table 4.1) except for the replacement throughout of 0.02M tris/citrate by 0.02M phosphate buffer, pH6.0. The results are shown in Table 4.2, and it can be seen that they follow a pattern similar to Table 4.1. In the absence of ATP and $MgCl_2$ there is little sedimentable or acid-soluble radioactivity, and the lysosomes remain substantially intact for the duration of the experiments.

In the presence of ATP and $MgCl_2$ there is a significant increase in the sedimentable radioactivity, and also in the acid-soluble radioactivity, independent of whether the albumin is added at the beginning or end of the incubation. There is also total disruption of lysosome integrity under these conditions, as judged by the free enzyme activity.

Since the results using phosphate are substantially similar to those obtained with tris/citrate as buffer, an effect of tris on the lysosomal membrane is unlikely. On the other hand, it is quite possible that ATP or $MgCl_2$ could be causing the disruption of the lysosomes. The effect of ATP and $MgCl_2$ on the stability of lysosome-enriched suspensions was therefore studied further.

Table 4.2 Acid-soluble radioactivities and sedimentable radioactivities measured after incubation of lysosome-enriched fractions with ^{125}I-labelled dBSA for the times shown

Each figure is the mean ± SEM of three experiments

All of the lysosome-enriched fractions were suspended in 0.25M sucrose, 0.02M phosphate buffer, pH 6.0, with 2μg/ml ^{125}I-labelled dBSA. $MgCl_2$ and ATP were both 3.3mM. In the experiment blanks the radiolabelled albumin was added directly to the tubes used for counting the radioactivity (see Figure 4.1).

Conditions	Temp. °C.	Min. incubn.	% free N-Ac-β-D-glucosaminidase	Sedimentable radioactivity, % of total	Acid-soluble radioactivity, % of total
Control	37	0	9.9 ± 0.2	7.1 ± 1.2	2.1 - 0.2
		30	n.d	8.0 ± 1.2	1.8 ± 0.1
		60	16.2 ± 2.8	9.2 ± 0.6	1.8 ± 0.1
Control	4	0	9.9 ± 0.2	7.5 ± 0.2	1.9 ± 0.1
		30	n.d	8.0 ± 0.2	1.9 ± 0.1
		60	13.3 ± 1.2	6.7 ± 1.4	1.9 ± 0.1
+ATP +$MgCl_2$	37	0	12.7 ± 3.0	49.1 ± 5.3	2.0 ± 0.4
		30	n.d	62.0 ± 1.2	7.3 ± 1.4
		60	108.3 ± 5.3	67.8 ± 0.6	9.0 ± 1.0
Blank (+ATP, +$MgCl_2$)	37	0	12.7 ± 3.0	34.7 ± 2.0	1.8 ± 0.1
		30	n.d	34.7 ± 2.5	11.7 ± 3.1
		60	100.7 ± 2.3	42.9 ± 0.8	8.1 ± 2.1

4.3.4 The effect of ATP and $MgCl_2$ on the free enzyme activity of a lysosome-enriched suspension

A lysosome-enriched suspension, prepared as in Section 2.1.1, was divided into two parts. One part was suspended in 0.25M sucrose, containing 0.02M tris, adjusted with citric acid to pH 6.0 and the other part contained, in addition, 3.3mM ATP and 3.3mM $MgCl_2$. Both suspensions were incubated for 120min at 37°C. At time intervals throughout the incubation the free and total N-acetyl-β-D-glucosaminidase activities were assayed (as in 4.2.2) and the percentage free enzyme activity calculated. The results of one experiment are shown in Figure 4.3 (a second experiment omitting $MgCl_2$ gave quantitatively similar results).

Clearly the ATP causes a rise in the percentage free activity of the enzyme which ultimately leads to the abolition of latent enzyme activity. At the end of the experiment, the pH of the suspensions were measured. The pH of the control was found to be 6.10, whereas that of the suspension containing ATP and $MgCl_2$ was 5.15.

In subsequent experiments, in an effort to overcome this problem, the solutions of ATP and $MgCl_2$ were adjusted to pH 6.0 with NaOH prior to use. When the experiment described above in this Section was repeated using a "pH adjusted" solution of ATP, the free enzyme activity after 120min incubation at 37°C was 13.5% (see Figure 4.3). ATP appears to cause the breakage of lysosomes *in vitro* only when its inclusion in the medium lowers the pH of that medium.

4.3.5 The effect of "pH adjusted" ATP and $MgCl_2$ on the degradation of ^{125}I-labelled dBSA added to lysosome-enriched suspensions

Table 4.3 shows the results of experiments in which "pH adjusted" ATP and $MgCl_2$ were used. No large increases in sedimentable or acid-soluble radioactivities were detected, and there was no disruption of lysosomes.

Figure 4.3 The effect of 3.3mM ATP and 3.3mM $MgCl_2$ upon the percentage free N-acetyl-β-D-glucosaminidase activity of an intact lysosome-enriched suspension at 37°C

In each case, a lysosome-enriched fraction was suspended in 0.25M sucrose, 0.02M tris citrate buffered to pH 6.0.

Additions

●—·—· 3.3mM ATP, 3.3mM $MgCl_2$

▲—·—· 3.3mM "pH adjusted" ATP and $MgCl_2$

○—·—· no additions

Each line depicts one experiment only

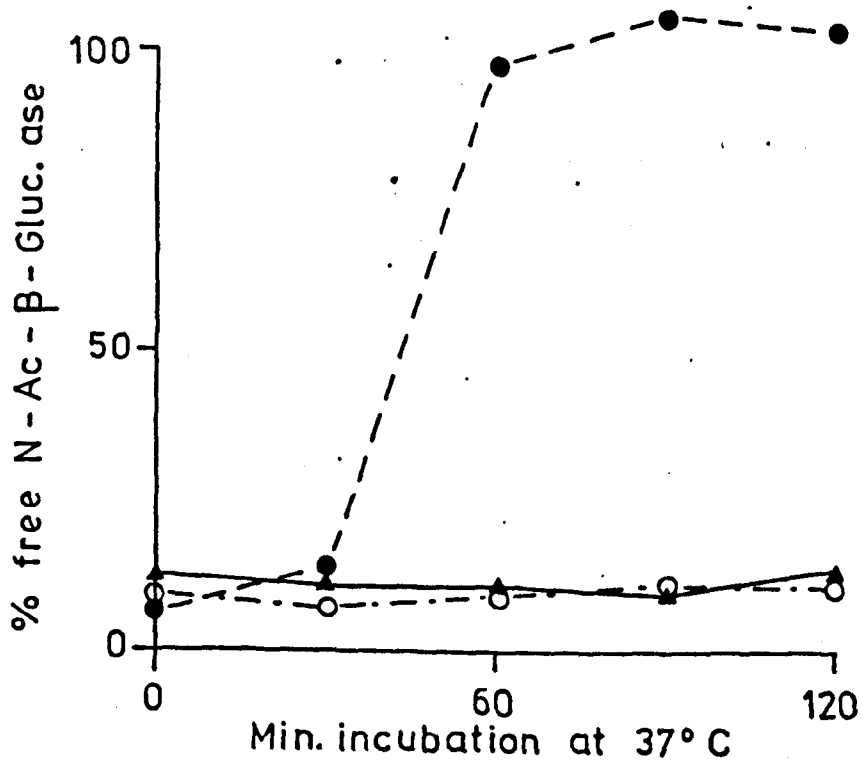

100
50
0
% free N-Ac-β-Gluc. ase
0
60
120
Min. incubation at 37° C

Table 4.3 Acid-soluble radioactivities and sedimentable radioactivities measured after incubation of lysosome-enriched fractions with ^{125}I-labelled dBSA for the times shown

Each figure (except those of the lower experiment blanks, where just one result is shown) is the mean ± SEM of three experiments

All of the lysosome-enriched fractions were suspended in 0.25M sucrose, 0.02M phosphate buffer, pH 6.0, with 2μg/ml ^{125}I-labelled dBSA. $MgCl_2$ and ATP (both "pH adjusted") were 3.3mM. In the experiment blanks the radiolabelled albumin was added directly to the tubes used for counting the readioactivity (see Figure 4.1).

Conditions	Temp. °C.	Min. incubn.	% free N-Ac-β-D-glucosaminidase	Sedimentable radioactivity, % of total	Acid-soluble radioactivity, % of total
+ATP		0	9.6 ± 0.3	7.0 ± 0.6	1.5 ± 0.1
"pH	37	30	n.d	6.2 ± 0.3	1.4 ± 0.1
adjusted"		60	11.4 ± 1.0	8.2 ± 1.4	1.3 ± 0.1
Blank (+ATP,		0	9.6 ± 0.3	10.2 ± 1.0	1.6 ± 0.1
"pH	37	30	n.d	11.0 ± 1.8	1.2 ± 0.1
adjusted")		60	11.3 ± 0.8	12.7 ± 4.0	1.0 ± 0.2
+ATP, $MgCl_2$		0	12.3 ± 3.1	6.0 ± 0.3	1.8 ± 0.1
"pH	37	30	n.d	6.1 ± 0.2	1.8 ± 0.1
adjusted"		60	12.5 ± 0.7	8.3 ± 0.1	2.0 ± 0.1
Blank (+ATP,		0	9.1	12.7	1.7
$MgCl_2$, "pH	37	30	n.d	17.5	1.4
adjusted")		60	15.3	9.8	1.8

4.3.6 Degradation of ^{125}I-labelled dBSA during counting procedure

The acid-soluble radioactivities measured in the experiments are depicted in Tables 4.1 - 4.3 as percentages of the total radioactivity recovered. Almost all of these are of the same order as the background level of acid-soluble radioactivity found in preparations of ^{125}I-labelled dBSA (i.e. 1-2%) that is due to a small amount of free [^{125}I] iodide. However, two rows of figures are significantly above this background level, those in experiments were "unadjusted" ATP was used with 0.02M phosphate buffer (Table 4.2). These figures cannot be entirely due to degradation of ^{125}I-labelled dBSA occurring during the incubation period, since the experiment blanks received no radiolabelled protein until the commencement of the centrifugation/counting procedure. Further analysis of the results showed that, owing to congestion on the γ-scintillation counter, the time elapsing before the addition of trichloroacetic acid was between 1 and 3h. During this time the 150,000g x min supernatant would have been at room temperature. This suspension would contain non-sedimentable lysosomal enzymes (arising from broken lysosomes) and also non-sedimentable ^{125}I-labelled dBSA. Hence, in those experiments where lysosome damage had been measured the conditions in the supernatant (i.e. the presence of lysosomal enzymes in a medium of pH approx. 5.0 - see Section 4.3.4) would have been suitable for degradation of ^{125}I-labelled dBSA to occur.

In a further experiment, a lysosome-enriched suspension was incubated for 60min at 37°C in 0.25M sucrose, 0.02M phosphate buffer, pH 6.0, 3.3mM ATP, 3.3mM $MgCl_2$ and 2µg/ml ^{125}I-labelled dBSA ("+ATP, +$MgCl_2$" in Table 4.2). Eight duplicate 1ml samples were taken for centrifugation and treated as described in Figure 4.1. All were treated identically apart from the time between the end of the 150,000g x min centrifugation to the moment of addition of 0.5ml 20% trichloroacetic acid, which was varied from 210min down to approx. zero. The resulting increase in

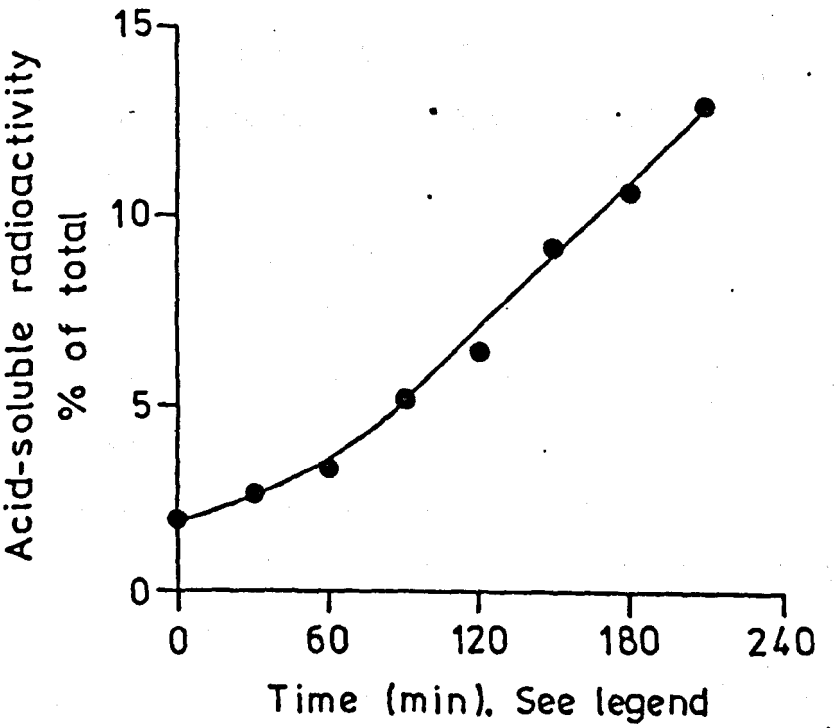
percentage trichloroacetic acid-soluble radioactivity with time (Figure 4.4) indicates that the increases in percentage acid-soluble ^{125}I-radioactivity in Table 4.2 are almost certainly due to the extralysosomal hydrolysis described above.

Returning to the data shown in the lower half of Table 4.2, it can be seen that the acid-soluble radioactivities recorded at the beginning of the incubation are normal. This is to be expected if the lysosomes are intact at this stage, and virtually no lysosomal enzymes would remain in the 500,000g x min supernant. At the other times, after lysosome damage has occurred, non-sedimentable lysosomal enzymes would appear in this supernatant which would thus be capable of digesting the ^{125}I-labelled dBSA under suitable conditions.

Figure 4.4 The appearance of acid-soluble radioactivity in a 150 000g x min supernatant of a lysosome-enriched fraction of rat liver

A lysosome-enriched suspension was incubated for 1h at 37°C in the following medium: 0.25M sucrose, 0.02M phosphate buffer, pH 6.0, 3.3mM ATP, 3.3mM $MgCl_2$, 2µg/ml ^{125}I-labelled dBSA. At the end of the incubation several 150,000g x min supernatants were prepared (see Figure 4.1) in 3ml disposable plastic tubes. These were immediately counted for ^{125}I-radioactivity and then left at room temperature. At various times the acid-soluble radioactivity was determined in duplicate tubes as shown in Figure 4.1.

The percentage acid-soluble radioactivity was plotted against the time elapsing between the end of the 150 000g x min centrifugation and the addition of 0.5ml 20% trichloroacetic acid.


Acid-soluble radioactivity
% of total
15
10
5
0
0
60
120
180
240
Time (min). See legend

4.4 Discussion

Solutions containing sucrose have been used in previous investigations into protein degradation by lysosomes (e.g. Mego et al., 1967 and 1972; Gregoriadis and Ryman, 1972; Hayashi et al., 1973) in order to preserve the integrity of lysosomes in vitro. Small changes in various parameters are easier to detect against a stable background provided by a stable preparation of lysosomes. The experiments described at the beginning of this Chapter attempted to ascertain the optimum conditions for the maintenance of an intact population of lysosomes at 37°C. The minimum percentage free enzyme activity of a lysosome-enriched suspension after incubation for 1h at 37°C was obtained using 0.25M sucrose, pH 6.0 (Figure 4.2). This result cannot be an artifact caused by an alteration of enzyme activity with the variation in pH, since the free enzyme activity was throughout calculated as a percentage of the total enzyme activity obtained under the same conditions.

This work compares interestingly with that of the previous Chapter. At 25°C there is only a slight difference in the permeability of the membrane of the lysosome to 0.25M mannitol at different pH values (Table 3.1), with the minimum occurring again at pH 6.0. Although lysosomes remain stable in 0.25M sucrose at both 25°C (Lloyd, 1969) and 37°C, the present results show they are stable in 0.25M mannitol only at 25°C. Presumably mannitol (m.wt. 182) is able to penetrate the membrane of the lysosome at 25°C, but only very slowly, whereas at 37°C the penetration occurs much more quickly. Sucrose (m.wt. 342) on the other hand, may be too large a molecule to permeate the lysosomal membrane at either 25° or 37°C.

The shape of the graph of "percentage released cathepsin activity" (Balasubramaniam and Deiss, 1965) of lysosomes incubated at various pH values in 0.25M sucrose resembles that of Figure 4.2, although the beef

thyroid lysosomes used were most stable at pH 5.0, a displacement of one pH unit. Other experimental evidence (Bertini et al., 1967; Mego, 1971) concerning the release of acid-precipitable protein by heterolysosomes at pH 4, 5, 7 and 8 points to a diminished stability of suspensions of lysosomes at pH 8.

ATP (3.3mM) causes the pH of a lysosome-enriched suspension to drop from 6.10 to 5.15, although the mechanism of this action is uncertain. This pH effect has been reported previously (Mego et al., 1972), and the adjustment of solutions of ATP with NaOH to the required pH (Mego, 1975) is similar to the treatment used in this Chapter. In causing a pH drop of one unit, 3.3mM ATP causes a rapid and complete loss of latency (Figure 4.3) which was not observed when "pH adjusted" ATP was used in an otherwise identical experiment.

Increases in the sedimentable radioactivity (presumably due to lysosome-associated ^{125}I-labelled dBSA) were only found in those experiments where ATP was used without pH adjustment. These increases were also detected in the corresponding control experiments where the radiolabelled protein was added directly to the sample tube prior to the commencement of the counting procedure. It seems likely, therefore, that the sedimentability of albumin in these experiments is influenced mainly by the status of the lysosomes. Interestingly, the albumin binds significantly only to disrupted lysosome membranes, possibly due to the molecules gaining access under these conditions to receptors which are carried to the inner surface of the lysosomal membrane following endocytosis.

Similarly, a significant increase in the acid-soluble radioactivity was only found in those experiments where ATP was used without pH adjustment. Degradation of albumin also occurred in experiment blanks, indicating that at least some of the proteolysis occurred after the incubation period. This was confirmed in a subsequent experiment

(Figure 4.4).

The work reported in the paper of Hayashi et al., (1973) was mostly carried out at pH 4.5, following an initial experiment which the authors interpreted as indicating that their preparations of lysosomes were stable at that pH. However, it is clear that what they were measuring in that experiment was not the percentage free activity of the lysosomal proteases during the experiment, but a cumulative figure of the degradative power of the enzymes released. In other words, rather than recording data similar to individual points in Figure 4.3, they recorded data equivalent to the area under Figure 4.3 bounded by zero and their assay time. Hence a low result in their experiments does not necessarily indicate a low degree of lysosome disruption, since a similar result would be obtained if all the lysosomes broke near the end of the incubation time.

Most of the remaining work in the paper (Hayashi et al., 1973) can be explained in terms of a lability of the membrane of the lysosome caused by ATP. The authors also assumed that the percentage free activity of an enzyme is the same as its non-sedimentable activity, which is not necessarily correct.

To conclude, the work reported in this Chapter, provides no evidence that ATP promotes the transport of substrates into intact isolated lysosomes. Indeed, some of the results suggest that ATP acts to decrease the pH of the suspending solution, and thus lower the stability of lysosomes. Huisman et al. (1974) have criticised the rationale of the experiments of Hayashi et al. (1973), but without reappraising the original method. Taken together, this work and that of Huisman et al., (1974) provide alternative explanations for all the phenomena observed in the original paper (Hayashi et al., 1973).

CHAPTER 5

The subcellular location of two lysosomal enzymes and ^{125}I-Labelled PVP, following differential centrifugation of rat liver homogenates in different solutions

5.1 Introduction

Bearing in mind that the aim of this Thesis is to study the fate of cystine residues in the lysosomal system, it is essential to set up an experimental system that can establish the location of substances within cells. This brief Chapter describes an investigation into the effect of different solutions on the recovery of ^{125}I-labelled PVP, N-acetyl-β-D-glucosaminidase and arylsulphatase from rat liver using differential centrifugation.

The reasons underlying the choice of ^{125}I-labelled PVP are given in Section 6.2.2 but, briefly, the substance is an easy to detect, non-degradable macromolecule. It can reasonably be expected, therefore, that ^{125}I-labelled PVP enters cells solely by endocytosis; indeed there is experimental evidence (Roberts et al., 1977) to suggest that in vitro it enters rat yolk sac by fluid-phase pinocytosis. It can thus be considered an appropriate marker for the pinosomes and secondary lysosomes.

To ascertain the subcellular location of injected material within the rat liver, two techniques are available. Light or electron microscopy of liver tissue would be useful only if the material was detectable in the sections produced by virtue of a staining property or its electron density.

Differential centrifugation of cell homogenates is probably the most widely used technique for investigating the subcellular distribution of a material in a tissue. In 1955, de Duve and coworkers published what is now regarded as a standard centrifugation procedure, which results in the isolation of five subcellular fractions from a rat liver homogenate. Separation is afforded by the use of centrifugation at successively increasing speeds, which isolates particles according to their size, the larger particles tending to sediment at slower centrifugation speeds. In this way, fractions enriched with nuclear, mitochondrial, lysosomal, microsomal and supernatant material respectively, can be isolated.

The different solutions were used in this study in an attempt to

maximize the lysosomal recovery of N-acetyl-β-D-glucosaminidase, aryl-sulphatase and injected ^{125}I-labelled PVP. Initially, 0.25M Sucrose was used and acted as a control against which the other solutions could be compared. The fifth solution, in which 5mM EGTA replaced 5mM EDTA, was tested subsequently to eliminate the possibility that EDTA interfered with the protein assay, perhaps by the formation of an EDTA-Cu^{2+} ion complex.

5.2 Experimental procedure

Male Wistar rats (250-350g) were injected with ^{125}I-labelled PVP (50µg/kg body weight) as described in Section 2.7. After 2h the animal was killed by a blow to the neck, and the liver treated as described in 5.2.1.

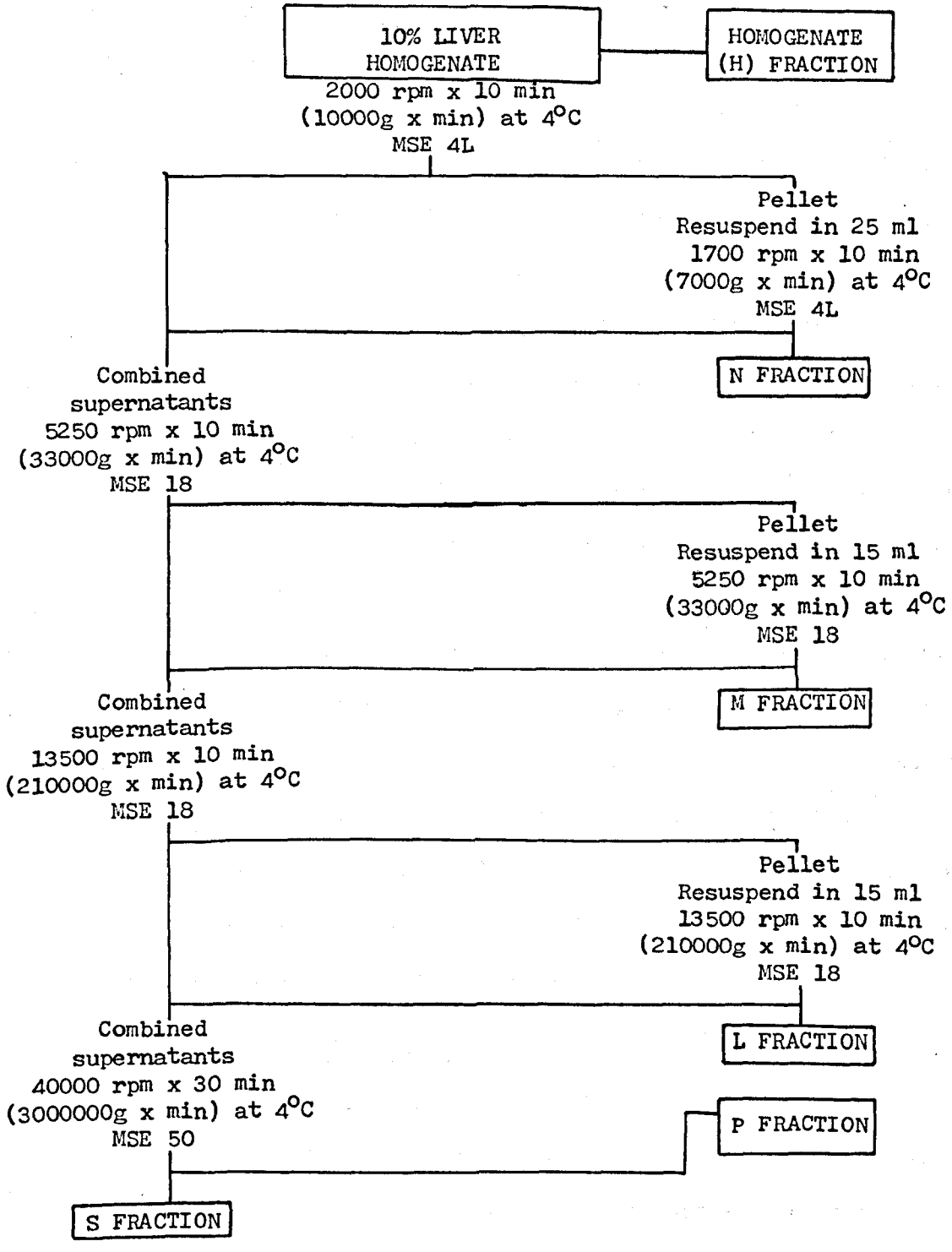
5.2.1 Differential subcellular fractionation of rat liver

The rat liver was exposed, and rapidly perfused with 20-30ml of the solution under test. This and all subsequent operations utilised ice-cold solutions. The liver, now pale brown, was excised, blotted and weighed, then immediately forced through a cooled stainless steel sieve (approx. 1.0mm^2 mesh) and subsequently reweighed.

The liver pulp (approx. 7-10g) was resuspended in 2.5ml/g pulp of the solution under test, and homogenised in a Potter-Elvejhem Teflon/glass homogeniser (Tri-R Instruments Inc., New York. 0.019cm clearance) at 3000rpm (setting 2.7) by three steady up and down strokes lasting in total about 30 seconds. The resulting suspension was then centrifuged at 4°C in an MSE 4L centrifuge at 250g x 5 min (rotor no. 34123-602). After this centrifugation, the supernatant was decanted and stored on ice, and the pellets rehomogenised in about 10ml of solution as before. The supernatant and rehomogenised suspension were made up to 10% (g pulp weight/volume) with ice-cold solution. Some of this homogenate was stored at -20°C for later assay, while a known volume of the remainder was subjected to the centrifugation scheme shown in Fig. 5.1, all operations being carried out on ice or at 4°C in precooled centrifuges. All resuspensions of pellets were carried out using the Potter-Elvejhem homogeniser at 1500rpm (speed setting 1.7). Each fraction isolated was deep frozen at -20°C for later assay of protein, radioactivity and assay of total lysosomal enzyme activities (see Chapter 2 for assay procedures). The time taken for the complete fractionation was approx. 3.5h.

Figure 5.1 Schematic representation of the differential centrifugation scheme employed to separate rat liver homogenates into five subcellular fractions

The fractionations were carried out (see Section 5.2.1) using precooled centrifuges and rotors 34183-602 (MSE 4L), 43114-106 (MSE High Speed 18) and 59113 (MSE Super Speed 50).


10% LIVER HOMOGENATE
HOMOGENATE (H) FRACTION
2000 rpm x 10 min (10000g x min) at 4°C MSE 4L
Pellet Resuspend in 25 ml 1700 rpm x 10 min (7000g x min) at 4°C MSE 4L
N FRACTION
Combined supernatants 5250 rpm x 10 min (33000g x min) at 4°C MSE 18
Pellet Resuspend in 15 ml 5250 rpm x 10 min (33000g x min) at 4°C MSE 18
M FRACTION
Combined supernatants 13500 rpm x 10 min (210000g x min) at 4°C MSE 18
Pellet Resuspend in 15 ml 13500 rpm x 10 min (210000g x min) at 4°C MSE 18
L FRACTION
Combined supernatants 40000 rpm x 30 min (3000000g x min) at 4°C MSE 50
P FRACTION
S FRACTION

5.3 Results and Discussion

5.3.1 Total recoveries of markers

The total recovered activity in all five fractions was expressed as a percentage of the activity measured in the homogenate. Table 5.1 shows that the recovered activities for both protein and N-acetyl-β-D-glucosaminidase were usually between 90 and 100%. The total recovery of ^{125}I-labelled PVP was usually slightly above the homogenate value. The mean recovery of arylsulphatase was initially estimated to be 143.5% of the activity in the homogenate. To investigate this anomaly further experiments were performed using the L and S fractions, reconstituted together as they were in the homogenate. The observed activity was 60.1 $\pm$ 2.2% (Mean $\pm$ S.E.M., 7 expts) of that expected from measurements of the individual fractions. This implies inhibition of arylsulphatase by some factor present in the cytosol. If the simple assumption is made that the homogenate as measured is similarly inhibited, then the recovery data in Table 5.1 can be corrected by multiplying by 0.6. This is, of course, only an approximate correction, but does give some indication of the recovery obtained. After this correction has been applied (see Table 5.1 last column) the recoveries do fall to a more acceptable level, with a mean of 86.2%, only a few per cent below the recovery of N-acetyl-β-D-glucosaminidase.

5.3.2 Expression of results

As the total recoveries (including, after adjustment, that of arylsulphatase) were all approx. 100%, the activity of each fraction was calculated as a percentage of the sum of the activities of the five fractions i.e. as a percentage of the amount recovered during centrifugation. It was assumed that the slight losses which occurred during centrifugation were distributed equally through the five fractions, since possible further corrections have a minimal effect on the overall result. Consequently, an estimate of the relative specific activities could be obtained by dividing

Table 5.1 Percentage recovery of ^{125}I-labelled PVP, N-acetyl-β-D-glucosaminidase and arylsulphatase after subcellular fractionation of rat liver

The recovery of protein, ^{125}I-labelled PVP, N-acetyl-β-D-glucosaminidase and arylsulphatase in each fraction is expressed as a percentage of the homogenate activity, after subcellular fractionation of a 10% liver homogenate in the solutions shown (see Section 5.2.1). Each rat had been injected with 50μg/kg body weight ^{125}I-labelled PVP 2h prior to sacrifice and the commencement of the isolation procedure.

(Results are mean ± S.E.M., or the range is shown).

Homogenising solution	Recovery (% of Homogenate Value)				
	Protein	^{125}I-labelled PVP	N-Ac-β-Gluc.ase	Aryl.S.ase	Aryl.S.ase (Corrected)
0.25M Sucrose	104.6 ± 2.9 (4)	94.9 ± 8.2 (3)	93.3 ± 6.0 (4)	152.5 ± 8.9 (4)	91.7 ± 5.4 (4)
0.3M Sucrose	94.3 ± 3.2 (4)	114.1(103.9-124.3) (2)	93.3 ± 4.0 (4)	145.9 ± 8.3 (4)	87.7 ± 5.0 (4)
0.3M Sucrose 5mM MOPS pH 7.4	92.9 ± 2.3 (3)	107.5(99.5-115.4) (2)	103.4 ± 3.5 (3)	141.0 ± 9.8 (3)	84.7 ± 5.9 (3)
0.3M Sucrose 5mM MOPS pH 7.4, 5mM EDTA	98.4 ± 1.6 (3)	106.0 ± 1.1 (3)	84.3 ± 7.1 (3)	100.8(94.0-107.6) (2)	60.6(56.5-64.7) (2)
0.3mM Sucrose 5mM MOPS pH 7.4, 5mM EGTA	91.8 ± 4.5 (3)	110.8 ± 8.3 (3)	91.0 ± 5.1 (3)	159.0 ± 6.8 (3)	95.6 ± 4.1 (3)
Total	97.5 ± 1.7 (17)	106.0 ± 3.3 (13)	93.1 ± 2.5 (17)	143.5 ± 5.6 (16)	86.2 ± 3.4 (16)

the percentage of material in a fraction by the percentage protein, without using data from the homogenate (see de Duve, 1967). Using this method, the total of protein, and each marker, is thus 100%, which makes comparison easier between the different markers.

It is usual to express the results of differential centrifugation experiments as histograms showing the specific activity of each fraction relative to a homogenate value of 1. However, either data from "typical experiments" must be used or, if mean results are shown, then no estimate of interexperimental variation can be given on the diagram, since the variation occurs in the area of each bar of the histogram, i.e. in two dimensions. Therefore, a preliminary table of the percentage recovery in each fraction was used, which enabled a measure of the variation to be shown, and from this a pictorial representation of relative specific activity was derived in order to show clearly the pattern of recovery and localization of materials.

5.3.3 Effects of using different homogenizing solutions

The results are shown in Table 5.2 and Figure 5.2. Table 5.2 shows that there is very little difference in the distribution of the two enzymes, whichever solution is used to prepare the homogenate and isolate the fractions. Between 40% and 50% of N-acetyl-β-D-glucosaminidase and arylsulphatase was recovered in the L fraction (with the exception of isolation in 0.3M sucrose containing 5mM MOPS, pH 7.4 and 5mM EDTA, where only 34.7% of arylsulphatase was localised in the L fraction).

The distribution of ^{125}I-labelled PVP alters slightly depending on the solution used, but apparently without pattern. The recovery of the radiolabel in the L fraction varied from 21.5% to 32.9% of the total recovered.

The recovery of protein in the five fractions was very similar in both 0.25M and 0.3M sucrose, and also in 0.3M sucrose containing 5mM MOPS pH 7.4. However, there was a startling change in the protein distributi[illegible]

Table 5.2 The effects of various media on the subcellular distribution of ^{125}I-labelled N-acetyl-β-D-glucosaminidase, arylsulphatase and protein in the rat liver, two hours following the injection of ^{125}I-labelled poly(vinylpyrrolidone) (5μg/100g b.wt.)

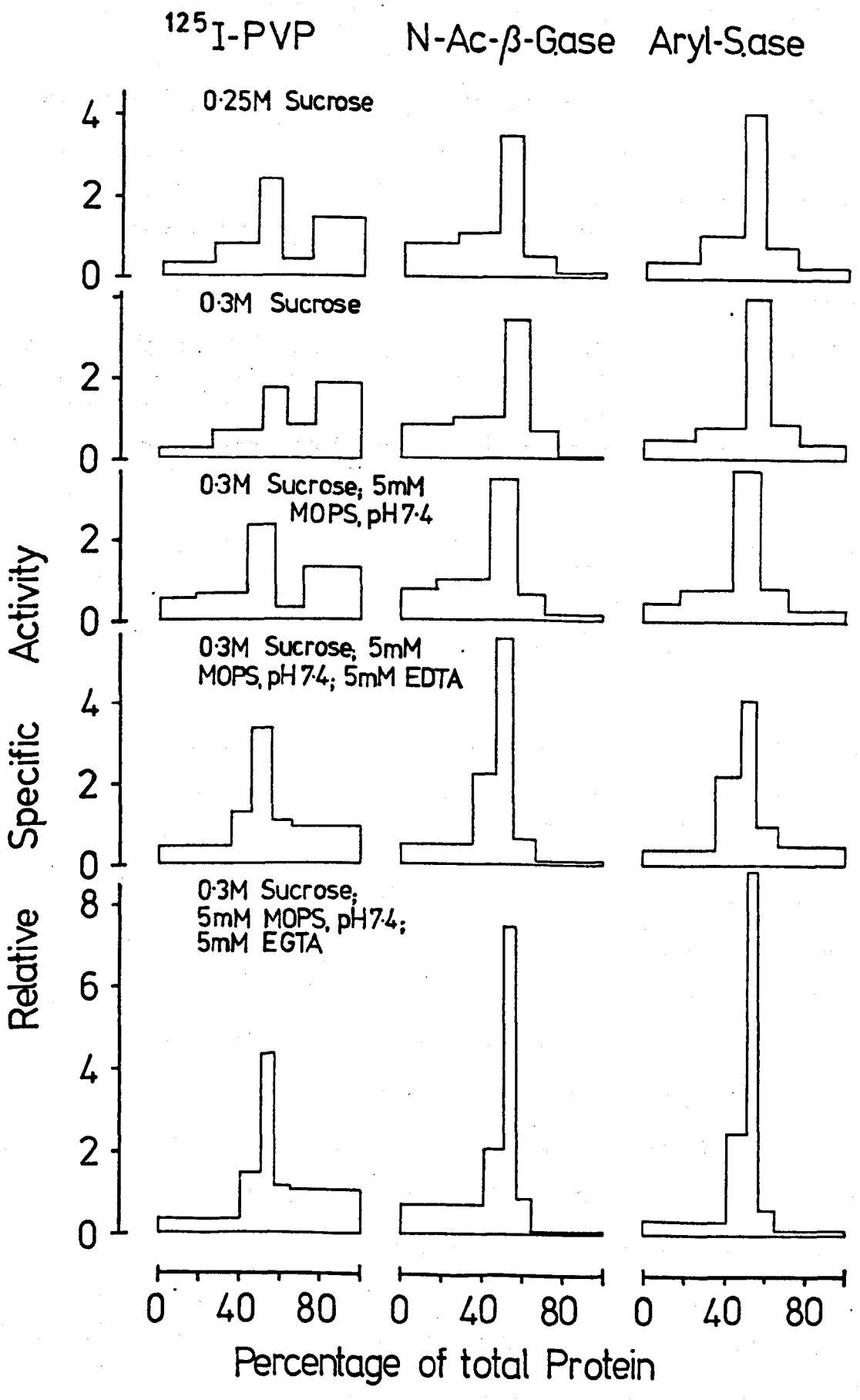
^{125}I-labelled PVP (50μg/kg body weight) was injected 2h prior to sacrifice. The rat liver was fractionated according to the scheme of Section 5.2.1. The amount of material in each fraction is expressed as a percentage of the sum of all five fractions (see Section 5.3.2). Results are mean ± S.E.M., or the range is shown.

