STUDIES ON THE FACTORS INVOLVED IN THE SECRETION OF ENZYMIC AND NON-ENZYMIC CONTENTS OF RAT LIVER LYSOSOMES

A Thesis submitted to the University of London for the Degree of Doctor of Philosophy in Biochemistry

by

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ABSTRACT

The aim of this present study has been to investigate the role of microfilaments in the secretion of lysosomal enzymes from rat liver cells and the effect, if any, on this process of changes in the intralysosomal environment.

Using the systems of short-term rat hepatocyte cultures and isolated rat liver prefusions, the effect of the microfilament poison, cytochalasin B, on the secretion of the lysosomal enzymes arylsulphatase A + B, β-galactosidase and, in some instances, β-N-acetyl-glucosaminidase has been examined. In addition the release of the cytosol enzyme lactate dehydrogenase was monitored to determine non-specific enzyme release due to cell damage.

The intralysosomal environment was modified by preloading the liver lysosomes <u>in vivo</u> with a variety of macromolecular materials, i.e. ^{125}I-polyvinylpyrrolidone, ^{3}H-dextran and ^{125}I-Triton WR-1339. The effect of this lysosomal modification on the release of the lysosomal enzymes and its modulation of the effect of microfilament disruption, as induced by cytochalasin B, as well as the effect of this disruption on the release of the preloaded materials, was determined.

In addition, the intracellular distribution of these preloaded materials in the rat liver was investigated before, and in some cases after, the livers had been perfused. Attempts were made to correlate the

heterogeneity of the density-gradient-centrifugation profiles of the preloaded, intralysosomal materials and those of the various lysosomal enzymes with their heterogeneous secretion patterns during perfusion in the presence and the absence of cytochalasin B.

Preliminary attempts were made to delineate the contributions of secretion (exocytosis) and reuptake (endocytosis) in the net "appearance" of both the enzymic and non-enzymic lysosomal contents in the perfusate.

Form the results obtained a hypothesis was formulated which suggested that the intralysosomal presence of non-enzymatic material, does indeed modulate not only the "base-line" secretion of lysosomal enzymes, but also the effect of microfilament disruption on this process. This modulation could involve modifications to the lysosomal membrane, such that it might fuse more readily with the plasma membrane.

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ABBREVIATIONS AND KEY TO FIGURES

The following abbreviations have been used throughout this thesis:

CB	cytochalasin B
CD	cytochalasin D
AS	arylsulphatase A + B
NAGA	β-N-acetylglcuosaminidase
β-GAL	β-galactosidase
LDH	lactate dehydrogenase
cyt-c-ox	cytochrome-c-oxidase
Gluc-6-P	Glucose-6-Phosphatase
^{125}I-PVP	^{125}I-polyvinylpyrrolidone
L-15	Leibovitz-15
PBS"A"	Phosphate buffered saline free of Ca^{2+}
RSA	Relative specific activity
SD	Standard deviation

The ordinates in the figures of the distribution profiles of all the parameters in sedimentation and isopycnic sucrose gradients represent the activities of the parameters in each fraction, expressed as the percentages of the activities recovered from the gradient. The fraction numbers (abscissa) are placed at the end of the corresponding fraction.

The ordinates in the figures of the release of the enzymes and radioactivity from hepatocytes in short-term cultures, represent the activities found in the medium

at each time point, expressed as the percentage of the total activity recovered i.e. the sum of the activities found in the medium and the cells at that time point.

The ordinates in the figures of the release of the enzymes and radioactivity from isolated perfused rat livers, represent the cumulative activities found in the perfusate at each time point, expressed as the percentage of the total activity recovered at the end of the perfusion i.e. the sum of the activity found in the liver and the cumulative activity found in the perfusate.

Absolute enzymic activities were defined as the μg of product/total test fraction/hour.

The relative specific activity of an enzyme was defined as the % activity of the enzyme over the % of protein found in the particular fraction.

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CHAPTER 1

INTRODUCTION

1.1 Lysosomes

1.1.1 General

The lysosomes are a heterogeneous group of intracellular organelles characterised as having a membrane-bound vesicular structure and containing a variety of hydrolytic enzymes, all with acid pH optima. They are involved in a range of catabolic processes reflecting the particular specificities of their enzyme complement.

They were first identified by de Duve and his co-workers (de Duve *et al.*, 1955) in rat liver, as a result of the sedimentation properties and structure-linked latency of acid phosphatase and a few other hydrolases. Cytochemical and ultrastructural studies have resulted in the identification of peribiliary dense bodies and other vacuoles in the liver as lysosomes (Novikoff, 1973). Since then, lysosomes have been identified in a great variety of animal cells and more than 40 acid hydrolases have been associated with them (Barrett, 1969).

The permeability properties of the lysosomal membrane confer to the enclosed enzymes the properties of latency. The lysosomal membrane completely prevents the escape of large molecules (e.g. enzymes) and also limits the access of certain lower molecular weight compounds (e.g. artificial enzyme substrates) to the interior of lysosomes. As a result when the lysosomal membrane is intact, little or no enzymic activity is observed, whereas its disruption leads to the manifestation of full activity. Within the cell the membrane protects the cytoplasmic components from the action of the lysosomal enzymes.

Studies of the permeability properties of the lysosomal membrane (Lloyd, 1969 and 1971) have shown that molecular weight and structure are important factors in determining the permeability of the membrane to a particular molecule. The permeability to inorganic salts and protons is still an unsolved problem (Mego et al., 1967; Lloyd, 1971; Allen and Lee, 1972). In particular, knowledge of the permeability of the protons would greatly contribute to our understanding of the mechanism by which the intralysosomal acid pH is maintained (Reijngoud and Tager, 1977).

One of the main problems associated with the study of lysosomes is their heterogeneity (Davies, 1975). The first indications of the lysosomal heterogeneity came from the sedimentation data for a variety of acid hydrolases (de Duve et al., 1955) which showed that lysosomes could have sizes ranging from 0.25 to 0.8 μm. Later biochemical

and morphological evidence suggested at least three types of heterogeneity: intracellular, intercellular and intralysosomal.

At least part of the intracellular heterogeneity of lysosomes arises as a result of the life cycle of the organelles. Thus in one cell there exist primary and secondary lysosomes and dense bodies. Primary lysosomes are newly formed lysosomes which have not received any substrate from outside the lysosome. The synthesis of their lysosomal enzymes is believed to involve the rough endoplasmic reticulum, the enzymes being then translocated through the smooth endoplasmic reticulum to the Golgi apparatus where packaging of the enzymes occurs (de Duve and Wattiaux, 1966) However, according to the GERL (Golgi-associated endoplasmic reticulum giving rise to lysosomes) concept proposed by Novikoff (1973), specialised regions of smooth endoplasmic reticulum, adjacent to, but distinct from Golgi can give rise to lysosomes. Secondary lysosomes are lysosomes that contain non-lysosomal material. They arise from the fusion of primary lysosomes with other intracellular vesicles. Dense bodies are secondary lysosomes characterised by the accumulation of residues of undigestible material e.g. lipofuscin (Novikoff, 1973) and they represent the final intracellular stage in the life cycle of a lysosome. Biochemical evidence for the concept of intracellular heterogeneity is provided by the finding that in the case of the liver, for example, different acid hydrolases associated with a specific cell type show different specific activities from each other. So parenchymal cells show a higher specific

activity of acid phosphatase (Lentz and Di Luzio, 1971) than arylsulphatase (Berg and Boman , 1973).

Intercellular heterogeneity in multicellular tissues was originally demonstrated by histochemical staining for acid hydrolases in situ. In particular, in the case of the liver, these studies have shown that differences in the size, distribution and number of acid-hydrolase-positive granules exist between Kupffer and parenchymal cells. For example, it has been shown that Kupffer cells stain more intensely for acid phosphatase (Novikoff and Essner, 1960) and β-N-acetyl-glucosaminidase (Ma and Biempica , 1971) than parenchymal cells, the combined results indicating that in general Kupffer cell lysosomes are larger and stain more densely than the lysosomes of parenchymal cells. Biochemical studies of isolated Kupffer and parenchymal cells have shown a wide variation in the distribution of different enzymes amongst these two cell populations (Arborgh et al., 1973; Berg and Boman, 1973). Also, heterogeneous distribution of lysosomal enzyme activity among different parenchymal liver cells in human (Ma and Biempica , 1971) and rat (Novikoff, 1963) liver, dependent on their position in the hepatic lobule, has been noted. The differences in the distribution patterns of different lysosomal enzymes obtained during subcellular distribution studies (Beaufay et al., 1959) using liver provide further support for the lysosomal heterogeneity occuring both intra- and intercellularly.

Finally, Baccino et al. (1971) have provided evidence, although not conclusive, for the third form of heterogeneity

arising from the fact that different lysosomal enzymes appear to be associated with the lysosomal membrane to different extents.

Lysosomes are involved in the intracellular digestion of both intra- and extracellular material. Extracellular macromolecular and particulate material is taken up by the cells through the process of endocytosis and enters the cytoplasm within vesicles, called endocytic vacuoles. Due to the permeability properties of the lysosomal membrane discussed earlier, lysosomal enzymes gain access to the material to be digested through the fusion of the endocytic vacuoles with either or both primary and secondary lysosomes.

Intracellular material is digested through the process of autophagy which seems to take place under both physiological and non-physiological conditions. The way by which the cytoplasmic components to be digested are sequestered within vesicles and are exposed to lysosomal enzymes is not clear (Ericsson, 1969; Novikoff, 1973). It has been proposed that intracellular components are sequestered by endoplasmic reticulum to form autophagic vacuoles which are encircled with lysosomal enzymes through fusion(s) with lysosomes. In view of the fact that lysosomes do not fuse with the endoplasmic reticulum, a change in the properties of the membrane of the endoplasmic reticulum derived vacuole would be presumed to occur before such a fusion is allowed (de Duve, 1966). Novikoff (1973) has proposed that the endoplasmic reticulum membrane alters its permeability to lysosomal enzymes and the accumulation

of a small amount of the enzymes somehow allows the fusion of the vacuole with lysosomes. On the other hand, it is possible that the lysosomal membrane invaginates allowing the sequestration of cytoplasmic areas inside the lysosomes (Wattiaux, 1977).

Finally, extracellular digestion of extracellular material can be achieved by the secretion of lysosomal enzymes (exocytosis). For example, in vitro studies have shown that lysosomal enzyme secretion in response to parathyroid hormone is important in the resorption of bone (Vaes, 1969).

Fig. 1 gives a schematic representation of the properties and functions of the lysosomes discussed above.

1.1.2 Lysosomes in Pathology

Lysosomal malfunction leading to excess or decreased lytic activity has been associated with a variety of pathological conditions (de Duve and Wattiaux, 1966).

Increased lytic activity can be the result of the labilization of the lysosomal membrane (Allison, 1969; Roels, 1969) and/or increased autophagy (Becker and Barron, 1961; Ashford and Porter, 1962; Trump et al., 1962).

Decreased lytic activity can result from the partial or total depletion of one or more lysosomal enzymes, e.g. in lysosomal storage diseases (Hers, 1973), the inability of the lysosomes to fuse with endocytic vacuoles containing the material to be digested, e.g. in infections with the

Mycobacterium Tuberculosis (Armstrong and d'Arcy Hart, 1971) or the absence of lysosomal enzymes capable of degrading the intralysosomally accumulated material e.g. the aging pigment lipofuscin (Novikoff, 1973). All the situations connected with decreased lysosomal lytic activities are associated with excessive intracellular accumulation of the undigested material. In the cases arising from the depletion or the absence of suitable lysosomal enzymes, the accumulated material will be stored intralysosomally.

1.1.2.1 Lysosomal Storage Diseases

Lysosomal storage diseases are inborn errors of metabolism resulting from the partial or total depletion of one or more lysosomal enzymes. They are characterised by the intralysosomal accumulation of all the macromolecules in the digestion of which the depleted enzyme is essential (Hers, 1965).

Lysosomal storage diseases are inherited mainly as autosomal recessive disorders. The enzymatic defect can result from either missense or nonsense mutations of structural genes, both of which could alter the kinetic, stability or other properties of the enzyme, rendering it catalytically inactive or partially inactive. Since most lysosomal enzymes are glycoproteins, it is also possible that missense or nonsense mutations in genes controlling the post-translational modifications of the enzyme protein may cause catalytic deficiencies. I-cell disease (Hickman and Neufeld, 1972) may be a result of such mutations since it has been shown that high levels of lysosomal enzymes

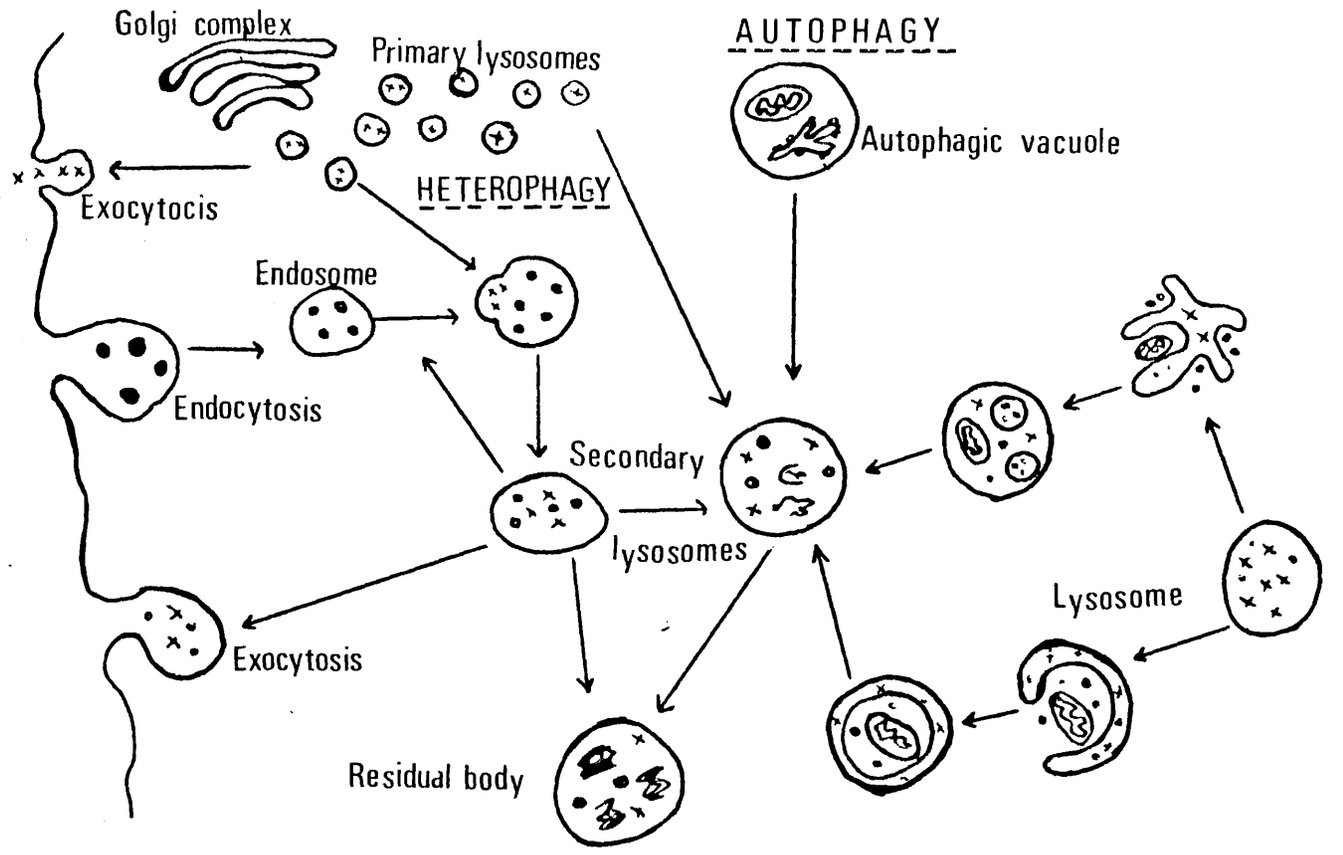

Fig. 1

A schematic representation of the heterophagic and the autophagic function of lysosomes.

are present extracellularly which lack the carbohydrate moieties responsible for their re-uptake by cells. Finally mutations of regulatory genes could occur (Desnick et al, 1976).