Medium	Fraction	% Protein	% ^{125}I-labelled PVP	% N-Ac-β-Gluc.ase	% ArylS.ase
0.25M	N	26.2 ± 2.6	9.1 ± 1.7	22.0 ± 4.8	10.6 ± 1.9
	M	21.3 ± 0.6	17.2 ± 2.4	23.8 ± 1.7	21.9 ± 2.1
Sucrose	L	11.8 ± 1.5	28.7 ± 3.6	42.0 ± 3.4	48.4 ± 0.6
	P	15.4 ± 1.4	7.1 ± 1.7	8.7 ± 1.2	12.1 ± 0.5
	S	25.4 ± 2.2	37.8 ± 6.3	3.6 ± 1.1	7.1 ± 1.9
No. of Expts		(4)	(3)	(4)	(4)
0.3M	N	25.7 ± 2.3	7.3 (6.7 - 7.8)	21.6 ± 1.7	11.8 ± 0.9
	M	25.3 ± 0.6	17.2 (15.8 - 18.6)	25.7 ± 1.5	19.5 ± 0.9
Sucrose	L	12.3 ± 1.7	21.5 (21.2 - 21.7)	42.5 ± 2.0	48.4 ± 3.0
	P	14.3 ± 0.9	11.7 (10.7 - 12.6)	9.7 ± 1.2	12.3 ± 1.5
	S	22.5 ± 1.6	42.5 (42.4 - 42.5)	0.8 ± 0.3	8.0 ± 1.5
No. of Expts		(4)	(2)	(4)	(4)
0.3M Sucrose	N	17.9 ± 1.4	9.8 (7.6 - 12.0)	13.2 ± 1.8	8.2 ± 1.5
	M	25.8 ± 1.2	16.8 (12.7 - 20.9)	25.2 ± 3.6	20.0 ± 2.4
5mM MOPS	L	14.1 ± 2.0	32.9 (31.3 - 34.5)	49.0 ± 6.1	52.4 ± 8.0
	P	14.1 ± 0.6	4.4 (2.2 - 6.6)	8.7 ± 1.6	11.4 ± 1.9
pH 7.4	S	28.1 ± 3.1	36.0 (29.1 - 42.9)	3.9 ± 2.3	8.1 ± 4.6
No. of Expts		(3)	(2)	(3)	(3)
0.3M Sucrose	N	35.5 ± 3.1	16.0 ± 2.0	17.5 ± 5.0	14.0 (6.4 - 21.6)
5mM MOPS	M	12.0 ± 2.7	15.0 ± 1.7	26.4 ± 3.7	26.3 (25.6 - 26.9)
pH 7.4	L	8.5 ± 0.8	28.2 ± 1.2	46.9 ± 0.6	34.7 (32.2 - 37.2)
5mM EDTA	P	10.3 ± 0.3	10.8 ± 2.5	6.3 ± 0.2	10.1 (9.9 - 10.3)
	S	33.7 ± 3.6	29.9 ± 4.7	3.0 ± 1.6	15.0 (9.4 - 20.5)
No. of Expts		(3)	(3)	(3)	(2)
0.3M Sucrose	N	41.0 ± 0.8	14.6 ± 1.5	28.9 ± 3.4	15.0 ± 1.7
5mM MOPS	M	10.4 ± 0.8	15.3 ± 4.0	21.2 ± 1.7	25.8 ± 1.2
pH 7.4	L	5.5 ± 0.4	23.9 ± 4.0	40.9 ± 3.1	48.4 ± 1.0
5mM EGTA	P	8.0 ± 0.5	9.0 ± 1.4	7.1 ± 0.6	5.3 ± 1.4
	S	35.1 ± 0.4	37.3 ± 10.4	2.0 ± 1.0	5.2 ± 1.1
No. of Expts		(3)	(3)	(3)	(3)

Figure 5.2 The effects of various media on the subcellular distributions of ^{125}I-labelled PVP, arylsulphatase and N-acetyl-β-D-glucosaminidase in rat liver

^{125}I-labelled PVP (50μg/kg body weight) was injected 2h before sacrifice. The rat liver was fractionated according to the scheme of Section 5.2.1. The histograms are calculated from the data of Table 5.2 as indicated in the text (5.3).

The term "specific activity" is potentially ambiguous when applied to the concentration of a radioactive substance such as ^{125}I-labelled PVP. In this thesis it denotes the measured counts per minute in a tissue homogenate or fraction, per mg protein.


^{125}I-PVP
N-Ac-β-Gase
Aryl-Sase
0·25M Sucrose
0·3M Sucrose
0·3M Sucrose; 5mM MOPS, pH 7·4
0·3M Sucrose; 5mM MOPS, pH 7·4; 5mM EDTA
0·3M Sucrose; 5mM MOPS, pH 7·4; 5mM EGTA
Relative Specific Activity
Percentage of total Protein
0
40
80

when the liver homogenate was suspended in 0.3M sucrose containing 5mM MOPS, pH 7.4 and either 5mM EDTA or 5mM EGTA. When either of these solutions were used, the percentage of protein in the middle three fractions (M, L and P) dropped to 25–30% of the total recovered, compared to 45–55% of the total in the other solutions used. In particular, the percentage of protein in the lysosome-enriched fraction dropped from approximately 12%, in 0.25M sucrose, to 5.5% in 0.3M sucrose containing 5mM MOPS, pH 7.4 and 5mM EGTA. The redistribution of protein results in an increased recovery of protein material in the first and last fractions (i.e. N and S).

Since similar protein distributions were observed with both the EDTA and the EGTA solutions, chelation with the Cu^{2+} ions of the protein assay is unlikely. A modified assay for protein (Section 2.4) using phosphotungstic acid to precipitate the protein and remove the EDTA with the supernatant, failed to detect any differences from the original assay (results not shown).

Presumably, the EDTA (and EGTA) cause an alteration in the protein distribution by complexing with Ca^{2+} ions. The effective removal of Ca^{2+} from solution might cause the formation of aggregates of macromolecules, or disrupt the binding of protein molecules to membranes. Since there is virtually no change in the distribution of the enzymes between the fractions, this removal of Ca^{2+} seems not to cause disruption of the lysosomes themselves, but rather removes non-lysosomal protein which would normally sediment with this fraction. Both de Duve *et al.* (1955) and Sawant *et al.* (1964) include 1mM EDTA in the homogenising solutions they use, de Duve *et al* (1955) commenting that 1mM EDTA "exerts a protective action on glucose-6-phosphatase, perhaps other enzymes and seems to have no undesirable effects". Davidson (1975a) found that the removal of metal ions with 1mM EDTA stimulated the digestion of ^{125}I-labelled ribonuclease by mouse kidney lysosomes at 37°C, presumably by increasing

the stability of the lysosomes. None of the authors comment on an alteration of the protein distribution caused by the addition of EDTA.

Figure 5.2 shows what the change in the protein distribution does to the specific activities of the enzymes and ^{125}I-labelled PVP. The increased specific activity in the L fraction when 0.3M sucrose containing 5mM MOPS, pH 7.4 and either 5mM EDTA or 5mM EGTA is used for the fractionation, is due almost solely to the alteration in protein distribution.

The histograms of N-Acetyl-β-D-glucosaminidase and arylsulphatase are similar to pattern III in the paper of de Duve et al. (1955), shown by acid phosphatase and cathepsin. It is reasonable to assume, therefore, that these enzymes are located in the lysosomes. The distribution of ^{125}I-labelled PVP is not so clear. The highest specific activity does occur in the L fraction, but a large amount is also found in the supernatant. The subcellular distribution of ^{125}I-labelled PVP is discussed in Chapter 8.

In all three cases, however, an L fraction containing the highest relative specific activity for the lysosomal enzymes is obtained following fractionation in 0.3M sucrose containing 5mM MOPS, pH 7.4 and 5mM EGTA. Therefore, this solution was used in almost all the later experiments. In a few of the experiments performed before the work described in this Chapter was finished, 0.3M sucrose containing 5mM MOPS, pH 7.4 and 5mM EDTA was used and it was assumed that these results were similar to those which would have been obtained using the solution containing EGTA. These few experiments are, however, clearly indicated in the Figure legends.

CHAPTER 6

The preparation and properties of liposomes containing entrapped substances

6.1 Introduction

The preparation and use of liposomes (spherules or vesicles of lipid in aqueous solution) was first described by Bangham et al. (1965) and they were widely used in the late sixties as a model system to elucidate the structural and permeability properties of biological membranes (see e.g. Papahadjopoulos and Miller, 1967; Papahadjopoulos and Watkins, 1967). The observation that liposomes are able to entrap certain solutes gave rise to the idea (Sessa and Weismann, 1969; Gregoriadis et al., 1971) that enzymes could be entrapped within liposomes, and that such liposomes might then be administered to patients with lysosomal storage diseases, as an enzyme replacement therapy (see 1.2.5.3).

Since then, a large volume of literature has appeared on the subject of the use of liposomes in various model schemes. As well as papers describing model systems relating to enzyme therapy (Gregoriadis and Ryman, 1972; Weissmann et al., 1975), there have been attempts to develop the use of liposomes containing anti-tumour drugs (Gregoriadis et al., 1974a; Dapergolas et al., 1976), hormones (Patel and Ryman, 1976; Dapergolas and Gregoriadis, 1976; Shaw et al., 1976; Patel et al., 1978) and chelating agents (Jonah et al., 1975, 1978).

This Introduction describes the structure, preparation and properties of liposomes of various types. In the later Sections, our attempts to entrap various substances within liposomes formed from dipalmitoylphosphatidylcholine (DPPC) are evaluated.

6.1.1 The structure and permeability of liposomes

Liposomes are vesicles of lipid in aqueous suspension. They can be either unilamellar, i.e. consisting of only one lipid bilayer, or multilamellar, i.e. built up of several concentric lipid bilayers. Each lipid bilayer is roughly spherical, and therefore encloses a volume of fluid. The ability of substances within the aqueous phase to escape from liposomes

will depend upon their ability to cross the bilayers. The degree of retention of substances by liposomes is affected by the nature and stability of the liposomes, the nature of the enclosed substances, and also the temperature.

6.1.1.1. The effect of lipid composition on liposome structure and permeability

The composition of lipid bilayers determines two major features of the resultant liposomes, viz. their charge, and also the general integrity and physical characteristics of the bilayer. Lipid bilayers exist in two forms. At lower temperatures the fatty acyl chains of the lipids exist in the solid state but, as the temperature is raised, these hydrophobic chains undergo a phase transition to the liquid state, resulting in a liquid-crystalline bilayer. Generally, any substance which causes an increased degree of order in the fatty acyl chains of the bilayer causes an increase in the thermal phase transition temperature. Also, the more ordered the bilayer, the less permeable are the resulting liposomes (see below).

Liposomes made from members of the homologous series of fatty acyl phosphatidylcholines show a progressive variation in permeability to different substances. As the chain length of the constituent fatty acids is increased, the corresponding liposomes become less permeable to glycerol and erythritol (de Gier et al., 1968) and cortisol octanoate (Shaw et al., 1976).

The liposomes most commonly used for studies in vivo are formed from egg phosphatidylcholine (see e.g. Gregoriadis and Ryman, 1972; Jonah et al., 1975) which contains a mixture of diacylphosphatidylcholines (Paphadjopoulos and Miller, 1967; Mason and Huang, 1978). The heterogeneity in the acyl chain lengths results in a lower degree of order of the bilayer, giving rise to an increase in the permeability of liposomes made from this lipid

(Shaw et al., 1976). However, the addition of a proportion (up to 50 Mole percent has been used) of cholesterol to egg phosphatidylcholine effectively raises the transition temperature by restoring the order of the hydrophobic region of the bilayer, and hence decreases the permeability of such liposomes (Ladbrooke et al., 1969). Shaw et al. (1976) made liposomes from a defined phosphatidylcholine and found that the inclusion of cholesterol made such liposomes more leaky, presumably because it disrupts the already ordered structure of the bilayers and lowers the effective phase transition temperature.

Charged lipids included in the bilayers have two properties. Firstly, by repulsion of like-charged lipids in adjacent bilayers, they tend to increase the interlamellar distance, and thus the volume of the aqueous compartment (Sessa and Weissmann, 1970). Secondly, charged lipids within the bilayer may electrostatically attract (or repel) substances so that the latter are held in close proximity to (or repelled from) the bilayer during formation of the liposomes, and thus increase (or decrease) the level of entrapment. The retention of charged substances can be improved by using a lipid of similar charge, since the interaction of the entrapped substance with the bilayers can be reduced.

6.1.1.2. The permeability of liposomes

The lipid composition of liposomes is largely responsible for their permeability properties. However, certain features of entrapped substances will also determine their rate of escape from liposomes. It is reasonable to expect that large molecules are much more efficiently retained than small ones. Indeed proteins and other macromolecules remain sequestered within liposomes after entrapment, providing the liposomes remain stable (Sessa and Weissmann, 1970 . See Tyrrell et al., 1976b and Finkelstein and Weissmann, 1978, for lists of macromolecules reportedly entrapped within liposomes). Liposomes are more permeable to inorganic anions (Bangham et al., 1965) than to cations (see Papahadjopoulos and

Kimèlberg, 1973), although negatively charged liposomes are the more permeable to cations (Scarpa and de Gier, 1971). Several small molecular weight molecules have been entrapped within liposomes, including glucose (Sessa and Weismann, 1970; Inoue, 1974; Batzri and Korn, 1975) and EDTA (Jonah et al., 1975). Inoue (1974) found that glucose was entrapped most efficiently by liposomes of DPPC. A similar study by de Gier et al. (1968) involving glycerol and erythritol has already been referred to in Section 6.1.1.1. Naoi et al. (1977) have shown that amino acids can permeate liposome membranes and observed that neutral amino acids were more permeant. The basic amino acids permeated slowest. The retention of the lipophilic substance cortisol was much improved by covalent attachment of the molecule to fatty acids (Shaw et al., 1976), suggesting that the acyl chain "anchored" the steroid to the bilayers. Amphiphilic substances are the most difficult to entrap successfully (Tyrrell et al., 1976b) since they have the ability to "hop" through the bilayers of the liposomes and escape. Some degree of success has been achieved in the entrapment of 5-fluorouracil (Tyrrell et al., 1976b) and actinomycin D and benzyl penicillin (Gregoriadis, 1973) using liposomes of DPPC.

6.1.1.3. The effect of temperature on the permeability of liposomes

In general, the permeability of liposomes is increased as the temperature is raised (de Gier et al., 1968). In particular, the permeability of some types of liposomes is increased markedly near the phase transition temperature of the components lipid(s) (Papahadjopoulos et al., 1973).

6.1.2 The preparation of liposomes

The lipids are first dispersed in an organic solvent. This mixture is then subjected to rotary evaporation to leave a thin film of lipid on the wall of a flask. An aqueous solution containing the substance to be entrapped is now used to wash the dried lipid film from the wall of the flask. During this procedure, the lipids swell to form liposomes.

Liposomes prepared in this way are very heterogeneous in size, ranging from 50-1000nm in diameter (Papahadjopoulos and Miller, 1967; Papahadjopoulos and Watkins, 1967), and are known as multilamellar vesicles (MLV).

The heterogeneous MLV described above can be subjected to ultrasonic irradiation to yield a more uniform population of small unilamellar vesicles or SUV (Huang, 1969). Sonication of liposomes can be carried out either in a bath-type (Papahadjopoulos and Miller, 1967; Papahadjopoulos and Watkins, 1967) or probe-type sonicator (Patel and Ryman, 1976; Patel et al., 1978) although the former may be more suitable where sterile preparations are required. SUV prepared in this way range in size from 50-100nm (Papahadjopoulos and Miller, 1967), but prolonged sonication (160min.) produces an even more uniform population of liposomes, diameter 25nm (Huang, 1969). Although such preparations are more homogeneous in size, substances are less efficiently entrapped in SUV because of the lower volume to surface area ratio.

Deamer and Bangham (1976) have described an alternative method of preparing liposomes. In this method, the lipids in solution in ether are injected into a warm aqueous solution. The method is claimed to be 10-100 times more efficient than other available methods and to be much quicker (Deamer, 1978). There is a range in the diameters of liposomes so produced from 50-250nm, although most of them are between 150 and 250nm (Deamer, 1978). Thus this method appears to be useful for preparing liposomes of intermediate size.

Large unilamellar vesicles (LUV) are prepared from SUV by the addition of Ca^{2+} ions. This causes fusion of the SUV into cochleate cylinders, and by the subsequent addition of EDTA, chelation of the Ca^{2+} ions leads to the formation of large unilamellar vesicles (Papahadjopoulos and Vail, 1978). In this way, phosphatidylserine has been used to prepare LUV with diameters between 200 and 1000nm (Papahadjopoulos and Vail, 1978), containing

entrapped ferritin (which was added immediately prior to EDTA). This method is claimed (Papahadjopoulos and Vail, 1978) to be more efficient than the original (MLV) method. Another advantage of this method is that the cochleate structures are extremely stable for long periods of time (Papahadjopoulos and Vail, 1978), and can thus be stored until required, when the substance to be entrapped, and then EDTA, is added to form LUV.

In all the methods outlined above, the final product is a preparation of liposomes suspended in a matrix which is identical to their interior. The purification of liposomes therefore involves the removal and replacement of this external matrix. Three methods are generally available for this separation stage.

Dialysis has a limited usefulness, since it can only be applied when the entrapped material is of low molecular weight. It is also the slowest of the three techniques.

Gel-filtration has been employed by several groups (Weissmann et al., 1975; Dapergolas and Gregoriadis, 1976; Sharma et al., 1977) to segregate liposomes from unentrapped material. The method is extremely effective for this separation task, although yield of purified liposomes may be lowered by adsorption of lipid to the gel.

Centrifugation has been used as a method for the purification of liposomes, but has the disadvantage that there is no clear separation of the liposomes from unentrapped solution. Some workers have used centrifugation merely as a means of separating liposomes from unentrapped material, whereas others have chosen centrifugation conditions that lead to the isolation of a selected portion of the total population of liposomes (see Tyrrell et al., 1976b, Table 1). The main advantage of this technique is its suitability for the preparation of sterile liposomes.

Additionally, it should be mentioned that Batzri and Korn (1975) carried out a preliminary concentration of liposomes using ultrafiltration

prior to purification using gel-filtration. Although apparently effective, this technique does not seem to have been used widely by other workers.

6.1.3 The interaction of liposomes with cells

This Section is concerned with the mode of interaction of liposomes with plasma membranes *in vitro*. Liposomes may interact with cell surfaces in several ways, some depicted in Figure 6.1. These are:

6.1.3.1 Endocytosis (Figure 6.1.i).

If liposomes enter cells by either fluid-phase or adsorptive endocytosis, the result is the intact transfer of all the liposomal material to the lysosomal system. Once liposomes are contained within secondary lysosomes they become subject to hydrolytic attack by the lysosomal enzymes.

The eventual fate of the components of endocytosed liposomes will depend on their susceptibility to degradation within the lysosomes and their ability to permeate the lysosomal membrane. One can envisage that "lysosomal enzyme-resistant" liposomes (and their contents) would remain intact within the secondary lysosomes and follow the fate of the lysosomes themselves. On the other hand, digestible lipids would release the contents of the liposomes into free solution so that they too may be broken down. If the liposomal contents are or become able to permeate the membrane of the lysosome, they will escape into the cytosol, but large, indigestible substances will remain trapped within the secondary lysosomes.

6.1.3.2 Fusion (Figure 6.1.ii and iii).

The mechanics of the interaction of both unilamellar and multilamellar liposomes with cells by fusion is similar, although the final result is different. The outer lipid bilayer fuses with the cell membrane, resulting in the incorporation of the outer bilayer into the plasma membrane, and the introduction of the contents of the liposome into the cytosol. In the case of unilamellar liposomes, all of the lipid becomes incorporated into the plasma membrane and the contents enters the

Figure 6.1 Cellular uptake of liposomes by phagocytosis and fusion

(i) Entry by phagocytosis

The fate of SUV and MLV is the same, i.e. the liposomes and contents are taken up into phagosomes, and are eventually localized within secondary lysosomes.

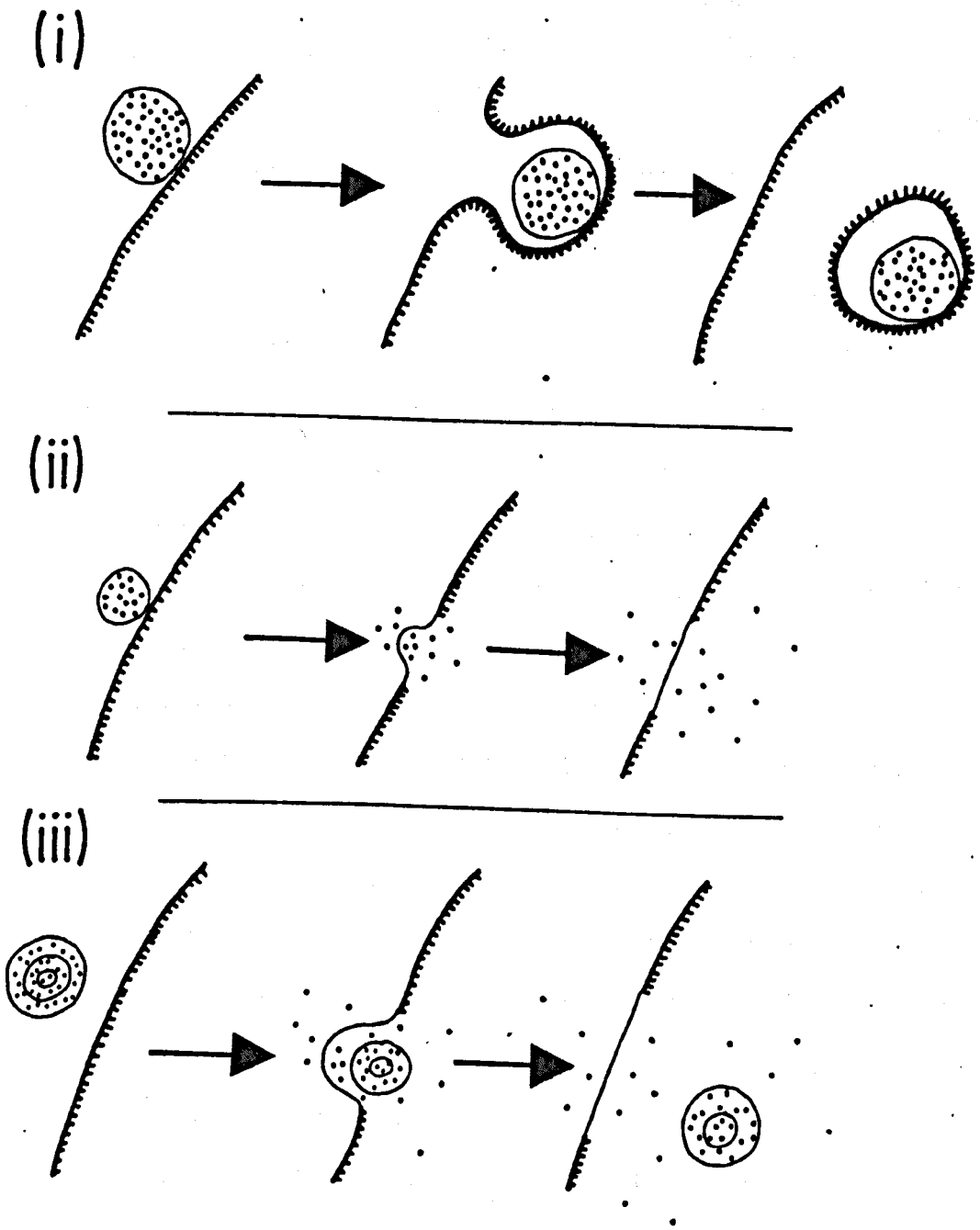
(ii) Entry by fusion with the plasma membrane
and
(iii) The interaction is similar for both SUV and MLV, but whereas the SUV bilayer is totally incorporated in the plasma membrane, only the outermost MLV bilayer is removed by the fusion process. The liposome contents enter the cytosol.

KEY:

Entrapped substance

Liposome bilayer

Plasma membrane


(i)
(ii)
(iii)

cytoplasm. On the other hand, only the outer lipid bilayer of multilamellar liposomes is incorporated into the plasma membrane, and a liposome comprising one less lamella enters the cytoplasm together with the material originally sequestered immediately within the outermost lamella. It has been postulated that such a process would cause the plasma membrane momentarily to become "leaky" (Batzri and Korn, 1975), and would lead to release of entrapped substances into the medium.

6.1.3.3. Adsorption.

The adsorption of liposomes to the surface of a cell may not be followed by any interiorization of extracellular material so that, although the liposomes and their contents are associated with the cell, they are not within the cell itself. This adsorption may be accompanied by changes in the cell membrane or the liposomes, which may allow some transfer of liposomal lipids to other areas, e.g. the plasma membrane or the cytosol.

6.1.3.4. Exchange of membrane components

Some components of the liposome lipid bilayer may exchange with parts of the plasma membrane, without there being any long-term interaction or adsorption between the two.

Although four distinct modes of interaction between liposomes and cells have been described above, there is no reason why more than one type of interaction may not occur at one cell surface at any one time. This makes it very difficult to know exactly what mechanisms are operating and conclusions drawn from results obtained with one cell type involving liposomes of a given composition may not be applicable in other instances.

6.1.4 Experimental evidence of the interaction of liposomes with cells

Throughout this Section the word uptake is used to describe the co-recovery of liposomes with cells, and does not necessarily indicate any internalization of liposomal material.

6.1.4.1. The effects of metabolic inhibitors and cytochalasin B.

The uptake of mammalian cells of "solid" (i.e. gel state) or uncharged or phospholipid vesicles is inhibited by cytochalasin B and metabolic inhibitors (Poste and Papahadjopoulos, 1976), both inhibitors of phagocytosis. The uptake of charged "fluid" vesicles is inhibited only slightly by metabolic inhibitors (Stendahl and Tagesson, 1977; Poste and Papahadjopoulos, 1976) and cytochalasin B (Poste and Papahadjopoulos, 1976; Weissmann et al., 1977). Poste and Papahadjopoulos (1976) reported that SUV and MLV having the same lipid composition behaved similarly in their experiments, suggesting that the type of lipid was more important than the size of the vesicles in determining the mode of interaction with cells.

Cyclic AMP had no effect on the growth of 3T3 cells when administered in free solution or contained within "solid" SUV (Papahadjopoulos et al., 1974) but, when given in "fluid" liposomes, cyclic AMP caused a 75% inhibition of cell growth (Papahadjopoulos et al., 1974). Presumably the cyclic AMP was entering the cytosol following fusion of the liposomes with the plasma membrane. Pagano et al. (1974) claimed that fusion is the major route of entry of DOPC (fluid) liposomes into Chinese hamster V79 fibroblasts, and subsequently stated (Pagano and Huang, 1975) that at 37°C about 90% of the phospholipid uptake could be accounted for by the fusion of DOPC liposomes with cells, the rest being due to lipid exchange.

The above results all suggest that, whereas "fluid" liposomes enter cells mainly by fusing with the cell membrane, "solid" vesicles enter cells by endocytosis.

In contrast, Batzri and Korn (1975), after following the fate of markers for both the aqueous and lipid parts of the liposomes, concluded that Amoeba castellanii internalizes "solid" vesicles by a fusion process and "fluid" vesicles by endocytosis. However, these authors used only 2,4-dinitrophenol as a metabolic inhibitor, and did not make use of cytochalasin B in their attempts to elucidate the possible mechanisms of

uptake.

Pagano et al. (1978) have shown that azide and deoxyglucose cause only slight inhibition of the uptake of egg PC, DMPC or DOPC (fluid) liposomes by fibroblasts and thymocytes. There was no inhibition of DPPC (solid) liposome uptake. Cytochalasin B slightly inhibited the uptake of egg PC liposomes by fibroblasts but there was no inhibition in thymocytes. Unfortunately the usefulness of this study is limited since, although a difference was found between the mode of uptake of liposomes in the two cell types, the use of cytochalasin B was restricted to the egg PC liposomes.

6.1.4.2. The effect of temperature

Two factors may alter the interaction of liposomes with cells at low temperatures. Firstly, the liposome lipids may undergo a phase transition, changing from the liquid-crystalline ("fluid") to the gel ("solid") state, and a similar transition may occur in the plasma membrane. Secondly, low temperatures may affect the metabolism of the cell, so that energy-dependent processes may be halted or seriously curtailed.

At low temperatures the uptake of charged "fluid" vesicles was only partially inhibited (Stendahl and Tagesson, 1977; Poste and Papahadjopoulos, 1976) whereas that of uncharged or "solid" vesicles is almost abolished under similar conditions (Poste and Papahadjopoulos, 1976). Studies by Batzri and Korn (1975), however, produced results indicating that the intake of "solid" DPPC liposomes by cells was only partially inhibited by low temperatures, whether SUV or MLV, although Poste and Papahadjopoulos (1978) comment that the prolonged sonication given to these vesicles may have altered their structure so that they were less stable.

It has been clearly shown (Poste and Papahadjopoulos, 1976) that the shape of the graph relating uptake to temperature depends on the composition of the liposomes, and not on whether they are MLV or SUV. The uptake of both PC/DSPC/DPPC and PC ("solid" and uncharged respectively)

vesicles increases linearly with temperature, but PS/PC ("fluid") liposomes show a distinct transition in uptake around 15°C. Poste and Papahadjopoulos (1978) argue that a continuous curve of uptake with temperature is indicative of endocytosis while the curves containing a clear transition point show entry of liposomes by a fusion mechanism. However, results obtained at Keele indicate that the uptake of ^{125}I-labelled PVP by both rat yolk sac (Duncan and Lloyd, 1978) and peritoneal macrophages (Pratten and Lloyd, 1979) is almost totally inhibited below 15-20°C. It appears that there is not only dispute over the mode of entry of the various types of liposome at low temperatures, but also over the effect of these low temperatures upon the membrane phenomena themselves.

Since both endocytosis and liposome-plasma membrane fusion are diminished at low temperatures, surface adsorption of liposomes (without any internalization) and lipid exchange between the vesicles and plasma membrane are likely to be responsible for a large part of the total lipid "uptake" (i.e. co-recovery) by cells at low temperature. Pagano and Huang (1975) found that at 2°C there was more rapid uptake by V79 cells of [^{3}H]DOPC than of [^{14}C]cholesterol when both were included in the lipid phase of liposomes. In the same paper they also showed that V79 cells accumulated [^{14}C] DOPC without the uptake of entrapped [^{3}H]insulin. Such disparate uptake of hydrophilic and lipophilic substances from the same liposomes points to the occurrence of lipid exchange between the liposomes and the plasma membrane. However, it is more difficult to prove that co-recovery of all components with cells at low temperatures is due to solely surface adsorption, even though there is presumably little endocytosis or liposome-membrane fusion under these conditions.

6.1.4.3. Electron microscopic evidence

Both Magee et al. (1974) and Batzri and Korn (1975) have produced electron micrographs of objects resembling liposomes fusing with plasma membranes but, since electron micrographs present only a static picture,

it is difficult to be sure that this is in fact what is depicted. Following the incubation of liposomes containing horseradish peroxidase or radiolabelled substances with cells, micrographs showing stained material in the cytoplasm (Magee et al., 1974; Weissmann et al., 1977) or within secondary lysosomes (Magee et al., 1974; Batzri and Korn, 1975) have been obtained, which offer evidence of the events leading up to the time depicted, deduced from the location of the materials within the cells. In none of these papers is the micrographic evidence quoted alone, suggesting that this technique is of only secondary importance, having only a supportive role.

6.1.4.4. Summary

Liposomes have been widely used in recent years to investigate many membrane phenomena and their interactions with cells have been extensively studied. Several reviews have recently appeared (Tyrell et al., 1976b; Finkelstein and Weissmann, 1978, and also a collection edited by Papahadjopoulos, 1978) which give a fuller picture of the properties and potential uses of liposomes.

The study of the interaction of liposomes with cells is now an area of intense activity, but several questions remain unanswered. However, there are indications that at normal temperatures, liposomes are taken up into cells by endocytosis or by fusion with the plasma membrane. At temperatures just above $0^{\circ}C$ this uptake becomes almost negligible, and the main interactions between liposomes and cells take the form of surface adsorption or lipid exchange, without actual internalization of liposomes. It seems probable that charged "solid" and also neutral liposomes enter cells mainly by endocytosis, whereas charged "fluid" liposomes are capable of fusion with the plasma membrane (Papahadjopoulos et al., 1975, Poste and Papahadjopoulos, 1978).

6.2 The preparation of liposomes used in this work

6.2.1 Theoretical requirements of the liposomes

The eventual aim of the work described in this Thesis was to use liposomes to investigate aspects of lysosome function, and so at the outset it was desirable to choose to make liposomes with properties which would favour their interaction with the lysosomal system. This is probably best achievable by using liposomes which are taken up by endocytosis, so that the complete liposome is presented to the lysosomes. The second factor that may influence the design of these liposomes is their ability to hold low molecular weight solutes.

Although it is difficult to prove that liposomes enter cells solely by one route e.g. endocytosis, it does seem likely that charged "solid" liposomes enter cells mainly by an endocytic mechanism (Papahadjopoulos et al., 1975, Poste and Papahadjopoulos, 1978). From the point of view of minimizing permeability of the bilayers, it would seem that an ordered structure is desirable (de Gier et al., 1968; Shaw et al., 1976). This may be easily and consistently attained by the use of defined lipids that are below their thermal phase transition temperature (Shaw et al., 1976; Tyrrell et al., 1976b). It was therefore decided to prepare liposomes using synthetic dipalmitoylphosphatidylcholine (DPPC) with small amounts of dipalmitoylphosphatidic acid (DPPA) to give a negative charge to the bilayers.

Since the study was primarily concerned to investigate the release of substances from lysosomes, it was felt that there was little justification for an intense survey of the interactions of various types of DPPC liposomes with cells. Therefore, large multilamellar vesicles were prepared, so that the population of liposomes used in experiments was of the widest size range. In this way, there would be a fair chance that some of the liposomes would be a suitable size for endocytosis.

6.2.2 Selection of substances for entrapment within liposomes

It was desired to observe the release of cystine from lysosomes, following their entrapment within liposomes and administration to rats. However, because of the insolubility and difficulty of detection of cystine, it was necessary to use ^{125}I-labelled PVP for the first experiments, in which the method of preparing liposomes was established and developed, and the experimental procedures devised. There were several reasons for this choice. First , ^{125}I-labelled PVP is a macromolecule, which makes it unlikely that it will be able to escape from the liposomes once entrapped. Secondly, the molecule is easy to detect by virtue of the gamma emission of ^{125}I-iodide. Thirdly, ^{125}I-labelled PVP is not broken down by cells so that one may be sure that PVP is being monitored, and not its breakdown products. Finally, the molecule is water soluble, which further aids the efficiency of its entrapment. Thus ^{125}I-labelled PVP may be useful in determining the validity of the experimental systems and provide a control against which the behaviour of other entrapped substances may be compared.

The second substance chosen for study was[U-^{14}C]sucrose, which was selected as a small (molecular weight 342) substance which, according to several reports (Cohn and Enrenreich, 1979; Lloyd, 1969; Lee, 1970) could not permeate the lysosomal membrane. Sucrose is much smaller than PVP and is, by comparison, very similar in size to cystine (molecular weight 240). Studies with the sucrose would thus provide some evidence of the feasibility of continuing the study to include cystine.

Finally, [^{35}S]cystine itself was used to investigate the feasibility of this system for following the escape of disulphides from lysosomes.

6.2.3 Preparation of liposomes containing ^{125}I-labelled PVP

Multilamellar liposomes were prepared using the method of Shaw *et al.* (1976) with minor modifications. DPPC (9mg) and DPPA (1mg) were suspended in 5ml chloroform and placed in a "Quickfit" pear shaped 100ml

flask under nitrogen. The suspension was rotary-evaporated in vacuo at 61°C (20°C above the phase transition temperature of DPPC) to yield a thin film of lipid on the wall of the flask.

The lipid film was removed, and the liposomes formed, by adding 5mM phosphate-buffered saline, pH7.4 (see Section 2.2), and agitating on a vortex mixer. The phosphate-buffered saline contained the ^{125}I-labelled PVP to be entrapped. Both the volume of saline and the concentration of radiolabel were varied in the first series of experiments, in order to observe any possible alterations in the recovery of liposomes. This suspension was then left for 1h at 21°C.

The purification of the liposomes was achieved using three centrifugation steps. Following each centrifugation (50000g for 10min in an MSE Superspeed 50 centrifuge at 21°C, rotor no. 59113) the unentrapped ^{125}I-labelled PVP was decanted, and the pellet containing the liposomes was resuspended in 2ml of the saline. The supernatant from the first centrifugation was recycled for use in later experiments.

6.2.3.1. Estimation of the degree of entrapment of ^{125}I-labelled PVP

The percentage of initial entrapment was estimated by comparing the amount of radiolabel recovered in the final washed liposome preparation with the total radiolabel contained in the phosphate-buffered saline added to the lipid film in the flask. The radiolabel in the liposome preparations was measured by taking small (10-20µl) duplicate samples into disposable plastic tubes. The samples were then diluted to 1ml with the saline. Similar samples were taken from the first supernatant to estimate the total radioactivity present, and both were counted as described in Section 2.6.1. The radioactivity in the total volumes of both the initial saline solution and the final suspension of liposomes was then calculated, and the ^{125}I-labelled PVP entrapped initially expressed as a percentage. The initial entrapment was also expressed in terms of the amount of aqueous phase entrapped per unit amount of lipid

i.e. as µl/µMole phospholipid (in these experiments there were 1.34µMole/mg lipid). This unit is a more useful one for the purpose of comparing results using different materials (Tyrrell et al., 1976b), but does not, of course, take into account any alterations in the concentration of the PVP which may occur during the preparation e.g. by adsorption of PVP to the lipids.

6.2.3.2. The entrapment of ^{125}I-labelled PVP within liposomes of DPPC

Table 6.1 shows the results of the series of experiments in which the concentration of ^{125}I-labelled PVP and the total volume of phosphate-buffered saline added to the lipid film were both varied. The Table shows two important trends. Firstly, moving down the columns, it can be seen that the percentage entrapment drops by approx. half each line, with the result that the entrapment expressed in µl/µMole remains almost constant in each column. These figures imply that a similar population of liposomes is produced which ever volume of the saline is used. The smaller results (µl/µMole) obtained when 0.5ml saline was used point to the practical difficulties of using such small volumes.

Secondly, moving across the Table, the entrapment decreases from left to right, i.e. with increasing concentration of ^{125}I-labelled PVP. This is difficult to account for, although it may be similar to the effect that Tyrrell and Ryman (1976) commented on when experimenting with resealed erythrocyte ghosts. Sessa and Weissmann (1970) reported that the inter-lamellar distance of their liposomes was 5-12nm, which should be compared with the sp^3 hybridized carbon-carbon bond length (of ethane) which is 0.15nm (Maham, 1971). Thus, a linear ^{125}I-labelled PVP molecule of 40 monomers would just bridge the interlamellar space. Such a molecule would have a molecular weight of around 4000, below the specified average molecular weight range of 30000-40000. Clearly, there are many assumptions in this calculation, but they illustrate the large size of the molecules of PVP in comparison to the interlamellar distance, so that some form of

Table 6.1 Initial entrapment of ^{125}I-labelled PVP by multilamellar DPPC/DPPA liposomes

The liposomes were prepared from 10mg lipid as described in the text, using the volume of saline indicated to wash the lipid film from the wall of the flask. The concentration of ^{125}I-labelled PVP in the saline is indicated. Entrapment is calculated both as a percentage of the total radiolabel and as µl aqueous phase/ µMole phospholipid. Each result is the mean $\pm$ S.E.M. of the number of experiments (shown in parentheses).

Volume phosphate-buffered saline (ml)	Concentration of ^{125}I-labelled PVP (µg/ml) in the phosphate buffered saline					
	250		500		1000	
	Entrapment		Entrapment		Entrapment	
	%	µl/µMole	%	µl/µMole	%	µl/µMole
0.5	4.82 ± 0.48 (3)	1.76 ± 0.18 (3)	3.73 ± 0.31 (3)	1.36 ± 0.19 (3)	2.80 ± 0.26 (4)	1.02 ± 0.10 (4)
1.0	2.65 ± 0.19 (3)	1.93 ± 0.14 (3)	2.09 ± 0.35 (4)	1.53 ± 0.25 (4)	-	-
2.0	1.32 ± 0.11 (4)	1.92 ± 0.17 (4)	1.05 ± 0.19 (3)	1.53 ± 0.26 (3)	-	-

steric hindrance may occur at high concentrations of PVP. It is interesting that Wacker et al. (1976) calculated the SUV they prepared contained only 1-3 aminopeptidase dimers per liposome.

In choosing the concentrations and volumes to be employed in subsequent experiments, it is important to avoid unnecessary wastage of materials. Since the liposomes contained similar amounts of radiolabel when the lipid film was resuspended in either 1 or 2ml of the saline, it was decided to use 1ml in future experiments. It was also decided to use the ^{125}I-labelled PVP at a concentration of 500µg/ml, as this was found more suitable for the purposes for which the liposomes were subsequently needed. The preparation of liposomes, including the final volumes and concentrations used, is described in Section 2.2.

6.2.3.3. The temperature stability of liposomes containing ^{125}I-labelled PVP

The retention of radiolabel by liposomes was studied over a range of temperatures in order to ascertain their suitability for administration to animals. Liposomes containing ^{125}I-labelled PVP, prepared as described in Section 2.2 from 40mg lipids, were diluted to 4ml with phosphate-buffered saline, pH 7.4, and divided into 4 x 1ml aliquots. To each of these were added 14ml of the saline, and the suspensions were then incubated at different temperatures. At intervals, a 2ml sample was taken from each suspension and centrifuged at 50000g for 10min (rotor no. 59113) in an MSE Superspeed 50 at 21°C. Two 0.5ml samples of the supernatant were then removed and placed in disposable plastic tubes. Saline (0.5ml) was added, and the ^{125}I-radioactivity counted as in Section 2.6.1. This value was expressed as a percentage of the total radioactivity in the suspension, and was taken to represent the amount of radiolabel that had escaped from the liposomes.

Figure 6.2 shows that DPPC/DPPA liposomes incubated at 45°C rapidly

Figure 6.2 Release of entrapped ^{125}I-labelled PVP from multilamellar DPPC/DPPA liposomes at different temperatures

The freshly prepared liposomes were diluted with saline, divided into four aliquots, and each incubated at a different temperature. At various times, the percentage non-sedimentable radioactivity was measured as described in Section 6.2.3.3. Each point is the mean of two experiments.

KEY:

—●— : 4°C

--o-- : 25°C

—·▲·— : 37°C

·····Δ····· : 45°C

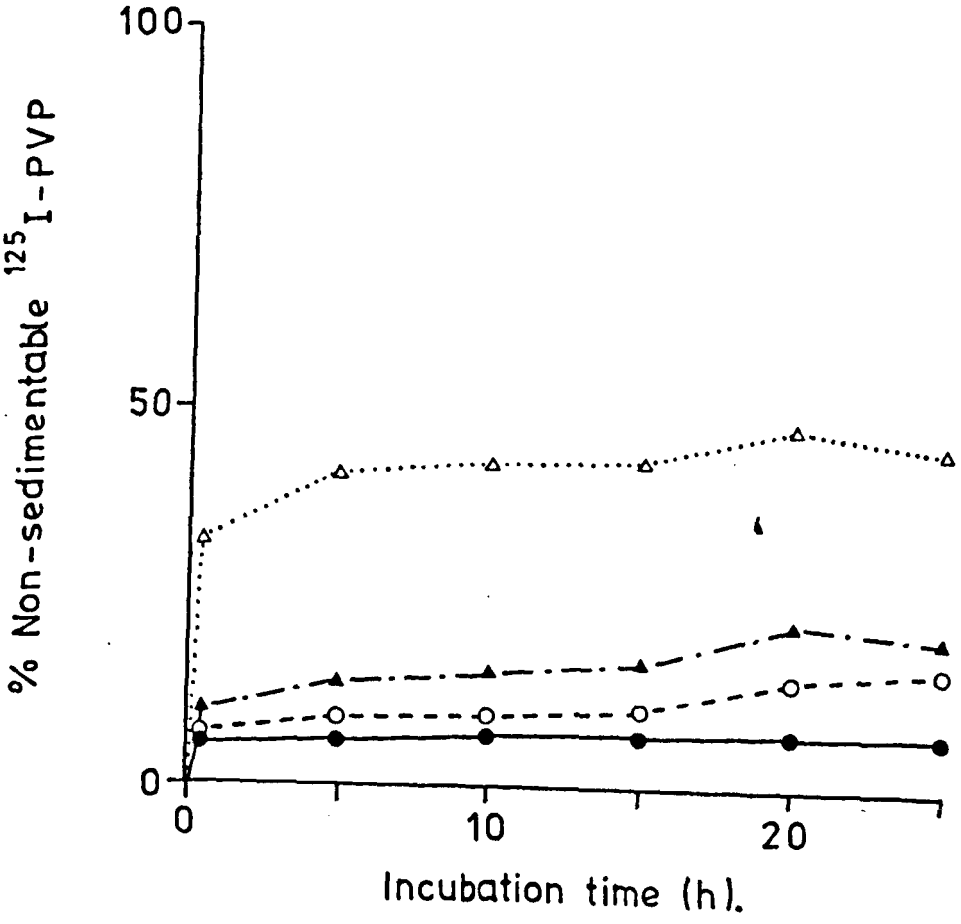

100
50
0
0
10
20
% Non-sedimentable ^{125}I-PVP
Incubation time (h).

Table 6.2 The effect of Triton X-100 on the release of ^{125}I-labelled PVP from multilamellar DPPC/DPPA liposomes

Two otherwise identical preparations were incubated at 37°C as described in Section 6.2.3.4. One suspension contained 0.1% Triton X-100. At 0 and 30min, the percentage non-sedimentable radioactivity was measured (see Section 6.2.3.3).

Results are mean ± S.E.M of a number of experiments (shown in parentheses)

Incubation time (min) at 37°C	Percentage of non-sedimentable ^{125}I-labelled PVP	
	Control	+ 0.1% Triton X-100
0	7.9 ± 2.1 (6)	81.7 ± 6.7 (6)
30	9.9 ± 0.5 (3)	104.5 ± 1.9 (3)

lose nearly half of the ^{125}I-labelled PVP initially entrapped within them. In contrast, those liposomes incubated below the transition temperature of the lipid (41°C) retained at least 75% of the initially entrapped radiolabel. Since most of the loss of entrapped material occurred within the first 5h of incubation, future experiments of this type were limited to 5h duration.

6.2.3.4. The effect of Triton X-100 on the release of ^{125}I-labelled PVP from liposomes

The effect of 0.1% v/v Triton X-100 upon the entrapment of radiolabel was investigated by preparing two identical diluted suspensions of liposomes similar to those of Section 6.2.3.3. One suspension, however, contained 0.1% Triton X-100. Immediately, and also after 30min incubation at 37°C, a 2ml sample was taken from each suspension, and the percentage of non-sedimentable radiolabel estimated, as in 6.2.3.3. The results are shown in Table 6.2. The addition of Triton X-100 to the liposome suspensions causes the release of the entrapped radiolabel, implying that the radiolabel is held in a lipid structure of some kind.

6.2.3.5. The entrapment of ^{125}I-labelled PVP within positively charged liposomes

Positively charged liposomes were prepared by the replacement of DPPA by stearylamine (octadecylamine) in the description in Section 2.2., thus altering the concentration of lipid to 1.60μMole/mg lipid. The initial entrapment of ^{125}I-labelled PVP within liposomes of this type is shown in Table 6.3 and shows that there is little initial difference between liposomes made from either DPPC/DPPA or DPPC/stearylamine in this respect.

6.2.3.6. The temperature stability of positively charged liposomes containing ^{125}I-labelled PVP

The stability of these liposomes, measured as in Section 6.2.3.3, is shown in Figure 6.3, which shows the percentage non-sedimentable radio-

Table 6.3 Initial entrapment of ^{125}I-labelled PVP by liposomes of different composition

In the preparation of positively charged liposomes, DPPA was replaced by an equal amount (mg) of stearylamine, and the liposomes so prepared were examined for their initial entrapment of radiolabel as described in Section 6.2.3.1. Results are mean $\pm$ S.E.M. of a number of experiments (shown in parentheses).

Composition of liposomes	Initial entrapment of ^{125}I-labelled PVP in liposomes prepared from:			
	20mg lipid		40mg lipid	
	%	µl/µMole	%	µl/µMole
DPPC/DPPA (9:1) (-ve charge)	1.97 ± 0.09 (19)	1.44 ± 0.07 (19)	2.00 ± 0.25 (8)	1.46 ± 0.18 (8)
DPPC/stearylamine (9:1) (+ve charge)	2.20 ± 0.29 (3)	1.38 ± 0.18 (3)	2.47 ± 0.28 (6)	1.54 ± 0.18 (6)

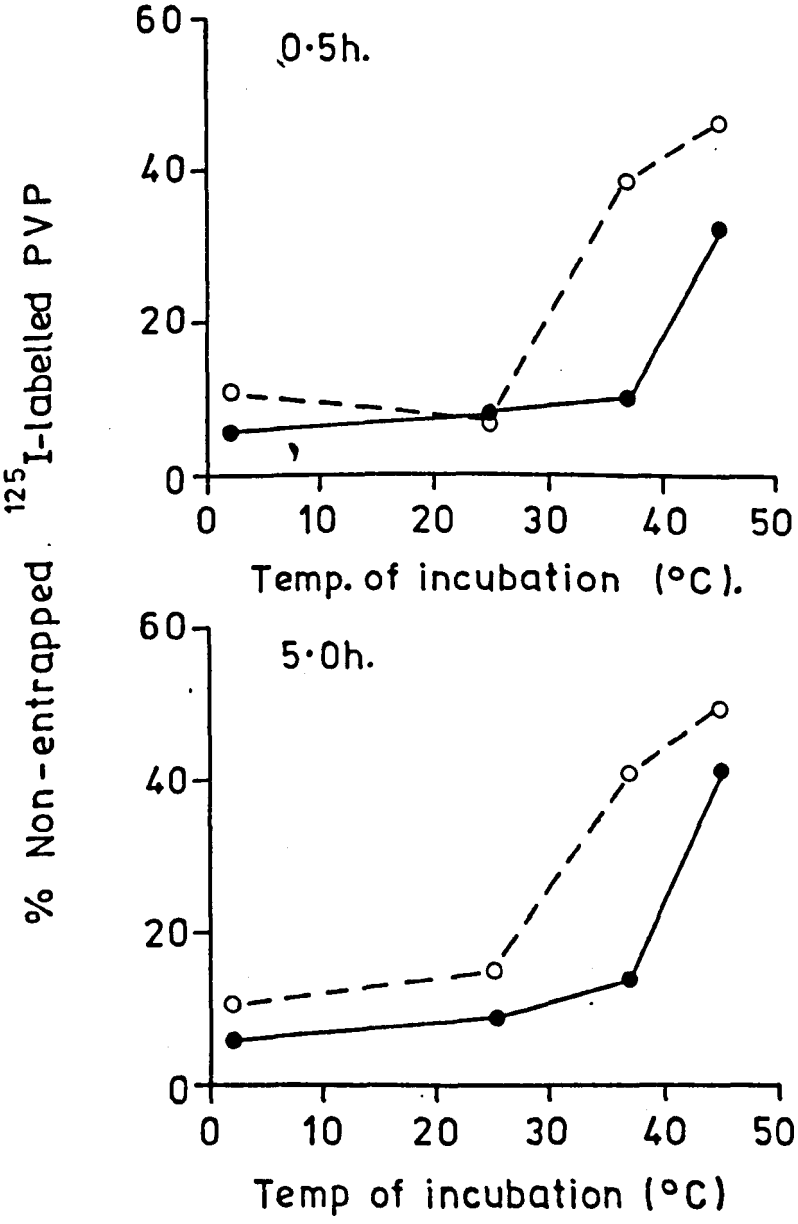
Figure 6.3 Comparison of the ability of DPPC/DPPA (negatively charged) and DPPC/stearylamine (positively charged) liposomes to retain ^{125}I-labelled PVP at different temperatures

A freshly prepared suspension of multilamellar DPPC/stearylamine liposomes was diluted with saline, divided into four aliquots, and each incubated at a different temperature. At 0.5 and 5.0h the percentage non-sedimentable radioactivity was measured as described in Section 6.2.3.3. The data for DPPC/DPPA liposomes is calculated from Figure 6.2. Each point is the mean of two experiments.

KEY:

—●— : DPPC/DPPA liposomes

--o--: DPPC/stearylamine liposomes


0·5h.
5·0h.
% Non-entrapped ^{125}I-labelled PVP
Temp. of incubation (°C).
Temp of incubation (°C)
0
20
40
60
0
10
20
30
40
50

activity released at different temperatures after 0.5h and 5.0h incubations. Clearly, the positively charged liposomes release more entrapped ^{125}I-labelled PVP, particularly at 37°C, than do negatively charged liposomes. Whereas the DPPC/DPPA liposomes remain stable up to above 37°C, the DPPC/stearylamine liposomes lose their stability below 37°C. It is likely that the highly ordered structure of the bilayers of the DPPC/DPPA liposomes enabled the crystalline state to be maintained until very near the normal transition temperature of DPPC, i.e. 41°C. On the other hand stearylamine might well have disrupted the bilayer sufficiently to cause the phase transition temperature to drop below 37°C, causing the loss of stability which was observed.

6.2.4 The entrapment of [U-^{14}C]sucrose and [^{35}S]cystine (as dihydrochloride) within liposomes, and the temperature stability of these liposomes

The method used to entrap [U-^{14}C]sucrose and [^{35}S]cystine within liposomes was similar to that already described for the entrapment of ^{125}I-labelled PVP.

Liposomes containing [U-^{14}C]sucrose were prepared from DPPC/DPPA as described in Section 2.2, but with the sucrose at a concentration of 500µg/ml replacing ^{125}I-labelled PVP. The estimation of entrapment and stability were measured as for liposomes containing ^{125}I-labelled PVP, except that the duplicate 1ml samples were taken into glass phials for liquid scintillation counting, as described in Section 2.6.2.

[^{35}S]Cystine was received as the dihydrochloride, in 0.1M HCl and initially liposomes were prepared using this solution diluted to 150µg/ml with 0.1M HCl in place of the phosphate-buffered saline. In the subsequent washing stages, however, the saline was used. Such a procedure might result in liposomes containing cystine in an acid solution suspended in a medium of saline at pH7.4.

Table 6.4 Initial entrapment of [U-^{14}C] sucrose and [^{35}S] cystine within multilamellar DPPC/DPPA liposomes

Liposomes (prepared from 30mg lipid) were formed by washing the film from the flask walls with 3.0ml of the solutions indicated. The initial level of entrapment was estimated as described in Section 6.2.3.1.

Results are mean $\pm$ S.E.M. of several experiments (number shown in parentheses).

Radiolabel entrapped	Initial incorporation of radiolabel in liposomes	
	%	µl/µ Mole
^{125}I-labelled PVP, 500µg/ml in phosphate-buffered saline, pH7.4	3.04 ± 0.28 (4)	2.22 ± 0.20 (4)
[U-^{14}C] sucrose, 500µg/ml in phosphate-buffered saline, pH7.4	3.94 ± 0.27 (6)	2.88 ± 0.20 (6)
[^{35}S] cystine diHCl, 150µg/ml in 0.1M HCl	1.80 ± 0.23 (3)	1.31 ± 0.17 (3)
[^{35}S] cystine, 100µg/ml in 67mM NaCl	4.62 ± 0.46 (3)	3.37 ± 0.34 (3)

Subsequently, experiments were performed using [^{35}S] cystine dihydrochloride which was diluted with NaOH to yield a solution of 100µg/ml [^{35}S] cystine in 67mM NaCl. In theory such a solution of cystine would be soluble, being below the maximum solubility. The difficulty is that such a concentrated saline may cause an osmotic instability in the liposomes, but to dilute the radiolabel further would make it very difficult to detect in some experiments.

The initial level of entrapment of these substances is shown in Table 6.4. The percentage entrapment of sucrose and cystine was a little higher than that of PVP except for that of cystine dihydrochloride in 0.1M HCl. The results are all in the range 1.3 - 3.4µl/µMole, values broadly in agreement with other work, e.g. 2.5 - 3.7µl/µMole using amyloglucosidase (Gregoriadis et al., 1971), 3.8µl/µMole using insulin (Patel and Ryman, 1976), 2.5 - 3.8µl/µMole using glucose and CrO_4^{2-} (Weissman et al., 1975), and with the average value of 1.8µl/µMole quoted by Finkelstein and Weissmann (1978).

The stability of these liposomes was measured as described in Section 6.2.3.3, the percentage non-sedimentable radiolabel being measured after 0.5 and 5.0h incubation at the various temperatures. The results are shown in Figures 6.4 ([U-^{14}C] sucrose) and 6.5 ([^{35}S] cystine dihydrochloride). These Figures show that in every instance the liposomes were stable at 4°C, i.e., there was no release of entrapped radioactivity after 5h incubation at this temperature. Similarly, the liposomes were fairly stable at 25°C, only those liposomes containing [^{35}S] cystine dihydrochloride in 0.1M HCl releasing more than 20% of the initially entrapped radioactivity. At 37°C, only [U-^{14}C] sucrose remained entrapped within negatively charged liposomes. At this temperature DPPC/DPPA liposomes could not retain [^{35}S] cystine dihydrochloride, (whether initially in 0.1M HCl or 67mM NaCl). At 45°C a maximum of approx. 60% of the radiolabel remained entrapped after 5h incubation, indicating that there was widespread disruption of the liposomes at this temperature.

Figure 6.4 Release of entrapped [U-^{14}C] sucrose from multilamellar DPPC/DPPA liposomes at different temperatures

The freshly prepared liposomes were diluted with saline, divided into four aliquots, and each incubated at a different temperature. At 0.5 and 5.0h the percentage non-sedimentable radioactivity was measured as described in Section 6.2.3.3. Each point is the mean of two experiments.

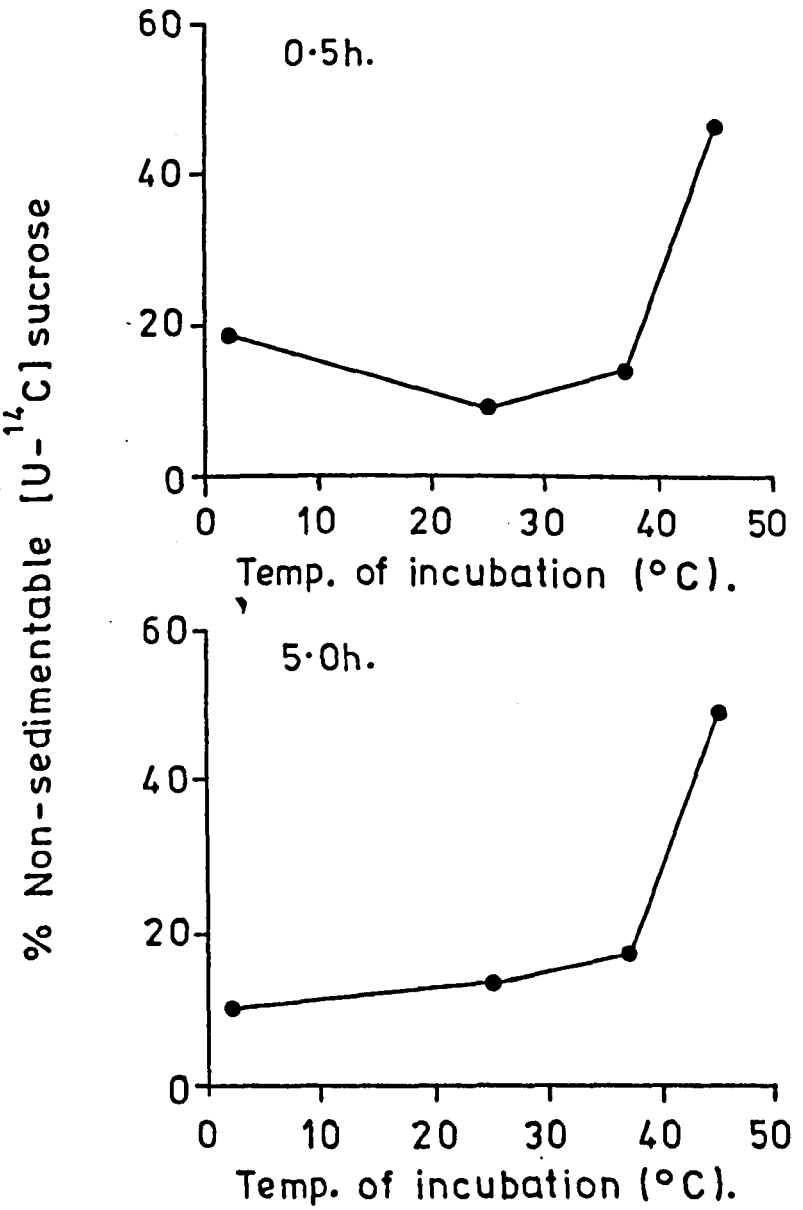

0·5h.
5·0h.
% Non-sedimentable [U-^{14}C] sucrose
Temp. of incubation (°C).
Temp. of incubation (°C).
0
20
40
60
0
10
20
30
40
50

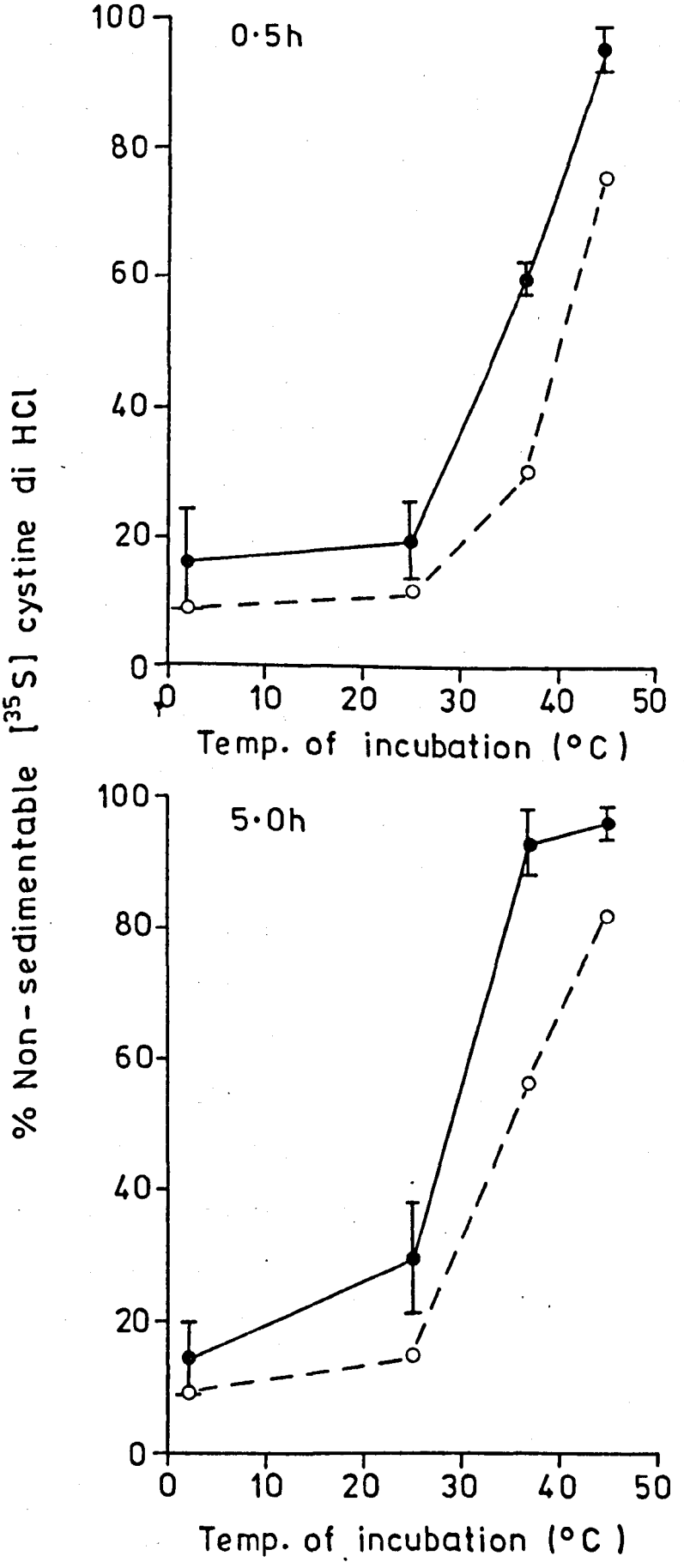
Figure 6.5 Release of entrapped [^{35}S]cystine from multilamellar DPPC/DPPA liposomes at different temperatures

The freshly prepared liposomes were diluted with saline, divided into four aliquots, and each incubated at a different temperature. At 0.5 and 5.0h the percentage non-sedimentable radioactivity was measured as described in Section 6.2.3.3.

KEY:

—●— : Liposomes prepared containing [^{35}S]cystine dihydrochloride (150µg/ml) in 0.1MHCl. (Mean ± S.E.M., 3 experiments)

--o-- : Liposomes prepared containing [^{35}S]cystine (100µg/ml) in 67mM NaCl (Mean of two experiments)


0·5h
5·0h
% Non-sedimentable [35S] cystine di HCl
Temp. of incubation (°C)
Temp. of incubation (°C)
0
20
40
60
80
100
0
10
20
30
40
50

6.3 Discussion

The experimental evidence given in this Chapter had a significant part to play in deciding the subsequent direction of the work.

The first series of experiments showed the feasibility of preparing liposomes from either a DPPC/DPPA or DPPC/stearylamine lipid film, and that ^{125}I-labelled PVP could be entrapped within such liposomes. These liposomes were disrupted by 0.1% Triton X-100. In addition, the physical limitations of the technique were established, e.g. the fall-off in the entrapment when volumes below 1.0ml were used to resuspend the lipid film.

The ability of these liposomes to retain entrapped ^{125}I-labelled PVP depends both on the charged lipid (DPPA or stearylamine) and the temperature. The negatively charged DPPC/DPPA liposomes were stable as judged by the non-sedimentable activity of ^{125}I-labelled PVP at temperatures up to and including 37°C, whereas the positively charged (DPPC/stearylamine) ones released much of the entrapped radiolabel at 37°C and above. This lowering of the thermal stability is probably the result of a perturbation of the lipid bilayers by stearylamine, which lowers the thermal phase transition temperature of the bilayer from around 41°C to below 37°C.

The later comparative study showed that [U-^{14}C] sucrose and [^{35}S] cystine dihydrochloride could also be entrapped within negatively charged DPPC/DPPA liposomes. The liposomes were less able to retain [^{35}S] cystine dihydrochloride, possibly because of the osmotic or pH imbalance between the interior of the liposomes and the external matrix. The thermal stability of the liposomes containing [U-^{14}C] sucrose was similar to that of similar liposomes containing ^{125}I-labelled PVP, suggesting that there was no alteration of the thermal phase transition temperature in sucrose-containing liposomes.

The levels of entrapment attained in all the experiments are of the same order as published data (see Section 6.2.4), though perhaps a little on the low side. A high level of entrapment (µl/µMole lipid) is due to

adsorption of material to the lipid as well as actual entrapment of material as solute in the aqueous phase. It is probable, therefore, that results which are slightly lower than reported values are indicative of entrapment of material solely as solute in the aqueous phase.

The stability of the DPPC/DPPA liposomes containing ^{125}I-labelled PVP or [U-^{14}C]sucrose at 37^{o}C means that there should be no thermal instability of these liposomes when administered to the living animal. There may, however, be release of radiolabel from liposomes containing [^{35}S]cystine dihydrochloride under similar conditions.

CHAPTER 7

The blood clearance and tissue distributions of non-entrapped and liposome-entrapped substances injected into rats

7.1 Introduction

Three conditions must be met if any uptake of liposomal material into cells is to occur *in vivo*. First, the liposomes must remain intact until they have been interiorized. Secondly, liposomes administered to animals must reach the vicinity of cells to be taken up by them; and finally, the liposomes must interact with the surface of the cells in some way, in order to initiate one of the various possible modes of uptake discussed in Section 6.1.3.

The stability of liposomes can be estimated *in vitro*, as has been done in Chapter 6, but such experiments can only be a guide to the stability of the liposomes *in vivo*. Papers have been published reporting the stability *in vivo* of liposomes administered by the intravenous (Gregoriadis and Ryman, 1971; Gregoriadis, 1975), intraperitoneal (Dapergolas *et al.*, 1976) and intragastric (Dapergolas *et al.*, 1976; Patel and Ryman, 1977) routes.

Although it is a fairly straightforward task to ascertain the stability of liposomes, it is more difficult to deduce from experimental evidence whether either of the two other conditions have been met. The main reason for this difficulty has been the lack of a technique sensitive enough to monitor the distribution of liposomes throughout the living animal. The use of ^{99m}Tc-labelled liposomes followed by external scanning using a γ-camera (Ryman *et al.*, 1978) is one technique that could provide information on where the liposomes travel before they become localised in particular tissues, so that the ability of tissues to take up liposomes can be seen in relation to the concentration of liposomes in the vicinity of the tissue. The majority of the literature, however, deals with the recovery of liposomal materials in tissues, which implies that all three conditions have been met. It cannot be inferred, however, that those tissues that do not take up liposomes have not had the opportunity to do s

Gregoriadis and coworkers have investigated the effect of the route of administration on the subsequent distribution of liposomes in the rat (Dapergolas et al., 1976; Gregoriadis et al., 1976). They found that intravenous administration of radiolabelled liposomes led to a much increased recovery of radiolabel in the liver, compared with uptake of unentrapped radiolabel. Entrapment in MLV resulted in maximal hepatic uptake, although uptake into tumour tissue was greatest with SUV. There was little hepatic uptake of intramuscularly administered radiolabel, whether within liposomes or not. A lowering of the blood glucose levels followed the intragastric administration of liposomes containing entrapped insulin to diabetic rats (Dapergolas and Gregoriadis, 1976), an observation interpreted as showing that intact insulin had entered the bloodstream. In a similar experiment 0.17% of the dose of intragastrically administered ^{125}I-labelled PVP was recovered in the liver, more than double the recovery of unentrapped radiolabel.