Considerable clinical heterogeneity (e.g. severity and age of onset) is found within particular lysosomal storage diseases and in some cases at least, this heterogeneity is determined by the extent of the depletion of the enzyme concerned. An example is offered by the three types of glucocerebrosidoses (Gaucher's disease) (Brady and King, 1973) in which the glucosylceramidase activity can vary from 0% in the case of the infantile Gaucher, to 30% of the normal activity in the case of the adult Gaucher, with the juvenile Gaucher exhibiting intermediate levels of residual activity.

The identification of the primary enzymatic defect in a variety of lysosomal storage diseases has been hindered by secondary enzymatic defects, possibly resulting from the accumulation of the undigested macromolecules. For example, although the primary enzymatic defect in mucopolysacharidosis type I (Hurler disease) is in α-iduronidase, partial deficiency of α- and β-galactosidase has also been reported (Van Hoof, 1973).

In some other cases where several lysosomal isoenzymes exist, the deficiency of one can be masked by the presence of the others which are, however, unable to compensate entirely for the deficient enzyme due to their slightly different specificities. So immunological studies

have shown that Tay-Sachs disease results from the deficient activity of β-hexosaminidase A (O'Brien, 1973), whereas Sandhoff's disease results from the deficient activities of both β-hexosaminidase A and B (Sandhoff and Harzer, 1973).

Very often the defect in one enzyme results in the accumulation of more than one related macromolecules, for example in GM_1 gangliosidosis in addition to the GM_1-ganglioside, its asialo derivative and keratan-sulphate-like-polysaccharides also accumulate, all of which require β-galactosidase to initiate their degradation (Van Hoof, 1973). Another type of heterogeneity in the lysosomal storage material will result from the inhibition of a variety of enzymes by the accumulated material. For example in mucopolysaccharidosis Type II (Hunter's disease), the accumulated dermatan sulphate inhibits arylsulphatase A, α-galactosidase and β-galactosidase resulting in the accumulation of their respective substrates.

The intralysosomal material that accumulates in the lysosomal storage diseases enters the lysosomes both through endocytosis and autophagy, the relative contributions of each being determined by the nature of the particular cell (Desnick et al., 1976).

Attempts to treat lysosomal storage diseases have been based largely on the replacement of the deficient enzyme. The replacement has been attempted either by injecting the purified enzyme (Brady et al., 1974), by injecting the enzyme entrapped in liposomes (Gregoriadis, 1973)

by the transfusion of plasma (di Ferrante et al., 1971), the injection of normal leucocytes (Knudson et al., 1971) and finally by the transplantation of normal fibroblasts (Dean et al., 1976). The difficulties associated with the above trials have been (1) the short half-lives of the administered enzyme, (2) the targetting of the enzymes to specific tissues and lysosomes and (3) the presence of immunological complications.

1.2 Microtubules and Microfilaments

Microtubules and microfilaments are two of the components of what is generally described as the cyto-skeletal system of cells (Nicolson, 1976).

In the following sections an account of the characterstics of microtubules and microfilaments and of their role in the processes of endo- and exocytosis will be given.

1.2.1 Characteristics of Microtubules and Microfilaments

Microtubules were first visualised as such as the major component of the "9 + 2" elements of flagella and cilia. Later it was shown that the mitotic spindle was also composed of microtubules.

It was observed that the mitotic spindle could be disrupted by certain drugs such as colchicine and the Vinca alkaloids, which arrested cells at metaphase. The effect of the use of mitotic-spindle inhibitors has revealed the existence of what is known as cytoplasmic microtubules (Stephens and Edds, 1976).

Microtubules, as the name implies, are tubules, the walls of which are composed of thirteen protofilaments, each protofilament being a linear polymer of the protein tubulin. Tubulin, as shown by polyacrylamide gel electrophoresis, is a dimer composed of two subunits, α and β , which show slightly different electrophoretic mobilities (Wilson, 1975). Tubulin copurifies with a variety of proteins, of both higher and lower molecular weight than the tubulin dimer, known as the microtubule-associated proteins (Sandoval and Cuatrecasas, 1976).

There appears to be an equilibrium between tubulin dimers and polymers. Low temperature, Ca^{2+}, colchicine and the Vinca alkaloids both disrupt and prevent the formation of the cytoplasmic and the mitotic-spindle microtubules. The above agents can only prevent the formation of the flagella and cilia microtubules, but have no effect on them once they are formed. Colchicine and the Vinca alkaloids can bind to tubulin dimers and are believed to disrupt microtubules by shifting the equilibrium towards the free dimer (Olmsted and Borrisy, 1973). Although the way in which Ca^{2+} interacts with tubulin polymerization is not known, it appears that the sensitivity of microtubules to Ca^{2+} is greater in the absence of microtubule associated protein than in their presence (Rebhun et al., 1980).

Microfilaments are polymers of an actin-like monomer. The existence of actin in non-muscle cells has been shown both by biochemical and ultrastructural methods. The biochemical approach has shown that a protein of

molecular weight about 45,000, with an actin-like amino acid composition and the same peptide maps as actin, was present in non-muscle cells (Fine and Bray, 1971; Pollard and Korn , 1972).

Ultrastructural evidence of the actin-like nature of microfilaments has been obtained using the method of heavy meromyosin binding, originally used for skeletal muscle F-actin (Huxley, 1963). The results showed that microfilaments could bind and become "decorated" with heavy meromyosin (Senda et al., 1969; Allison et al., 1971; Behnke et al., 1971; Pollard and Korn, 1971).

Microfilaments are mostly present directly adjacent to the interior of the plasma membrane. In the case of macrophages, they comprise an approximately 400-600 Å thick filamentous zone (Reaven and Axline, 1973). In some cases, it has been shown that microfilaments are closely associated with cell membranes. For example, actin-like filaments have been shown to be associated with purified plasma membranes from Acanthamoeba (Pollard and Korn, 1971) and hepatocyte cell membranes (Riordam and Alon, 1977).

Along with the actin, a whole system of proteins comparable to that found in muscle has been identified in non-muscle cells; myosin-like molecules, (Pollard et al., 1975, Hartwig and Stossel, 1975), a protein cofactor which is necessary for the full manifestation of the Mg^{2+}-ATPase activity (Stossel and Hartwig, 1976) and an "actin-binding protein" (Hartwig and Stossel, 1976). This latter protein

iscapable of binding actin filaments into branching, interconnecting bundles and can cause the gelation of filaments in solution at room temperature (Hartwig and Stossel, 1976). The myosin-like filaments of neutrophils and macrophages are easily extracted from these cells by gentle solvents, indicating that the protein is either localised in the cytoplasm or that there exists a loose association with membranes (Stossel, 1977).

Microfilaments are sensitive to certain mould metabolites known as cytochalasins (Carter, 1967). The different types of cytochalasins interfere to different extents with the microfilament structure and amongst the most studied ones are cytochalasin D (CD) and cytochalasin B (CB), the former being more potent than the latter. In vitro experiments have shown that, depending on the specific experimental conditions, the cytochalasins can have different effects on the structure of actin filaments. For example, using proteins isolated from macrophages, CB (1 μM) inhibited the actin-binding-protein-induced assembly of actin in sucrose solutions and the gelation of actin-binding protein (Stossel, 1977). CB also dissolved the actin plus actin-binding protein gels without depolymerising the actin filaments (Stossel, 1977). CD (0.02-2 μM) in 0.5 mM $MgCl_2$ has been shown to inhibit the initial rate of polymerisation and lower the final viscosity of muscle-actin preparations. In 30 mM KCl, however, the same concentrations of CB increased the initial rates of polymerization while inhibiting the final

viscosities (Brenner and Korn, 1979). On the other hand, CB (0.2 μM) in 0.5 M $MgCl_2$ only slightly decreased the initial rates of polymerisation without having any effect on the final viscosities, whereas in 30 mM KCl, the effect of CB, although quantitatively less active, was qualitatively similar to that of CD, mentioned above (Brenner and Korn, 1979). CB (0.02 μM) and CD (0.002 μM) in 0.1 M KCl and 0.1 M $MgCl_2$ decreased the rate of assembly of actin isolated from Dictyostelium discoideum without reducing the final viscosities of the preparation (Brown and Spudich, 1979). Higher concentrations of CB (5 mM) however, increased the assembly rate of actin (Brown and Spudich, 1979).

The above experimental results indicate that cytochalasins do bind to actin, but clearly the effects of the binding appear to be determined by a variety of factors.

Finally, biochemical and morphological studies suggest that the structural, and consequently functional, state of microfilaments depends on the structural state of microtubules. For example, it has been shown (Griffith and Pollard, 1978) that the viscosities of mixtures of co-polymerised actin filaments and microtubules are higher than those expected from the viscosities of each of the components on their own and also that microtubule-associated proteins were essential for the formation of such high viscosity mixtures. The above observations were interpreted as evidence for actin-microtubule interactions mediated by microtubule-associated proteins.

Ultrastructural studies using human blood polymorphonuclear leucocytes, monocytes and lymphocytes have shown that the disassembly of microtubules was associated with a marked redistribution of microfilaments from a relatively uniform submembranous distribution into a localised protuberance (Berlin and Oliver, 1978).

1.2.2 Microtubules and microfilaments in endo- and exocytosis

The evidence indicating the involvement of microtubules and microfilaments in the processes of endo- and exocytosis has been provided by studies of the sensitivity of these processes to microtubule- and microfilament-disrupting agents.

The uptake of extracellular material during the process of endocytosis, can be the result of phagocytosis, macro- and micropinocytosis. Phagocytosis is the process by which particles with a size of 1 μm or more, e.g. bacteria, immune complexes, are ingested. Particles of sizes smaller than 1 μm are ingested either by macro- or micropinocytosis. By definition, macropinocytosis will result in the formation of vesicles visible by light microscopy (200-700 nm in diameter) whereas micropinocytosis will result in the formation of vesicles visible by electron microscopy (60-70 nm in diameter) (Allison, 1977). Both phagocytosis and macropinocytosis are energy requiring processes whereas micropinocytosis is energy independent (Allison, 1977).

Pinocytosed material can enter the cell either in solution, a non-selective process or bound to the plasma membrane, a selective process, or by a combination of both (Jacques, 1969).

CB has been shown to inhibit the phagocytosis of E. coli by mouse peritoneal macrophages (Allison et al., 1971) and rabbit peritoneal polymorphonuclear leucocytes (Davies et al., 1973c), the phagocytosis of opsonized sheep erythrocytes by mouse peritoneal macrophages (Klauss, 1973) and the pinocytosis of sucrose by Chang liver cells (Wagner et al., 1971). However, CB only partly inhibited the uptake of colloidal ^{198}Au by macrophages (Davies et al., 1973b) and had no effect on the uptake of proteins by macrophages (Klauss, 1973) and the uptake of horseradish peroxidase by BHK-21 fibroblasts (Goldman et al., 1973). The results indicated that although CB inhibited phagocytosis and macropinocytosis, it did not have any effect on micropinocytosis. The interpretation of the above differential action of CB was complicated by the fact that CB was found to inhibit the glucose transport across plasma membranes (Estensen and Plagemann, 1972) which made it possible that CB acted by inhibiting energy production rather than by interfering with microfilaments directly. However, accumulated evidence would seem to suggest that the CB effect is linked to microfilament disorganization rather than to the inhibition of cellular metabolism. It has been shown (Zigmond and Hirsch, 1972), that at CB concentrations capable of almost totally inhibiting the phagocytosis of bacteria by polymorphonuclear leucocytes, lactate production and glucose utilization were not inhibited

to any significant extent. Cells treated with CB (up to 100 μM) for relatively long periods continue to synthesise proteins at rates not significantly different from normal (Parkhouse and Allison, 1972). In addition, CD, which does not affect the hexose uptake and thus the energy production, has the same effects as CB (Allison, 1977). Further support for the notion of a direct effect of the drug on microfilaments is provided by recent studies which have revealed the existence of high affinity binding sites for CB in human, bovine and rabbit red blood cells located on membrane proteins exposed to the cytoplasmic side of the cell membrane and which are not related to glucose transport (Lin and Snyder, 1977); also by the high affinity binding of CB to purified actin *in vitro* (Brown and Spudich, 1979; Brenner and Korn, 1979) and isolation of a complex from erythrocyte membranes which binds CB with high affinity and contains actin, spectrin and other minor components (Lin and Lin, 1979).

Microtubules do not seem to be essential for endocytosis since microtubule-disrupting agents do not inhibit the process (Malawista and Bodel, 1967). But, since the microtubule-disruption has been shown to lead to the non-selective internalization of membrane receptors (Berlin, 1975), it seems likely that microtubules are involved in the regulation of the composition of the plasma membrane areas that are internalised. It was originally proposed that this occured through the direct connection of membrane receptors to underlying microtubules or complexes of microtubules and microfilaments termed the "surface

modulating assembly" (Yahara and Edelman, 1975; Edelman et al., 1976). Recently, however, it has been suggested that microtubules do not act as anchoring devices and that rather the gathering of membrane receptors is determined by the existence of local regions depleted of microtubules and enriched in microfilaments (Berlin and Oliver, 1978).