Jonah et al. (1975), using liposomes of various compositions, found that 1h after the intravenous injection of liposome-entrapped [^{14}C]EDTA into mice, 36% - 43% of the dose was recovered in the liver. The lowest uptake was with positively charged, the highest with neutral liposomes. In addition the spleen took up between 5 and 8% and the bone marrow 5% - 7% of the dose (maximum with negatively charged liposomes). Uptake by the lungs was 10% using positively charged liposomes and around 5% using the other types, and uptake by the kidneys reached 1% using charged liposomes. Recovery of radioactivity in the brain was very low, but followed the pattern of uptake by the lungs. In a similar survey, Richardson et al. (1977), found that 29h after injection of ^{99m}Tc-labelled liposomes, 24% of the radiolabel from positively charged or neutral liposomes and 12% from negatively charged liposomes was recovered in the liver. Subsequently, an investigation of the effect of size in the distribution of negatively charged liposomes (Sharma et al., 1977) found that recovery in the liver,

spleen and lungs followed the order LUV > MLV > SUV.

In an effort to increase the uptake of liposomes by tissues other than the liver, liposomes have been produced which incorporate specific antibodies into the outer lipid bilayer. Gregoriadis and Neerunjun (1975) found that the uptake of liposomes by cells *in vitro* was enhanced when the liposomes were associated with IgG immunoglobulins raised against the specific cell strain. The uptake by dogfish phagocytes of liposomes coated with aggregated IgG was much higher than that of uncoated vesicles (Weissmann *et al.*, 1975). When liposomes containing IgG raised against a tumour were used *in vivo* the modest increase in tumour tissue uptake was overshadowed by large rises in the recoveries of liposomal material in both the liver and spleen (Neerunjun *et al.*, 1977). The general stimulation in uptake was probably due to the low specificity of the immunoglobulins used.

The eventual fate of liposomes administered to living animals depends upon both the nature of the liposomes and the route of administration. Intravenously administered liposomes accumulate mainly in the liver (Jonah *et al.*, 1975; Dapergolas *et al.*, 1976; Gregoriadis *et al.*, 1976; Richardson *et al.*, 1977), although there is a slight variation in the degree of uptake depending on the charge and size of liposomes used. Where more restricted routes of administration are used, i.e. the intraperitoneal, intramuscular and intragastric routes, the uptake of the liposomes is more limited (Dapergolas *et al.*, 1976; Gregoriadis *et al.*, 1976) although the liver still apparently accounts for the majority of the uptake that occurs. It seems that, following the intramuscular injection of liposomes, only radiolabel entrapped within SUV is able to reach the blood, and a larger area of the body (Dapergolas *et al.*, 1976). It may be possible to exploit this limited mobility of liposomes administered to some sites to restrict the uptake of MLV to cells of a certain type, although this could only be utilised in certain cases, e.g. the treatment of rheumatoid arthritis by intra-articular

injection of drug-containing liposomes (Shaw et al., 1976).

In this Chapter, the clearance and the tissue distribution of intravenously administered MLV containing ^{125}I-labelled PVP, [U-^{14}C] sucrose and [^{35}S] cystine are studied, and the results compared with those obtained using simple solutions of the radiolabels. The findings are then discussed in relation to the literature.

7.2 The clearance of intravenously injected substances from the bloodstream of the rat

The substances in 5mM phosphate-buffered saline, pH 7.4, were injected into male Wistar rats weighing 250-350g as described in Section 2.7. At successive time intervals, a sample of blood was taken (see 2.8), and treated in order to count the contained radioactivity (Sections 2.8.1 - 2.8.3). This radioactivity was expressed as a percentage of the dose administered, using the formula of Benacerraf *et al.* (1957), who estimated that the blood volume of the rat was 7.2cc/100g body weight.

7.2.1 ^{125}I-labelled PVP

The solution of ^{125}I-labelled PVP used in these experiments contained 50µg/ml, and was injected to a concentration of 50µg/kg body weight. The liposome-entrapped ^{125}I-labelled PVP was also injected to a concentration of 50µg/kg body weight, and was prepared as described in Section 2.2. Control experiments were performed by injecting unentrapped ^{125}I-labelled PVP (50µg/kg body weight) together with liposomes prepared using saline without any radiolabel. The results are shown in Figure 7.1. The clearance of unentrapped ^{125}I-labelled PVP is biphasic. Initially, there is a rapid removal of radiolabel from the blood, until after 10 - 15 min approx. 60% of the injected dose remains. After this point further clearance is much slower, so that even after 120 min (not shown) approx. 40% remains in the blood. The clearance of DPPC/DPPA (negatively-charged) liposomally entrapped radiolabel follows a smooth curve. When plotted on a semi logarithmic scale (Figure 7.2) this yields a straight line, which implies that the clearance follows first order kinetics. The clearance of unentrapped ^{125}I-labelled PVP in the presence of saline-filled liposomes was similar to that of unentrapped radiolabel alone, indicating that the clearance of liposome-entrapped radiolabel was due to the uptake by tissues of intact liposomes, and not merely to lipid-associated radiolabel.

Figure 7.1 Clearance of injected non-entrapped and liposome-entrapped ^{125}I-labelled PVP from rat bloodstream

^{125}I-Labelled PVP (approx. 50µg/ml) was injected into rats (see Section 2.7) to a concentration of 50µg/kg body weight, whether or not it was entrapped within multilamellar DPPC /DPPA liposomes. At the times shown, 50µl blood was taken from the right foot pad of the rat and the ^{125}I-radioactivity counted (see Sections 2.8 and 2.8.1)

KEY

....O..... : Non-entrapped ^{125}I-labelled PVP (mean ± S.E.M., 3 expts.)

—■—·— : Multilamellar DPPC/DPPA liposome-entrapped ^{125}I-labelled PVP (Mean ± S.E.M., 4 experiments)

—●— : Non-entrapped ^{125}I-labelled PVP plus saline-filled multilamellar DPPC/DPPA liposomes (Mean ± S.E.M., 3 expts.)

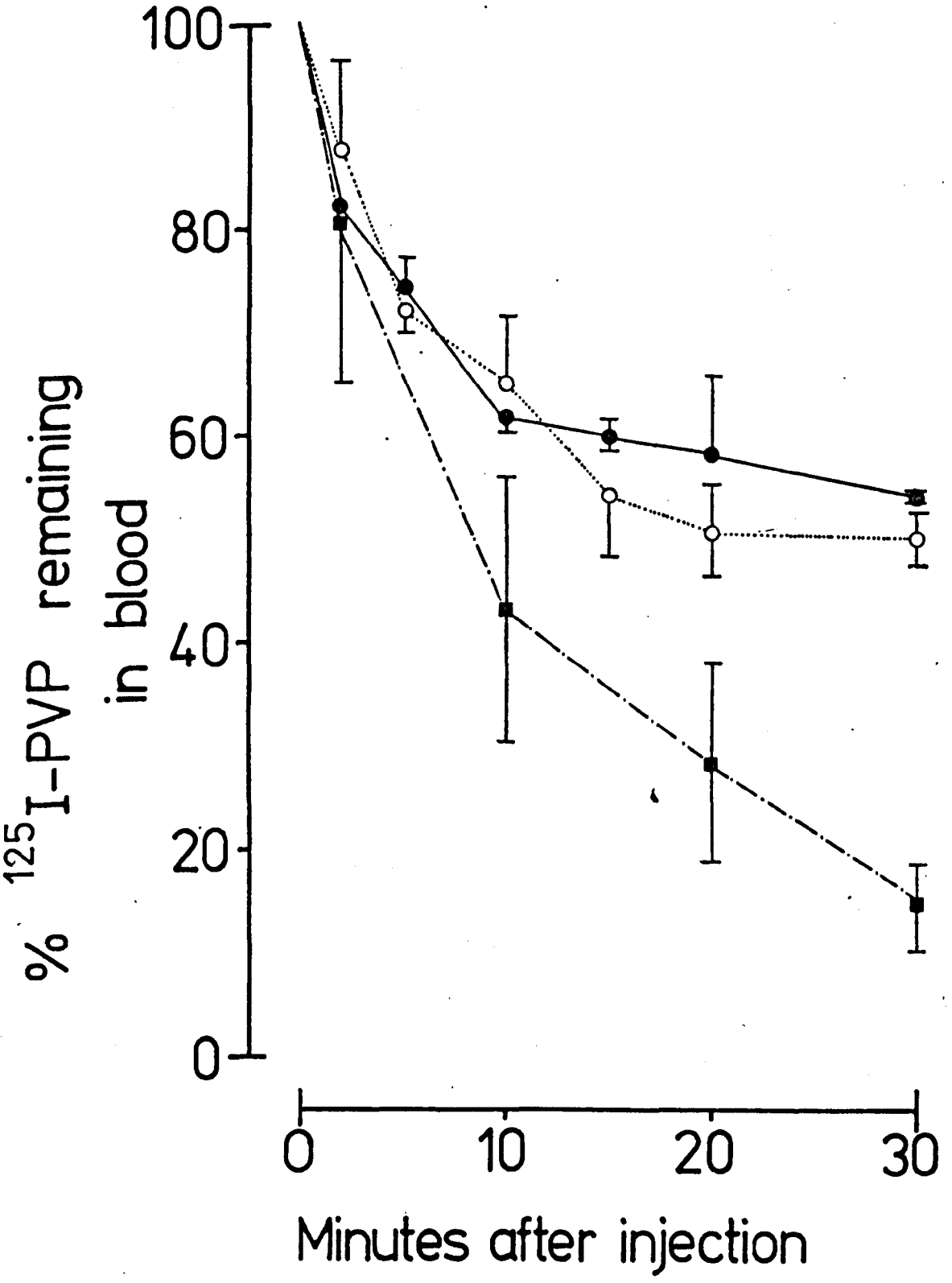
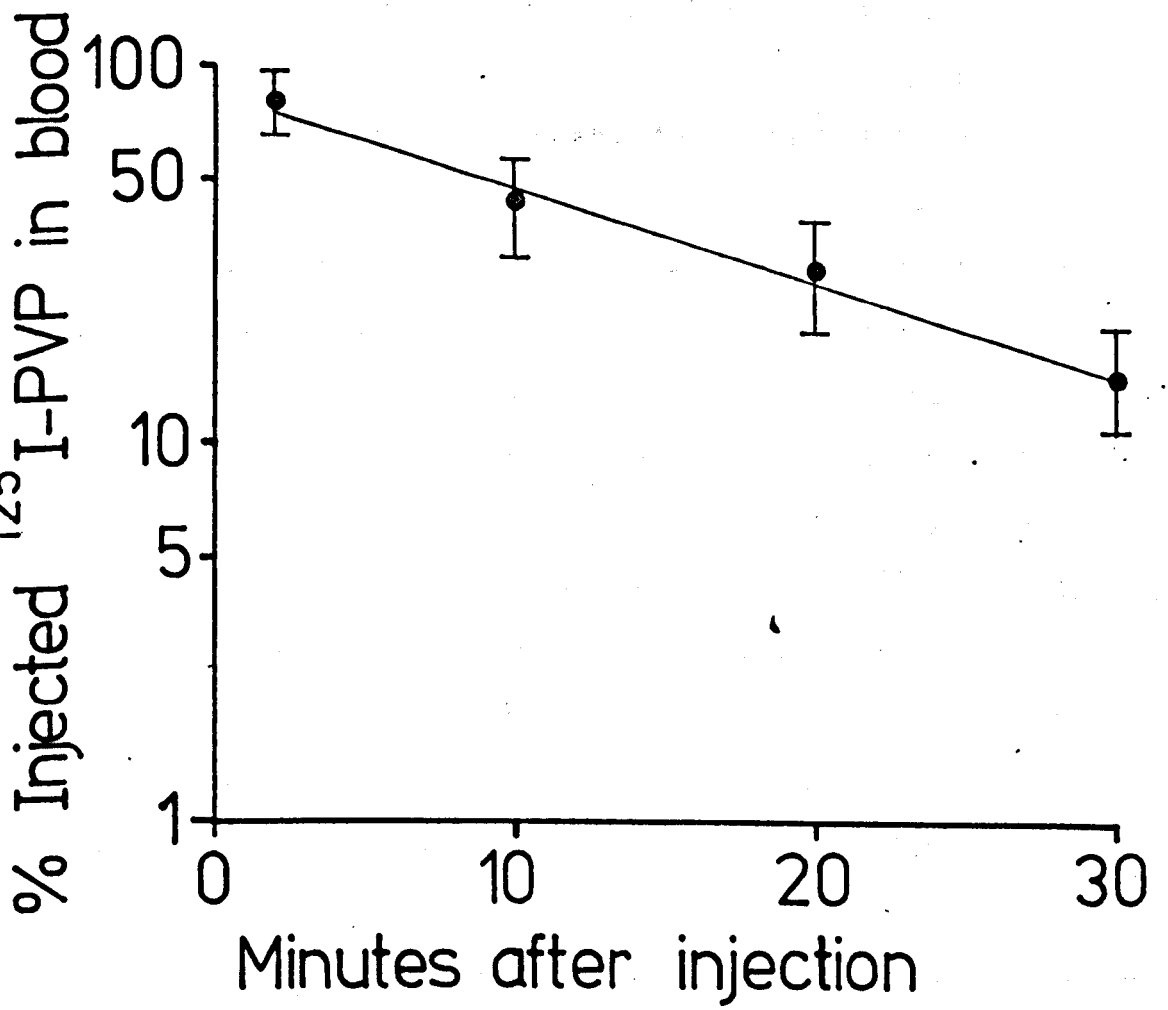

100
80
60
40
20
0
% 125I-PVP remaining in blood
0
10
20
30
Minutes after injection

Figure 7.2 Clearance of injected liposome-entrapped ^{125}I-labelled PVP from rat bloodstream

Data from Figure 7.1 relating to the clearance of multilamellar DPPC/DPPA liposome-entrapped ^{125}I-labelled PVP (■) replotted to a semilogarithmic scale.


% Injected ^{125}I-PVP in blood
100
50
10
5
1
0
10
20
30
Minutes after injection

The clearance of ^{125}I-labelled PVP entrapped in DPPC/stearylamine (positively charged) liposomes was also measured. Between 25% and 30% of the injected radioactivity was detected in the blood between 2min and 2h after the injection of the liposomes. Clearly, the PVP behaves differently when entrapped within positively charged liposomes. There are three possible explanations for this. It is possible, but unlikely, that in each of the experiments using positively charged liposomes, the bulk of the radioactivity was not injected into the bloodstream. Secondly, since the DPPC/stearylamine liposomes are less able to retain ^{125}I-labelled PVP at 37°C than DPPC/DPPA liposomes, the clearance observed might be due to unentrapped PVP, though this does not explain the rapid drop to only 30% remaining 2min after injection. Finally, the positively charged liposomes may have become aggregated so that they did not enter the circulation immediately, and the radioactivity was removed from the bloodstream at approximately the same rate as the aggregation was broken down.

Returning to the biphasic clearance of unentrapped ^{125}I-labelled PVP, enquiries to The Radiochemical Centre at Amersham requesting information about the molecular weight range of ^{125}I-labelled PVP and the distribution of ^{125}I-radioactivity throughout this range produced the graph shown in Figure 7.3 upper. Although the stated mean molecular weight is approx. 30,000 - 40,000, a large proportion of the molecules are shown as being less than 20,000, and the distribution has a pronounced skew. We wondered if the large range of molecular weights involved (8,000 - 80,000) might be sufficient to allow the smallest molecules to pass through the glomeruli of the kidney, whilst the larger molecules remained in the circulation. This would explain the biphasic clearance of ^{125}I-labelled PVP, since there would be a rapid initial clearance of the smaller molecules through the kidney, followed by slower removal of the remaining larger molecules.

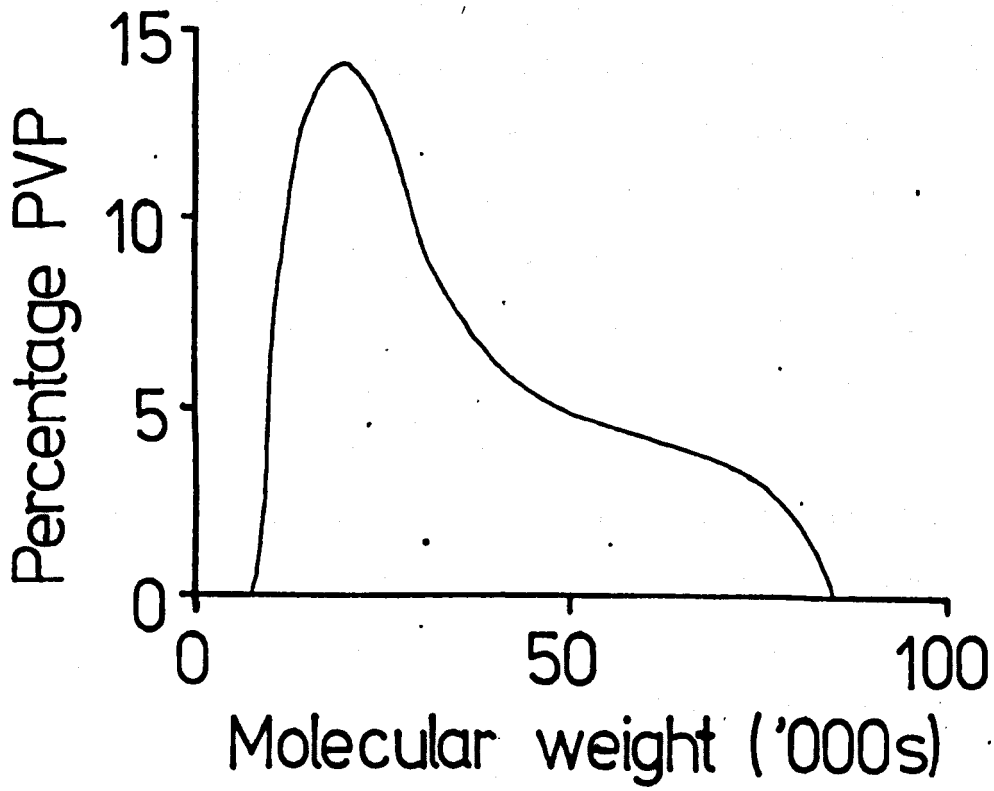
In order to obtain an estimate of the "cut off point" i.e. the largest molecular weight of molecule which could traverse the glomerulus, the data

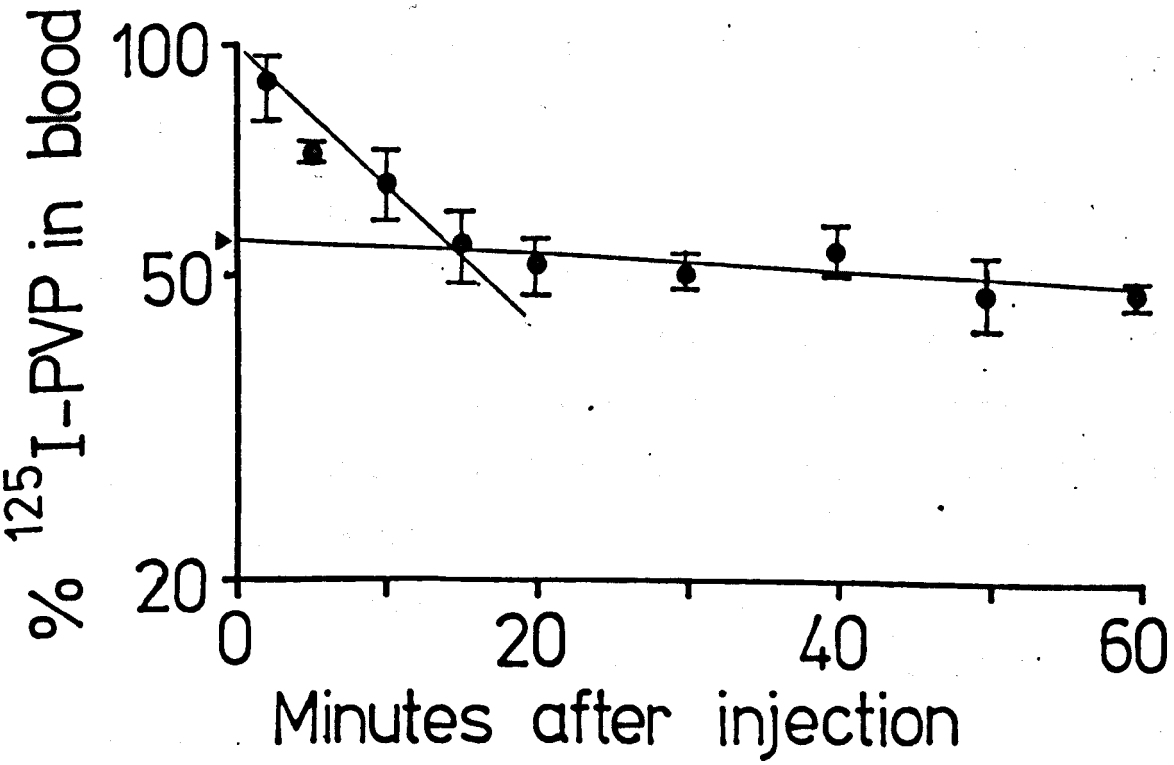
Figure 7.3 (Upper) Molecular weight distribution of ^{125}I-labelled PVP

Supplied by The Radiochemical Centre, Amersham.

Figure 7.3 (Lower) Clearance of injected non-entrapped ^{125}I-labelled PVP from rat bloodstream

Data from Figure 7.1 relating to the clearance of non-entrapped ^{125}I-labelled PVP (O) replotted to a semilogarithmic scale.


15
10
5
0
Percentage PVP
0
50
100
Molecular weight ('000s)


100
50
20
% 125I-PVP in blood
0
20
40
60
Minutes after injection

of Figure 7.1 relating to unentrapped ^{125}I-labelled PVP was recalculated on a semilogarithmic graph (Figure 7.3 lower). A straight line drawn through the points between 15 and 60min will intercept the y-axis at a point corresponding to the amount of ^{125}I-labelled PVP in the "larger molecular weight pool", since almost all of the "low molecular weight pool" has presumably been cleared by 15min. This line intercepts at 55.6%, so that presumably 44.4% of the ^{125}I-labelled PVP is capable of passing through the glomeruli. In order to transfer this estimate into a molecular weight limit for the rat glomerulus, the lower molecular weight 44.4% of the area under the curve of Figure 7.3 (upper) must be identified. This fraction includes PVP up to molecular weight approx. 27,500, a figure in close agreement with the statement made by Gartner et al. (1968), that "the pores in the glomerular capillaries hardly allow PVP molecules of a molecular weight of 25,000 to pass." More recent work by Morgan and Soothill (1975) has shown that the excreted ^{125}I-labelled PVP found in the urine is of lower molecular weight than that which remains in the body.

7.2.2 [U-^{14}C] Sucrose

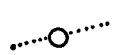
The concentrations of sucrose administered in these experiments were identical to those of ^{125}I-labelled PVP used in Section 7.2.1: a 50µg/ml suspension was injected to a concentration of 50µg/kg body weight. The clearance of unentrapped and liposome-entrapped [U-^{14}C] sucrose, and also of unentrapped [U-^{14}C] sucrose in the presence of saline-filled liposomes are shown in Figure 7.4.

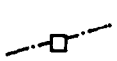
As for ^{125}I-labelled PVP, the clearance of unentrapped sucrose was the same whether or not saline-filled liposomes were also injected. In both cases over 80% of the radiolabel was removed from the serum within 10min but the remaining radiolabel was cleared only slowly. Liposome-entrapped [U-^{14}C] sucrose was cleared much more slowly and, when these data were replotted on a semilogarithmic graph (Figure 7.5), the clearance

Figure 7.4 Clearance of injected non-entrapped and liposome-entrapped [U-^{14}C] sucrose from rat bloodstream

[U-^{14}C] Sucrose (approx. 50μg/ml) was injected into rats (see Section 2.7) to a concentration of 50μg/kg body weight, whether or not it was entrapped within multilamellar DPPC/DPPA liposomes. At the times shown, 50μl blood was taken from the right foot pad of the rat and the ^{14}C-radioactivity counted (see Sections 2.8 and 2.8.2)

KEY


: Non-entrapped [U-^{14}C] sucrose (Mean ± S.E.M., 3 expts.)


: Multilamellar DPPC/DPPA liposome-entrapped [U-^{14}C] sucrose (Mean ± S.E.M., 3 expts.)


: Non-entrapped [U-^{14}C] sucrose plus saline-filled multilamellar DPPC/DPPA liposomes (Mean ± S.E.M., 3 expts.)

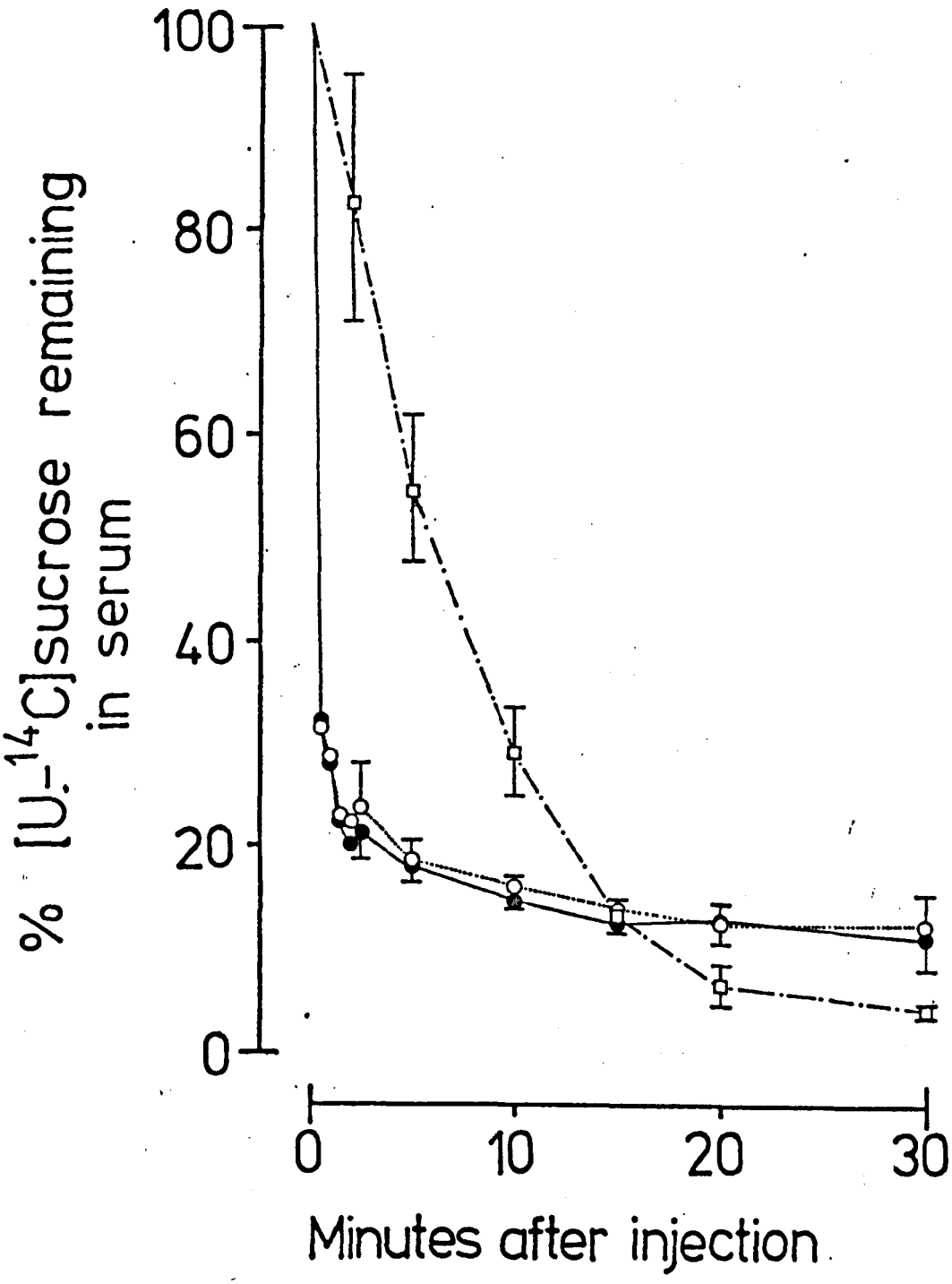
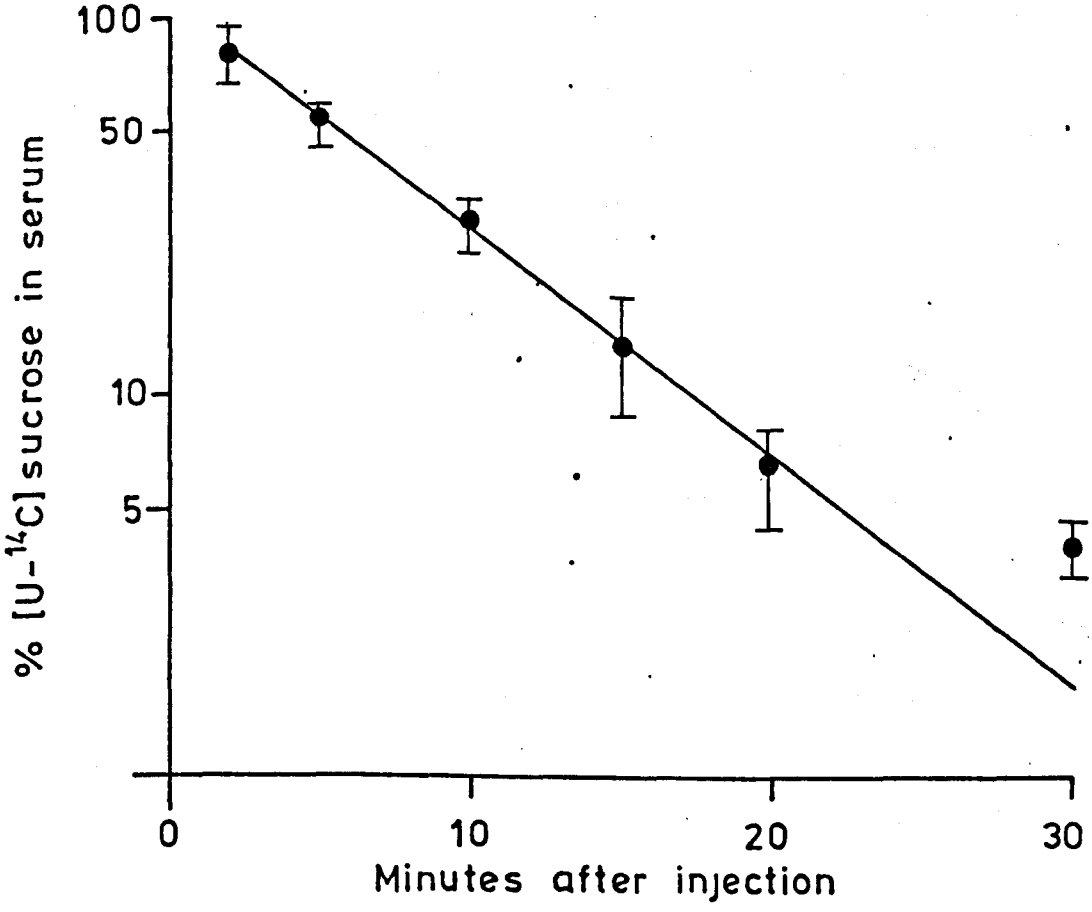

100
80
60
40
20
0
% [U-14C]sucrose remaining in serum
0
10
20
30
Minutes after injection

Figure 7.5 Clearance of injected liposome-entrapped [U-^{14}C] sucrose from rat bloodstream

Data from Figure 7.4 relating to the clearance of multilamellar DPPC/DPPA liposome-entrapped [U-^{14}C] sucrose (□) replotted to a semilogarithmic scale.


100
50
10
5
% [U-14C] sucrose in serum
0
10
20
30
Minutes after injection

was found to be first order. Again, the entrapment of the radiolabel within liposomes has altered the rate of removal of the radiolabel from the blood. That the difference is due to entrapment and not just association of the radiolabel with the lipids is shown by the clearance of radiolabel observed in the control experiments with saline-filled liposomes.

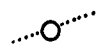
7.2.3 [^{35}S]Cystine

In this series of experiments, [^{35}S]cystine was injected to a concentration of 10μg/kg body weight, whether or not it was entrapped within liposomes. The results obtained (shown in Figure 7.6) were almost identical to those obtained using [U-^{14}C]sucrose. There was rapid removal of 80% of the injected unentrapped radiolabel, whether alone or in the presence of saline-filled liposomes. The liposomally entrapped [^{35}S]cystine was cleared more slowly, and again this clearance appeared as a straight line when replotted in Figure 7.7, indicating a first order clearance.

Figure 7.6 Clearance of injected non-entrapped and liposome-entrapped [^{35}S] cystine from rat bloodstream

[^{35}S] Cystine (approx. 10μg/ml) was injected into rats (see Section 2.7) to a concentration of 10μg/kg body weight, whether or not it was entrapped within multilamellar DPPC/DPPA liposomes. At the times shown, 50μl blood was taken from the right foot pad of the rat and the ^{35}S-radioactivity counted (see Sections 2.8 and 2.8.3). (Each point is the mean ± S.E.M. of three experiments.)

KEY



: Non-entrapped [^{35}S] cystine

: Multilamellar DPPC/DPPA liposome-entrapped [^{35}S] cystine (prepared from 100μg/ml [^{35}S] cystine in 67mM NaCl).

: Non-entrapped [^{35}S] cystine plus saline-filled multilamellar DPPC/DPPA liposomes

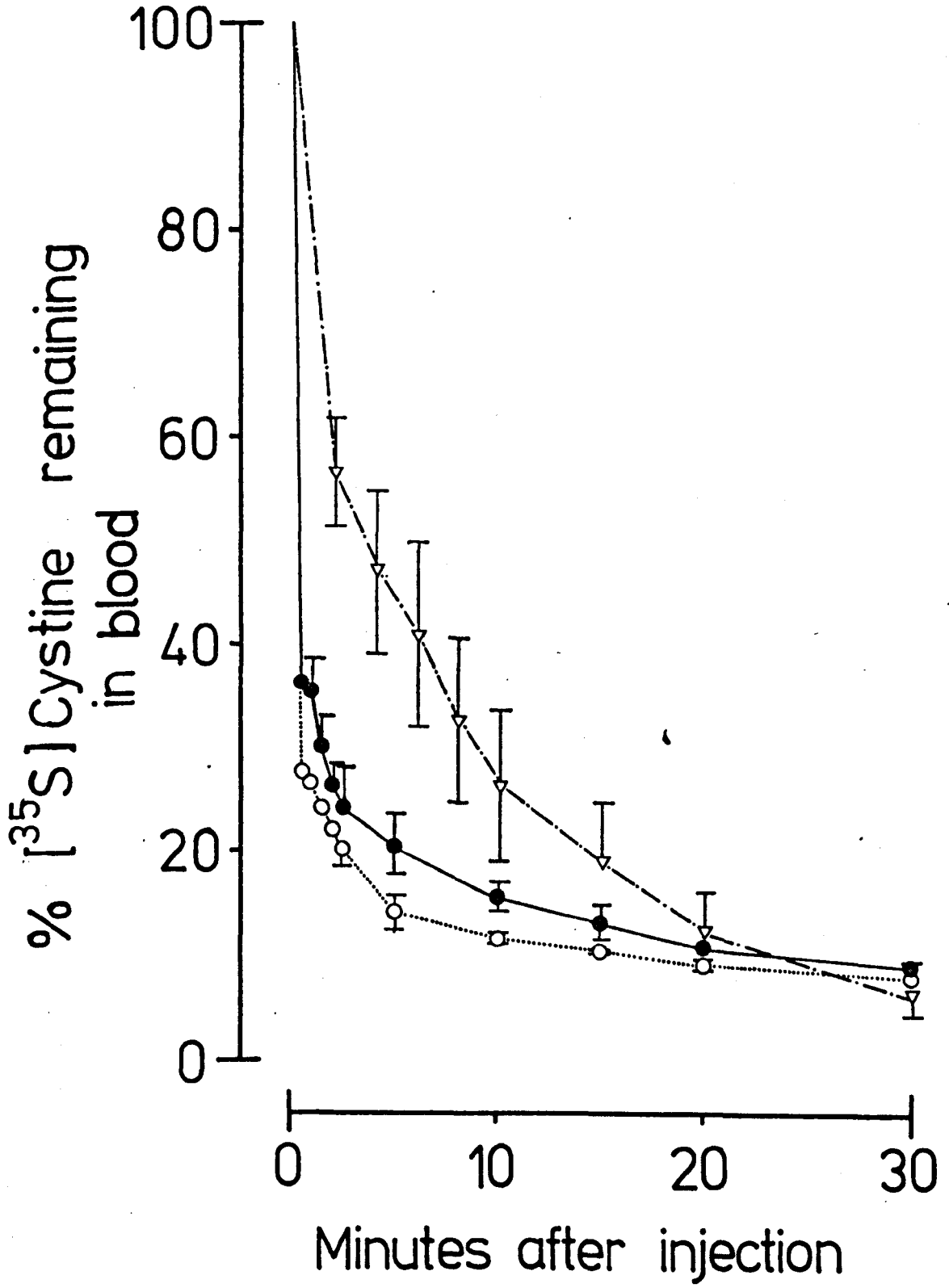
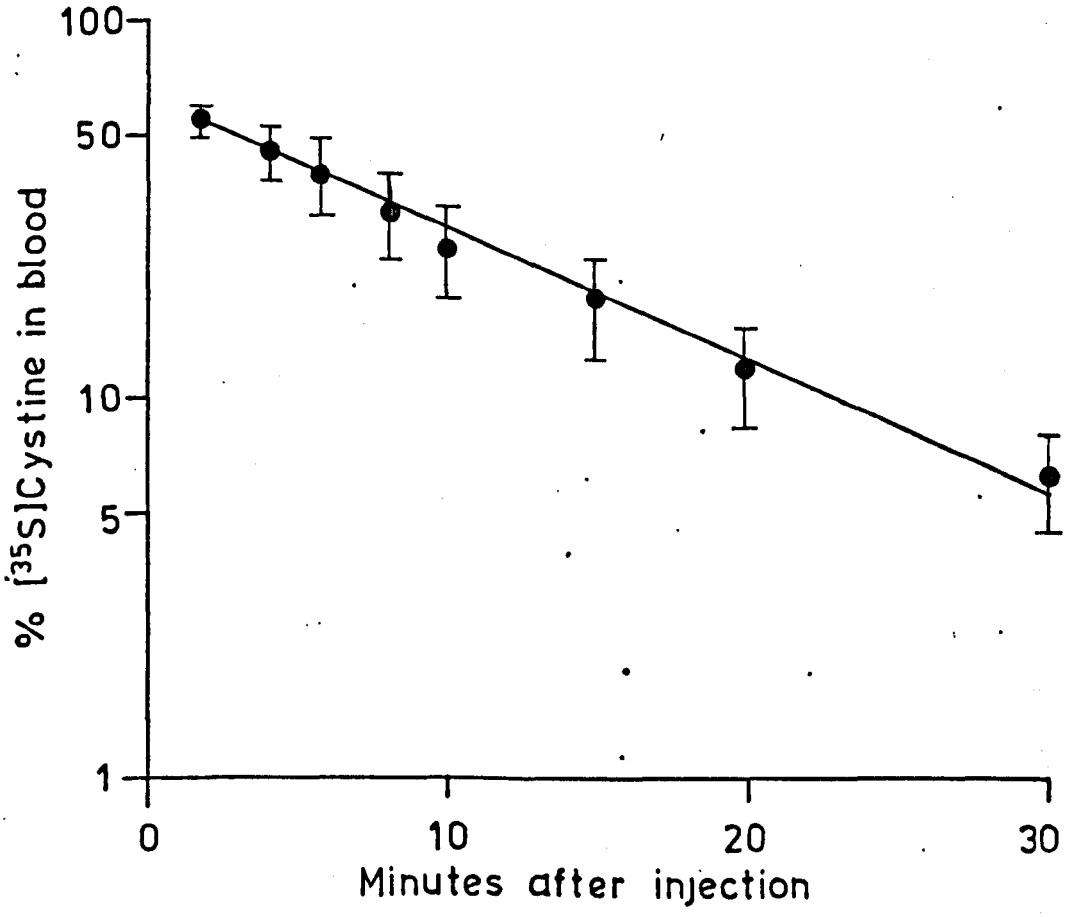

100
80
60
40
20
0
% [35S] Cystine remaining in blood
0
10
20
30
Minutes after injection

Figure 7.7 Clearance of injected liposome-entrapped [^{35}S] cystine from rat bloodstream

Data from Figure 7.6 relating to the clearance multilamellar DPPC/DPPA liposome-entrapped [^{35}S] cystine (▽) replotted to a semilogarithmic scale.


100
50
10
5
1
% [35S]Cystine in blood
0
10
20
30
Minutes after injection

7.3 The tissue distribution of substances intravenously injected into the rat

The substances are injected, as described in Section 2.7, into male Wistar rats weighing 250 - 350g. All the rats were fasted for 18h before sacrifice, at which point the tissues were removed for estimation of the radioactivity, as described in Sections 2.9.1 - 2.9.3. For the urine and faeces, the cumulative total radioactivity was estimated over the time from injection of radioactivity to sacrifice of the animal. Thus the data for urine and faeces give the total excretion, whereas for the tissues, the content of radioactivity at the point of sacrifice is estimated. All data is the total radioactivity in the tissue (or urine or faeces) expressed as a percentage of the injected radioactivity.

7.3.1 ^{125}I-labelled PVP

The results of the experiments to determine the tissue distribution of injected ^{125}I-labelled PVP (50µg/kg body weight) under different conditions are shown in Table 7.1. The distributions fall into three classes.

Whenever non-entrapped ^{125}I-labelled PVP was injected, most of the radiolabel was recovered in the urine and also in the blood. The recoveries of non-entrapped ^{125}I-labelled PVP in various tissues is consistent with there being little uptake of PVP by cells, so that the radiolabel remains in the bloodstream for a long period. Consequently, the smaller molecules of PVP are liable to be excreted by the kidneys resulting in high recoveries in the kidneys (initially) and urine. This fits in with the fate of injected ^{125}I-labelled PVP that was proposed in Section 7.2.1, namely rapid clearance of the smaller molecular weight fraction of PVP, followed by slower clearance of the larger molecules.

It is clear that entrapment of ^{125}I-labelled PVP within negatively charged MLV significantly affects its tissue distribution following

Table 7.1 Tissue distributions of non-entrapped and liposome-entrapped ^{125}I-labelled PVP injected into rats

^{125}I-Labelled PVP (approx. 50µg/ml) was injected into rats (see Section 2.7) to a concentration of 50µg/kg body weight, whether or not it was entrapped within liposomes. At the times shown after injection, the rat was sacrificed and the ^{125}I-radioactivity of the various tissues determined as described in Section 2.9.1. The data for urine and faeces are therefore a cummulative figure of the total excreted radiolabel. Figures are the mean ± S.E.M. of the number of experiments (in parentheses).

Notes -ve liposomes: multilamellar DPPC/DPPA liposomes

+ve liposomes: multilamellar DPPC/stearylamine liposomes

* ^{125}I-labelled PVP (unentrapped) plus saline-filled -ve liposomes (2h). Data for radioactivity in kidney and urine combined is 31.20 ± 3.93%.

Substance Injected	Time after Injection (h)	Percentage of Dose in Tissue						
		Liver	Kidney	Spleen	Thyroid	Urine	Faeces	Blood
^{125}I-labelled PVP (unentrapped)	2	2.09±0.20 (16)	3.21±1.03 (4)	0.22±0.02 (4)	0.18 (1)	37.98±2.88 (4)	n.d.	37.79±3.39 (3)
^{125}I-labelled PVP (unentrapped)	24	8.14±1.38 (4)	1.27±0.22 (4)	1.45±0.14 (4)	0.43±0.10 (3)	49.87±2.92 (4)	5.20±0.72 (4)	n.d.
^{125}I-labelled PVP (in -ve liposomes)	2	61.81±6.08 (5)	2.10±0.55 (4)	5.99±1.32 (4)	0.01 (1)	1.94±0.70 (4)	n.d.	4.95±1.37 (4)
^{125}I-labelled PVP (in -ve liposomes)	24	55.65±7.75 (4)	0.53±0.07 (4)	7.86±0.83 (4)	0.33±0.21 (3)	20.9 ±2.22 (4)	1.58±0.50 (4)	n.d.
^{125}I-labelled PVP (in -ve liposomes)	48	54.82±4.57 (3)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
^{125}I-labelled PVP (unentrapped) plus saline-filled -ve liposomes	2	2.11±0.17 (3)	12.00±8.92* (3)	0.26±0.03 (3)	0.06 (2)	19.20±8.62* (3)	n.d.	45.61±2.40 (3)
^{125}I-labelled PVP (in +ve liposomes)	2	33.69±8.25 (3)	3.56±0.29 (3)	0.87±0.17 (3)	0.05±0.04 (3)	10.07±4.01 (3)	n.d.	18.84±5.00 (3)

injection of the liposomes. There is a massive uptake of radiolabel by the liver, and uptake into the spleen also occurs. The levels of radioactivity in the liver decrease slightly from 2h to 48h after injection, while the amount in the spleen increases slightly over the period from 2h to 24h. The amount of radiolabel accumulating in the urine is 20.9% after 24h, which indicates a large loss of PVP from unidentified tissues between 2h and 24h.

An intermediate distribution was found when the ^{125}I-labelled PVP was injected entrapped within positively charged liposomes. This may have been due to the fragile nature of these liposomes (see Sections 6.2.3.6 and 7.2.1).

7.3.2 [U-^{14}C] sucrose

Table 7.2 shows the tissue distributions found following the injection of [U-^{14}C] sucrose (50µg/kg body weight) under different conditions. Unlike PVP, sucrose can be slowly metabolised by the rat, so that there is no guarantee that the radiolabel is always [U-^{14}C] sucrose. Non-entrapped sucrose rapidly appeared in the kidneys, and after 48h approx. 80% of the radiolabel was recovered in the urine. It is interesting to note that there was more radiolabel recovered in the blood after 48h than after 2h, suggesting that at least some of the later radioactivity is due to metabolized [U-^{14}C] sucrose. No significant accumulation of radiolabel was observed in either the liver or spleen at either 2 or 48h.

When [U-^{14}C] sucrose entrapped within negatively charged liposomes was injected into rats : over half of the radioactivity was recovered within the liver 2h after administration. This uptake was coupled with an increased recovery of radiolabel in the spleen. After 48h, there was still approx. 5% of the radioactivity associated with the liver, and urinary excretion was about half that observed when non-entrapped sucrose had been injected.

Table 7.2 Tissue distributions of non-entrapped and liposome-entrapped [U-^{14}C] sucrose injected into rats

[U-^{14}C] Sucrose (approx. 50μg/ml) was injected into rats (see Section 2.7) to a concentration of 50μg/kg body weight, whether or not it was entrapped within liposomes. At the times shown after injection, the rat was sacrificed and the ^{14}C-radioactivity of the various tissues determined as described in Section 2.9.2. The data for urine and faeces are therefore a cummulative figure of the total excreted radiolabel. Figures are the mean ± S.E.M. of the number of experiments (in parentheses).

Notes -ve liposomes: multilamellar DPPC/DPPA liposomes

Substance Injected	Time after Injection (h)	Percentage of Dose in Tissue					
		Liver	Kidney	Spleen	Urine	Faeces	Blood
[U-^{14}C]Sucrose (unentrapped)	2	0.84±0.23 (3)	47.58±8.95 (3)	0.09±0.02 (3)	12.39±6.29 (3)	n.d.	2.37±0.78 (3)
[U-^{14}C]Sucrose (unentrapped)	48	0.12±0.11 (3)	0.07±0.04 (3)	0.01±0.01 (3)	80.08±6.67 (3)	1.76±1.22 (3)	5.73±2.55 (3)
[U-^{14}C]Sucrose (in -ve liposomes)	2	57.96±4.04 (3)	3.72±1.60 (3)	3.73±1.53 (3)	4.02±0.63 (3)	n.d.	2.26±0.48 (3)
[U-^{14}C]Sucrose (in -ve liposomes)	48	4.64±0.55 (4)	0.26±0.13 (4)	0.45±0.09 (4)	38.03±5.14 (4)	3.20±0.10 (4)	1.67±1.41 (4)
[U-^{14}C]Sucrose (unentrapped) plus saline-filled -ve liposomes	2	2.71±0.54 (3)	38.71±0.36 (3)	0.25±0.10 (3)	11.97±2.96 (3)	n.d.	4.75±0.22 (3)

In control experiments, where non-entrapped [U-^{14}C] sucrose was injected together with saline-filled negatively charged liposomes, the tissue distribution was similar to that observed when non-entrapped sucrose was injected alone. Slightly raised values for the recovery of radiolabel in liver and spleen were, however, noted.

7.3.3 [^{35}S] Cystine

Radiolabelled cystine was injected in these experiments to a concentration of 10μg/kg body weight. The results are shown in Table 7.3. Unlike the other two substances, cystine was taken up significantly by the liver after injection in the non-entrapped form. After 48h, there was little radiolabel recovered in any of the tissues examined, and there was only approx. 30% of the radiolabel in the urine.

When [^{35}S] cystine entrapped within negatively charged MLV was injected, nearly three times as much radioactivity was recovered in the liver (approx. 35%) compared with the recovery following injection of non-entrapped cystine. Again, this was accompanied by an increased uptake into the spleen, although, after 48h, there was little radiolabel remaining in either the liver (approx. 3%) or spleen.

The tissue distribution of [^{35}S] cystine 2h after the injection of non-entrapped cystine with saline-filled liposomes was almost identical to that of the experiments where only non-entrapped cystine was injected.

It should be pointed out that in all of these experiments the maximum recovery of radiolabel in the tissues examined was only approx. 40% of that injected, which contrasts with both ^{125}I-labelled PVP and [U-^{14}C] sucrose.

Table 7.3 Tissue distributions of non-entrapped and liposome-entrapped [^{35}S] cystine injected into rats

[^{35}S] Cystine (approx. 10μg/ml) was injected into rats (see Section 2.7) to a concentration of 10μg/kg body weight, whether or not it was entrapped within multilamellar DPPC/DPPA liposomes (prepared from 100μg/ml [^{35}S] cystine in 67mM NaCl). At the time shown after injection, the rat was sacrificed and the ^{35}S-radioactivity of the various tissues determined as described in Section 2.9.3. The data for urine and faeces are therefore a cummulative figure of the total excreted radiolabel. Figures are the mean ± S.E.M. of the number of experiments (in parenthesis).

Note -ve liposomes: multilamellar DPPC/DPPA liposomes

Substance Injected	Time after Injection (h)	Percentage of Dose in Tissue					
		Liver	Kidney	Spleen	Urine	Faeces	Blood
[^{35}S] Cystine (unentrapped)	2	13.16±0.91 (3)	4.23±0.47 (3)	0.45±0.03 (3)	1.79±1.17 (3)	n.d.	8.37±0.60 (3)
[^{35}S] Cystine (unentrapped)	48	1.81±0.84 (3)	0.62±0.27 (3)	0.17±0.07 (3)	29.77±4.44 (3)	1.30±0.40 (3)	2.53±1.33 (3)
[^{35}S] Cystine (in -ve liposomes)	2	34.46±2.24 (4)	2.02±0.41 (4)	1.20±0.08 (4)	0.45±0.32 (4)	n.d.	3.55±0.31 (4)
[^{35}S] Cystine (in -ve liposomes)	48	3.17±0.82 (3)	0.82±0.12 (3)	0.28±0.03 (3)	24.34±2.11 (3)	3.54±1.61 (3)	3.03±0.47 (3)
[^{35}S] Cystine (unentrapped) plus saline-filled -ve liposomes	2	11.76±1.49 (3)	4.79±0.68 (3)	0.50±0.04 (3)	2.09±1.86 (3)	n.d.	6.83±0.22 (3)

7.4 Discussion

The initial clearance from the rat bloodstream of negatively charged liposomes containing either ^{125}I-labelled PVP, [U-^{14}C] sucrose or [^{35}S]-cystine were very similar. The half-times for the clearances were 11.5, 5.0 and 8.5 min respectively for the period 0 - 30 min after injection of the liposomes. After this time there was a slower clearance of the remaining 5 - 15% of the radiolabel. It may be that the residual, slow clearing fraction of radiolabel is due to material that has become non-entrapped, owing either to breakage of some liposomes,or to leakage from the liposomes. One would then expect to see this slower clearance following the same pattern as the clearance of the non-entrapped substance, and this is in fact the case (see Figures 7.1, 7.4 and 7.6). There are few accounts in the literature of studies of the clearance of liposomes from the blood over the first two hours after injection. A two-phase clearance of liposomes was observed by Gregoriadis and Neerunjun (1974), who linked the rate of clearance of the different liposomes tested to their rate of uptake by the liver. Rapid clearance of PC/cholesterol liposomes from the bloodstream of mice was found by Jonah *et al.* (1975). After 30 min, only 10 - 20% of the injected liposome-entrapped [^{14}C]EDTA remained in the bloodstream.

Apart from comparison of the clearance of the liposomes with published data, the two "internal" controls carried out for each substance provide evidence that the substances were entrapped within liposomes. The significant differences in clearance between liposome-entrapped and non-entrapped substance implies that liposomes were able to retain the entrapped substances at least for long enough to affect their clearance. Also, the mere association of the substances with saline-filled liposomes was not sufficient to significantly alter the rate of clearance of any of the substances.

The clearance of non-entrapped ^{125}I-labelled PVP is almost identical to that found by Regoeczi (1976) in rabbits, although he separated the first 2h clearance into three phases having half-lives of 0.12h, 0.9h and 3.4h respectively (compared with 0.26h and 1.94h half-lives calculated from Figure 7.3 lower). The two rapid initial clearance phases were attributed to equilibration into the extravascular space, and to renal clearance of a fraction of the PVP. The renal clearance of PVP observed by Ammon and Braunschmidt (1949) after its injection into dogs was also explained in terms of an extravascular equilibration and clearance of a low molecular weight fraction.

Wattiaux (1966) followed the clearance of sucrose from the rat bloodstream over a period of several days, and estimated that approx. 20% of the injected sucrose remained after 2h. This contrasts with the present estimate of 2.37%, possibly because Wattiaux administered a larger amount of sucrose (8ml 50% sucrose, in rats weighing 250g c.f. these experiments 12.5µg for rats of this size) that was injected intraperitoneally.

The tissue distribution of non-entrapped ^{125}I-labelled PVP found in this study is consistent with the work of Regoeczi (1976), though he used a biologically screened preparation of PVP, resulting in little urinary excretion of the macromolecule. Wattiaux (1966) monitored the levels of sucrose in rat liver following intraperitoneal injection of 8ml 50% sucrose. He found that after 2h there was 0.6%, and after 48h, 2.1% of the injected sucrose in the liver.

The tissue distributions of both ^{125}I-labelled PVP and [U-^{14}C] sucrose, 2h after their administration to the animal in negatively charged liposomes, were very similar. More than half the radiolabel was recovered in the liver at this time. However, whereas the concentration of DPPC/DPPA (negatively charged) liposome-entrapped ^{125}I-labelled PVP in the liver does not alter dramatically over the next 46h, only 4.64% of the ^{14}C-radioactivity (initially as sucrose) remains 48h after injection, and 38% was recovered

in the urine. It seems that, although both the PVP and sucrose are initially entrapped within the negatively charged liposomes (and thus exhibit a similar tissue distribution), the radiolabel of the sucrose can escape from the liver whereas the iodine-125 cannot. Since the liposomes used were identical in their method of preparation, and have similar properties *in vitro* (see Figures 6.3 and 6.4) and *in vivo* (i.e. similar distribution after 2h), it is unlikely that the liposomes remain stable in one case but not in the other. It is much more plausible that the liposomes release their contents intracellularly in both cases, but that the PVP (and therefore the ^{125}I-radioactivity) remains immobile whilst the sucrose becomes mobile. Although membranes are generally considered to be "impermeable" to both sucrose and PVP, sucrose may nevertheless be expected to permeate a membrane quicker than the much larger PVP molecule. Equally, both sucrose and PVP are considered to be indigestible by intracellular enzymes but some of the intracellular glycosidases might have some weak residual activity against sucrose, and thus liberate glucose and fructose which are both permeable and capable of being further metabolized by cells. PVP, on the other hand, requires many reactions to be digested to the monomeric form, and so complete digestion is much less likely to take place. It is conceivable, therefore, that low concentrations of intracellular sucrose might be removed quicker than similar amounts of PVP.

^{125}I-labelled PVP entrapped within DPPC/stearylamine (positively charged) liposomes, and [^{35}S] cystine entrapped within DPPC/DPPA (negatively charged) liposomes both shared a somewhat similar tissue distribution. In both cases, liver uptake of radiolabel after 2h was approx. 34% , with approx. 1% in the spleen. Renal excretion of the radiolabels was different, and so were the blood levels. Evidence was presented in Chapter 6 (Figures 6.3 and 6.5) that these particular liposome preparations were unable to retain their contents at 37°C *in vitro*. It is likely,

therefore, that these tissue distributions reflect the instability of these liposomes *in vivo*. If this were so, one might expect to find that ^{125}I-labelled PVP entrapped with positively charged liposomes showed a pattern of uptake and excretion resembling that of unentrapped ^{125}I-labelled PVP. Similarly, [^{35}S] cystine initially entrapped with negatively charged liposomes would show a distribution resembling that of the unentrapped amino acid. For each of the radiolabels, the appropriate distribution was observed.

As was stated in the Introduction to the Chapter, a large proportion of injected liposomes are localized in the liver and spleen. The results obtained using liposome-entrapped ^{125}I-labelled PVP, [U-^{14}C] sucrose and [^{35}S] cystine are in agreement with this general statement. In view of the high hepatic recoveries of these substances obtained after their entrapment within liposomes, it was decided to look more closely at their subcellular distribution within the liver.

CHAPTER 8

The subcellular distribution of some liposome-entrapped exogenous substances in rat liver

8.1 Introduction

In Chapter 7, the requirements for uptake of liposomes into tissues were presented, together with experimental data that indicated there was a sizeable hepatic recovery of radiolabel following the administration of liposome-entrapped substances to rats. It is difficult to ascertain from these data alone the mode of entry of the substances into the cells, and so further studies were carried out to identify the subcellular location of the substances within the liver.

If entry of the liposome-entrapped substances was by endocytosis, one would expect to recover a large proportion of the substance in a sedimentable fraction of the liver, since it would presumably be localized within the lysosomes. Conversely, if entry of the liposomes was by a fusion mechanism, one might expect to find most of the radiolabelled substance in the supernatant and in fractions that sediment only at very high speed, since this mode of entry would result in the free substance together with free liposomes entering the cytosol.

In this Chapter, differential centrifugation has been used to prepare subcellular fractions of rat liver (as described in Chapter 5), following the injection of substances into rats, in an effort to elucidate the subcellular location of the substances and from this their mode of entry into the cells.

8.2. Experimental procedure

This has been described in detail in Chapter 5. The final concentrations of injected ^{125}I-labelled PVP, [U-^{14}C]sucrose and [^{35}S]cystine dihydrochloride are noted in the text. The differential centrifugations were carried out in 0.3M sucrose containing 5mM MOPS (pH 7.4) and 5mM EGTA, unless otherwise stated.

8.3 Results and discussion

8.3.1 The subcellular location of injected ^{125}I-labelled PVP

The results of experiments using ^{125}I-labelled PVP are presented in three stages: first, the subcellular fractionations of rat livers following the injection of non-entrapped ^{125}I-labelled PVP (50µg/kg body weight); secondly, the subcellular fractionations following the injection of liposome-entrapped ^{125}I-labelled PVP (50µg/kg body weight); and finally, the data from some control experiments.

(i) Non-entrapped ^{125}I-labelled PVP

Table 8.1 shows the recoveries of protein, ^{125}I-labelled PVP, N-acetyl-β-D-glucosaminidase and arylsulphatase in the five fractions of rat liver, expressed as a percentage of the total recovered, following the injection of non-entrapped ^{125}I-labelled PVP. These data are expressed as histograms in Figure 8.1. The distribution of protein is very similar 2h and 24h after the injection of the radiolabel, and the location of the marker enzymes also varies little over the same period compared with uninjected controls (see below, Table 8.3 and Figure 8.3). The recovery of radiolabel does, however, vary over this time. At 2h over a third of the ^{125}I-labelled PVP is recovered in the supernatant fraction, and about 40% in the M and L fractions. One day after the injection, little radiolabel is found in the supernatant, and nearly 60% is in the M and L fractions.

The percentage radioactivity found in the supernatant at 2h is surprisingly high. The data apparently indicate that the ^{125}I-labelled PVP initially enters the cytosol, and subsequently enters the lysosomes, perhaps by autophagy. It is difficult to see how a molecule as large as PVP (30,000 - 40,000 molecular weight) would possibly enter the cytosol by traversing the plasma membrane, so this explanation is unlikely. It is also possible, but not likely, that the radiolabel is removed from the polymer, so that the location of ^{125}I-radioactivity does not reflect the subcellular position of the PVP molecule.

Table 8.1 The recovery of protein, ^{125}I-labelled PVP, N-acetyl-β-D-glucosaminidase and arylsulphatase in subcellular fractions of rat liver following the injection of non-entrapped ^{125}I-labelled PVP

Non-entrapped ^{125}I-labelled PVP (approx. 50μg/ml) was injected into rats (see Section 2.7) to a concentration of 50μg/kg body weight. After the time shown, the rat was sacrificed and the liver subjected to the scheme of subcellular fractionation described in Chapter 5. The results are the mean ± S.E.M., or the range is shown.

N.B. Centrifugation media:

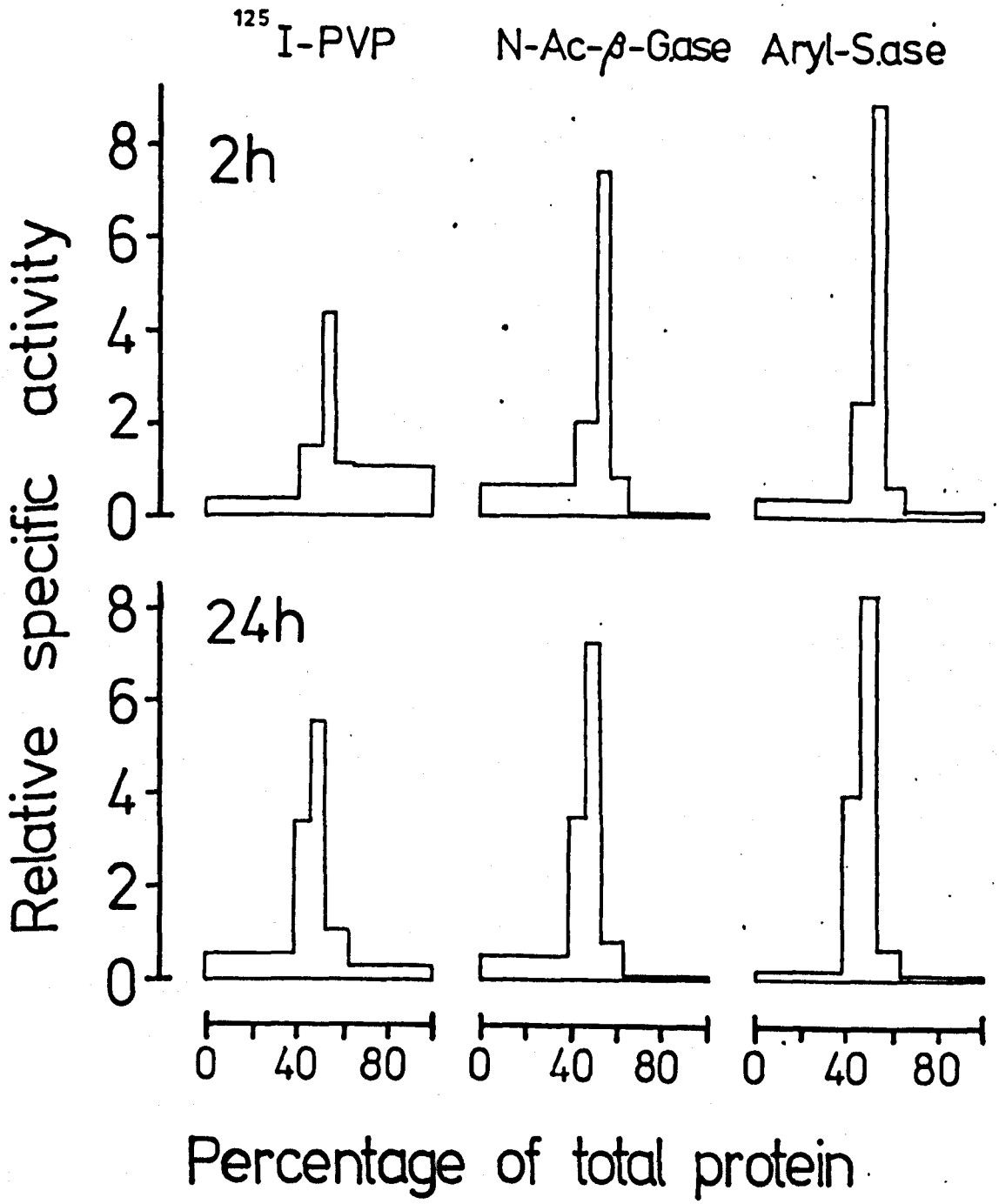
2h. 0.3M Sucrose, 5mM MOPS pH 7.4, 5mM EGTA

24h. 0.3M Sucrose, 5mM MOPS pH 7.4, 5mM EDTA

Time after injection (h)	Fracn	% Protein	% Radioactivity	% N-Ac-β-glucosaminidase	% Arylsulphatase
2	N	41.0 ± 0.8	14.6 ± 1.5	28.9 ± 3.4	15.0 ± 1.7
	M	10.4 ± 0.8	15.3 ± 4.0	21.2 ± 1.7	25.8 ± 1.2
	L	5.5 ± 0.4	23.9 ± 4.0	40.9 ± 3.1	48.8 ± 1.0
	P	8.0 ± 0.5	9.0 ± 1.4	7.1 ± 0.6	5.3 ± 1.4
	S	35.1 ± 0.4	37.3 ± 10.4	2.0 ± 1.0	5.2 ± 1.1
		(3)	(3)	(3)	(3)
24	N	39.3 ± 3.2	21.8 ± 1.7	19.8 ± 4.3	8.5 ± 0.9
	M	7.2 ± 1.7	24.3 ± 1.4	25.3 ± 2.8	28.8 ± 2.8
	L	6.0 ± 0.9	33.1 ± 0.8	43.5 ± 3.0	49.9 ± 2.0
	P	10.2 ± 0.9	10.6 ± 1.6	8.1 ± 0.5	7.0 ± 0.1
	S	37.4 ± 4.6	10.2 ± 1.9	4.3 ± 1.3	5.7 ± 0.4
		(4)	(4)	(4)	(3)

Figure 8.1 The subcellular distributions of ^{125}I-labelled PVP, N-acetyl-β-D-glucosaminidase and arylsulphatase in rat liver following the injection of non-entrapped ^{125}I-labelled PVP

Data of Table 8.1.


^{125}I-PVP
N-Ac-β-Gase
Aryl-Sase
2h
24h
Relative specific activity
0
2
4
6
8
0
40
80
Percentage of total protein

Two other explanations are between them probably sufficient to account for the initial distribution of ^{125}I-labelled PVP and its change by 24h. First, if the PVP enters the cells by endocytosis, then it will be contained in either a pinosome or a phagosome prior to gaining access to the lysosomes. It may be that these pre-digestive vesicles are more susceptible to breakage during the centrifugation process. If this were so, then it would account for the high recovery of radiolabel in the supernatant fraction 2h after the injection of ^{125}I-labelled PVP. There was no large recovery of either of the enzymes in the supernatant fraction, so that the supernatant radioactivity could not have arisen from broken secondary lysosomes. Secondly, by referring to Table 7.1, it can be seen that, whereas only 2% of the injected radiolabel was recovered in the liver 2h after injection, this figure had risen to 8% after 24h. In the intervening time, therefore, more radiolabel was taken up by the liver, and to compare the two sets of data in terms of absolute amounts of radioactivity, the second set (24h) should be multiplied by four. Such multiplication gives N, 84.9; M, 94.6; L, 128.9; P, 41.3; S, 39.7. These figures show that the recovery of PVP in the supernatant fraction has not decreased, but that the recovery in the heavier fractions has increased markedly. Such an increase is probably due to the continued uptake of the radiolabel, and its progressive accumulation in the lysosomes. The relatively constant amount of radioactivity in the S fraction is further evidence that the S fraction radioactivity is derived from fragile pinosomes being formed and destroyed (by conversion to secondary lysosomes) at a constant rate.

(ii) Liposome-entrapped ^{125}I-labelled PVP

Table 8.2 and Figure 8.2 show the results of this series of experiments, where liposome-entrapped ^{125}I-labelled PVP was injected, and subcellular fractions of rat liver obtained. Using negatively charged (DPPC/DPPA) liposomes, there was little alteration over two days in the

Table 8.2 The recovery of protein, ^{125}I-labelled PVP, N-acetyl-β-D-glucosaminidase and arylsulphatase in subcellular fractions of rat liver following the injection of liposome-entrapped ^{125}I-labelled PVP

Liposome-entrapped ^{125}I-labelled PVP (approx. 50μg/ml) was injected into rats (see Section 2.7) to a concentration of 50μg/kg body weight. After the time shown, the rat was sacrificed and the liver subjected to the scheme of subcellular fractionation described in Chapter 5. The results are the mean ± S.E.M., or the range is shown.

Notes -ve liposomes: multilamellar DPPC/DPPA liposomes

+ve liposomes: multilamellar DPPC/stearylamine liposomes

N.B.