The term exocytosis is used for the extracellular release of both secretory products and lysosomal enzymes which involves the fusion of the membrane of the lysosome and/or the secretory vesicle with the plasma membrane, and is not associated with the extracellular release of cytosolic constituents.

It has been observed that while inhibiting the phagocytosis of bacteria by polymorphonuclear leucocytes, CB did not reduce the selective release of lysosomal enzymes that normally accompanies phagocytosis, instead the selective release of lysosomal enzymes was increased (Davies et al., 1973c). The release of lysosomal enzymes during phagocytosis is believed to be the result of the fusion of lysosomes with the phagosome before this is enclosed completely by the plasma membrane (Weissmann et al., 1975). When phagocytosis is inhibited, the lysosomes fuse with the plasma membrane at the sites of the attachment of the phagocytosed particle, but these sites are never interiorized, so that lysosomal enzymes are released into the extracellular environment (Davies et al., 1973b). However, CB alone did not augment the release of lysosomal enzymes from human peripheral blood polymorphonuclear

leucocytes (Zurier et al., 1973). On the other hand, rabbit peritoneal polymorphonuclear leucocytes induced by glycogen, release their lysosomal enzymes in the presence of CB alone (Davies et al., 1973b). CB has been shown to increase the release of lysosomal enzymes by macrophages both in the presence and the absence of a phagocytic stimulus (Temple et al., 1973; Davies et al., 1973a).

It appears that CB can stimulate the exocytosis of secretory products by some cell types, inhibit it in others and have no effect at all in some instances. For example, it has no effect on the release of immunoglobulins by plasma cells (Parkhouse and Allison, 1972), the release of which is believed to proceed through a process equivalent to micropinocytosis. It increases the release of insulin from pancreatic β-cells (Orci et al., 1972) and inhibits the discharge of histamine from rat peritoneal mast cells triggered by cell-bound antibody and antigen, the drug 48/30 or the calcium ionophore A23187 (Orci et al., 1972). Clearly then, no universal model for the involvement of microfilaments in exocytosis can be offered. In the cases where CB enhances basal and/or stimulated exocytosis, it has been proposed that microfilaments act as a barrier that restricts the access of cytoplasmic particles, including lysosomes, to the plasma membrane. Loss of the integrity of the microfilaments in cells treated with CB allows easier access of lysosomes and packaged secretions to the plasma membrane (Allison, 1973).

However, such an explanation could not account for the inhibition by CB of the stimulus-coupled exocytosis observed in some cases. A model that could probably account for the above observation, was suggested by Allison (1973). It proposes that vesicles are released by sling-like mechanisms in which relative concentrations of filaments attached to or around the granule contracts after appropriate stimulation pulling the granule onto the plasma membrane, allowing the fusion of the two membranes and the release of the vesicle contents. CB could act in this model by inhibiting the contractile properties of microfilaments.

The results concerning the effect of microtubule disruption, as opposed to those obtained for microfilaments, on exocytosis of both lysosomal enzymes and secretory products are less equivocal. For instance, colchicine inhibited the release of lysosomal enzymes from human leucocytes in the presence of zymosan particles (Weissmann et al., 1971), the throbin induced release of β-glucuronidase from human platelets (Kenney and Chao, 1978) and the secretion of proteoglycans by chondrocytes (Lohmander et al., 1974).

Microtubules are supposed to act by directing the movement of the vesicles from the cytoplasm to the cell membrane (Hoffstein et al., 1977) rather than having a direct effect on the fusion of the vesicle with the cell membrane (Malawista and Bodel, 1967). However, microtubules may indirectly affect the fusion step through their effect on microfilaments as suggested by Berlin and Oliver

(1978) who, as mentioned earlier, obtained morphological evidence that the disruption of microtubules led to the formation of microfilament-rich protuberances from which secretory vesicles are excluded.

1.3 Rationale Behind the Present Project

From the review it can be seen that much work has been done on the role of microfilaments and microtubules in the exocytosis of lysosomal enzymes and secretory products, but little or no work has been done on their role, if any, in the release of intralysosomal, non-enzymatic material. It has been proposed, (de Duve and Wattiaux, 1966), however, that the extracellular release of lysosomal contents into the bile is the way by which liver cells dispose of undigested intralysosomally-accumulated material.

With the long-term aim of assessing the feasibility of a novel form of treatment for the lysosomal storage diseases based on the principle of stimulated exocytosis of the accumulated intralysosomal storage product, the aim of the present study has been to investigate the release of both the enzymatic and non-enzymatic contents of lysosomes from rat livers, with and without preloading with a variety of indigestible macromolecules, and some of the factors involved in this phenomenon. The involvement of the microfilament component of the cytoskeleton has been examined using cytochalasin B (CB).

The liver was the organ used in the present study because it is the organ most consistantly grossly affected in lysosomal storage diseases and it would be expected to be the prime target for a therapy based on stimulated exocytic disgorgement.

Isolated perfused livers were used because they provide an *in vitro* system in which the structural and presumably the functional integrity of the organ is maintained. However, to help differentiate between the contributions of the different cell types, some experiments were also carried out using short-term hepatocyte cultures.

Conditions similar to lysosomal storage diseases were induced by the *in vivo* administration of ^{125}I-polyvinylpyrrolidone (PVP), ^{125}I-Triton WR-1339 and ^{3}H-dextran. The choice of the injected material was based on their fulfilling the following conditions:

1. They should be of high molecular weight and therefore unable to permeate through the lysosomal membrane.

2. They should accumulate intralysosomally.

3. They should be indigestible or only slowly degraded.

All the three materials used, i.e. ^{125}I-PVP, ^{125}I-Triton WR-1339 and ^{3}H-dextran, had high molecular weights (40,000, 100,000 and 82,000 respectively), fulfilling the first condition.

In the present study the intralysosomal localisation of ^{125}I-PVP was established and that of ^{3}H-dextran was confirmed by subcellular distribution studies of the label and marker enzymes, whereas the previous studies (Wattiaux *et al.*, 1963) have shown that ^{125}I-Triton accumulates intralysosomally.

Previous studies had established that ^{125}I-PVP and ^{125}I-Triton WR-1339 are indigestible, whereas ^{3}H-dextan is degraded very slowly (Colley and Ryman, 1976).

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Equipment

The low-speed centrifugations were carried out in a MSE 6L centrifuge using a swing-out rotor head and the high speed ones in a MSE superspeed 65 centrifuge using a 8 x 50 ml MSE angle rotor head, with the exception of the isopycnic-gradient centrifugations which were carried out using a 3x70ml swing-out MSE rotor head.

The colour development in the case of the lysosomal and microsomal markers, protein and phosphate, was measured using a Bausch and Lomb Spectronic 710 spectrophotometer, whereas the rate of reaction in the case of the mitochondrial and cytosol marker was recorded using a Unicam SP-500 spectrophotometer connected to an SP 45 chart recorder.

The sucrose refractive index was measured using an Abbé refractometer, purchased from Gallenkamp.

^{3}H radioactivity was measured using a Nuclear Chicago Liquid Scintillation Counter, mark 1.

^{125}I radioactivity was measured using an LKB Wallac 80,000 Gamma Sample Counter.

Potassium was measured using an Instrumentation Laboratory 543 Digital Flame Photometer.

2.1.2 Chemicals

Supplier	Chemical
Sigma London Chemical Company	Enzymes and enzyme substrates (cytochrome *c* type III from horse heart, collagenase type I from *clostridium histolyticum*, Hyaluronidase type I from bovine testes).
	Bovine Serum Albumin fraction V powder.
Flow Laboratories	Leibovitz-15 medium.
	Foetal calf serum.
Glaxo	Penicillin/streptomyosin.
May and Baker Ltd	Pentobarbitone Sodium.
Paines and Byrne Ltd	Heparin injection.
BDH	Glutamine.
	All other chemicals.

Radiochemical Centre Amersham	PVP (^{125}I) injection. Radioactive concentration: 125μCi/ml, specific activity: 20-60μCi/mg PVP, average molecular weight: 30,000-40,000
	Dextran (^{3}H) solid, specific activity: 10mCi/g, average molecular weight: 82,000.

2.2 METHODS

2.2.1 Fractionation Procedures

The experimental animals used were Sprague-Dawley male rats weighing between 400-500 g. They were maintained on a "Spratts Rodent Breeding" diet, food and water being allowed *ad libitum*.

The rats were killed by dislocation of the cervical vertebrae under light ether anaesthesia. The abdomen was then opened and the inferior vena cava (IVC) was ligatured between the hepatic vein and the renal vein. Subsequently the thorax was cut open, a canula was passed into the right auricle down the IVC and the hepatic portal vein was sectioned. In order to remove the blood, the liver was perfused *in situ* with NaCl (0.9%) at 4 ^{o}C containing heparin (500 IU/ml) over a period of 5 min. The liver was then removed and washed briefly in sucrose (0.25 M) at 4 ^{o}C. The tissue was then forced by hand through a metal grid (mesh size, 0.1 mm) and was homogenised in sucrose (0.25 M) in a Potter-Elvenjheim apparatus using three up and down strokes of the motor driven Teflon pestle at 10,000 RPM. The homogenate was diluted with sucrose (0.25 M) to a final concentration of one gram weight of liver in 10 ml (10% w/v).

2.2.1.1 Differential centrifugation

The scheme used (Fig. 2) was a modification of that described by Dampure (1971). All centrifugations were carried out at 4 ^{o}C.

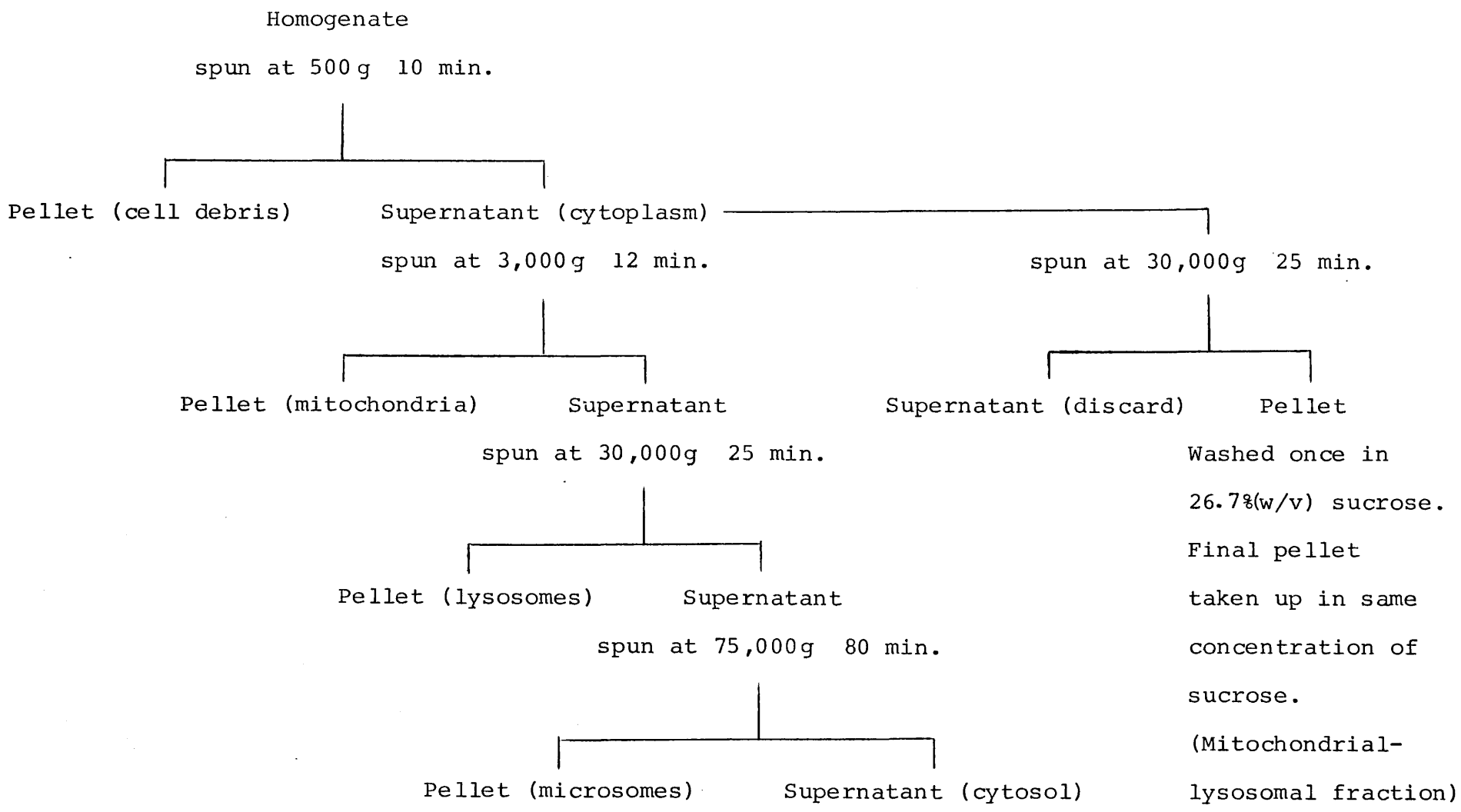

Fig. 2 The differential centrifugation scheme.

The liver homogenate was spun at 500 g for 10 min. to give a supernatant (cytoplasmic fraction) and a pellet mainly containing unbroken cells, nuclei and plasm membranes (cell debris fraction). The cytoplasmic fraction was spun successively at 3,000 g for 12 min., 30,000 g for 25 min. and 75,000 g for 80 min., to give mitochondrial, lysosomal and microsomal pellets respectively, which were resuspended in known volumes of sucrose (0.25 M). The final supernatant gave the cytosol fraction.

For the isopycnic gradients, a mitochondrial-lysosomal pellet was prepared by spinning the cytoplasmic fraction at 30,000 for 25 min. The pellet obtained was resuspended in a sucrose solution having the same concentration as the lighter sucrose solution used in the preperation of the isopycnic gradient (ie 26.7% w/v) and was washed once. The final pellet was resuspended as above.

A mitochondrial-lysosomal pellet prepared as above, but resuspended in Leibovitz L-15 medium was used for the preparation of lysosomal enzymes in a crude form. The suspension was sonicated and was centrifuged at 75,000 g for 80 min. to obtain a pellet containing unbroken organelles and membrane fragments and a supernatant which was used as the crude lysosomal enzyme preparation.