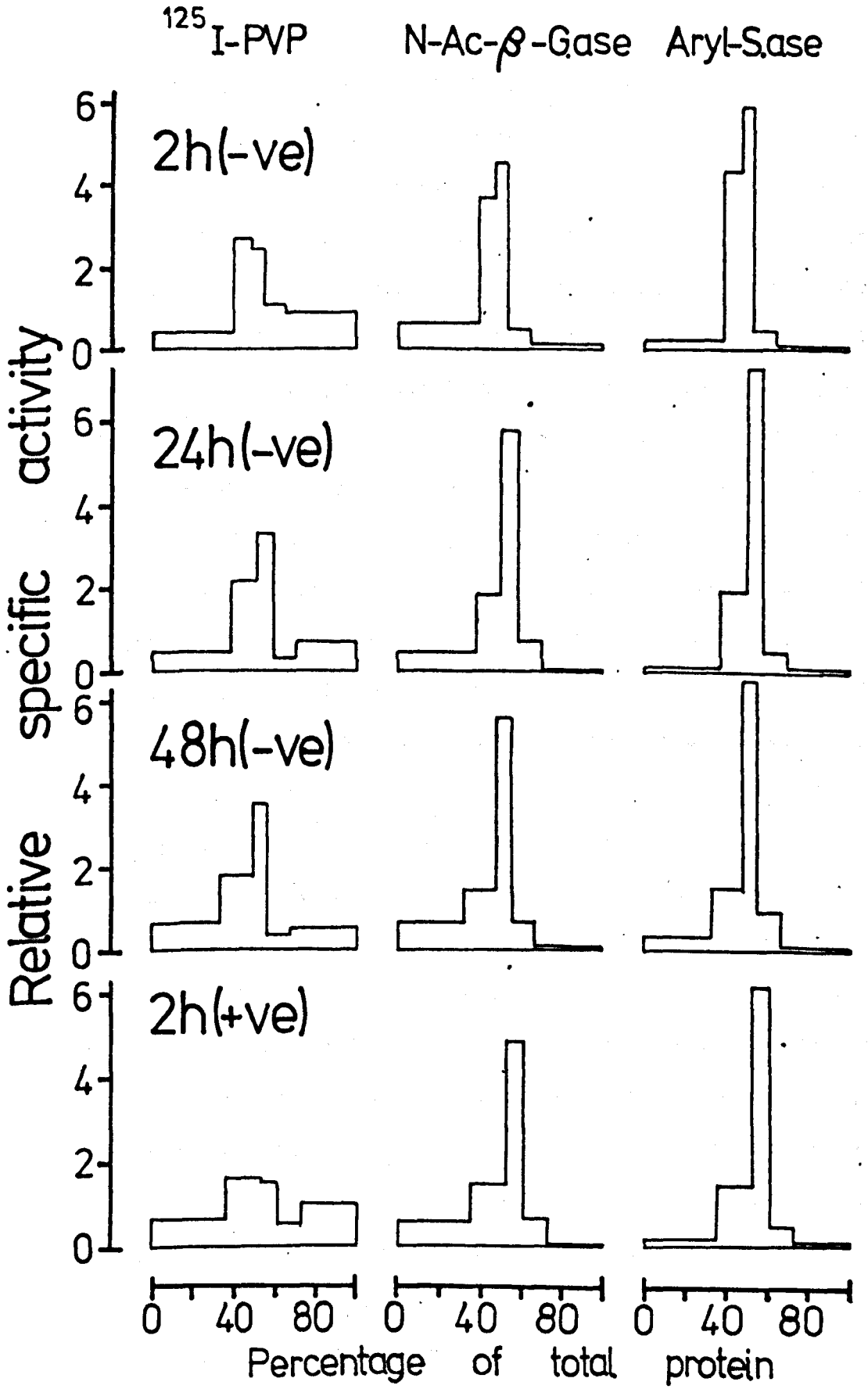
Centrifugation media:

0.3M Sucrose, 5mM MOPS pH 7.4, 5mM EGTA, except for 24h., in which 0,3M sucrose, 5mM MOPS pH 7.4, 5mM EDTA was used.

Time after injection (h)	Charge of Liposomes	Frac^{n}	% Protein	% Radioactivity	% N-Ac-β-glucosaminidase	% Arylsulphatase
2	(-)	N	39.5 ± 1.5	16.7 ± 2.3	24.9 ± 2.6	10.8 ± 2.8
		M	9.0 ± 1.0	24.3 ± 2.8	33.2 ± 2.7	39.2 ± 1.6
		L	6.7 ± 0.3	16.0 ± 2.1	30.5 ± 2.5	39.9 ± 2.9
		P	10.4 ± 1.1	11.3 ± 1.9	5.6 ± 0.9	5.4 ± 1.1
		S	34.5 ± 1.9	31.6 ± 3.7	5.7 ± 0.8	4.7 ± 1.4
			(5)	(5)	(5)	(5)
24	(-)	N	38.2 ± 2.0	18.9 ± 0.5	18.5 ± 2.8	6.4 ± 1.5
		M	13.3 ± 1.0	28.8 ± 1.2	25.0 ± 2.7	26.1 ± 3.0
		L	8.0 ± 0.3	26.5 ± 2.0	46.5 ± 2.4	58.2 ± 1.9
		P	11.1 ± 0.7	3.9 ± 0.4	8.3 ± 0.4	5.5 ± 0.9
		S	29.3 ± 0.3	21.8 ± 0.7	1.6 ± 1.1	3.8 ± 0.8
			(3)	(3)	(3)	(3)
48	(-)	N	33.9 ± 1.2	22.2 ± 0.2	22.6 ± 0.7	12.9 ± 0.6
		M	15.8 ± 1.0	28.8 ± 1.1	22.8 ± 1.3	23.1 ± 1.2
		L	7.8 ± 0.1	27.5 ± 0.7	43.8 ± 2.1	50.9 ± 0.7
		P	10.7 ± 0.6	4.1 ± 0.4	7.9 ± 0.5	9.7 ± 1.6
		S	31.9 ± 0.6	17.3 ± 0.5	2.8 ± 0.7	3.4 ± 0.3
			(3)	(3)	(3)	(3)
2	(+)	N	35.4 ± 1.5	24.0 ± 5.4	20.9 ± 2.0	8.5 ± 0.9
		M	17.2 ± 1.5	28.2 ± 4.8	25.1 ± 2.1	25.4 ± 1.5
		L	9.1 ± 0.6	14.1 ± 2.6	44.1 ± 0.1	56.2 ± 0.8
		P	11.4 ± 0.4	6.4 ± 1.7	7.7 ± 0.3	5.6 ± 0.7
		S	26.8 ± 1.1	27.3 ± 7.4	2.0 ± 1.2	4.3 ± 1.1
			(3)	(3)	(3)	(3)

Figure 8.2 The subcellular distributions of ^{125}I-labelled PVP, N-acetyl-β-D-glucosaminidase and arylsulphatase in rat liver following the injection of liposome-entrapped ^{125}I-labelled PVP

Data of Table 8.2.


125I-PVP
N-Ac-β-Gase
Aryl-Sase
2h(-ve)
24h(-ve)
48h(-ve)
2h(+ve)
Relative specific activity
0
2
4
6
0
40
80
Percentage of total protein

recovery of protein in the fractions. Table 7.1 shows that there is little difference in the amount of radiolabel in the liver at 2h, 24h and 48h after injection, and so the data in Table 8.2 and Figure 8.2 relate to similar levels of radioactivity. The amount of radiolabel recovered in the supernatant fraction progressively decreased over the two days. Meanwhile, the recovery in both the M and L fractions increased from 40% at 2h to 56% at 48h. The most interesting finding was the alterations in the marker enzyme distributions which occured simultaneously with those of the radiolabel. Compared with control experiments (see below, Table 8.3 and Figure 8.3), a shift in the recovery of both N-acetyl-β-D-glucosaminidase and arylsulphatase from the L fraction to the M fraction was observed 2h after the injection of liposome-entrapped ^{125}I-labelled PVP. At 24h after injection, the distribution had returned to normal.

In contrast to experiments where non-entrapped ^{125}I-labelled PVP was injected, by 2h almost all of the radiolabel is removed from the blood (see Chapter 7). There is therefore no constant interiorization of ^{125}I-labelled PVP into the cells, since most of the liposomes are cleared from the blood in the first 30 min. The ^{125}I-labelled PVP is in very large particles compared with the non-entrapped form, and the MLV used are large enough to increase significantly the initial volume of any secondary lysosomes which might be formed. If this occurred, then it is quite probable that these particles would exhibit an increased sedimentation in the M fraction, rather than the L fraction. This explanation is supported by the data for the location of radiolabel after 2h, most being in the M fraction. A similar shift of lysosomal enzyme activity from the L to the M fraction was observed in rat liver by Wattiaux (1966), 2h after the injection of massive amounts of sucrose, and this was attributed to a large increase in the volume of the lysosomes.

Interestingly, although the distribution of the two enzymes returned to normal by 24h (see Table 8.3), the radiolabel remained localised in the M fraction, while the recovery in the L fraction increased towards the M

fraction recovery. The total activity of both the enzymes did not alter significantly in any of the experiments in this Chapter (results not shown) and so it is unlikely that vast amountsof newly-synthesised enzymes appearing only in the L fraction, could be responsible for reasserting the control distribution. An alternative explanation is that the degradation of the lysosomal enzymes themselves (i.e. intracellular protein turnover) takes place at such a rate that the enzymes are degraded within 24h and replaced by newly synthesised enzymes, so that the subcellular distribution returns to normal.

It is just possible that the multilamellar liposomes might enter the cells by fusion with the plasma membrane, and that the free liposomes thus in the cytosol then enter the lysosomal system by autophagy. This process would lead to the formation of large secondary lysosomes, and also localization of some liposome-entrapped and unentrapped ^{125}I-labelled PVP in the cytosol. However, the alteration of the marker enzyme distribution implies rapid formation of liposome-containing secondary lysosomes, consistent with entry by phagocytosis.

The results obtained following the injection of ^{125}I-labelled PVP entrapped within positively charged (DPPC/stearylamine) liposomes are shown at the bottom of Table 8.2. There is no perturbation of the two enzyme distributions like that found using liposomes of negative charge, but there was a higher recovery of protein in the M fraction 2h after injection. The radiolabel was distributed in a similar way to that of negatively charged (DPPC/DPPA) liposome-entrapped ^{125}I-labelled PVP. The stability of the positively charged liposomes at 37^{o}C *in vitro* (see Section 6.2.3.6) leads one to expect that they will not remain intact for any length of time after injection into animals. It is possible, therefore, that both non-entrapped and liposome-entrapped ^{125}I-labelled PVP would be taken up by cells in such experiments, and that a composite subcellular distribution might be observed. Unfortunately, the results obtained were

not sufficiently clear-cut to substantiate this interpretation, even though the levels of ^{125}I-labelled PVP recovered in the liver (Table 7.1) show that the uptake of radiolabel in these experiments was intermediate between non-entrapped and DPPC/DPPA liposome-entrapped ^{125}I-labelled PVP.

Since the DPPC/stearylamine liposomes are more leaky, the bilayers may be in the liquid-crystalline state. If this were so, an alternative explanation, in accord with the work of Papahadjopoulos *et al.* (1975) would be that these liposomes were internalized by a fusion mechanism. A significant proportion of the ^{125}I-labelled PVP will leak from these liposomes before interaction of the latter with cells, and so will remain in the bloodstream. The radiolabel entering the cell would be localized in the cytosol, and so would not affect the distribution of lysosomal marker enzymes. If such a course of events took place, one would expect to find the radiolabel localized largely in the S fraction whether or not the liposomes remained intact within the cytosol (see below, and Table 8.3 and Figure 8.3).

On balance, these data are as difficult to interpret concisely as the clearance data for this type of liposomes. Clearly, positively charged (DPPC/stearylamine) liposomes are treated differently by the rat than those that are negatively charged (DPPC/DPPA). The difficulty in interpretation of data relating to positively charged liposomes and also the lower uptake of radiolabel by the liver after injection of these liposomes led to the decision to continue the study using only negatively charged (DPPC/DPPA) liposomes.

(iii) Control experiments using ^{125}I-labelled PVP

These experiments are detailed in Table 8.3 and Figure 8.3. In experiment A, non-entrapped ^{125}I-labelled PVP was injected together with saline-filled liposomes. After 2h, the distributions of the two enzymes and ^{125}I-labelled PVP were altered in a similar way to those in the experiments where liposome-entrapped ^{125}I-labelled PVP was injected

Table 8.3 The recovery of protein, ^{125}I-labelled PVP, N-acetyl-β-D-glucosaminidase and arylsulphatase in subcellular fractions of rat liver. Some control experiments

A. Non-entrapped ^{125}I-labelled PVP (approx. 50μg/ml) was injected into rats (see Section 2.7) to a concentration of 50μg/mg body weight, together with saline-filled multi-lamellar DPPC/DPPA liposomes. After 2h, the rat was sacrificed and the liver subjected to the scheme of sub-cellular fractionation described in Chapter 5. The results are the mean ± S.E.M.

B. Multilamellar DPPC/DPPA liposome entrapped ^{125}I-labelled PVP (10μg) added to the liver homogenate immediately prior to centrifugation. This concentration is of the same order as that recovered in the liver 2h after the injection of multi-lamellar DPPC/DPPA liposome-entrapped ^{125}I-labelled PVP. The results are the mean of two experiments (the range is shown).

C. Non-entrapped ^{125}I-labelled PVP (< 1μg) added to the liver homogenate immediately prior to centrifugation. The results are the mean of two experiments (the range is shown).

N.B. Centrifugation media:

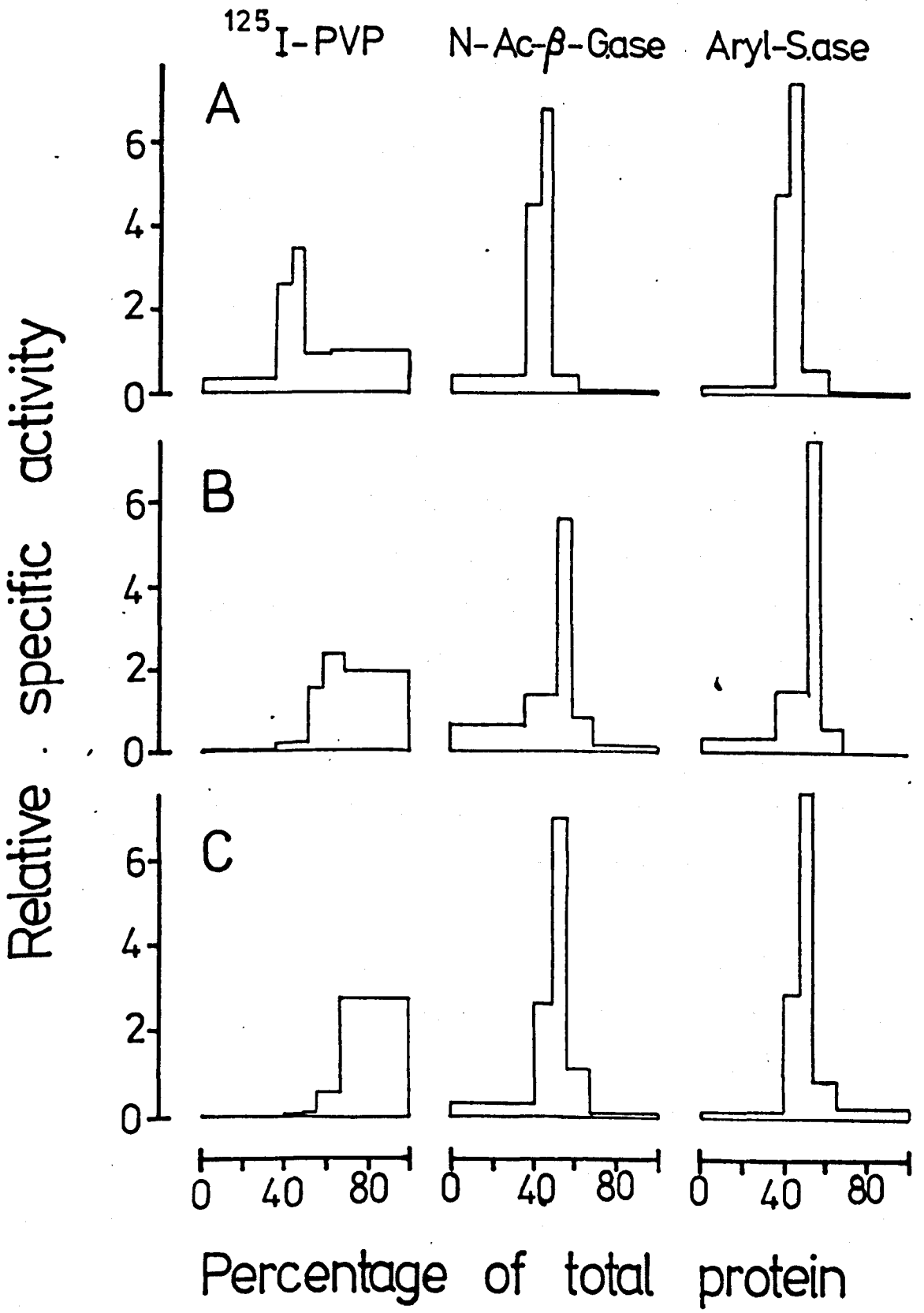
A, C 0.3M Sucrose, 5mM MOPS pH 7.4, 5mM EDTA

B 0.3M Sucrose, 5mM MOPS pH 7.4, 5mM EGTA

Experiment	Frac^{n}	% Protein	% Radioactivity	% N-Ac-β-glucosaminidase	% Arylsulphatase
A	N	35.7 ± 4.3	12.4 ± 1.1	15.0 ± 2.4	7.5 ± 1.1
	M	7.8 ± 2.8	20.4 ± 11.0	35.1 ± 9.0	37.1 ± 3.2
	L	5.9 ± 0.8	17.1 ± 6.2	40.1 ± 4.7	43.8 ± 1.5
	P	13.3 ± 1.7	12.1 ± 1.1	6.3 ± 2.4	7.8 ± 0.9
	S	37.4 ± 4.6	38.0 ± 13.0	3.5 ± 3.2	3.6 ± 0.2
		(3)	(3)	(3)	(3)
B	N	35.0 (34.8 - 35.1)	1.2 (1.1 - 1.3)	23.2 (22.1 - 24.2)	13.3 (12.6 - 13.9)
	M	16.0 (15.8 - 16.2)	3.7 (3.3 - 4.0)	22.0 (19.7 - 24.3)	23.3 (22.2 - 24.4)
	L	7.6 (7.5 - 7.6)	11.6 (10.2 - 13.0)	42.3 (41.4 - 43.2)	56.9 (56.3 - 57.4)
	P	9.8 (9.2 - 10.4)	23.0 (17.9 - 28.1)	8.1 (7.9 - 8.3)	5.9 (5.2 - 6.5)
	S	31.7 (31.0 - 32.3)	60.6 (57.2 - 64.0)	4.4 (4.3 - 4.5)	0.8 (0 - 1.5)
		(2)	(2)	(2)	(2)
C	N	39.9 (38.4 - 41.4)	0.6 (0.2 - 1.0)	13.0 (9.3 - 16.7)	5.7 (5.1 - 6.3)
	M	8.9 (7.9 - 9.9)	0.5 (0.4 - 0.5)	23.5 (21.2 - 25.7)	25.8 (20.8 - 30.8)
	L	6.8 (6.7 - 6.8)	0.6 (0.2 - 0.9)	47.7 (43.4 - 51.9)	52.1 (51.7 - 52.5)
	P	11.2 (10.4 - 12.0)	6.6 (1.8 - 11.3)	12.7 (9.0 - 16.4)	9.6 (8.6 - 10.6)
	S	33.3 (32.9 - 33.7)	91.9 (87.8 - 95.9)	3.3 (1.3 - 5.3)	6.8 (3.0 - 10.6)
		(2)	(2)	(2)	(2)

Figure 8.3 The subcellular distributions of ^{125}I-labelled PVP, N-acetyl-β-D-glucosaminidase and arylsulphatase in rat liver. Some control experiments

Data of Table 8.3.


^{125}I-PVP
N-Ac-β-Gase
Aryl-Sase
A
B
C
6
4
2
0
0
40
80
Relative specific activity
Percentage of total protein

(Table 8.2 and Figure 8.2) even though the level of uptake by the liver was only 2.1% of the dose (Table 7.1). These similarities demonstrate the inability of liver cells to distinguish between liposomes of the same lipid composition which contain different aqueous phases. Non-entrapped ^{125}I-labelled PVP would enter the cells by fluid phase pinocytosis, but still end up in the large secondary lysosomes, together with the saline-filled liposomes.

Experiments B and C were carried out in order to determine the distribution of comparable amounts of DPPC/DPPA liposome-entrapped and non-entrapped ^{125}I-labelled PVP added to the liver homogenate. The majority of the liposome-entrapped radiolabel was recovered in the S fraction, with smaller amounts in the L and P fractions. Any liposomes released from phagosomes or secondary lysosomes, would thus be localised in these fractions. Non-entrapped radiolabel was recovered almost exclusively in the S fraction, so that again, if any pinosomes or secondary lysosomes were to be disrupted during the isolation procedure, one would expect most of the radiolabel to appear in the S fraction. The results of these control experiments emphasise the membrane-bounded nature of the ^{125}I-labelled PVP recovered in the heavier fractions.

The distributions of N-acetyl-β-D-glucosaminidase and arylsulphatase in experiment C were taken to be the usual subcellular distributions of these enzymes for the purposes of comparison with other parts of this Chapter. Both the enzymes were localized chiefly in the L fraction, with some activity in the M fraction. The distributions of the enzymes were not identical, since in almost every individual experiment, a higher percentage of N-acetyl-β-D-glucosaminidase than arylsulphatase was recovered in the N fraction, and vice-versa for the S fraction. This fits in with other data on these enzymes, since the non-sedimentable activity of arylsulphatase is generally higher than that of N-acetyl-β-D-glucosaminidase measured under the same conditions. This could be

interpreted as evidence that arylsulphatase dissociates more readily from broken lysosomes. It would appear that under the conditions of these experiments, arylsulphatase behaves as a soluble lysosomal enzyme, while N-acetyl-β-D-glucosaminidase behaves as if it was bound to the lysosomal membrane.

8.3.2. The subcellular location of injected [U-^{14}C] sucrose

The experiments involving the subcellular fractionation of liver homogenates containing [U-^{14}C] sucrose are shown in Table 8.4 and also in Figure 8.4. As the total recovery in the liver was very low following the injection of non-entrapped [U-^{14}C] sucrose (see Table 7.2), only one subcellular fractionation of this type was carried out. The concentration of injected [U-^{14}C] sucrose was 50μg/kg body weight in all experiments.

The uptake of DPPC/DPPA liposome-entrapped [U-^{14}C] sucrose by the liver after 2h (approx. 55% of dose administered) is almost the same as that of liposome-entrapped ^{125}I-labelled PVP, but the subcellular distribution is very different: the recovery in the S fraction is higher, and that of the N and M fractions is lower. Also, there is only a slight alteration in the distributions of the two marker enzymes relative to controls. It should be noted that the raised M fraction recovery of N-acetyl-β-D-glucosaminidase does not stand out in the histogram of Figure 8.4 because of the high protein content of the M fraction. After 48h, nearly 80% of the radioactivity is recovered in the S fraction, but the total liver radioactivity is only approx. 5% of the dose (see Table 7.2). If the figures for the levels of ^{14}C-radioactivity found after 48h are adjusted as was done in Section 8.3.1 for non-entrapped ^{125}I-labelled PVP (by dividing by 12.5), one obtains N, 0.7; M, 0.3; L, 0.4; P, 0.2; S, 6.4, so that there has been movement of radiolabel from all the fractions, but particularly from the heavier ones (i.e. N, M and L).

Table 8.4 The recovery of protein, [U-^{14}C]sucrose, N-acetyl-β-D-glucosaminidase and arylsulphatase in subcellular fractions of rat liver following the injection of liposome-entrapped [U-^{14}C]sucrose

2h and 48h. Multilamellar DPPC/DPPA liposome-entrapped [U-^{14}C]sucrose (approx. 50μg/ml) was injected into rats (see Section 2.7) to a concentration of 50μg/kg body weight. After the time shown, the rat was sacrificed and the liver subjected to the scheme of subcellular fractionation described in Chapter 5. The results are the mean ± S.E.M.

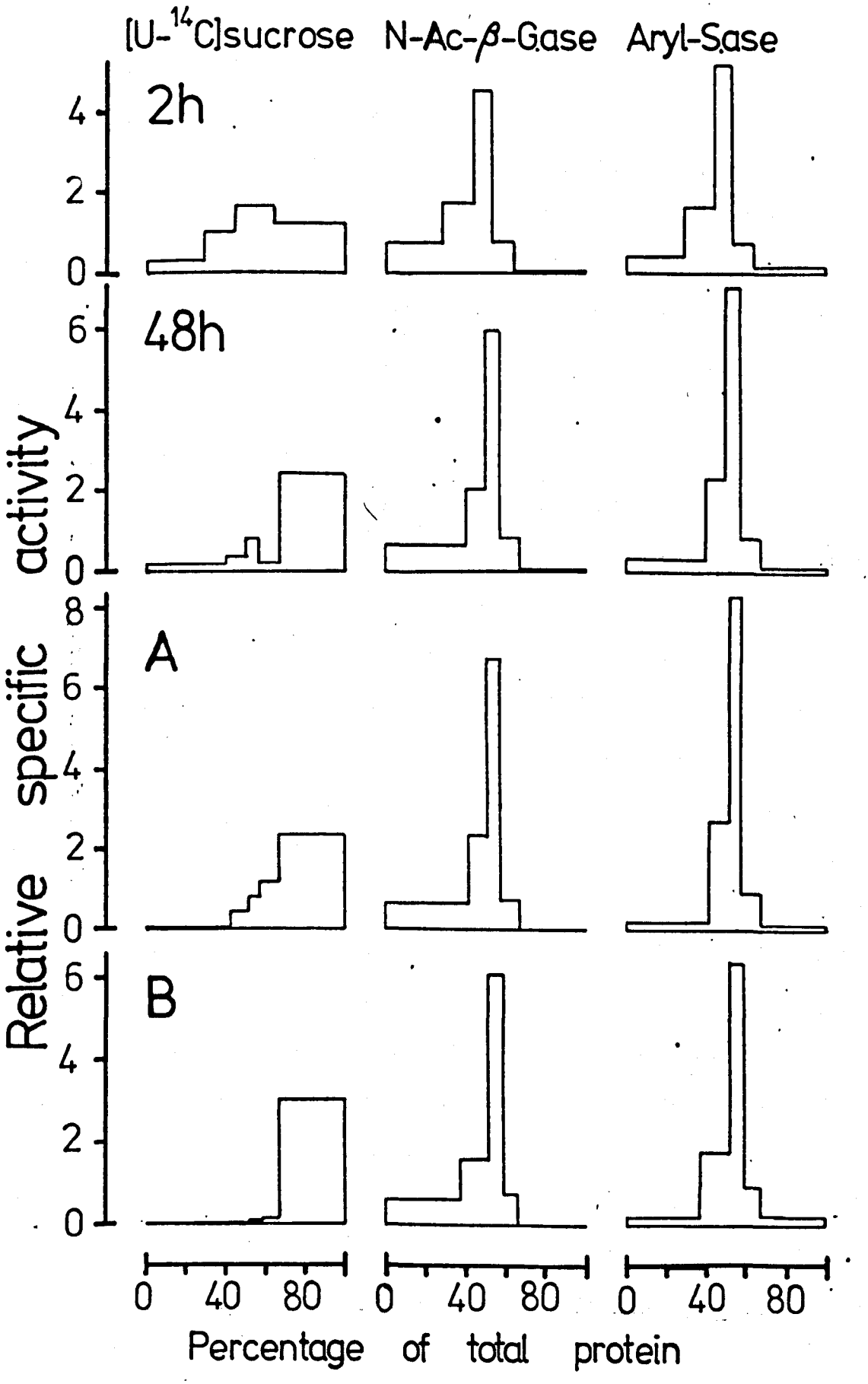
A and B. Multilamellar DPPC/DPPA liposome-entrapped [U-^{14}C]sucrose (A) or non-entrapped [U-^{14}C]sucrose (B), both approx. 10μg, were added to the liver homogenate immediately prior to centrifugation. The results are the mean of two experiments (the range is shown).

All experiments utilized 0.3M sucrose, 5mM MOPS pH 7.4, 5mM EGTA.

Time after Injection (h)	Fracn	% Protein	% Radioactivity	% N-Ac-β-glucosaminidase	% Arylsulphatase
2	N	28.4 ± 6.3	8.0 ± 0.6	21.3 ± 0.7	12.1 ± 0.6
	M	16.2 ± 1.3	15.9 ± 1.2	28.3 ± 0.6	27.1 ± 0.8
	L	8.6 ± 0.3	14.0 ± 3.3	39.3 ± 0.6	45.3 ± 2.2
	P	11.7 ± 1.6	19.0 ± 2.6	9.5 ± 0.1	8.8 ± 0.1
	S	35.0 ± 3.7	43.0 ± 6.8	1.6 ± 1.1	6.7 ± 0.8
		(3)	(3)	(3)	(3)
48	N	40.4 ± 2.9	88.4 ± 2.7	29.1 ± 5.3	15.5 ± 3.3
	M	10.0 ± 0.9	4.0 ± 0.5	20.7 ± 1.7	23.7 ± 1.3
	L	6.6 ± 0.4	5.6 ± 1.2	39.7 ± 2.0	46.8 ± 1.3
	P	10.3 ± 0.8	2.3 ± 1.0	8.9 ± 0.9	8.5 ± 0.7
	S	32.7 ± 3.0	79.6 ± 3.0	1.7 ± 1.0	5.4 ± 0.8
		(4)	(4)	(4)	(4)
A	N	41.2 (40.9 - 41.5)	1.9 (1.8 - 2.0)	26.1 (24.6 - 27.5)	8.7 (6.4 - 10.9)
	M	9.3 (8.4 - 10.2)	4.0 (2.6 - 5.3)	21.9 (20.6 - 23.2)	25.4 (25.2 - 25.6)
	L	6.6 (6.1 - 7.0)	5.1 (3.8 - 6.3)	44.6 (44.3 - 44.8)	54.8 (48.7 - 60.9)
	P	9.4 (9.2 - 9.5)	10.9 (10.6 - 11.1)	7.0 (6.4 - 7.6)	8.8 (8.3 - 9.2)
	S	33.6 (33.3 - 33.8)	78.3 (75.8 - 80.8)	0.5 (0 - 1.0)	5.5 (5.1 - 5.9)
		(2)	(2)	(2)	(2)
B	N	37.6 (33.5 - 41.6)	1.1 (1.0 - 1.1)	23.1 (22.7 - 23.5)	9.1 (7.2 - 10.9)
	M	14.0 (12.1 - 15.9)	0.5 (0.2 - 0.7)	22.1 (15.7 - 29.1)	25.1 (19.6 - 30.5)
	L	7.8 (7.4 - 8.1)	0.5 (0.4 - 0.5)	47.7 (44.0 - 51.3)	49.6 (44.5 - 54.6)
	P	9.1 (8.0 - 10.1)	1.6 (1.4 - 1.7)	6.9 (4.2 - 9.6)	8.6 (7.8 - 10.3)
	S	31.7 (30.9 - 32.4)	96.5 (96.4 - 96.5)	0	7.3 (6.3 - 8.2)
		(2)	(2)	(2)	(2)

Figure 8.4 The subcellular distributions of [U-^{14}C] sucrose, N-acetyl-β-D-glucosaminidase and arylsulphatase in rat liver following the injection of liposome-entrapped [U-^{14}C] sucrose

Data of Table 8.4.


[U-14C]sucrose
N-Ac-β-Gase
Aryl-Sase
2h
48h
A
B
Relative specific activity
0
2
4
6
8
0
40
80
Percentage of total protein

Two types of control experiments were carried out with [U-^{14}C]sucrose, both involving the addition of the radiolabel to the liver homogenate. In the first (A of Table 8.4 and Figure 8.4), DPPC/DPPA liposome entrapped [U-^{14}C]sucrose was added to the homogenate in an experiment similar to control B using ^{125}I-labelled PVP (Table 8.3 and Figure 8.3). The majority of the ^{14}C-radioactivity was localized in the S fraction, with some in the P fraction, indicating that this radioactivity is less sedimentable than ^{125}I-labelled PVP under similar conditions. In the second experiment (B of Table 8.4 and Figure 8.4), non-entrapped [U-^{14}C]sucrose was added to the homogenate, and was found to be localized almost exclusively in the S fraction. The two control experiments show that [U-^{14}C]sucrose behaves similarly to ^{125}I-labelled PVP when added directly to liver homogenate, although it is more non-sedimentable, owing to the small size of the sucrose molecule in comparison to PVP.

It is difficult to deduce the course of events following the injection of DPPC/DPPA liposomes containing [U-^{14}C]sucrose into rats. The evidence of earlier Chapters (6 and 7) suggests that DPPC/DPPA liposomes are indistinguishable whether they contain ^{125}I-labelled PVP or [U-^{14}C]sucrose. If this is so, one might reasonably expect the initial stages of uptake by cells to be identical. Since the subcellular location of [U-^{14}C]sucrose is different from that of ^{125}I-labelled PVP 2h after the injection of entrapped radiolabel, something must have happened prior to this time, but after uptake by the cells, that would have different effects on sucrose and PVP. If the uptake of the liposomes was by phagocytosis, they would end up in secondary lysosomes, and would then be in the presence of lysosomal lipases. The ordered nature of the DPPC/DPPA bilayers probably makes them very difficult to digest, and so the integrity of the liposomes would diminish only slowly, but eventually the bilayers would be disrupted and the liposomes degraded.

The sucrose molecule might behave differently from PVP during these events,

since it is considerably smaller than PVP, and would presumably be able to escape from liposomes which were only slightly digested, long before the damage was sufficient to allow the passage of PVP through the bilayers. It is much more difficult to envisage differences in the treatment of PVP and sucrose once they are free in the lysosomes. PVP is of course indigestible, but so is sucrose. PVP is a large molecule and would not be expected to cross the lysosomal membrane, but neither can sucrose. Nevertheless, it is likely that the indigestibility and inpenetrability of sucrose are not as absolute as PVP. It is also likely that the lysosomal glycosidases have some residual activity towards sucrose, so that sucrose can escape from the lysosomes after its hydrolysis.

It is worth mentioning at this stage the results of the one experiment where subcellular fractionation of rat liver was carried out 2h after the injection of non-entrapped [U-^{14}C]sucrose. Of the 1.27% recovered in the liver, the following distribution was found: N, 6.5%; M, 14.2%, L, 7.2%; P, 2.5%; S, 69.7%. In contrast, in the same experiment using unentrapped ^{125}I-labelled PVP, only 37.3% of the hepatic radioactivity was found in the S fraction. If sucrose can only enter the liver cells by pinocytosis, this result reflects an ability of the radiolabelled sucrose to escape from the lysosomes in some way.

8.3.3 The subcellular location of injected [^{35}S]cystine

Table 8.5 and Figure 8.5 show the results of experiments where subcellular fractionation of the liver was carried out following the injection of [^{35}S]cystine into rats. The radiolabel was supplied as [^{35}S]cystine dihydrochloride (see Section 2.10), but this material was diluted, neutralized and injected as 10µg/kg body weight in a saline solution, and was thus probably injected as the free amino acid. Three types of experiment were performed using [^{35}S]cystine.

Negatively charged (DPPA/DPPC) liposomes cannot retain cystine at

Table 8.5 The recovery of protein, [^{35}S] cystine, N-acetyl-β-D-glucosaminidase and arylsulphatase in subcellular fractions of rat liver following the injection of liposome-entrapped [^{35}S] cystine

2h and 48h. Multilamellar DPPC/DPPA liposome-entrapped [^{35}S] cystine (approx. 10μg/ml) was injected into rats (see Section 2.7) to a concentration of 10μg/kg body weight. After the time shown, the rat was sacrificed and the liver subjected to the scheme of subcellular fractionation described in Chapter 5. The results are the mean ± S.E.M.