In order to investigate the sedimentability of different lysosomal enzymes and also for liberating the intralysosomally accumulated material, in the case of lysosomes loaded with ^{125}I-PVP, ^{125}I-Triton WR-1339 or ^{3}H-dextran, a mitochondrial-lysosomal pellet was prepared

as above. The organelles were lysed by suspending the pellet in hypotonic sucrose (0.085%) and incubating at 37 ^{o}C for 1 hr. At the end of the incubation period, the suspension was centrifuged at 75,000 g for 60 min.

2.2.1.2 Gradient Centrifugation

All the gradients used were continuous and were made up from different concentrations of sucrose solutions employing an MSE gradient generator.

2.2.1.2.1 Sedimentation Gradients

Sedimentation gradients designed to separate organelles primarily on the basis of their size were generated as follows. On top of a dense sucrose shelf (3.5 ml, 82% w/v) a continuous gradient of 36.5 ml was generated, constructed from 40% (w/v) and 8.5% (w/v) sucrose. 5 ml of the cytoplasmic fraction was layered on top of the gradient which was then centrifuged at 1,350 g for 120 min., in a swing-out head. 3 ml fractions were collected by piercing a hole in the bottom of the tube.

2.2.1.2.2 Isopycnic Gradients

The technique of isopycnic-gradient centrifugation was used to separate mitochondria and lysosomes on the basis of their buoyant densities. The isopycnic gradients used were continuous and were normally constructed without a "shelf", using 25 ml of 82% or 75% (w/v) and 25.5 ml of 26.7% (w/v) sucrose solution. 2 ml of the mitochondrial-lysosomal fraction was layered on top of the gradients and

they were centrifuged at 75,000 g for 210 min. 3 ml fractions were collected through a hole pierced in the bottom of the tube.

2.2.2 Liver Cell Cultures

Liver cell cultures were set up by a modification of the method described by Berry and Friend (1969). Male Sprague-Dawley rats were injected intraperitoneally with 0.5 ml sodium pentobarbitone (60 mg/ml) and heparin (2,000 USP). The abdomen was shaved, washed with iodine (Povidone surgical scrub) and cut open. The inferior vena cava was ligatured between the kidney and the liver and the hepatic portal vein was cannulated. The thorax was cut open and a canula was passed into the right auricle and down the inferior vena cava.

In order to wash the blood away and also to disrupt the desmosomes, the liver was perfused *in situ* with 150 ml of PBS"A". This was followed by the perfusion of 350 ml of Leibovitz 15 (L-15) medium containing collagenase (0.05%) and hyaluronidase (0.1%), to disrupt the connective tissue. Both perfusion media were oxygenated, kept at 37 ^{o}C and the perfusion rate was 40 ml/min. To ensure the maximum possible sterility, sterilized dissecting instruments and media were used. The enzyme containing medium was sterilized by passing through a 0.25 μm Millipore filter.

At the end of the perfusion, the liver was removed into 50 ml of enzyme-containing L-15 medium kept at 4 ^{o}C.

It was gently dispersed, bubbled with O_2 and was incubated with shaking for 20 min. at 37 ^{o}C. After the incubation the cells were spun down (50 g for 4 min.) and the cell pellet was resuspended in L-15 medium supplemented with foetal calf serum (17.5%), glutamine (0.06%) and penicilin-streptomyosin (100 IU/ml each), (complete medium) and were washed once. The final pellet was resuspended in complete medium at a final concentration of 1.5 x 10^6 cells/ml. The cells were either plated out in glass petri dishes (60 mm diameter, 2 ml/petri dish) or were incubated for 40 min. at 37 ^{o}C with mild shaking. At the end of the incubation the cells were spun down and the cell pellet was washed once in complete medium. The final cell pellet was resuspended in complete medium at a final concentration of 1.5 x 10^6 cells/ml and 2 ml of the suspension were plated out per glass petri dish. The petri dishes were incubated in an atmosphere of oxygen at 37 ^{o}C in a LEEC incubator. Cytochalasin B (CB) was dissolved in dimethylsulphoxide (DMSO) and added to some of the petri dishes 90 min. after the beginning of the incubation at a final concentration of 25 μg/ml (1% v/v DMSO). An equivalent set of petri dishes acting as controls were treated with DMSO (1% v/v) only. At set time intervals petri dishes were removed from the incubator, the cells were spun down (50 g for 10 min.) and the cell pellet was resuspended in L-15 medium. Both the cell suspension and the medium were assayed for lysosomal enzymes and, in the case of cultures set up from animals pre-injected with radioactivite material, for radioactivity.

The viability of the hepatocytes after the pre-incubation step was estimated by trypan blue exclusion studies. The hepatocyte preparation was diluted six times in trypan blue (0.4% in 0.9% sodium chloride) and left for 5 min. at 23 ^{o}C and the trypan blue-excluding cells were counted using a hematocytometer (Laishes and Williams, 1976).

2.2.3 Liver Perfusions

The apparatus used is shown in Fig. 3 and it was kept in a cabinet maintained at 37 ^{o}C. The perfusion medium used was Leibovitz L-15 medium gassed with O_2, supplemented with bovine serum albumin (5%) and glutamine (0.06%) and containing streptomycin and penicillin (both 100 units/ml).

The operation procedure (Fig. 4) employed was as follows: male Sprague-Dawley rats were kept under ether anaesthesia. The abdomen was washed with iodine (Povidone surgical scrub) and the abdominal wall was cut open along the mid line. The liver was freed from connective tissue, the oesophagus was ligatured and was sectioned anteriorly to the tie (Fig. 4; 1,2). Loose ties were put round the bile duct close to the duodenum (Fig. 4; 3), the tributary vein from the intestine to the portal vein (Fig. 4; 4) and round the portal vein, one close to the liver and another about 1 cm from the first (Fig. 4; 5,6). The connections between the caudate lobe and the inferior vena cava (IVC) were cut and two loose ties were put round the IVC anterior to the

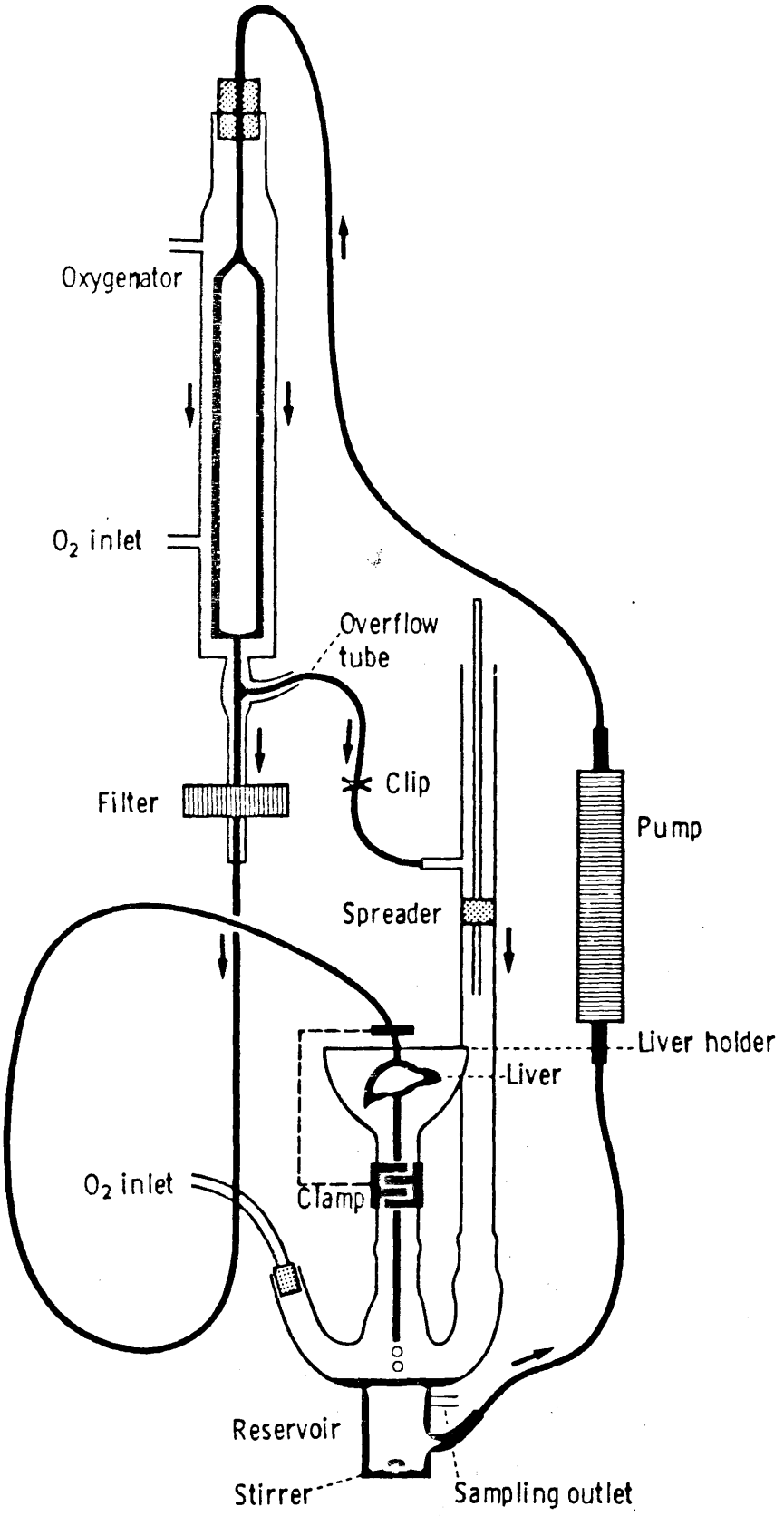

Fig. 3: APPARATUS FOR THE ISOLATED RAT LIVER PERFUSION

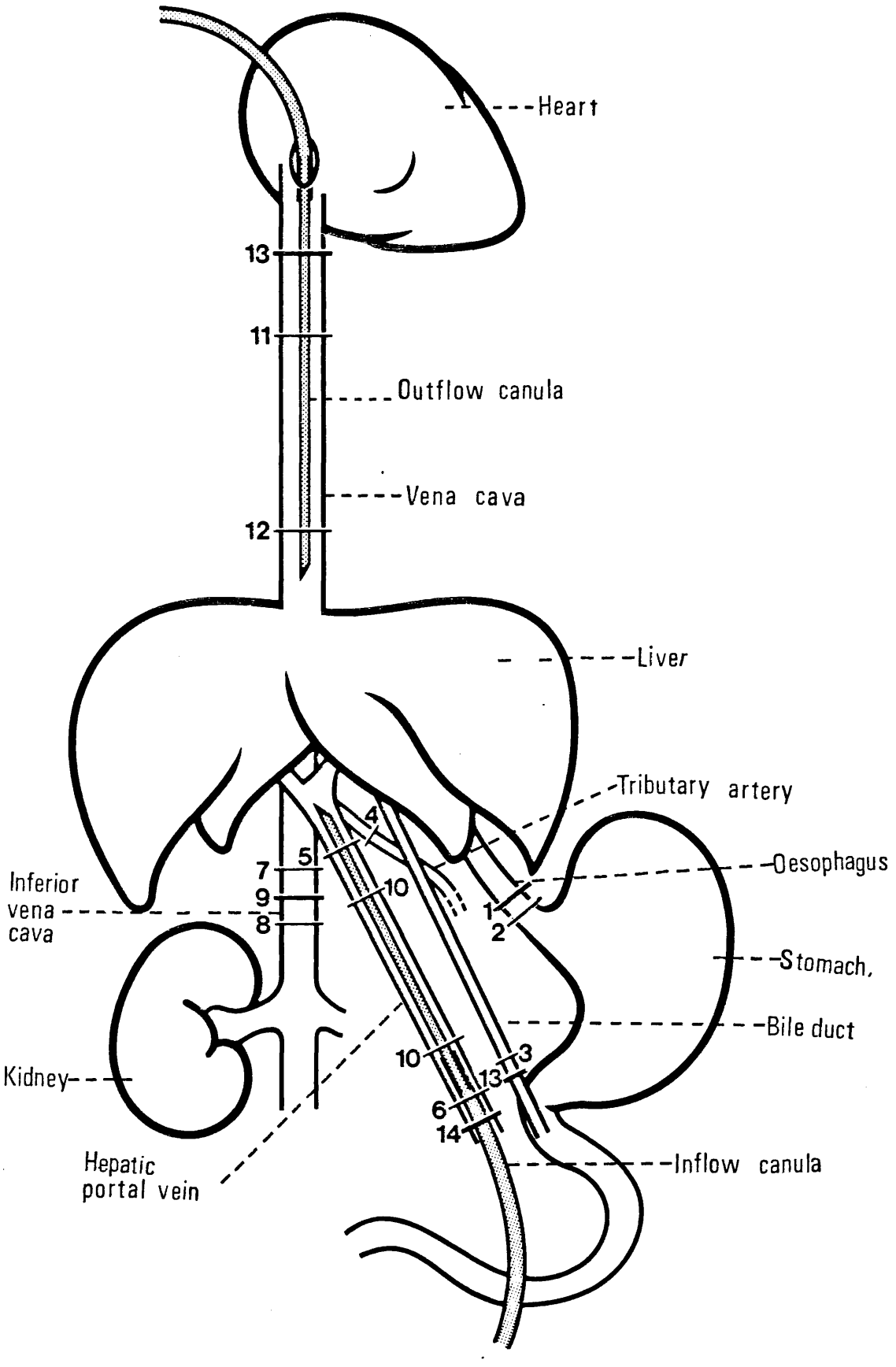

Fig. 4 - see overleaf

Fig. 4

The operation procedure followed for the isolation of rat livers. (▬) represent sectioning points and (___) ligatures. The numbers represent the order of each step.

kidney and posterior to the liver, (Fig. 4; 7,8). 0.5 ml heparin (500 IU/ml) solution was injected into the penile vein and after 5 min. the IVC was tied off and sectioned between the ties (Fig. 4; 9).

The perfusion cabinet (Fig. 3) was opened, the canula was removed from the reservoir, making sure clip B was closed, and was cleared of any air bubbles present. The tributary artery and the bile duct were tied off. The tie round the portal vein further from the liver was also tied off and a small hole was opened in the vein anterior to it. The canula was inserted in the portal vein and was firmly tied into position (Fig. 4; 10). At this stage the thorax was opened, cutting through the ribs and round the diaphragm. Two loose ties were put around the vena cava (Fig. 4; 11,12) and a plastic canula was inserted down the right auricle and tied firmly in place. The attachments of the liver to the stomach, the intestine and the heart were cut. A scoop was placed under the liver and was raised slightly to enable all the remaining connections to be cut. The liver was removed from the carcass and 50 ml of the perfusate were allowed to pass through it before the liver was put in the holder that was filled with liquid parafin at 37 ^{o}C (Fig. 3). After making sure that the rate of flow was reasonable (at least 80 drops/min.) the holder was put in the cabinet.

In the case of cytochalasin B (CB)-treated livers, the CB dissolved in DMSO (100 μg/ml, 1% v/v DMSO) was added 90 min. after the beginning of the perfusion. The

control livers were treated with DMSO (1% v/v). At 30 min. intervals, the rate at which perfusate was passing through the liver (drops/min) was recorded. Samples were assayed for lysosomal enzymes and lactate dehydrogenase. In addition, radioactivity was determined when livers preloaded with indigestable material were used. At the end of the perfusion the liver was homogenised in sucrose (0.25 M) and the homogenate was assayed for the same parameters.

2.2.4 Analytical methods

2.2.4.1 Enzymes

The following marker enzymes were assayed:

Subcellular fraction	Enzyme
Lysosomes	Arylsulphatases A & B
	β-Galactosidase
	β-N-acetyl-Glucosaminidase
Mitochondria	Cytochrome c oxidase
Microsomes	Glucose-6-Phosphatase
Cytosol	Lactate dehydrogenase

Prior to assaying the above enzymes, the samples were sonicated, frozen and thawed once. All the methods used were spectrophotometric.

The blanks used consisted of the test solution and all the reagents except the substrate. The latter was added after the addition of the reaction-terminating solution. In the case of cytochrome-c-oxidase and lactate dehydrogenase buffer blanks were used.

2.2.4.1.1 Arylsulphatase E.C. 3.1.6.1.

Arylsulphatases A and B were assayed by the method of Dodgson *et al*. (1955). To 0.1 ml of the test solution was added 0.2 ml of the substrate, p-nitrocatechol sulphate (0.02 M) in acetate buffer (0.5 M), pH 6.0. After incubating for 60 min. at 37 ^{o}C (5 hrs at 37 ^{o}C in the case of samples from perfused liver studies), the reaction was stopped by the addition of 3.5 ml of NaOH (1 M). The

optical density was measured at 515 nm. The standard used was p-nitrocatechol.

2.2.4.1.2 β-Galactosidase E.C. 3.2.1.23.

The enzyme was assayed by the method described by Barrett(1972). To 0.5 ml of test solution was added 0.5 ml of p-nitrophenyl-β-D-galactopyranoside (7.5 mM) in sodium citrate buffer (0.3 M), pH 3.6 and 0.5 ml of water. After 60 min. incubation at 37 ^{o}C, the reaction was stopped by the addition of 1.5 ml of sodium carbonate-bicarbonate buffer (0.5 M), pH 10. The precipitate formed was spun down and the optical density was measured at 420 nm. p-nitrophenol was used as the standard.

2.2.4.1.3 β-N-Acetyl-Glucosaminidase E.C. 3.2.1.30.

The enzyme was assayed by the method described by Barrett(1972). To 0.5 ml of the test solution was added 0.5 ml distilled water and 0.5 ml of p-nitrophenyl-β-D-glucosaminide (7.5 mM) in sodium citrate buffer (0.3 M) pH 4.5. After incubating for 60 min. at 37 ^{o}C the reaction was stopped by the addition of 1.5 ml of sodium carbonate-bicarbonate buffer (0.5 M) pH 10. The precipitate formed was spun down and the optical density was measured at 420 nm. p-nitrophenol was the standard used.

2.2.4.1.4 Lactate dehydrogenase E.C. 1.1.1.27.

The enzyme was assayed by the method described by Plummer and Wilkinson (1963). 0.1 ml of test solution was added to 2.9 ml of buffer/substrate solution containing NADH (10^{-4} M), sodium pyruvate (0.7 mM) in Tris-HCl

buffer (0.05 M), pH 7.4. The rate of change in the optical density at 340 nm was recorded at 25 ^{o}C using a buffer blank.

2.2.4.1.5 Cytochrome-c-Oxidase E.C. 1.9.3.1.

The enzyme was assayed by a modification of the method of Cooperstein and Lazarow (1951) similar to that of Appelmans et al. (1955). 150 ml of cytochrome c (5.0×10^{-5} M) in phosphate buffer (0.03 M) pH 7.4, was reduced with 0.5 ml of sodium hydrosulphite (0.6 M) and shaken for 1 hr. To 3 ml of this solution in a 1 cm cuvette was added 0.01 to 0.1 ml of test solution. The rate change in the optical density at 550 nm was measured at 25 ^{o}C using a buffer blank. To minimise the effect of substrate limitation, the volume of enzyme was adjusted until the rate of decrease in the optical density was linear.

2.2.4.1.6 Glucose-6-Phosphatase E.C. 3.1.3.9.

The enzyme was assayed by the method of Hers et al. (1953). To 0.2 ml of test solution was added 2 ml of disodiumglucose-6-phosphate (4.3×10^{-3} M) in sodium cacodylate buffer (0.033 M), pH 6.5, containing ethylenediaminetetra-acetic acid (EDTA, 0.001 M). After incubating for 30 min. at 37 ^{o}C the reaction was stopped by the addition of 5 ml tricholoacetic acid (TCA, 8%). The precipitate was spun off and the supernatant was assayed for phosphate (see 2.2.4.2.2).

2.2.4.2 Miscellaneous

2.2.4.2.1 Protein was measured by a modification of the method of Lowry et al. (1951). 0.1 ml of test solution was made up to 1 ml with distilled water, to it was added 5 ml of freshly prepared solution containing sodium carbonate (2%), copper sulphate (0.01%) and sodium potassium tartarate (0.02%) in NaOH (0.1 M). After standing for 5 min., 0.5 ml of Folin-Ciolcateu reagent was added and was immediately mixed. After standing for 30 min. at room temperature, the optical density was measured at 750 nm. The standard used was an aqueous solution of bovine serum albumin.

2.2.4.2.2 Phosphate was determined by a modification of the method of Fiske and Subbarow (1925) similar to that of King and Wootton (1959). The total material from the glucose-6-phosphatase assays (about 5 ml) was used and to it was added successively 0.4 ml conc. $HClO_4$, 0.4 ml ammonium molybdate solution (5%) and 0.4 ml ascorbic acid solution (0.5%), mixing after each addition. The solution was left to stand for 120 min. and the optical density was measured at 820 nm. The standard used was an aqueous solution of potassium dihydrogen orthophosphate.

2.2.4.2.3 Potassium was estimated using flame photometry (Wootton,1974). To compensate for random changes in flame temperature and various chemical interferences that could have caused fluctuations in the emission signal, Li was used as an internal standard.

2.2.4.2.4 Sucrose concentrations (% w/w) were determined by measuring the refractive index of various gradient fractions.

2.2.4.2.5 Radioactivity from ^{3}H was assayed by liquid scintillation counting. The scintillation fluid used consisted of toluene, containing 125 mg p-Bis-2-(5-phenyloxazolyl)-benzene and 10 g of 2,5-diphenyloxazole in 2.5 ℓ of toluene, and triton X-100 in the ratio 1:2. 9 ml of the above scintillant were mixed with 1 ml of test solution.

The radioactivity from ^{125}I was assayed by gamma counting.

2.2.5 Miscellaneous

2.2.5.1 The Iodination of Triton WR-1339

^{125}I-Triton WR-1339 was prepared as follows. 20 µl of Triton WR-1339 solution (1 mg/20 µl) in phosphate buffer (0.5 M) pH 7.4 was mixed with 10 µl of Na^{125}I (1 mCi) and 10 µl of a chloramine T solution (10 µg/10 µl) in phosphate buffer (0.05 M) pH 7.4. The mixture was vortexed for 60 sec. at the end of which 10 µl of a sodium metabisulphite in phosphate buffer (10 µg/10 µl) were added to stop the reaction. The mixture was layered on a G-25 Sephadex column and was eluted with phosphate buffer pH 7.4 (0.5 M). 1 ml samples were collected and the profile of the distribution of the radioactivity is shown in Fig. 5. Fractions 4-7 were pooled together and were used as the injection material.

The recovery of radioactivity from the column was 64.4% and from the recovered radioactivity 27.9% was associated with the pooled fractions.

2.2.5.2 Gel Chromatography Studies

Unless otherwise stated, G-200 Sephadex columns (150 x 80 cm) were used. The gel slurry was prepared as described in the Pharmacia manual. The samples were eluted using Tris-HCl buffer (10 mM), pH 7.4, at 4 ^{o}C.

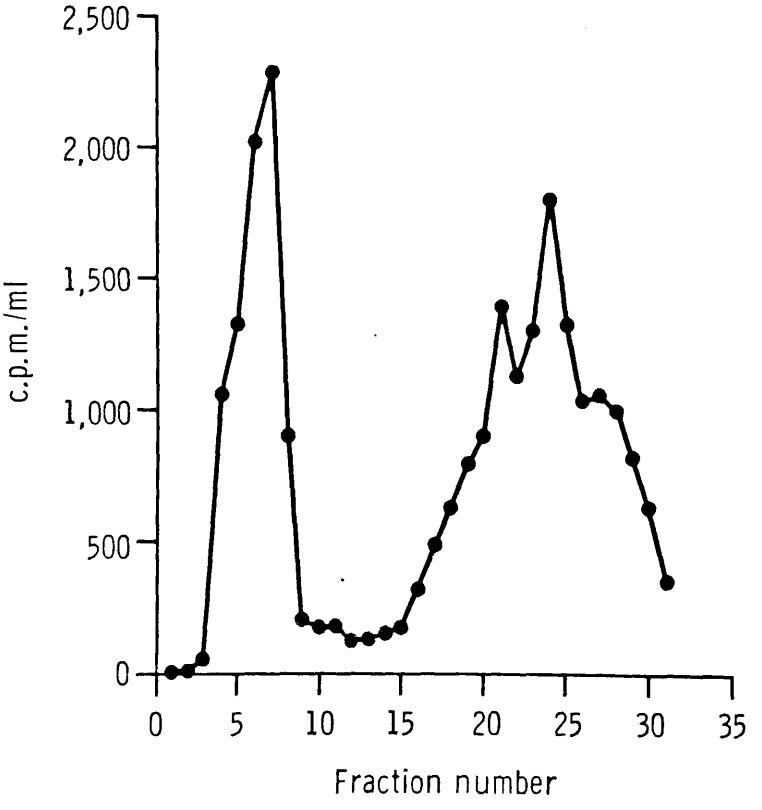

Fig. 5

The elution profile obtained after passing the reaction mixture (40 μl) of the iodination of Triton WR-1339 down a Sephadex G-25 (coarse) column.

2.2.5.3 Injection Solutions

^{125}Iodine polyvinylpyrrolidone(^{125}I-PVP)

The animals were injected through the tail vein with 100 mg/100 g body weight of a PVP (average molecular weight 40,000) solution (250 mg/ml in saline) together with 65 μCi ^{125}I-PVP.

^{3}H-Dextran

The animals were injected intraperitoneally with 200 mg/100 g body weight of a dextran (average molecular weight 80,000) solution (250 mg/ml in saline) together with 100 μCi of ^{3}H-Dextran.

Triton WR-1339

The animals were injected intraperitoneally with 85 mg/100 g body weight of a Triton WR-1339 solution (170 mg/ml in saline). When required 50 μCi of ^{125}I-Triton WR-1339 were simultaneously injected.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Non-loaded Livers

3.1.1 Introduction

The existence of a cytoskeleton, composed of microtubules and filaments, in liver cells has been shown both in biochemical and morphological studies (Oda et al., 1974; French and Davies, 1975; Jeanrenaud et al., 1977). The use of known poisons of the cytoskeleton has produced evidence for its role in secretory and other processes characteristic of liver cells. For example, microtubules have been shown to be necessary for the secretion of newly-synthesized albumin (Le Marchand et al., 1974), the release of very-low-density lipoproteins (Le Marchand et al., 1973; Stein and Stein, 1977) and in bile lipid secretion (Gregory et al., 1978). Their role in the latter process however, is still a matter of dispute (Stein et al., 1974).

Liver cell microfilaments have been shown to be involved in the secretion of lipoproteins (Prentki et al., 1979) and also in the maintenance of the cell shape (Prentki et al., 1979) and bile flow (Phillips et al., 1976).

In the present study, the *in vitro* systems of short-term hepatocyte cultures and isolated liver perfusions have been used to investigate the involvement of microfilaments in the control of the release of lysosomal enzymes, as judged by the sensitivity of this process to cytochalasin B. Subcellular distribution studies were also carried out, employing the techniques of differential fractionation and both sedimentation and isopycnic gradient centrifugations, in order to investigate possible changes in the subcellular distribution of marker enzymes during the perfusion and also for comparison with the subsequent subcellular fractionation studies of livers preloaded with undigestible materials (see 3.2, 3.3 and 3.4).

3.1.2 Results

3.1.2.1 Subcellular Distribution Studies in Non-perfused Livers

The distribution of intracellular marker enzymes was investigated using the techniques of differential fractionation, sedimentation and isopycnic gradient centrifugation.

Table 1 shows the activities of different enzyme markers found in the cell debris pellet and the cytosol.

The method by which the gradients were generated provided reproducible, linear gradients, whether for sedimentation (Fig. 6) or isopycnic (Fig. 6) gradient centrifugation.

A good separation of the subcellular components was provided by the sedimentation gradients (Fig. 7). The majority of the mitochondria (cytochrome c oxidase) sedimented over the dense sucrose shelf in fraction 2, whereas the endoplasmic reticulum (glucose-6-phosphatase) remained near the top of the gradients, showing a maximum in fractions 12-13. The majority of the non-particulate cytoplasm, as judged by the distribution of lactate dehydrogenase, remained in the top 2-3 fractions.

The distribution of lysosomal enzymes was more diffuse. A major peak was observed between fractions 9-13, but a "pseudo" peak was also found over the dense sucrose "shelf". The profiles of the distribution of the different lysosomal enzymes assayed, showed minor differences

	Cell debris fraction		Cytosol	
	RSA	%	RSA	%
AS	1.2	38.5	0.02	0.5
β-GAL	0.9	28.5	0.03	0.9
NAGA	1.0	31.5	0.02	0.4
LDH	0.6	18.5	2.70	78.3
Cyt-c-ox	1.4	44.5	0.0	0.0
Gluc-6-P	1.5	48.9	0.01	2.1
Protein	1.0	32.0	1.0	29.0

Table 1

The RSA and the percentage activities of marker enzymes and protein associated with the cell debris and the cytosol fractions obtained from rat liver homogenates.