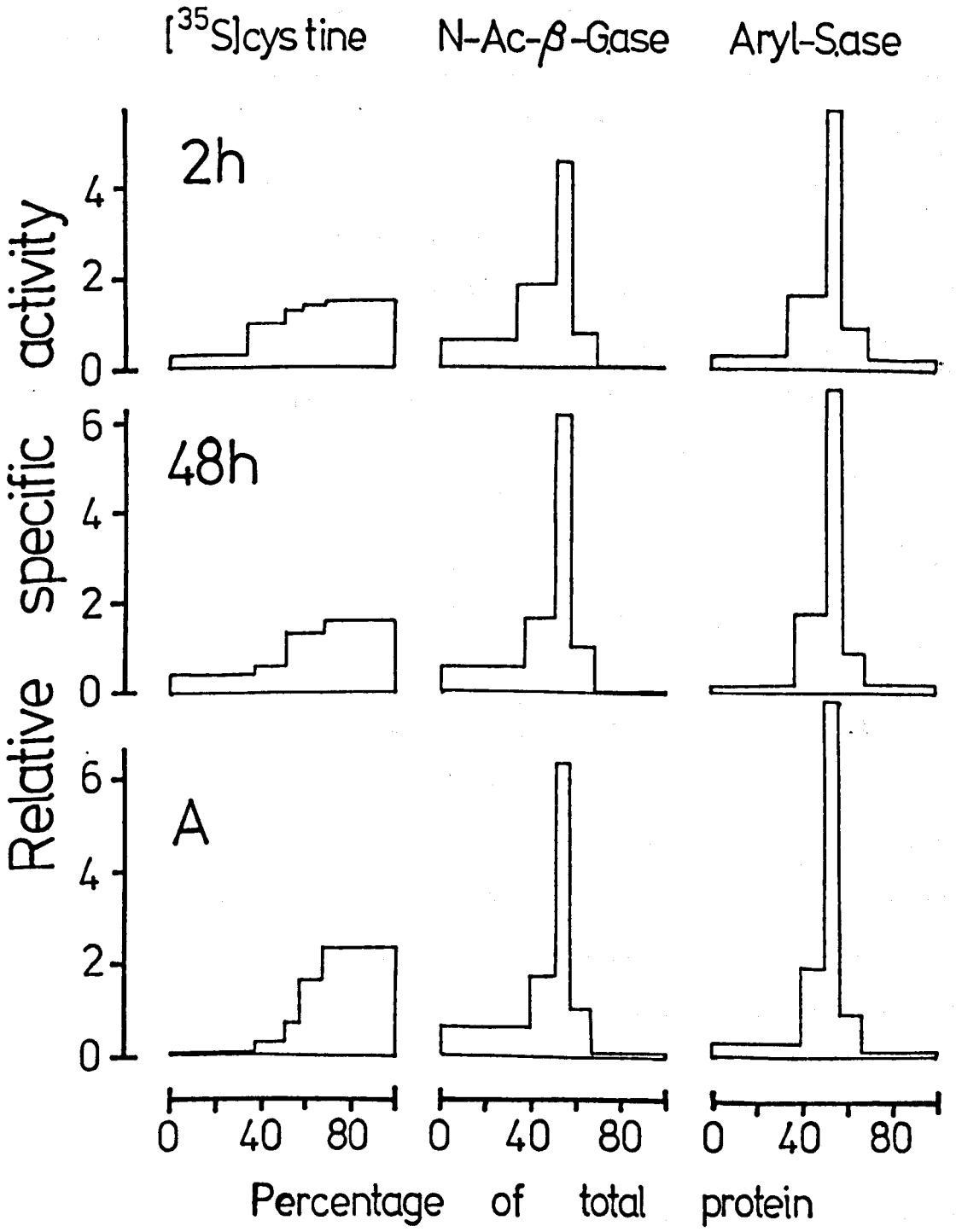
A. Multilamellar DPPC/DPPA liposome-entrapped [^{35}S] cystine (1.5μg) added to the liver homogenate immediately prior to centrifugation. The results are the mean of two experiments (the range is shown).

All experiments utilized 0.3M sucrose, 5mM MOPS, 5mM EGTA.

Time after Injection (h)	Frac^{n}	% Protein	% Radioactivity	% N-Ac-β-glucosaminidase	% Arylsulphatase
2	N	33.9 ± 1.4	11.3 ± 0.8	22.8 ± 1.8	9.6 ± 3.1
	M	16.9 ± 1.7	16.7 ± 2.5	31.5 ± 3.7	27.3 ± 3.1
	L	8.1 ± 0.4	10.7 ± 0.8	36.8 ± 3.9	46.0 ± 5.0
	P	10.7 ± 0.8	15.6 ± 2.4	8.1 ± 1.2	10.0 ± 1.5
	S	30.3 ± 2.4	45.6 ± 5.5	0.9 ± 0.5	7.2 ± 1.2
		(3)	(3)	(3)	(3)
48	N	37.0 ± 1.0	17.5 ± 1.7	21.6 ± 1.0	9.5 ± 1.2
	M	14.1 ± 0.5	9.4 ± 1.7	23.2 ± 2.3	25.3 ± 1.9
	L	7.3 ± 0.5	9.6 ± 1.6	45.0 ± 1.9	49.4 ± 2.2
	P	9.8 ± 0.4	12.7 ± 4.8	9.7 ± 0.6	9.4 ± 0.8
	S	31.7 ± 1.0	50.8 ± 6.1	0.4 ± 0.4	6.4 ± 0.7
		(3)	(3)	(3)	(3)
A	N	38.4 (37.7 - 39.1)	1.5 (1.4 - 1.6)	25.6 (20.7 - 28.0)	11.0 (8.6 - 13.3)
	M	12.9 (12.0 - 13.7)	3.8 (2.5 - 4.0)	22.1 (20.7 - 23.4)	24.7 (24.6 - 24.7)
	L	6.7 (6.5 - 6.9)	4.6 (3.7 - 5.4)	42.1 (40.7 - 43.5)	51.6 (50.0 - 53.1)
	P	9.5 (9.3 - 9.6)	15.0 (14.9 - 15.1)	9.2 (9.1 - 9.3)	9.2 (8.0 - 10.3)
	S	32.6 (30.7 - 34.5)	75.7 (73.9 - 77.5)	1.3 (1.3 - 1.3)	3.7 (3.4 - 4.0)
		(2)	(2)	(2)	(2)

Figure 8.5 The subcellular distributions of [^{35}S]cystine, N-acetyl-β-D-glucosaminidase and arylsulphatase in rat liver following the injection of liposome-entrapped [^{35}S]cystine

Data of Table 8.5.


[35S]cystine
N-Ac-β-Gase
Aryl-Sase
2h
48h
A
Relative specific activity
0
2
4
6
0
40
80
Percentage of total protein

37°C *in vitro* (see Section 6.2.4), and the tissue distribution of such liposomes in the rat leads one to believe that they do not remain intact for very long *in vivo* (Section 7.3.3). The subcellular distributions of protein, radiolabel and the two marker enzymes 2h after the injection of DPPC/DPPA liposome-entrapped [^{35}S]cystine were very similar to those found when entrapped [U-^{14}C]sucrose was injected. The recovery of N-acetyl-β-D-glucosaminidase, and to a lesser extent, arylsulphatase, was increased in the M fraction, at the expense of the L fraction. After a further 46h, most of the remaining radioactivity is recovered in the S fraction, and the enzyme distributions are normal, again a similar result to that obtained using entrapped [U-^{14}C]sucrose.

In the control experiment, DPPC/DPPA liposomes containing [^{35}S]cystine were added directly to the liver homogenate. Three quarters of the radiolabel was recovered in the S fraction, a result similar to that of the equivalent experiments using liposome-entrapped [U-^{14}C]sucrose.

In view of the similarity of the subcellular distributions between experiments using entrapped [U-^{14}C]sucrose and entrapped [^{35}S]cystine, a similar explanation can be proposed. The uptake of these liposomes by the cells could involve the same mechanism that operates in the uptake of the DPPC/DPPA liposome-entrapped ^{125}I-labelled PVP. After uptake into the lysosomal system, the [^{35}S]cystine leaks into the lysosomal interior, and then escapes into the cytosol. The escape of cystine from the lysosomes might be by one of several possible routes, which have been discussed in Chapter 1 (see Figure 1.1). If the cystine could not escape from the lysosomes, it would presumably show a similar distribution to ^{125}I-labelled PVP.

8.3.4 Summary

Evidence has been presented that is compatible with the entry of (DPPC/DPPA) negatively-charged liposomes by a phagocytic mechanism into liver cells

of the rat. The 41.0% of DPPC/DPPA liposome-entrapped ^{125}I-labelled PVP recovered in the liver M and L fractions 2h after injection compares favourably with a figure of 43.1% given by Gregoriadis and Ryman (1972) for β-fructofuranosidase entrapped within negatively charged (egg PC/ cholesterol/PA) liposomes recovered in a mitochondrial-lysosomal (M + L) fraction after a similar time. After 24h in the experiments reported here, 55.3% of the liver ^{125}I-radioactivity was in the M and L fractions, compared with 34.4% of the liver β-fructofuranosidase after 21h (Gregoriadis and Ryman, 1972), although some of the enzyme may have been digested in this time. In a similar study using liposome-entrapped neuraminidase, Gregoriadis et al. (1974b) found 60 - 69% of the total in the liver localized in the lysosomal fraction 5h after injection.

In a more recent survey (Steger and Desnick, 1977), the distributions of liposomally-entrapped bovine β-glucuronidase in N, M + L, and supernatant fractions of mouse liver were reported. The experiments were complicated by the necessity to distinguish between the endogenous and the exogenous β-glucuronidase. When entrapped within negatively-charged (DPPC/cholesterol/PA) MLV, approx. 75% of the exogenous enzyme in the liver was recovered in the M + L fraction, and approx. 20% in the S fraction, after both 1h and 24h. For positively charged liposomes (DPPC/ cholesterol/stearylamine) the figures were approx. 45% in the M + L and 45% in the S fractions after 1h. When the liposomes were added to the liver homogenate, 88% of the negatively charged liposomes were recovered in the supernatant. These results are in general agreement with those of this Chapter.

Steger and Desnick (1977) also examined the distributions of a series of endogenous lysosomal hydrolases. The mean percentage of α-galactosidase in the M + L fraction was reduced 1h after injection of negatively or positively charged liposomes, but the M + L recoveries of β-glucosidase and α-mannosidase were both increased. The other enzymes studied

(including arylsulphatase) showed no change at this time. The authors did, however, notice a more long-term increase in the supernatant levels of the enzymes 1-3 days after the injection of positively-charged liposomes, and suggested these were due to a deleterious effect of stearylamine on the stability of the lysosomal membrane. Steger and Desnick did not examine the enzyme distributions 2h after injection, and so it is difficult to draw any conclusions as to the mode of entry of the liposome-entrapped β-glucuronidase into the cells. There is little doubt that the material was eventually localized in the lysosomes.

From the present data it is impossible to ascertain which types of liver cell are involved in the uptake of the liposomes. Hepatocytes make up around 60% of the number of liver cells, the remainder of non-parenchymal cells being Kupffer cells (approx. 15 - 20%) and others (Berg and Boman, 1973; Munthe-Kaas et al., (1976). In terms of volume the respective figures are: hepatocytes, 78%, Kupffer cells 2.1% (Blouin et al., 1977). Kupffer cells are likely to account for the bulk of uptake of injected vesicles (Hoekstra et al., 1978) because of their high endocytic activity, and also because of their position in the liver sinusoids.

The Kupffer cell lysosomes account for approx. 25% of the total lysosomal volume (Blouin et al., 1977). Munthe-Kaas et al. (1976) have estimated that the specific activities of arylsulphatase and N-acetyl-β-D-glucosaminidase are 7 and 4 times higher respectively in the nonparenchymal cells than in the hepatocytes. Recently, Kooistra (1979) has made use of the widely different distributions within the liver of two lysosomal enzymes in an attempt to pinpoint the cell type involved in the uptake of non-entrapped ^{125}I-labelled PVP. By measuring the distributions of acid DNAase (almost 30% of this enzyme is found in non-parenchymal cells) and β-glucerophosphatase (mainly in hepatocytes), he was able to infer from the differences in subcellular distribution of the enzymes that the

majority of the radioactivity was located in the Kupffer cells.

A reevaluation of the data of this Chapter was attempted, in the light of the work of Kooistra (1979), but the differences between the locations of the two enzymes studied were not sufficient (see Munthe-Kaas et al., 1976) to determine which of the two cell types was involved in the uptake of non-entrapped ^{125}I-labelled PVP. If the uptake of materials involves only the Kupffer cells, it may nevertheless affect the overall lysosomal enzyme distribution, but the present data do not allow a confident prediction of the cell type involved. Further experiments utilizing either the separation of different cell types immediately prior to centrifugation or the technique of Kooistra may be required before any progress can be made in this direction.

CHAPTER 9

The Release of ^{125}I-labelled PVP from rat liver lysosomes *in vitro*, following the injection of liposome-entrapped ^{125}I-labelled PVP

9.1 Introduction

As explained in Chapter 1, there are only three techniques at present available for measuring the permeability of the lysosome membrane to small substances. The first of these, the osmotic protection method, is somewhat limited in its usefulness by the need to prepare a 0.25M solution of the test substance. Using this method, the permeability is deduced from the degree of lysosome disruption caused by permeation of the substance into the lysosomes. The two techniques that depend upon estimating the ability of substances to escape from lysosomes are both limited in the range of substances for which they can be used. The method used by Cohn and Ehrenreich (1969) relates gross changes in lysosome morphology of cells in culture to the pinocytic uptake of non-permeant substances. Mego and McQueen (1965) while investigating the digestion of ^{131}I-labelled albumin by rat liver secondary lysosomes, incidentally provide evidence that iodotyrosine (the major radiolabelled digestion product) and possibly dipeptides containing iodotyrosine can, but that radiolabelled albumin cannot escape from intact secondary lysosomes. The radiolabelled albumin could be regarded as a carrier for iodotyrosine in these experiments and, obviously, such a technique would have much potential in elucidating the permeability properties of lysosomes, although this has not been pursued. The major difficulty with this technique applied to the investigation of lysosome membrane permeability to small molecules is in loading the lysosomes with enough of the material to enable examination to be feasible.

Small molecules are difficult to direct specifically to the lysosomes, because they can enter cells by routes (diffusion and active transport) that are unavailable to macromolecules. Also, there is little, if any adsorptive pinocytosis (and therefore little uptake into lysosomes) of small molecules. The use of liposomes to localize small substances specifically within the lysosomes of rat liver offers a significant advance in the procedures available for the study of lysosome permeability,

because they provide a means of making cells treat small molecules as if they were macromolecules.

9.2 Experimental procedure

Negatively-charged (DPPC/DPPA) liposomes containing entrapped ^{125}I-labelled PVP were prepared as in Section 2.2. The liposomes were injected into rats (see Section 2.7) to a concentration of 50µg ^{125}I-labelled PVP/kg body weight. These rats were starved 18h before sacrifice, when a lysosome-enriched fraction of the liver was prepared as in 2.1.1. The two pellets prepared from one liver were gently resuspended, one in 0.25M sucrose and the other in 0.25M glucose (both 25ml), and then each was incubated in a water bath at 25^{o}C.

Immediately, and after 1, 2 and 4h, duplicate 2ml samples were removed from each suspension, and centrifuged at 4^{o}C in an MSE Superspeed 50 centrifuge at 150,000g x 30min (50,000rpm; rotor no. 59113). The supernatant was assayed for arylsulphatase activity, and 1ml was taken for the estimation of ^{125}I-radioactivity (see 2.6.1), both being termed the 'non-sedimentable activity'. At the same times, the free and total arylsulphatase activity of the suspension was also measured. At the start of the incubation, duplicate 1ml samples were taken for the estimation of total ^{125}I-radioactivity (see 2.6.1). Arylsulphatase was measured according to the method described in Section 2.3.3. The percentage of non-sedimentable radioactivity, non-sedimentable arylsulphatase and free arylsulphatase were plotted versus time for each suspension.

9.3 Results and Discussion

In the first, exploratory experiments, some of the conditions were altered from those described in 9.2, which were derived after considering the early results.

Several things were different in the first experiment, in which the rat was killed 2h after the injection of the liposomes. Only the percentage non-sedimentable radioactivity was followed during the incubation; enzyme activities were not measured. The non-sedimentable fraction was a 210,000g x min supernatant (13,500rpm x 10min, MSE 18 refrigerated centrifuge, rotor no. 43114-106) from the lysosome-enriched suspension. Also the incubation temperature was 37°C. After 1h incubation 21.0% of the radiolabel from the suspension in sucrose and 27.8% of that from the suspension in glucose had become non-sedimentable. The initial non-sedimentable radioactivities for the same suspensions were 18.4% and 12.9% respectively. The increase in the non-sedimentability of radiolabel in sucrose is very little: understandably so, since the lysosomes would remain intact. In glucose, the rise over 1h. was only approx. 15% of the total radioactivity, under conditions in which probably the majority of lysosomes would break within 1h. The low rise in non-sedimentable radioactivity may have been because the ^{125}I-labelled PVP was still entrapped within liposomes (only 2 - 3h after injection), and was still sedimentable. This sedimentability could not have been wholly a property of the liposomes themselves since, under the same conditions, approx. 55% and approx. 48% respectively of liposomes added directly to the suspensions were non-sedimentable. It may be that although the structural integrity of the lysosomes was diminished the liposomes were in some way bound to the lysosomes, so that they sedimented with the lysosomal membranes during the centrifugation. In an attempt to investigate this explanation, in the second experiment, the liposomes were injected 24h before death,

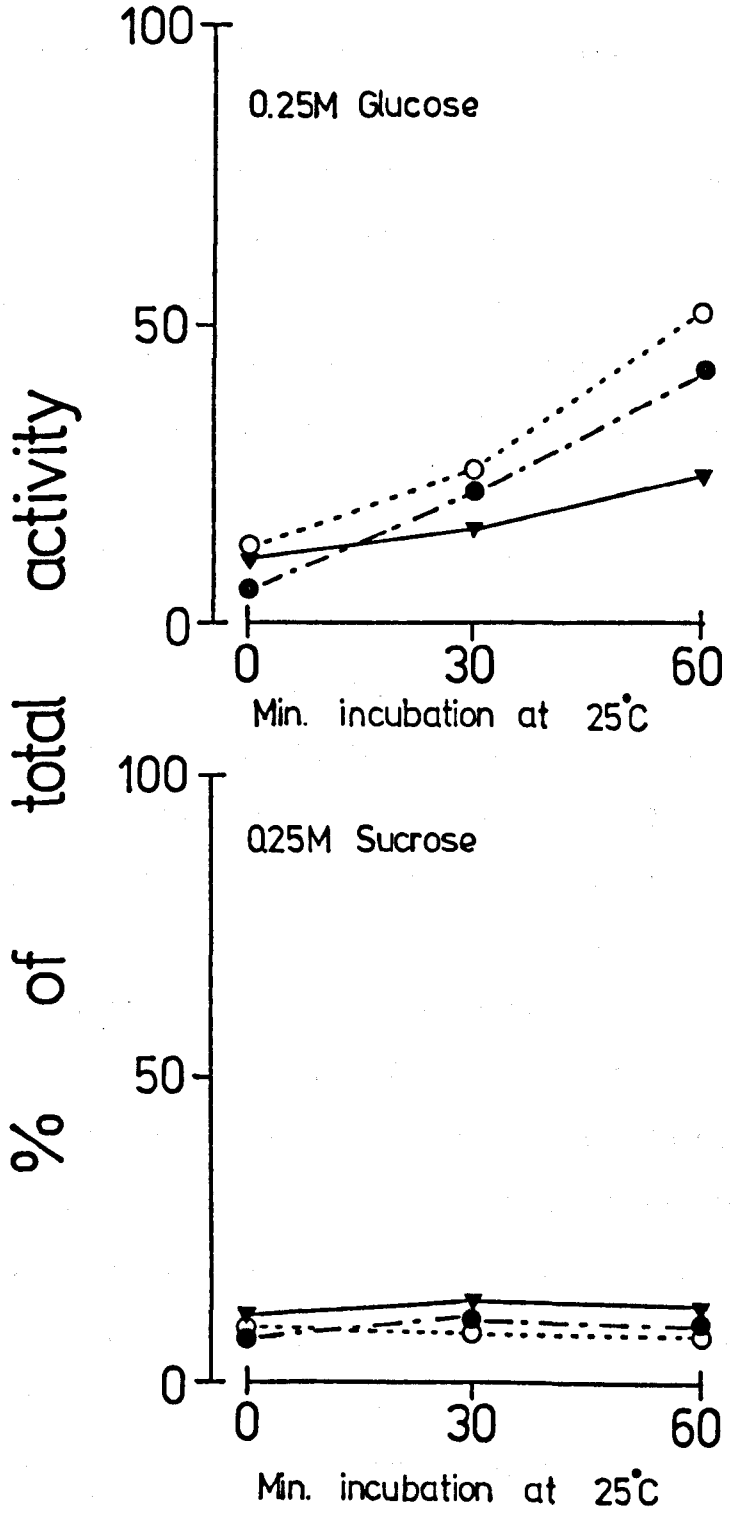
after which time it was hoped that the liposomes would have been digested by the lysosomal lipases.

The result of this experiment is shown in Figure 9.1. The incubation was carried out at 25°C to make the experiment (and the following ones) directly comparable with those of Chapter 3. Figure 9.1 shows clearly that during incubation in 0.25M sucrose there is no significant rise in the free or non-sedimentable enzyme activity, or in the non-sedimentable radioactivity. This indicates first, that the lysosomes remain stable in this solution and, secondly, that no ^{125}I-labelled PVP is escaping from the lysosomes. In 0.25M glucose there is a progressive rise in both the free and non-sedimentable aryl-sulphatase activities during the incubation, showing that the lysosomes were not stable in this solution. The non-sedimentable enzyme activity is always slightly less than the corresponding free activity since, while the latter is a measure of lysosome disruption, the former shows the degree of dissociation of the enzyme from the lysosomes. The non-sedimentable radioactivity also rose progressively, but reached only 25.5% after 1h incubation, compared with the percentage non-sedimentable enzyme activity of 58.8%. These data are insufficient to enable a definite conclusion to be drawn, since there are at least two possible explanations for the observed non-sedimentable radioactivity in 0.25M glucose. Certainly, at least 25.5% of the ^{125}I-labelled PVP is dissociated from the lysosomes after 1h incubation, but there is no indication as to whether or not this radiolabel is still liposome-entrapped after it has escaped from the lysosomes. It is just possible that more than 25% of the radiolabel escaped from the lysosomes, but that some of this was still in a sedimentable form, and so would have been detected as a lysosome-associated radioactivity. However, a more likely explanation is that the ^{125}I-labelled PVP is unable to escape from lysosomes as readily as arylsulphatase, either because the enzyme is smaller than

Figure 9.1 Release of arylsulphatase and ^{125}I-labelled PVP from lysosome-enriched suspensions in 0.25M glucose and 0.25M sucrose, 24h after the injection of liposome-entrapped ^{125}I-labelled PVP into rats

A rat was injected (see Section 2.7) with multilamellar DPPC/DPPA liposome-entrapped ^{125}I-labelled PVP (50µg/kg body weight) and, after 24h, a lysosome-enriched fraction of the liver was isolated (see Section 2.1.1), suspended in either 0.25M glucose or 0.25M sucrose and incubated at 25°C. At the times shown, a 210,000g x min supernatant was isolated, and the free and non-sedimentable arylsulphatase (Section 2.3.3) and non-sedimentable radioactivity (Section 2.6.1) measured. One experiment only.

KEY


100
0.25M Glucose
50
0
0
30
60
Min. incubation at 25°C
100
0.25M Sucrose
50
0
0
30
60
Min. incubation at 25°C
% of total activity

^{125}I-labelled PVP, or because the radiolabel is still liposome-entrapped.

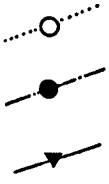
In a control experiment (Figure 9.2), the liposome-entrapped ^{125}I-labelled PVP was added directly to the lysosome-enriched suspension at the start of the incubation. Again, in 0.25M sucrose, the free and non-sedimentable enzyme activities remained low, while in 0.25M glucose they rose progressively during the incubation. The differences between the increases in enzyme activities in 0.25M glucose observed in this experiment and the one before are probably due to experimental variation. The non-sedimentable ^{125}I-radioactivity remained between 65% and 75% of the total radioactivity throughout the incubation, showing that the liposomes did not behave differently in the two solutions. The non-sedimentable nature of the liposomes in this experiment means that one can only speculate on the form (unentrapped or liposome-entrapped) of the ^{125}I-labelled PVP which became non-sedimentable in 0.25M glucose in the previous experiment (Figure 9.1). Had the liposome-entrapped ^{125}I-labelled PVP been largely sedimentable, one could have fairly confidently concluded that the radiolabel which became non-sedimentable in 0.25M glucose was not liposome-entrapped.

Three further alterations were made to the procedure to arrive at that described in Section 9.2. First, in an effort to reduce further the possibility of any intact liposomes remaining within the secondary lysosomes, the animal was killed 48h after the injection of the liposomes. As shown in Table 7.1, there is little reduction in the amount of ^{125}I-radioactivity in the liver after this time, and Table 8.2 and Figure 8.2 show that most of this radioactivity is localized in the M and L fractions. (The combined M and L fractions are approx. the same as the lysosome-enriched fraction used in this Chapter). To increase the sedimentability of the liposome-entrapped radioactivity, the centrifugation speed was increased to 150,000g x 30min (see Section 9.2). Finally, the incubation period was extended so that the suspensions were monitored over 4h.

Figure 9.2 Release of arysulphatase from lysosome-enriched suspensions in 0.25M glucose and 0.25M sucrose in the presence of liposome-entrapped ^{125}I-labelled PVP

A lysosome-enriched fraction of rat liver was prepared as described in Section 2.1.1, suspended in either 0.25M glucose or 0.25M sucrose and incubated at 25°C. At the beginning of the incubation, multilamellar DPPC/DPPA liposome-entrapped ^{125}I-labelled PVP (approx. 2µg) was added to each suspension (an amount equal to the radioactivity present in the lysosome-enriched suspensions prepared 24h after the injection of multilamellar DPPC/DPPA liposome-entrapped ^{125}I-labelled PVP, Figure 9.1). Centrifugations and assays are as for Figure 9.1. One experiment only.

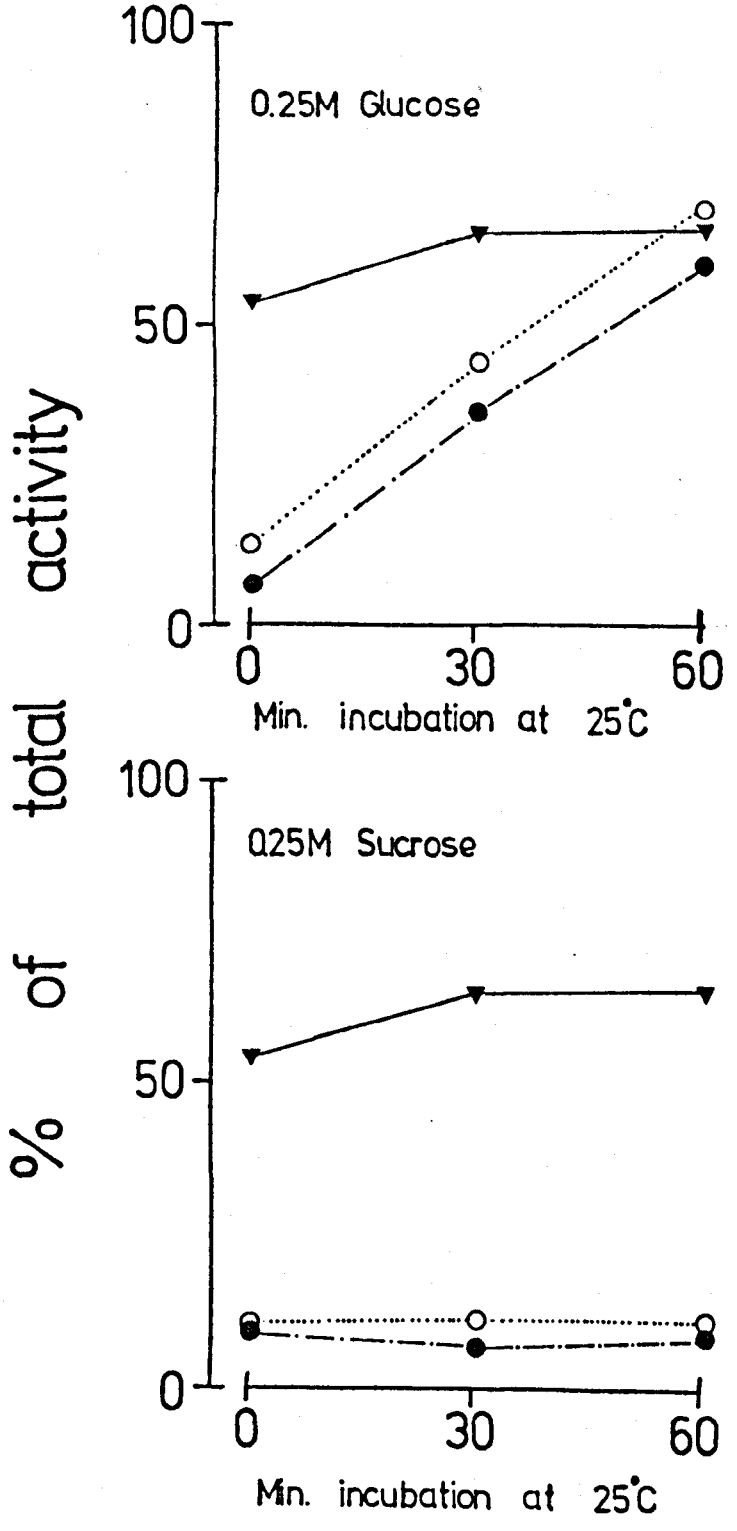
KEY



Free arylsulphatase activity

Non-sedimentable arylsulphatase activity

Non-sedimentable ^{125}I-labelled PVP


100
0.25M Glucose
50
0
0
30
60
Min. incubation at 25°C
% of total activity
100
0.25M Sucrose
50
0
0
30
60
Min. incubation at 25°C

When liposome-entrapped ^{125}I-labelled PVP was injected 48h before sacrifice, and the lysosome-enriched suspension then prepared was incubated at 25°C for 4h in 0.25M sucrose, there was little or no increase in any of the measured activities (see Figure 9.3). This indicates that, first, the lysosomes remain intact over the 4h incubation and, secondly, that there is no release of radiolabel from the intact lysosomes. However, since it is not known whether the ^{125}I-labelled PVP is still liposome-entrapped, it is impossible to say whether the lysosomal membrane is impermeable to ^{125}I-labelled PVP from this result alone.

When the lysosome-enriched suspension is suspended in 0.25M glucose, there is a progressive rise in both the free and the non-sedimentable arylsulphatase activities, indicating breakage of lysosomes during the incubation (see Figure 9.3). The non-sedimentable enzyme activity is always much less than the free activity measured at the same time, probably because the disruption of the lysosomes, while sufficient to allow the permeation of substrate molecules, does not permit the escape of the much larger enzyme molecules. The rise in non-sedimentable arylsulphatase activity during incubation in 0.25M glucose was closely paralleled by an increase in non-sedimentable radioactivity. This indicates that release of ^{125}I-labelled PVP from the lysosomes was at least as great as the release of arylsulphatase. It is unlikely, but just possible that some or all of the released ^{125}I-labelled PVP was still liposome-entrapped; if it was, some of the released radiolabel from broken lysosomes might be sedimentable, and thus decrease the measured non-sedimentable radioactivity.

In order to establish more fully the nature of the non-sedimentable ^{125}I-labelled PVP (i.e. whether or not it is liposome-entrapped), two series of control experiments were carried out. In the first (Figure 9.4), liposome-entrapped ^{125}I-labelled PVP was added directly to the lysosome-enriched suspension from an uninjected rat at the beginning of the incubation. From Figure 9.4, it can be seen that the liposomes are

Figure 9.3 Release of arylsulphatase and ^{125}I-labelled PVP from lysosome-enriched suspensions in 0.25M glucose and 0.25M sucrose, 48h after the injection of liposome-entrapped ^{125}I-labelled PVP into rats

A rat was injected (see Section 2.7) with multilamellar DPPC/DPPA liposome-entrapped ^{125}I-labelled PVP (50µg/kg body weight) and, after 48h, a lysosome-enriched fraction of the liver was isolated (see Section 2.1.1), suspended in either 0.25M glucose or 0.25M sucrose and incubated at 25°C. At the times shown, a 150,000g x 30min supernatant was isolated, and the free and non-sedimentable arylsulphatase (Section 2.3.3) and non-sedimentable radioactivity (Section 2.6.1) measured. One experiment only. Results are mean ± S.E.M. of three experiments.

KEY

Free arylsulphatase activity

Non-sedimentable arylsulphatase activity

Non-sedimentable ^{125}I-labelled PVP

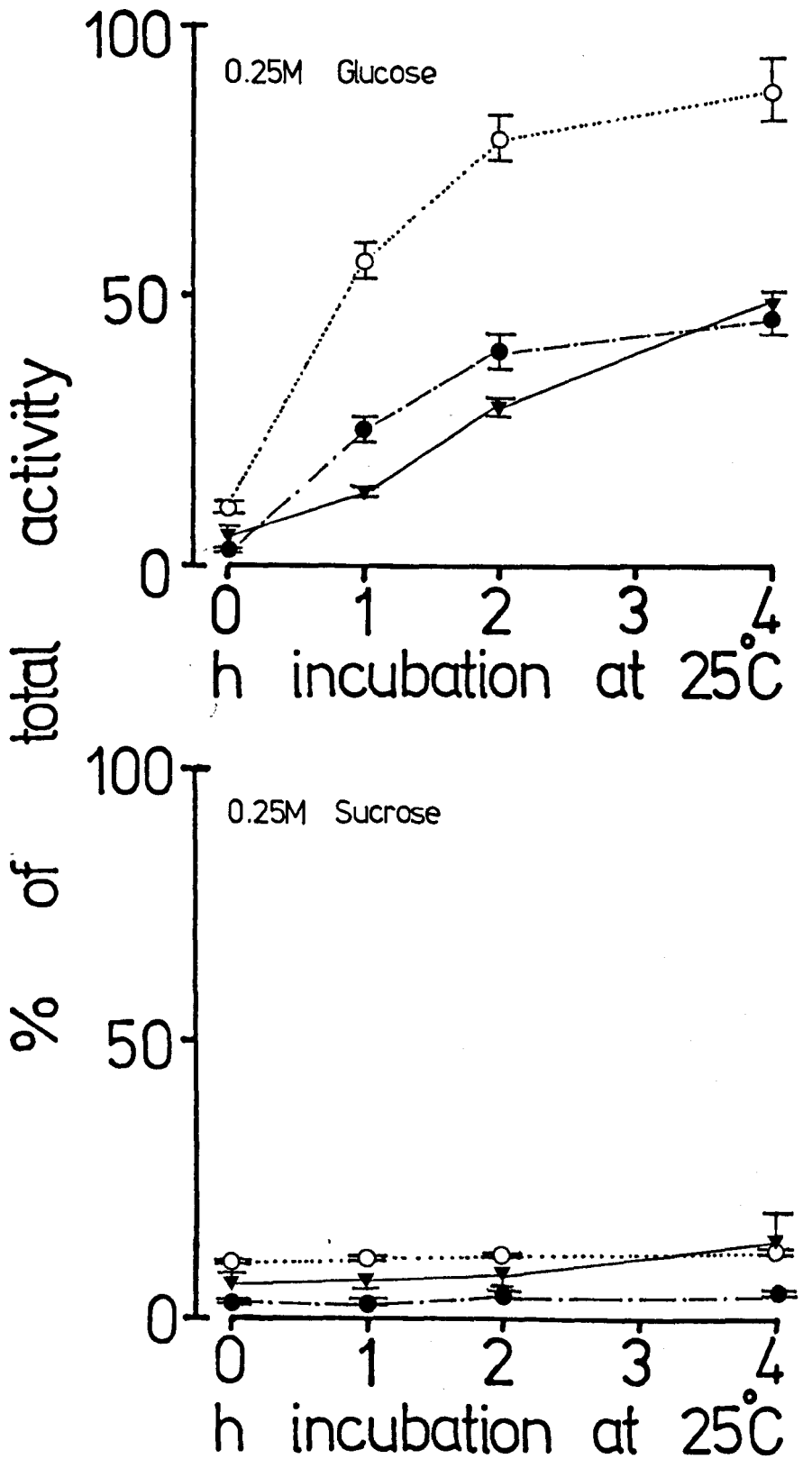

100
50
0
0.25M Glucose
% of total activity
0 1 2 3 4
h incubation at 25°C
100
50
0
0.25M Sucrose
0 1 2 3 4
h incubation at 25°C

Figure 9.4 Release of arylsulphatase from lysosome-enriched suspensions in 0.25M glucose and 0.25M sucrose in the presence of liposome-entrapped ^{125}I-labelled PVP

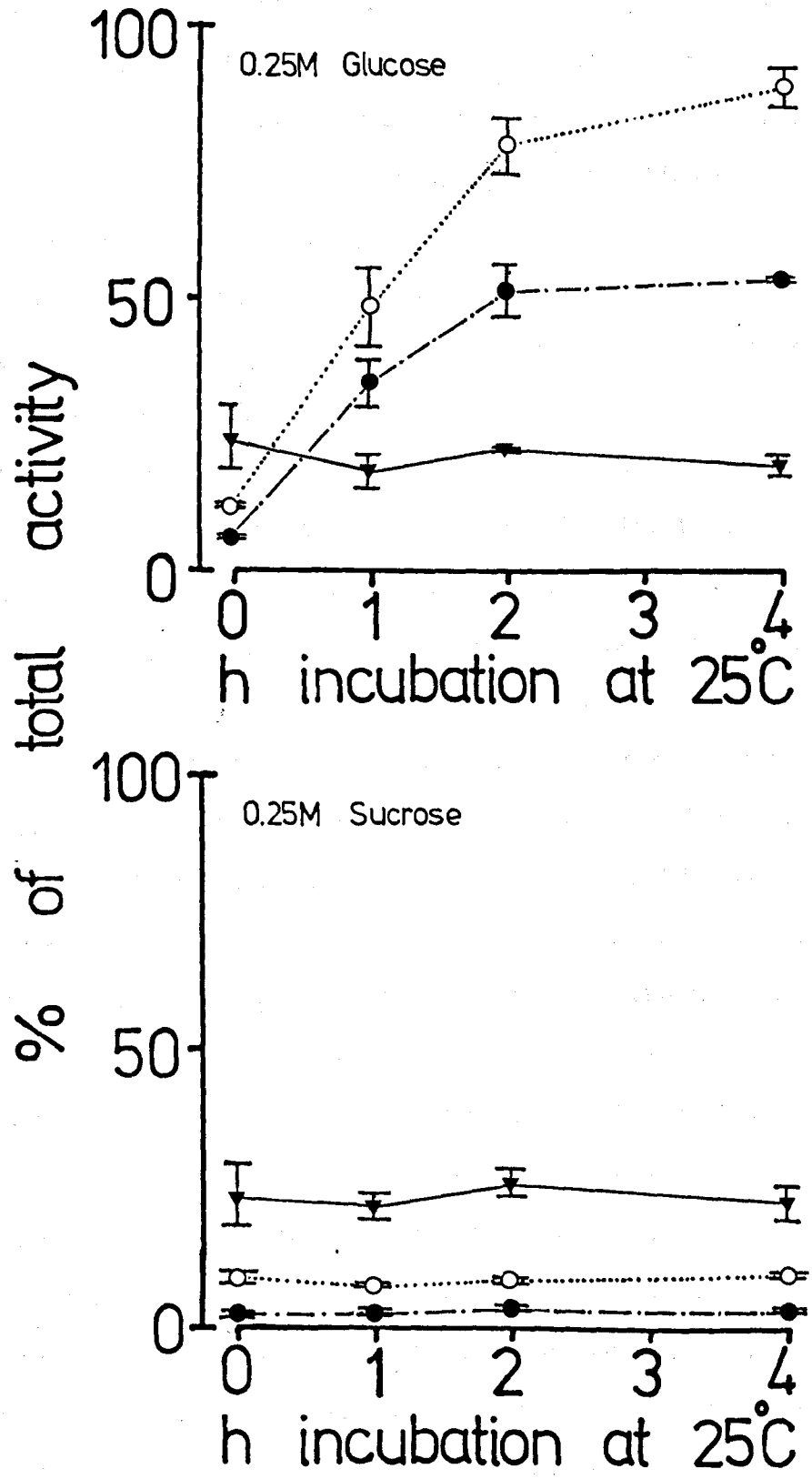
A lysosome-enriched fraction of rat liver was prepared as described in Section 2.1.1, suspended in either 0.25M glucose or 0.25M sucrose and incubated at 25°C. At the beginning of the incubation, multilamellar DPPC/DPPA liposome-entrapped ^{125}I-labelled PVP (approx. 5µg) was added to each suspension (an amount double that of the radioactivity present in the lysosome-enriched suspensions prepared 48h after the injection of multilamellar DPPC/DPPA liposome-entrapped ^{125}I-labelled PVP, Figure 9.3). Centrifugations and assays are as for Figure 9.3. Results are the mean ± S.E.M. of three experiments.