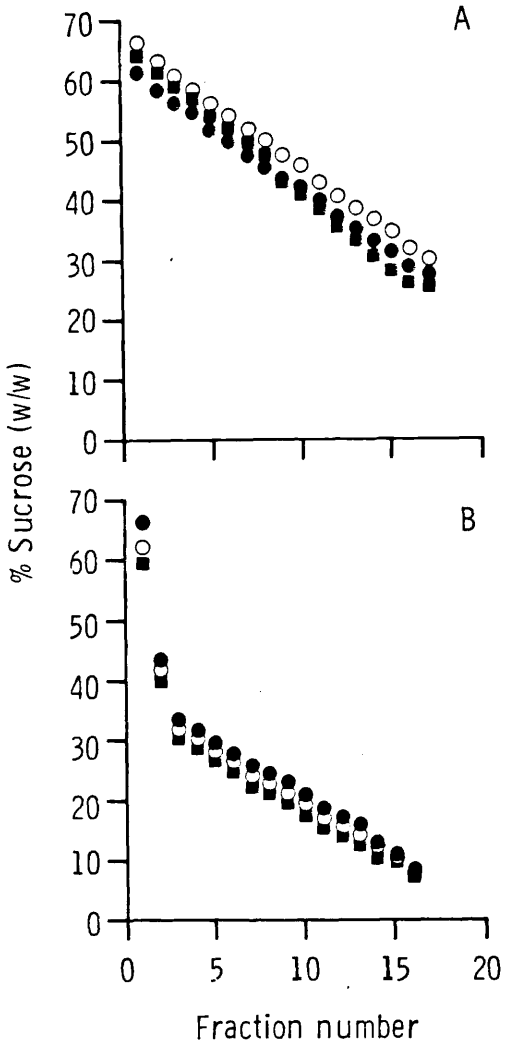

Fig. 6

The characteristics of three (o o, ● ●, ■ ■) nominally 26.7-82% w/v sucrose density gradients (isopycnic) (A) and three (o o, ● ●, ■ ■) nominally 8.5-40% w/v sucrose density gradients (sedimentation) (B). The ordinates represent the concentration of sucrose (w/w) in each fraction.

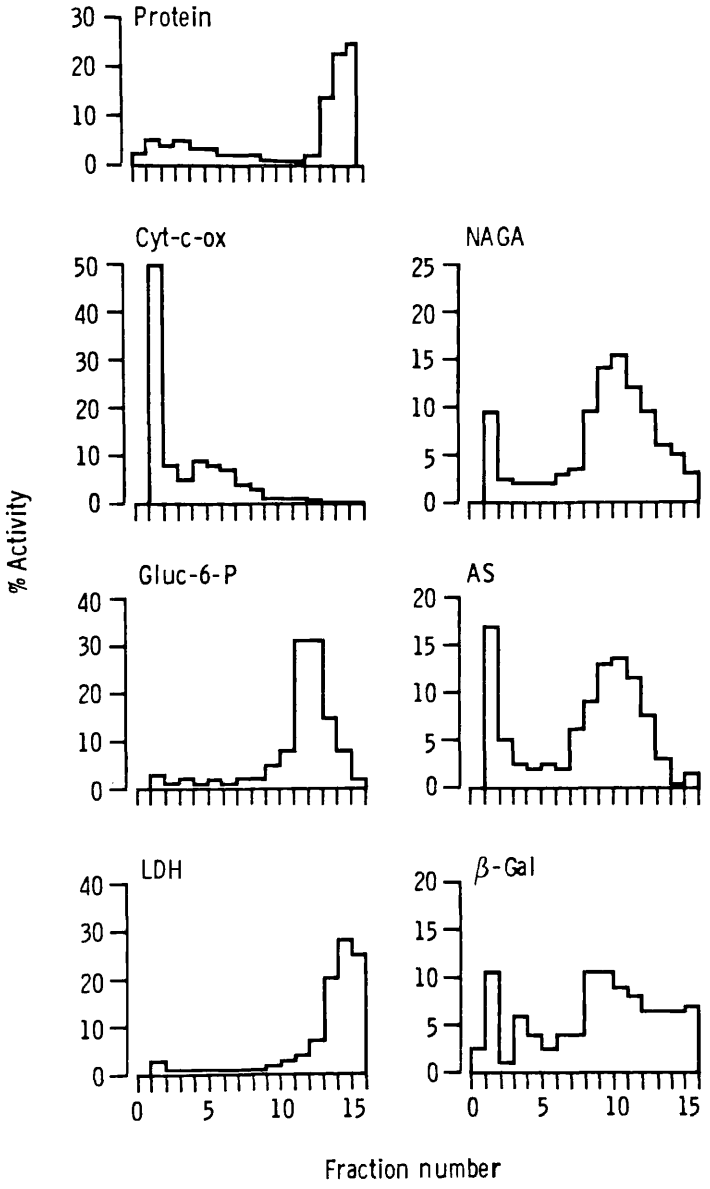

Fig. 7

The subcellular distribution of marker enzymes and protein on a 8.5-40.0% w/v sucrose sedimentation gradient obtained from a non-loaded rat liver.

which reflect the heterogeneity of the liver lysosomal population.

The profile of the distribution of the protein showed a maximum in the lightest sucrose fractions which coincided with the lactate dehydrogenase maximum. A peak was also observed over the dense sucrose "shelf". The observed protein distribution indicated that the cytosolic and mitochondrial proteins constitute the majority of the cytoplasmic protein.

Fig. 8 shows the distribution of lysosomes, mitochondria and protein in isopycnic gradients. The profiles of lysosomal enzymes practically coincided with that of cytochrome-c-oxidase. The densities of the lysosomal and the mitochondrial peaks were 1.21 and 1.19 respectively, in good agreement with the findings of de Duve (1966) and Wattiaux. The protein and cytochrome-c-oxidase profiles coincided. This was expected since the majority of the protein in the mitochondrial-lysosomal preparation used for the isopycnic gradient centrifugation would be expected to be provided by the mitochondria.

It has been shown that the intralysosomal accumulation of *in vivo* injected Triton WR-1339 leads to a reduction in the buoyant density of the lysosomes, providing the means of separating lysosomes from mitochondria on isopycnic gradients (Wattiaux *et al.* 1963). The effect of Triton WR-1339 on the profile of the distribution of lysosomes and mitochondria on the isopycnic gradients employed in the present study is shown in Fig. 9.

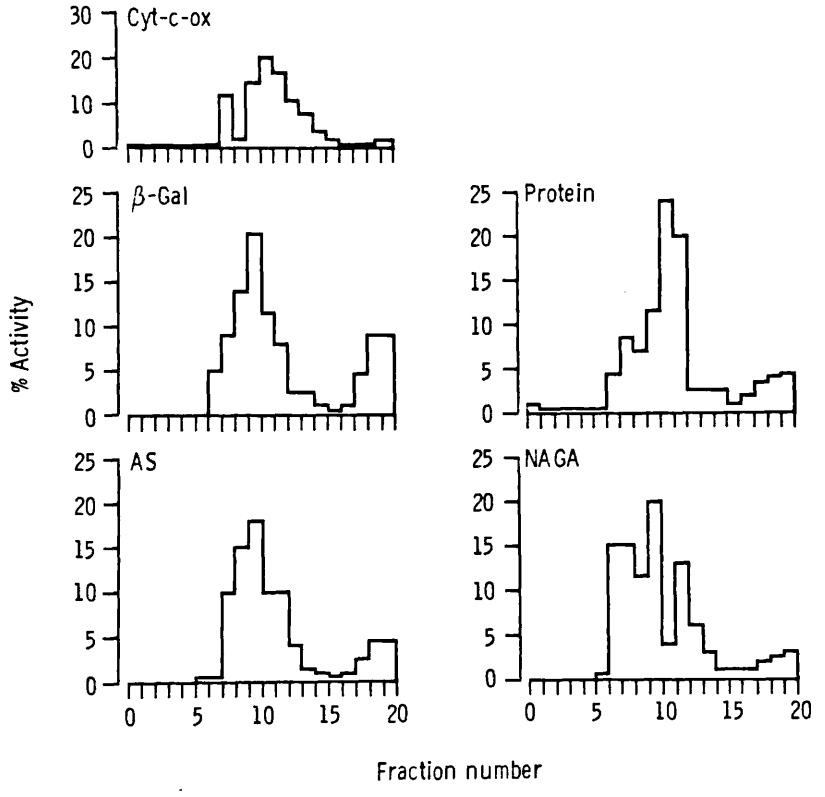

Fig. 8

The distribution of marker enzymes and protein on a 26.7-82.0% w/v sucrose isopycnic gradient obtained from a non-loaded rat liver.

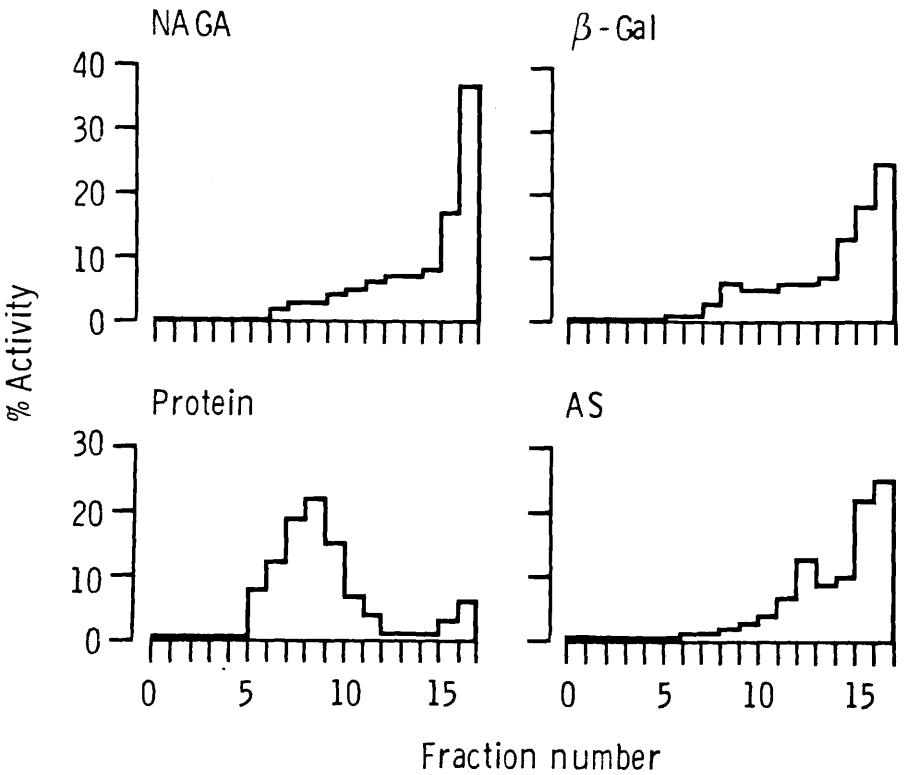

Fig. 9

The effect of Triton WR-1339 on the distribution profiles of lysosomal enzymes on a 26.7-82% (w/v) sucrose isopycnic gradient. The detergent was injected 4 days earlier.

In the presence of the detergent 43.5% of the β-galactosidase, 47.1% of the arylsulphatase and 54.3% of the β-N-acetyl-glucosaminidase activity was found in the top three fractions of the gradient, as opposed to 3.5%, 4.2% and 3.5% respectively, found at the top in its absence. Triton WR-1339 had no effect either on the profile of the distribution of protein or that of cytochrome-c-oxidase.

The recoveries of the various enzymes and protein from both the sedimentation and the isopycnic gradients are listed in Table 2.

	Sedimentation Gradient			Isopycnic Gradient		
	Mean	Range	No	Mean	Range	No
AS	93	88-106	6	84	72-100	6
β-GAL	92	84-103	5	96	93-103	4
NAGA	99	83-108	8	80	71-96	5
Cyt-c-ox	67	55-85	4	65	55-80	5
Gluc-6-P	69	59-80	4	-	-	-
LDH	92	76-106	5	-	-	-
Protein	107	98-113	4	101	91-105	5

Table 2

The activities recovered from the sedimentation and isopycnic gradients expressed as the percentage of the loaded activities.

3.1.2.2 Hepatocyte Cultures

The release of lysosomal enzymes from freshly-isolated rat hepatocytes kept in culture was examined both in the presence and in the absence of the microfilament poison CB.

The number of cells present at different steps in the isolation procedure is shown in Table 3. Only 13% of the cells present, after passing the tissue suspension through the nylon mesh, were present after the final step of the isolation procedure. It appears that the majority (77.3%) of the initially-isolated cells were lost during the washing step, whereas the majority of the cells (approximately 5.8%) survived the final preincubation step. The activities of lysosomal enzymes and lactate dehydrogenase associated with the final cell preparation were in agreement with the estimation of an approximately 40% cell loss during the preincubation step (Table 4). Also the final cell preparation did not appear to be specifically enriched in any of the enzymes assayed (Table 5). Trypan blue exclusion studies revealed that 90% of the isolated rat hepatocytes did not stain.

Freshly isolated hepatocytes in culture were found to release both lysosomal enzymes and lactate dehydrogenase (Table 6).

In preliminary experiments in which the final preincubation step had been omitted, hepatocytes thus prepared released even higher amounts of lactate dehydrogenase and lysosomal enzymes. So, at zero time the culture

Number of hepatocytes isolated			
	Mean	Range	No
A	327×10^6	100×10^6-623×10^6	5
B	74×10^6	33×10^6-128×10^6	5
C	43×10^6	23×10^6- 53×10^6	5

Table 3

The number of hepatocytes present at each step of the isolation procedure:

A: Number of cells after the mesh

B: Number of cells after the washing

C: Number of cells after the preincubation.

% Activity associated with cells after the preincubation step			
	Mean	Range	No
AS	56	54-65	5
NAGA	56	51-62	5
β-GAL	48	43-52	5
LDH	46	38-49	5

Table 4

The activities of lysosomal enzymes and lactate dehydrogenase associated with the hepatocytes after the preincubation step, expressed as the percentage of the activities after the washing step.

	A			B			C
	Mean	Range	No	Mean	Range	No	
AS	156776	99000-176000	9	7838	4300-11250	5	5.0
β-GAL	42500	38000-50000	4	2550	1600-2800	5	6.0
NAGA	235360	213000-266600	4	15300	10300-26300	5	6.5
LDH	11012	7600-13600	6	330	56-895	5	3.0

Table 5

The activities of lysosomal enzymes and lactate dehydrogenase associated with liver homogenates (A) and the hepatocytes after the preincubation step, expressed as absolute activities (B) and (C) as the percentage of (A).

	Controls			CB treated			p
	Mean	Range	No	Mean	Range	No	values
AS	14	12-18	4	18	16-20	4	$p<0.01$
NAGA	16	12-20	4	18	14-23	4	$p<0.05$
β-GAL	32	25-36	4	37	31-42	4	$p<0.025$
LDH	30	24-43	4	30	25-35	4	$p<0.3$

Table 6

The amounts of lysosomal enzymes and lactate dehydrogenase associated with the medium of liver cell cultures at the end of the incubation period, expressed as the percentage of total recovered activity, in the absence and the presence of CB and the "p" values obtained from paired "t" test analysis of corresponding control and CB treated cultures.

medium of non-preincubated hepatocytes contained 40%, 43%, 60% and 50% of the total activities of arylsulphatase, β-galactosidase, β-N-acetyl-glucosaminidase and lactate dehydrogenase respectively. The high levels of all the enzymes released at zero time made the evaluation of any subsequent release difficult and the preincubation step was introduced in all the subsequent hepatocyte preparations in order to reduce the initial loss of enzymes from the cells. Unless otherwise stated, the preincubation step was included in the preparation of the hepatocytes used in all the experiments described in the following study.

The release of lysosomal enzymes within a particular experiment were more consistent than those of lactate dehydrogenase (Fig. 10). The release of arylsulphatase and β-N-acetyl-glucosaminidase followed similar patterns to each other and the enzyme levels found in the medium showed little net variation throughout the incubation period. On the other hand, the activity of β-galactosidase found in the medium slowly increased throughout the incubation period (Fig. 10). When the activities of the different enzymes associated with the cell pellet at the end of the incubation period were compared to those at the beginning of the incubation, it was shown that the reduction of β-galactosidase activity was higher than that of the other lysosomal enzymes, but similar in magnitude to the loss of lactate dehydrogenase (Table 7).

The addition of the microfilament poison CB did not cause any change in the overall shapes of the release

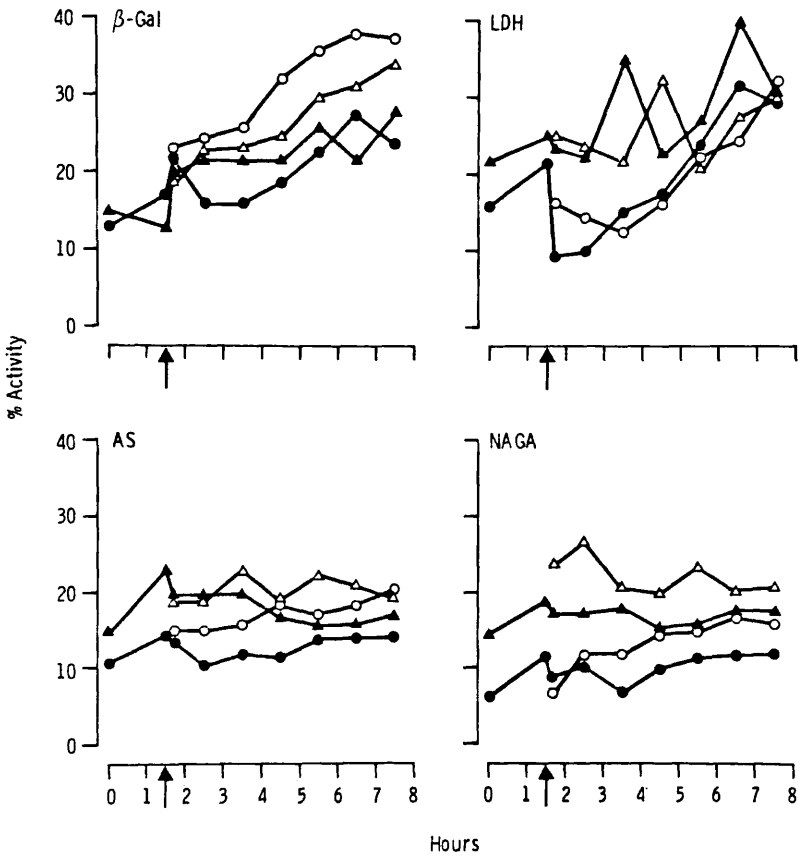

Fig. 10

The release of lactate dehydrogenase and lysosomal enzymes from DMSO (control) (closed symbols) and CB (open symbols) treated, non-loaded rat hepatocytes in short-term cultures. The arrows indicate the time points at which CB or DMSO were added. Two representative experiments are shown, indicated by triangles and circles.

Activities associated with the cell pellet						
	Control			CB Treated		
	Mean	Range	No	Mean	Range	No
AS	83.9	69.0-97.0	4	76.0	69.0-90.1	4
β-GAL	65.2	56.2-69.0	4	57.7	48.0-64.0	4
NAGA	87.0	71.3-98.0	4	81.0	68.0-89.0	4
LDH	66.0	53.0-73.0	4	75.0	58.0-100.0	4
Protein	90.0	83.0-93.4	4	91.7	88.5-94.5	4

Table 7

The activities of lysosomal enzymes, lactate dehydrogenase and protein associated with the cell pellet at the end of the culture period, expressed as the percentage of the respective activities at zero time.

of any of the enzymes measured. Also, although it had no effect on the levels of lactate dehydrogenase released, it did bring about a small but consistent increase in the release of the lysosomal enzymes which was, as judged by paired t test analysis, statistically significant (Table 6). In preliminary experiments, a second dose of CB was added 1 hr after the addition of the first CB dose. This second addition of CB did not further increase the observed release of lysosomal enzymes.

CB did not change the amount of protein lost from the cells (Table 7). The increases in the activities of the enzymes in the medium (Table 8) were consistantly less than the decreases in the activities associated with the cell pellet in both the CB-treated and non-treated cultures.

The total recovered activities at each examined time point were similar for both CB and control cultures (Fig. 11). Also although an initial decrease in the recoveries of the lysosomal enzymes measured was observed, these remained approximately constant for the last 3.5 hrs of the incubation period (approximately 90%).

In order to investigate to what extent the released enzymes were denatured during the incubation period, crude enzyme preparations, in the form of whole cytoplasm or as the supernatant obtained from a sonicated mitochondrial-lysosomal pellet suspension (Methods, 2.2.1.1) were incubated both in the presence and the absence of CB under the same conditions as the hepatocyte cultures. The results showed that there was no difference between the CB treated and non-treated preparations and that only β-galactosidase was inactivated to a significant extent (Table 9).

Changes in the activities associated with the cell pellet and the medium during cell culture												
	Cell pellet (decrease)						Cell medium (increase)					
	Control			CB treated			Control			CB treated		
	Mean	Range	No	Mean	Range	No	Mean	Range	No	Mean	Range	No
AS	81	11-174	4	116	57-202	4	19	8-30	4	32	11-49	4
NAGA	104	12-325	4	161	43-325	4	67	17-146	4	106	24-162	4
β-GAL	86	42-115	4	91	42-127	4	61	58-65	4	66	55-75	4
LDH	6	1.7-19.0	4	5	0.2-15.0	4	3.8	0.3-6.8	4	2.6	0.1-8.1	4

Table 8

The differences in the absolute activities of lysosomal enzymes and lactate dehydrogenase between 0 time and 7.5hr associated with the cell pellet and the cell medium both in the presence and in the absence of CB.

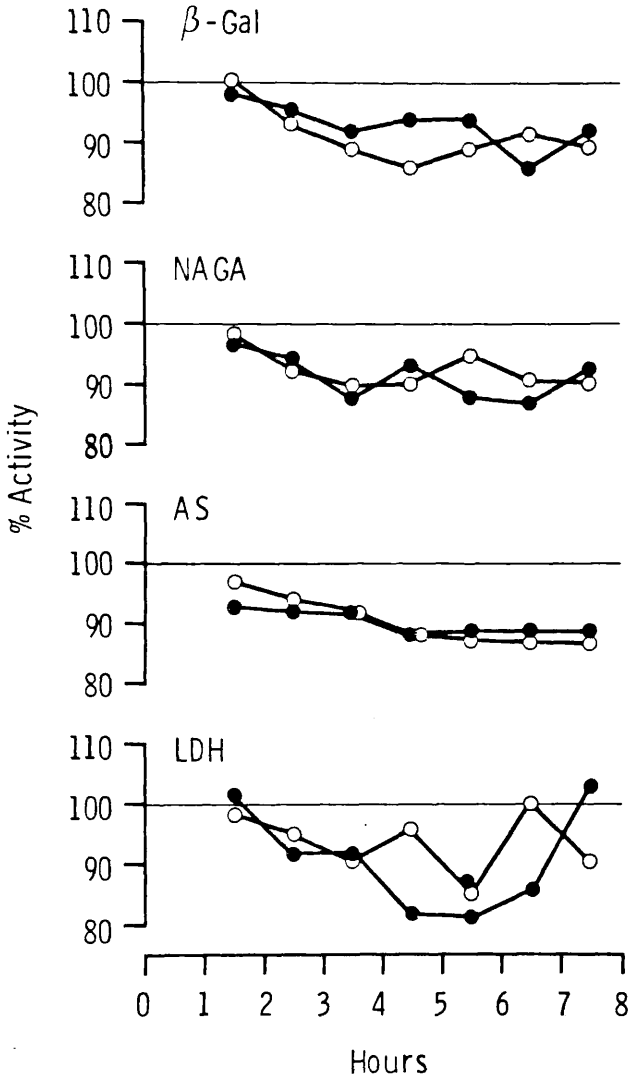

Fig. 11

The recoveries of lactate dehydrogenase, arylsulphatase, β-galactosidase and β-N-acetylglucosaminidase, expressed as the percentage of the total activity at zero time, at different time points during short-term hepatocyte cultures in the absence (● - ●) and the presence (o - o) of CB.

	Cytoplasm		M-L supernatant	
	Control	CB	Control	CB
AS	95	91	100	100
β-GAL	60	60	75	75
NAGA	98	93	93	91
LDH	109	117	-	-

Table 9

The activities of lysosomal enzymes and LDH, at the end of the incubation period under culture conditions, added in the form of cytoplasm or the supernatant of a sonicated mitochondrial-lysosomal pellet suspension, expressed as the % of the corresponding activities at zero time.

3.1.2.3 Isolated Liver Perfusions

The release of lysosomal enzymes from the isolated perfused rat liver was investigated both in the presence and absence of CB.

In the absence of the microfilament poison, the release of lysosomal enzymes and lactate dehydrogenase were low when compared with the release from hepatocyte cultures. The addition of CB brought about an increase in the release of both arylsulphatase and β-galactosidase in the perfusion medium while it had no effect on the release of lactate dehyrogenase (Fig. 12). Also the potassium lost during the perfusion, a measure of cell death, (D'Silva and Neil, 1954), represented a small percentage of that left in the liver which was similar in the presence and the absence of CB (Table 10). The magnitude of the effect of the CB was the same for both the lysosomal enzymes examined and their release in its presence was approximately twice that in its absence. The total activities of the enzymes assayed at the end of the perfusion were similar to those found in non-perfused livers (Table 11). However, the activities found in the perfusate in the presence of CB were significantly higher than those found in its absence (Table 12).

Preliminary experiments had shown that the addition of DMSO resulted in the presence of lower amounts of both arylsulphatase and β-galactosidase (0.7x and 0.5x respectively)

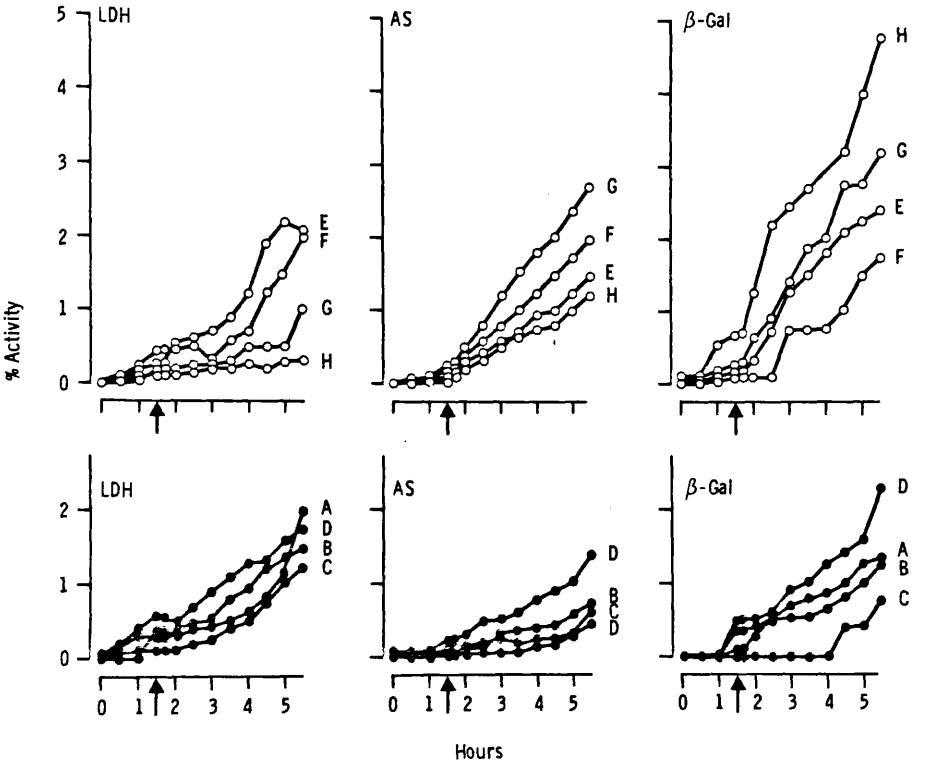

Fig. 12

The release of lactate dehydrogenase and lysosomal enzymes from DMSO controls (● - ●) and CB (o - o) treated isolated perfused non-loaded rat livers. The arrows represent the time points at which CB or DMSO were added. The letters correspond to different experiments.

		$[K^+]$		
		Mean	Range	n.
Control	Liver	8230	6600-10000	5
	Perfusate	0.9	0.7-1.0	5
CB	Liver	8700	7400-9700	5
	Perfusate	0.8	0.7-0.9	5

Table 10

The potassium concentration expressed in meq/total net weight in rat livers kept in perfusion for 5.5 hrs in the absence (control) and the presence of CB together with the potassium released (meq in total perfusate volume) under the same conditions.

Activity/wet liver weight						
	A			B		
	Mean	Range	No	Mean	Range	No
AS	7455	6070-10871	4	7001	5276-10028	4
β-GAL	2488	2230-2816	4	2457	2182-2759	4
LDH	577	566-594	4	538	401-680	4

	C		
	Mean	Range	No
AS	7838	4950-8800	9
β-GAL	2125	1900-2500	4
LDH	550	380-680	6

Table 11

A,B: The total activities recovered at the end of the perfusion in the presence of DMSO (A) or CB (B).

C: The activities associated with non-perfused livers.