KEY

....O..... Free arylsulphatase activity

—●— Non-sedimentable arylsulphatase activity

—▼— Non-sedimentable ^{125}I-labelled PVP


100
50
0
0.25M Glucose
% of total activity
0 1 2 3 4
h incubation at 25°C
100
50
0
0.25M Sucrose
0 1 2 3 4
h incubation at 25°C

sedimentable to the same degree in both 0.25M sucrose and 0.25M glucose. Also, the non-sedimentable radioactivity remains between 18 and 27% in both solutions throughout the incubation. This presumably indicates that the liposomes remain intact and mainly sedimentable in both solutions throughout the incubation. It should also be noted that the percentages of free and non-sedimentable arylsulphatase activities observed were very similar to those found in the preceding experiments (Figure 9.3) where the liposome-entrapped radiolabel was injected 48h before the rat was killed. This implies that there is no alteration of lysosome stability as a result of the injection of the liposomes.

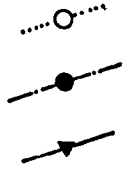
In the second series of control experiments, unentrapped ^{125}I-labelled PVP was added directly to the lysosome-enriched fraction from an uninjected rat at the beginning of the incubation. In both 0.25M sucrose and 0.25M glucose (see Figure 9.5) the non-sedimentable radioactivity was initially approx. 90%. Unexpectedly, however, this fell progressively during the incubation, so that after 4h incubation, 60% of the radioactivity in 0.25M glucose, and 78% in sucrose was non-sedimentable. The curves for free and non-sedimentable arylsulphatase activities observed in both sucrose and glucose were again similar to those of the previous experiments in which the radiolabel was injected 48h before the rat was killed. Although some of the ^{125}I-labelled PVP would become sedimentable by virtue of its location in the interstices of the pellet formed during the centrifugation, there is some sedimentability occurring in addition to this.

Finally, the experiments were carried out with a period of 2h between the injection of the liposomes and the isolation of the lysosome-enriched fraction. The results of these incubations are shown in Figure 9.6. In 0.25M sucrose there is no release of radiolabel or enzyme over the incubation period, as judged by the non-sedimentable radioactivity and the free and non-sedimentable arylsulphatase activities. This implies that the lysosomes remain substantially intact in 0.25M sucrose, and that the

Figure 9.5 Release of arylsulphatase from lysosome-enriched suspensions in 0.25M glucose and 0.25M sucrose in the presence of non-entrapped ^{125}I-labelled PVP

A lysosome-enriched fraction of rat liver was prepared as described in Section 2.1.1, suspended in either 0.25M glucose or 0.25M sucrose and incubated at 25°C. At the beginning of the incubation, non-entrapped ^{125}I-labelled PVP (approx. 2µg) was added to each suspension (an amount equal to the radioactivity present in the lysosome-enriched suspensions prepared 48h after the injection of multilamellar DPPC/DPPA liposome-entrapped ^{125}I-labelled PVP, Figure 9.3). Centrifugation and assays are as for Figure 9.3. Results are the mean ± S.E.M. of three experiments.

KEY



Free arylsulphatase activity

Non-sedimentable arylsulphatase activity

Non-sedimentable ^{125}I-labelled PVP

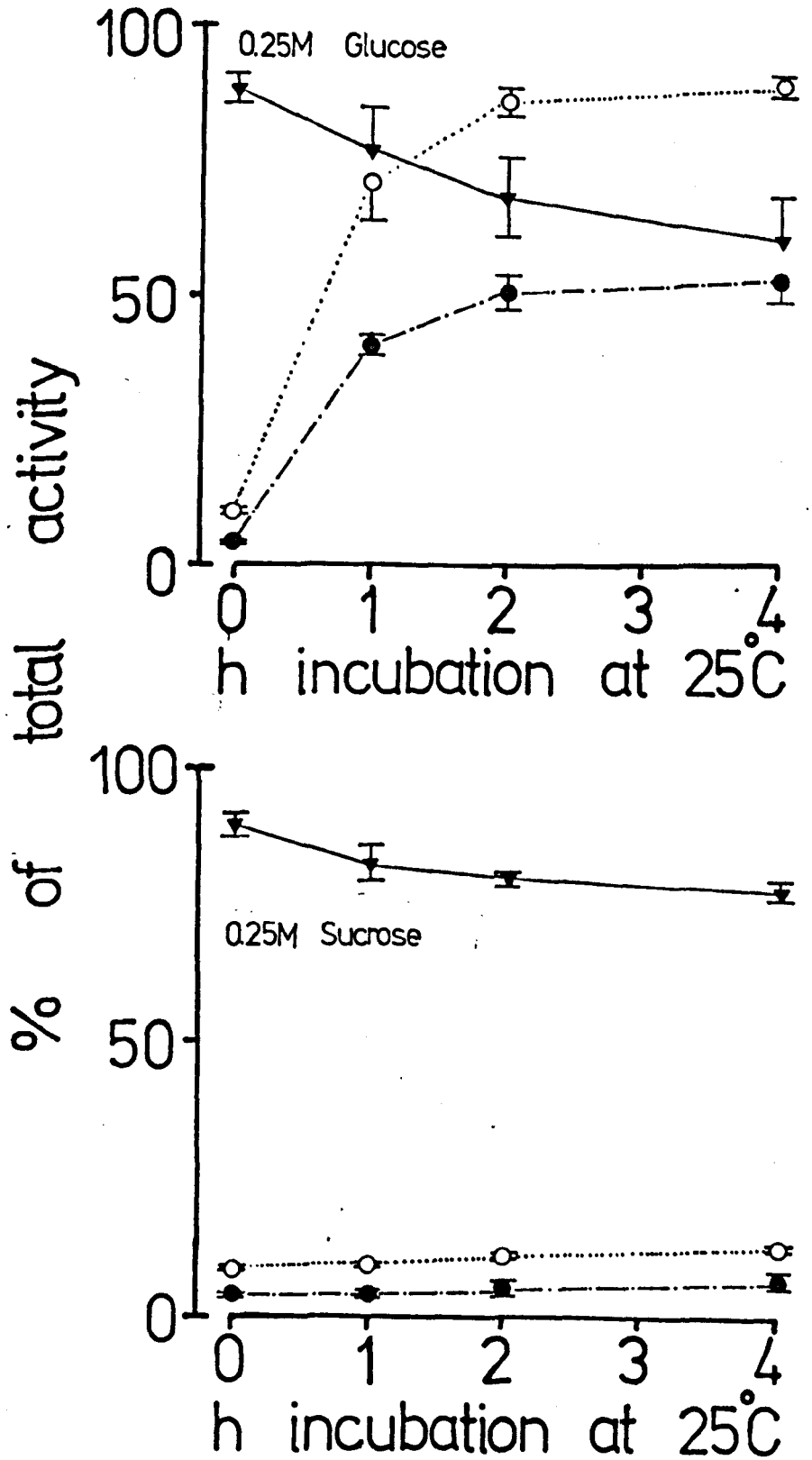
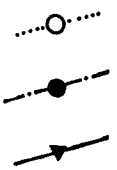

100
50
0
0.25M Glucose
% of total activity
0 1 2 3 4
h incubation at 25°C
100
50
0
0.25M Sucrose
0 1 2 3 4
h incubation at 25°C

Figure 9.6 Release of arylsulphatase and ^{125}I-labelled PVP from lysosome-enriched suspensions in 0.25M glucose and 0.25M sucrose, 2h after the injection of liposome-entrapped ^{125}I-labelled PVP into rats

A rat was injected (see Section 2.7) with multilamellar DPPC/DPPA liposome-entrapped ^{125}I-labelled PVP (50µg/kg body weight) and, after 2h, a lysosome-enriched fraction of the liver was isolated (see Section 2.1.1), suspended in either 0.25M glucose or 0.25M sucrose and incubated at 25°C. At the times shown, a 150,000g x 30min supernatant was isolated, and the free and non-sedimentable arylsulphatase (Section 2.3.3) and non-sedimentable radioactivity (Section 2.6.1) measured. One experiment only. Results are mean ± S.E.M. of three experiments.

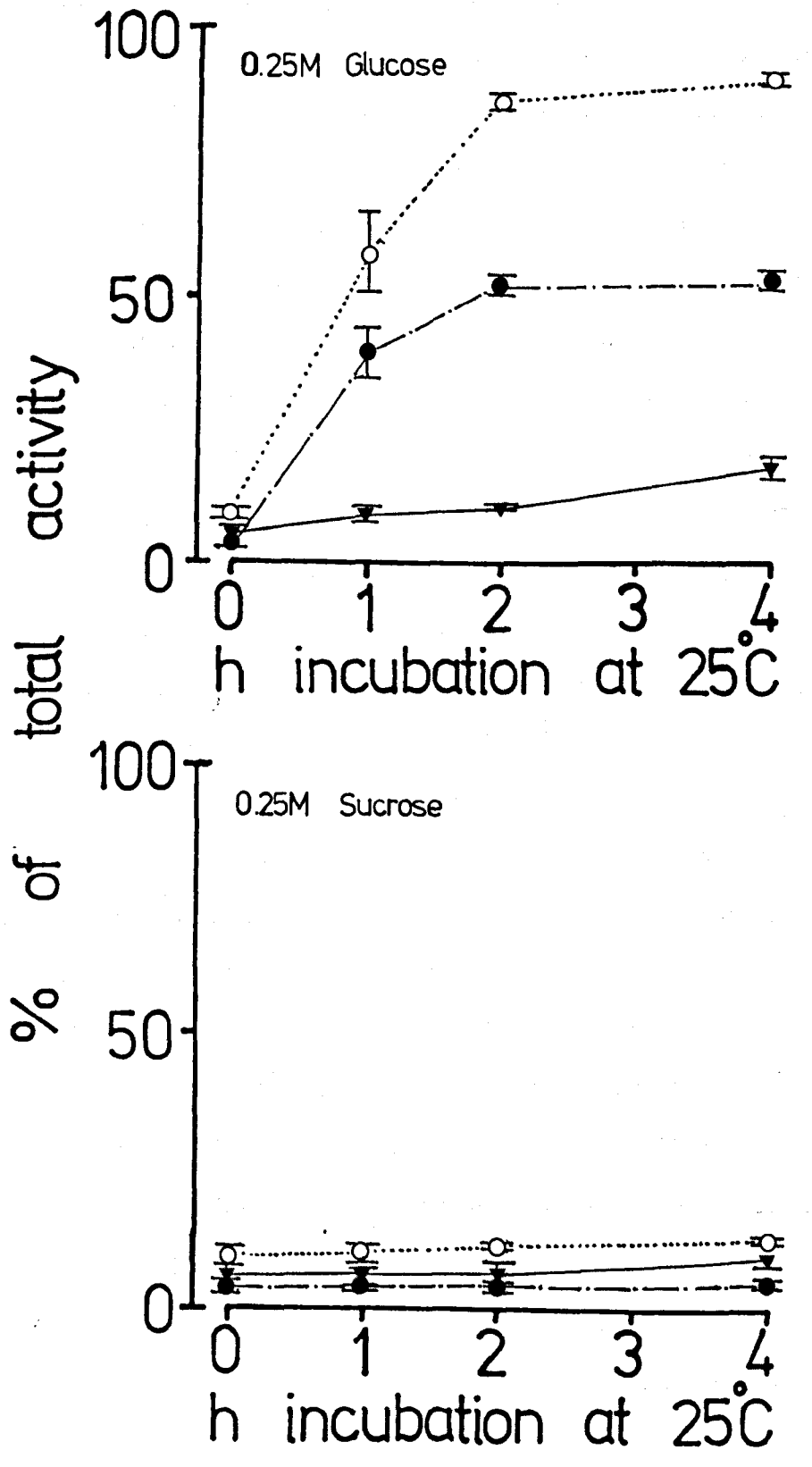
KEY



Free arylsulphatase activity

Non-sedimentable arylsulphatase activity

Non-sedimentable ^{125}I-labelled PVP


100
50
0
0.25M Glucose
% of total activity
0 1 2 3 4
h incubation at 25°C
100
50
0
0.25M Sucrose
0 1 2 3 4
h incubation at 25°C

^{125}I-labelled PVP cannot permeate the lysosomal membrane. After incubation in 0.25M glucose, there was a progressive rise in both the free and the non-sedimentable enzyme activities, so that after 4h they were 91.3% and 53.8% respectively (similar to the 88.9% and 46.3% respectively found in the similar experiments with 48h between injection and sacrifice). It would seem that there is little alteration in the permeability and fragility of the lysosomes over the period from 0, 2 and 48h after injection of the liposomes. There was a progressive rise in the non-sedimentable radioactivity in 0.25M glucose during the incubation, to 18.1% after 4h. This is much lower than the corresponding figure in experiments where the injection is 48h before death, but slightly higher than the equivalent figure following incubation in 0.25M sucrose. There are two possible explanations for this. First, the ^{125}I-labelled PVP may not be able to escape from disrupted lysosomes because of its size, or secondly, the ^{125}I-labelled PVP may escape from the lysosomes, but be in a sedimentable form. It has already been shown that liposome-entrapped ^{125}I-labelled PVP is largely sedimentable and that unentrapped ^{125}I-labelled PVP is largely non-sedimentable under these conditions. If the radiolabel does escape from the lysosomes in 0.25M glucose, it must therefore be liposome entrapped, otherwise it would become non-sedimentable. Also, if the ^{125}I-labelled PVP does not escape from the lysosomes in these experiments, it must be in some "multimolecular" form, since it does become non-sedimentable in similar experiments performed 48h after injection. There is strong evidence, therefore, that the ^{125}I-labelled PVP is still contained within liposomes 2h after injection. It is unclear from these experiments whether or not liposomes can dissociate from lysosomes as the latter break through osmotic damage. It is unlikely, however, that all the liposomes can escape when only half of the free arylsulphatase is non-sedimentable. Equally, it is unlikely that all the liposomes remain within the "shells" of disrupted lysosomes. The true picture is undoubtedly between these two extremes, since the lysosomes will be

disrupted to different degrees.

Since liposome-entrapped ^{125}I-labelled PVP is largely sedimentable (at 150,000g x 30min), and unentrapped ^{125}I-labelled PVP is not, the interpretation of most of the results is straightforward. When the lysosome-enriched fraction is prepared 48h after injection, most of the ^{125}I-labelled PVP is not within liposomes at this stage, since when the suspension is incubated in 0.25M glucose, much of the radiolabel becomes non-sedimentable (Figure 9.3). When the experiment is performed only 2h after injection of the liposomes, less ^{125}I-labelled PVP becomes non-sedimentable, even though the degree of lysosome damage is similar. This implies that most of the radiolabel is still largely liposome-entrapped at this stage, and is thus either unable to leave the lysosomes, or unable to become non-sedimentable. In 0.25M sucrose, there is no escape of ^{125}I-labelled PVP from intact lysosomes during the incubation.

The use of liposomes is a good method for the introduction of substances into the lysosomal system, providing that the substance under investigation can be efficiently entrapped. As it stands, this technique can be used to identify those substances that cannot permeate the lysosome membrane, because non-permeable substances remain within the lysosomes for a relatively long time, and there would be no release of the substance, even after prolonged incubation of the lysosomes in sucrose.

When a liposome-entrapped substance is administered to a rat, some of the liposomes are phagocytosed by the liver. Once within secondary lysosomes, the lysosomal enzymes will begin to digest the liposomes, and release the entrapped substance. Substances within the lysosomes are of two kinds, i.e. those that can escape intact or after intralysosomal digestion to permeable products, and those that cannot escape, either because they are too large or because their digestion products also cannot escape. If the substance cannot escape from the lysosomes,(e.g. PVP) it will remain within the lysosomes, and the subcellular distribution of the

substance will not alter significantly at any time after uptake by the liver. However, after the release of a permeable substance from the liposomes, it will immediately begin to escape from the lysosomes.

There are two fundamentally different types of experiments that could be carried out using liposomes to measure the lysosome permeability of small molecules. It might be possible to measure the permeability of substances after the liposomes have been digested. Alternatively, the rate of escape of substances could be monitored even though some of the liposomes were still intact within the lysosomes.

If one attempts to measure the rate of escape from the lysosomes of substances already released from the liposomes, it is necessary to wait until all of the liposomes have been digested. However, in the case of a lysosome-permeant substance, by the time that this has occurred there will probably be little of the substance left within the lysosomes. Thus the total amount of substance measured in experiments would be very small, and this would lead to a less accurate measurement of the permeability. Alternatively, if the experiments are carried out while a large amount of the substance is still within the lysosomes, much of the substance would still be liposome entrapped. In this case, the rate of escape of substance from the lysosomes is probably limited by the rate at which the substance is released from the liposomes as the latter are digested. The choice is thus between measuring small changes or slow changes in the total amount of substance present. Although in both cases the question of permeability can be answered, the rate of permeation of substance through the lysosome membrane cannot possibly be deduced from the results of the second type of experiment.

The data of Chapter 8 lead one to expect that experiments of the first type, involving permeability measurements after digestion of the liposomes, would necessitate the accurate measurement of very small amounts of radioactivity. It may, of course, be possible to raise the concentration

of the radiolabel that is entrapped within the liposomes, so that a higher level of radioactivity is present in the experiments.

The second method is probably the more appropriate one to use where the actual rate of permeability does not need to be calculated. There are two ways of increasing the sensitivity of this method of finding out whether or not a substance can permeate the lysosome membrane. First, the time between injection of the rat and the preparation of the lysosome-enriched fraction must be selected so that there is maximal recovery of the substance within secondary lysosomes. This may not be the same as the time when there is maximal recovery of the substance in the M and L fractions, since at this time much of the substance may still be within large phagosomes. Secondly, if the integrity of the lysosome-enriched suspensions could be retained throughout a longer incubation, then presumably more of any permeant substance would escape from the lysosomes during the experiment. Also, as for the first method above, it may be possible to increase the concentration of substance within the liposomes.

After the injection of liposome-entrapped ^{131}I-labelled albumin, there was release of acid-soluble radioactivity from lysosome-enriched fractions of rat liver (Gregoriadis and Ryman, 1972). In these experiments, the rats were killed only 30min after injection of the liposomes. Over a 2h incubation in 0.3M sucrose at 22^{o}C, the acid-soluble radioactivity rose from approx. 10% to 17.5% of the total. A similar time course might well be required in order to measure the ability of small substances to permeate the membrane of the lysosome.

If these sorts of experiments were carried out using substances that were employed in the preceding Chapters (i.e. [U-^{14}C]sucrose and [^{35}S]cystine), additional data would significantly increase their value. In the case of sucrose, it might be possible to assay for glucose in the non-sedimentable material. Detection of a rise in the non-sedimentable level of glucose, paralleled by a rise in the non-sedimentable radioactivity

would be evidence for the intralysosomal hydrolysis of sucrose. Similarly, for cystine, careful analysis of the sulphydryl levels in the non-sedimentable fractions would help to pinpoint the mechanism by which lysosomes deal with cystine residues.

Unfortunately, insufficient time was available to enable these experiments to be started but, clearly this technique shows great promise and, with minor adaptation could be put to good use in exploring lysosome membrane permeability to various substances.

CHAPTER 10

General Conclusions

The work described in this Thesis was carried out in an effort to ascertain the fate of cystine residues in the lysosomal system. Possible mechanisms involved have already been discussed in Section 1.3 and are shown in Figure 1.1. In this Chapter, some aspects of the possible mechanisms are re-examined.

A brief paper has recently appeared (Docherty, et al., 1979) that provides evidence that some monosaccharides enter rat liver lysosomes by facilitated diffusion. The authors used the osmotic protection method, and observed a significantly smaller increase in the free β-glycerophosphatase activity of lysosome-enriched suspensions incubated at 25°C in 0.25M glucose when either 1mM phlorrhizin or cytochalasin B was included. This report is the first to provide evidence of active transport of specific substances across the intact lysosome membrane, and must force a reconsideration of the possibility that, in normal lysosomes cystine escapes by a similar mechanism.

However, the work of Docherty et al. (1979) is unconfirmed and is but one aspect of a sharp controversy concerning possible ATPases in lysosomes. Several reports of a lysosomal "proton pump" (see Section 1.2.4.2), including two recent ones (Dell'Antone, 1979; Schneider, 1979), have been strongly disputed by Tager and coworkers (Reijngoud and Tager, 1973 and 1975; Hollemans et al., 1979) who propose an alternative mechanism for pH maintenance. We have shown that at least one report of an ATPase is mistaken (Chapter 4), and that ATP does not stimulate the uptake of proteins by lysosomes in vitro.

There would be no need to postulate a mechanism for the intralysosomal reduction of cystine residues if all protein disulphides were reduced prior to entry of the proteins into the lysosomes. Since the lysosomes receive material from both extra- and intracellular sources, the enzyme carrying out the pre-lysosomal reduction would have to be multi-sited within the cell. Also, any cystine residues that were delivered to the lysosomal interior intact would be unable to escape. This contrasts with the experimental evidence of Chapter 8

where, following the injection of DPPC/DPPA liposome-entrapped [^{35}S]cystine, most of the radioactivity in the liver was found in the S fraction after 2h. The distribution of N-acetyl-β-D-glucosaminidase suggests that entry into the cells was by phagocytosis, and so it is unlikely that prelysosomal reduction of disulphides is the only mechanism of escape of cystine residues from lysosomes.

If cystine within lysosomes is normally reduced before it can escape into the cytosol, then three fundamental questions need to be answered concerning the mechanism of the reduction. These are: (a) what is the enzyme involved? (b) what is the hydrogen donor? and (c) how is the hydrogen donor regenerated? These problems have been mentioned in Section 1.3, but some of the data of Chapter 3 enables a further discussion here.

One potential reducing agent, small enough to permeate the membrane of lysosomes is cysteamine. The present investigation showed that it could cross the lysosome membrane at all three pH values tested. In its oxidised form, cystamine, it exhibited a pH-dependent permeability. At high pH it was not able to penetrate the lysosome membrane, suggesting that perhaps it is unable to enter lysosomes <u>in vivo</u>. However, at low pH it is very permeant, so that it may be able to escape from the lysosome interior. It is thus possible that cysteamine could be the physiological reducing agent for the enzymic reduction of disulphides within normal lysosomes.

Administration of cysteamine may be a useful therapy in cystinosis since, once it is within lysosomes in large amounts, it would reduce the cystine, and alleviate the storage. A similar rationale was behind the recent clinical trial using ascorbic acid as reducing agent (see 1.1), although Kroll and Schneider (1974) discounted its action as a reducing agent since ascorbic acid has a lower redox potential than cystine. However, the redox potential may not be the only relevant parameter in

this instance since presumably any cysteine produced would be able to escape from the lysosomes and thus lower the cysteine/cystine ratio in favour of the desired reduction.

The present work, while not elucidating the mechanism by which cystine residues escape from lysosomes, has developed a technique that could be instrumental in ascertaining whether or not cystine is reduced prior to its escape. The rapid uptake of liposomes by the liver is particularly useful, since it enables the isolation of a lysosome-enriched fraction of liver containing a high concentration of exogenous material. Apart from the present goal, i.e. elucidation of the fate of cystine, similar procedures could be explored to investigate the effects of changes of intralysosomal pH on lysosomal membrane permeability, proteolysis within lysosomes or to ascertain the permeabilities of other types of substances. It would be unique, if, in the future, targetted liposomes containing the absent factor were used to alleviate cystinosis, after liposomes had been instrumental in pinpointing the nature of the defective protein.

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APPENDIX

Presentation of Data of Figures

These tables show the data presented graphically in the Thesis in those cases where it cannot be calculated from the information given. Each Table gives only the raw data, and should be interpreted in conjunction with the Figure and its Legend.

Figure 4.2

pH	○	●
5.0	51.2 ± 17.1	89.0 ± 7.3
5.5	11.0 ± 1.6	47.5 ± 6.3
6.0	10.3 ± 1.1	51.8 ± 9.4
6.5	12.6 ± 1.4	60.2 ± 10.0
7.0	20.7 ± 1.7	69.7 ± 8.3
7.5	30.1 ± 1.5	87.8 ± 4.9
8.0	41.7 ± 1.9	99.5 ± 3.2

Figure 4.3

Time (min.)	●	▲	○
0	6.1	12.5	8.4
30	14.7	11.3	7.3
60	97.8	10.0	9.9
90	105.6	10.7	11.0
120	103.4	13.5	11.0

Figure 4.4

Time (min.)	% acid-soluble radioactivity
0	1.9
30	2.6
60	3.3
90	5.2
120	6.4
150	9.1
180	10.7
210	12.9

Figure 6.2

Time (h)	●	○	▲	△
0.5	5.9	7.0	10.3	32.1
5.0	6.3	9.1	13.7	41.2
10.0	6.8	9.5	15.3	42.9
15.0	7.2	10.6	16.9	43.5
20.0	7.3	14.7	21.9	47.9
25.0	7.2	15.9	20.1	45.3

Figure 6.3

Temp.°C	0.5h○	0.5h●	5.0h○	5.0h●
4	11.0	5.9	10.5	6.3
25	7.9	8.0	15.0	9.1
37	38.5	10.3	41.0	13.7
45	46.2	32.1	49.6	41.2

Figure 6.4

Temp.°C	0.5h	5.0h
4	18.7	10.1
25	9.3	13.5
37	14.9	17.5
45	45.8	49.4

Figure 6.5

Temp.°C	0.5h○	0.5h●	5.0h○	5.0h●
4	8.9	16.6 ± 8.0	9.4	14.6 ± 5.5
25	11.9	19.6 ± 6.4	14.9	29.5 ± 8.3
37	30.3	59.5 ± 2.4	56.0	93.2 ± 5.2
45	75.6	95.0 ± 3.6	81.9	96.4 ± 2.7

Figure 7.1

Time (min.)	○	●	■
2	88.2 ± 8.2	82.5 ± 8.3	81.1 ± 15.7
5	72.3 ± 2.1	74.7 ± 2.7	n.d.
10	65.6 ± 6.2	62.1 ± 1.4	43.4 ± 12.8
15	54.5 ± 6.0	60.4 ± 1.6	n.d.
20	51.1 ± 4.5	58.4 ± 7.8	28.6 ± 9.6
30	50.5 ± 2.5	54.5 ± 0.4	15.3 ± 4.7
40	54.1 ± 4.0		
50	47.3 ± 5.2		
60	47.1 ± 1.9		

Figure 7.4

Time (min.)	○	●	□
0.5	31.3 ± 0.8	32.2 ± 2.6	n.d.
1	29.9 ± 1.2	27.7 ± 2.6	n.d.
1.5	22.8 ± 1.8	22.3 ± 1.9	n.d.
2	22.2 ± 0.9	20.0 ± 1.6	82.7 ± 12.0
2.5	23.7 ± 1.8	21.2 ± 2.6	n.d.
5	18.5 ± 2.0	18.0 ± 1.6	54.6 ± 7.0
10	15.9 ± 0.9	14.5 ± 0.7	29.1 ± 4.7
15	13.6 ± 1.1	12.4 ± 0.9	13.2 ± 4.2
20	12.4 ± 1.9	12.8 ± 1.5	6.5 ± 1.9
30	12.3 ± 3.0	11.1 ± 3.3	4.1 ± 0.7

Figure 7.6

Time (min.)	○	●
0.5	27.5 ± 1.0	36.3 ± 4.5
1	26.4 ± 1.4	35.3 ± 3.2
1.5	24.0 ± 1.5	30.1 ± 2.8
2	21.9 ± 1.2	26.4 ± 3.5
2.5	20.0 ± 1.6	24.2 ± 3.5
5	13.9 ± 1.6	20.7 ± 2.8
10	11.6 ± 0.4	15.6 ± 1.3
15	10.3 ± 0.2	13.2 ± 1.7
20	9.0 ± 0.6	10.8 ± 0.5
30	8.0 ± 0.4	9.2 ± 0.3

Time (min.)	▽
2	56.5 ± 5.3
4	46.9 ± 7.9
6	40.8 ± 8.9
8	32.5 ± 8.1
10	26.2 ± 7.3
15	18.9 ± 5.8
20	12.3 ± 3.7
30	6.5 ± 1.9

Figure 9.1

	0.25M Glucose		
Time (min.)	○	●	▼
0	13.5	6.4	12.1
30	27.2	22.8	16.7
60	52.3	43.0	25.5

Figure 9.1 contd.

0.25M Sucrose

Time (min.)	○	●	▼
0	9.6	7.8	11.2
30	8.6	11.1	13.7
60	8.7	9.3	13.1

Figure 9.2

0.25M Glucose

Time (min.)	○	●	▼
0	13.3	7.1	54.1
30	43.8	35.7	65.6
60	68.8	59.8	66.6

0.25M Sucrose

Time (min.)	○	●	▼
0	11.7	10.0	54.6
30	11.0	6.8	65.3
60	11.3	9.6	66.0

Figure 9.3

0.25M Glucose

Time (h)	○	●	▼
0	10.4 ± 1.1	2.6 ± 0.3	5.6 ± 1.3
1	56.8 ± 3.0	25.5 ± 1.9	13.8 ± 0.9
2	79.6 ± 4.1	39.5 ± 3.2	29.1 ± 1.6
4	88.9 ± 5.9	46.3 ± 3.4	49.8 ± 1.3

0.25M Sucrose

Time (h)	○	●	▼
0	10.2 ± 0.2	3.3 ± 0.1	6.1 ± 2.3
1	10.8 ± 0.4	3.0 ± 0.8	7.5 ± 2.2
2	11.4 ± 0.2	4.4 ± 0.7	8.9 ± 4.1
4	12.3 ± 0.8	5.2 ± 0.8	14.4 ± 4.9

Figure 9.4

0.25M Glucose

Time (h)	○	●	▼
0	11.5 ± 0.1	5.6 ± 0.2	23.8 ± 5.8
1	48.6 ± 6.8	34.3 ± 4.1	18.2 ± 2.9
2	78.4 ± 5.4	51.4 ± 4.9	22.1 ± 0.6
4	90.2 ± 3.5	54.8 ± 0.3	20.4 ± 1.9

0.25M Sucrose

Time (h)	○	●	▼
0	9.1 ± 1.1	2.8 ± 0.7	24.2 ± 5.8
1	8.2 ± 0.3	3.0 ± 0.6	22.5 ± 2.3
2	9.2 ± 1.1	4.1 ± 0.5	26.8 ± 2.2
4	10.5 ± 0.4	4.3 ± 0.2	24.0 ± 2.6

Figure 9.5

0.25M Glucose

Time (h)	○	●	▼
0	9.6 ± 0.6	3.7 ± 0.2	87.5 ± 3.0
1	69.9 ± 6.5	40.0 ± 1.7	75.9 ± 8.4
2	85.3 ± 2.6	50.2 ± 3.3	67.5 ± 7.0
4	88.5 ± 1.9	52.2 ± 3.6	60.3 ± 8.1

0.25M Sucrose

Time (h)	○	●	▼
0	8.3 ± 0.5	3.8 ± 0.4	89.5 ± 1.8
1	9.1 ± 0.1	4.1 ± 0.9	82.4 ± 3.3
2	10.6 ± 0.3	5.3 ± 1.2	79.6 ± 1.3
4	12.6 ± 0.8	6.3 ± 0.8	77.5 ± 1.7

Figure 9.6

0.25M Glucose

Time (h)	○	●	▼
0	9.1 ± 1.0	3.5 ± 0.5	4.2 ± 1.7
1	58.0 ± 7.4	39.3 ± 4.8	9.0 ± 1.5
2	86.6 ± 1.5	52.0 ± 2.1	10.4 ± 0.4
4	91.3 ± 0.9	53.8 ± 1.8	18.1 ± 2.3

0.25M Sucrose

Time (h)	○	●	▼
0	9.4 ± 1.7	3.6 ± 1.1	5.8 ± 1.6
1	10.3 ± 1.4	3.6 ± 0.5	5.9 ± 0.9
2	11.3 ± 0.8	4.3 ± 0.8	6.7 ± 1.1
4	12.9 ± 0.8	5.1 ± 0.5	9.8 ± 1.8