	DMSO			CB			P
	Mean	Range	No	Mean	Range	No	Values
AS	0.84	0.4-1.4	4	1.9	1.3-2.7	4	$p<0.025$
β-GAL	1.6	0.8-2.3	4	3.0	1.8-5.0	4	$p<0.05$
LDH	1.7	1.2-2.0	4	1.8	1.0-2.0	4	$p<0.70$

Table 12

The activities expressed as the percentages of total recovered found in the perfusate at the end of the perfusion period in the presence of either DMSO or CB and the statistical significance of their difference, obtained from the students' "t" test analysis of the date.

in the perfusate as compared to those in its absence, whereas there was no difference in the case of lactate dehydrogenase. The effect of DMSO, however, was not further investigated and therefore it is not clear whether DMSO interferes with the release of lysosomal enzymes or acts by inhibiting them.

In order to investigate the relative contributions of release and reuptake of lysosomal enzymes to their presence in the perfusate, two different types of experiments were conducted.

In the first, the effect of a mixture of known inhibitors of lysosomal enzyme uptake by the liver (Ullrich et al 1979) (α-methyl-mannoside (10 mM), N-acetyl-glucosamine (10 mM) and mannans (25.6 mg/100 ml)) on the amounts of β-galactosidase and arylsulphatase present in the perfusate, both in the presence and the absence of CB, was examined. The inhibitors were added at different points in the perfusion periods as shown in Fig. 13. The results obtained showed that the added inhibitors did not increase the amounts of enzyme present in the perfusate either in the presence or the absence of CB.

In the second experiment, the uptake by the isolated liver of exogenous lysosomal enzymes added to the perfusate in a crude form (i.e. a sonicated mitochondrial-lysosomal fraction high-speed supernatant (Methods 2.2.1.1)) was investigated. The enzyme preparation was added to the perfusate, 5 min. after the addition of either DMSO or CB in such amounts so that the exogenously added activity

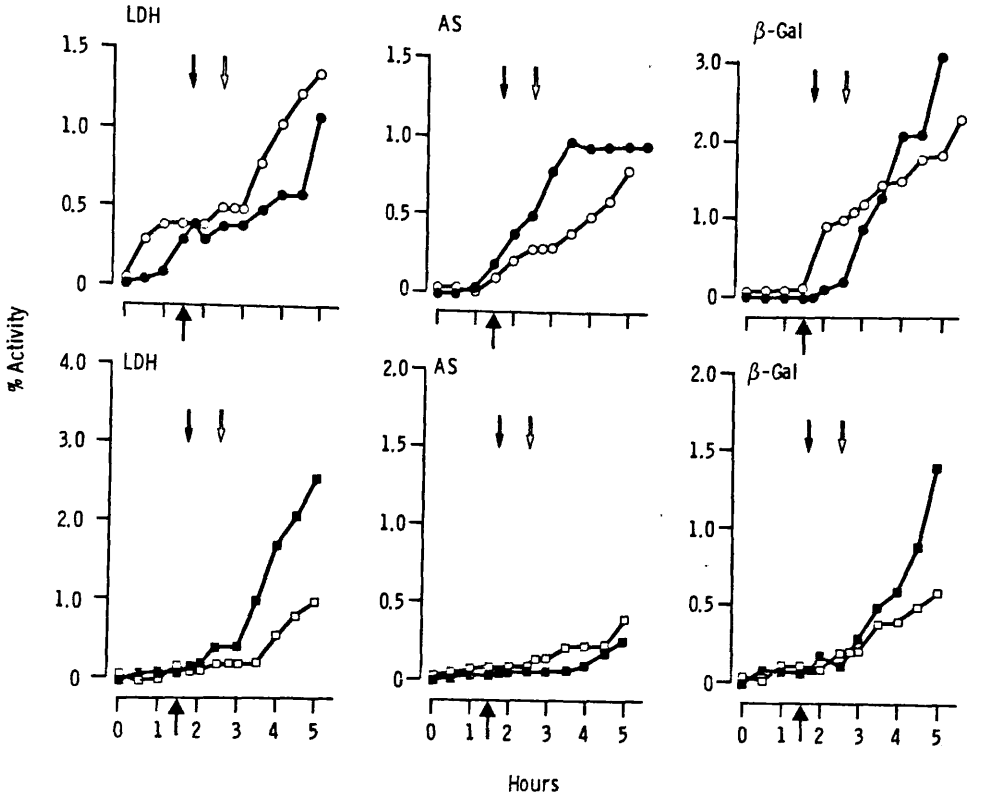

Fig. 13

The effect of the addition of a mixture of mannans (25.6 mg/100 ml perfusate), α-methyl-mannoside (10 mM) and β-N-acetyl-glucosamine (10 mM) after 110 min. (↓ and closed symbols) or 150 min. (⇓ and open symbols) on the release of lactate dehydrogenase and lysosomal enzymes from isolated perfused non-loaded rat livers treated with DMSO (■ - ■ , □ - □) or CB (● - ●, o - o) 90 min. from the beginning of the perfusion (↑). Each line is the mean of two experiments.

would roughly correspond to the activities released in the liver perfusion experiments. Due to the extent of inhibition of added arylsulphatase (80%) no conclusive studies could be made with reference to it, therefore the investigation was limited to the study of the uptake of exogenously added β-galactosidase. In the presence of DMSO an initial, slow disappearance of the exogenously added enzyme was observed (Fig. 14) so that 2 hrs after its addition approximately 20% (range 19%-22%) of the exogenously added enzyme had disappeared. Thereafter the activity associated with the perfusate started increasing and by the end of the perfusion it was approximately 128% (range 115%-135%) of the added activity. The rate of increase observed from the 2 hr point onwards was faster than the increase in the perfusate activity obtained in the absence of exogenously added enzyme. However, the levels of lactate dehydrogenase in the perfusate from the 2 hr point onwards and in the presence of exogenously added lysosomal enzymes were rising faster and finally reached higher values than those in the absence of added enzyme (Fig. 14). It was also observed that, over the small range of concentrations studied, the disappearance of β-galactosidase was independent of the amount of the enzyme added.

In the presence of CB, no initial disappearance of the enzyme was observed. On the contrary, its levels started rising from the zero time point. The rate of the observed increase was similar to that observed in the absence of exogenously added enzyme. In one experiment

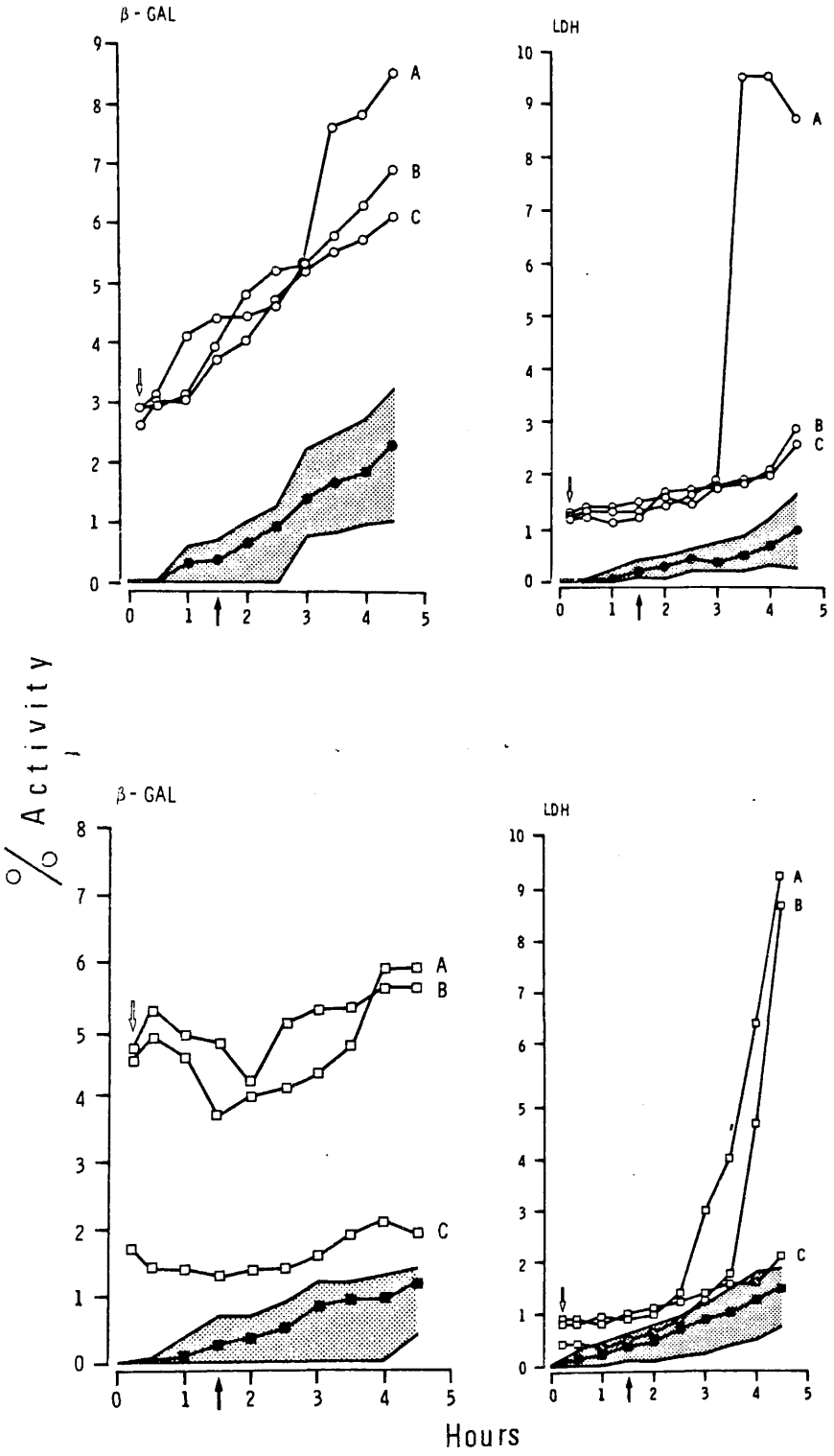

Fig. 14 - see overleaf

Fig. 14

The uptake of β-galactosidase and lactate dehydrogenase, added in the form of the high-speed supernatant of a lysed mitochondrial-lysosomal pellet preparation, by isolated perfused rat livers in the presence of DMSO (□ - □) and CB (o - o) added at ⇓ . The corresponding closed symbols represent the mean of the release of the enzymes in the absence of added enzyme preparation, the shaded areas representing the range of the results.

where this was not the case (experiment A, Fig. 14), the higher increase in the perfusate β-galactosidase activity in the presence of added enzyme was paralleled by a higher release of lactate dehydrogenase (Fig. 14).

In order to investigate whether the disappearance of added enzyme observed with the control (DMSO) liver was the result of a specific uptake mechanism, a preliminary experiment was carried out in which a mixture of known inhibitors of lysosomal enzyme uptake by the liver (α-Me-mannoside (10 mM), β-N-acetyl-glucosamine (10 mM) and mannans (25.6 mg/100 ml)) was added at zero time. The inhibitors failed to produce any change in the uptake of the enzyme.

In order to examine to what extent the subcellular distribution of marker enzymes had changed as a result of the perfusion, the techniques of differential and sedimentation-gradient centrifugation were employed. The homogenates of livers that had been kept in perfusion both in the presence and the absence of CB were used.

The sedimentation gradient profiles of lysosomal enzymes were similar for both DMSO and CB treated livers (Fig. 15, 16). When these profiles were compared to those obtained from non-perfused livers (Fig. 7), it was shown that there was an increase in the lysosomal enzymes found in the top fractions of the sedimentation gradients of the perfused livers, which coincided with the lactate dehydrogenase peak.

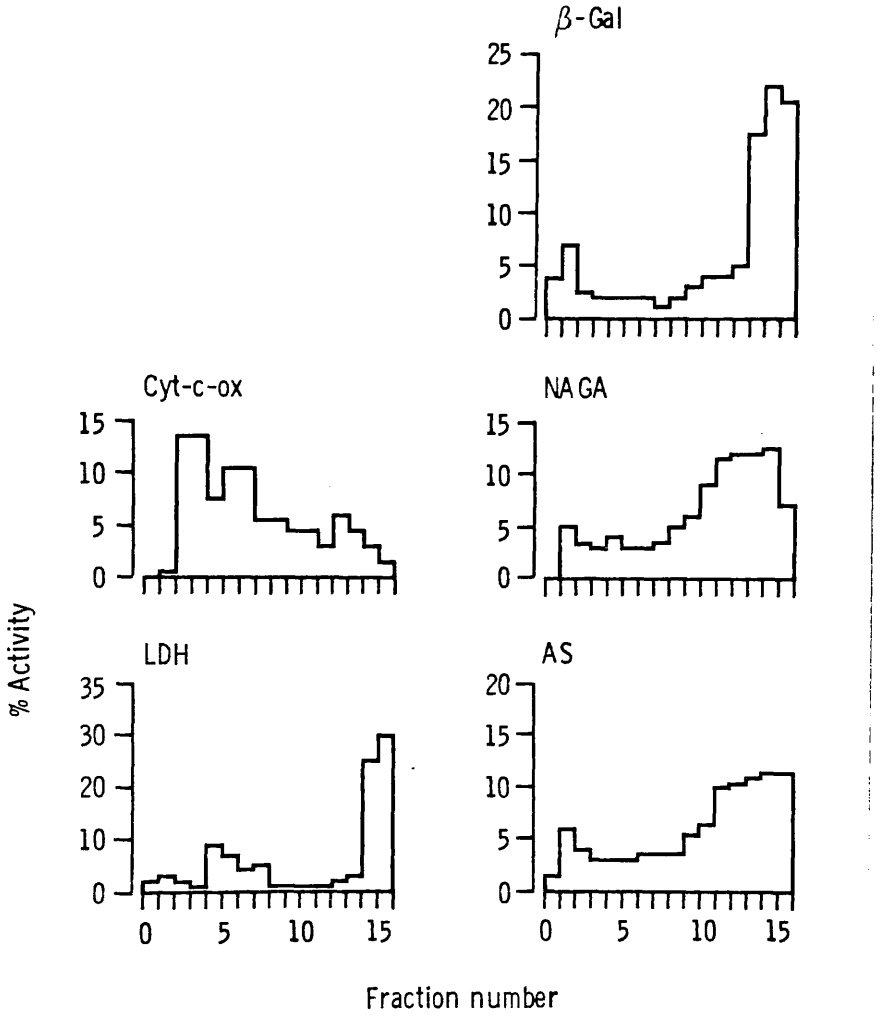

Fig. 15

The subcellular distribution of marker enzymes on a 8.5-40% w/v sucrose sedimentation gradient obtained from a non-loaded rat liver that had been perfused in the presence of DMSO (control).

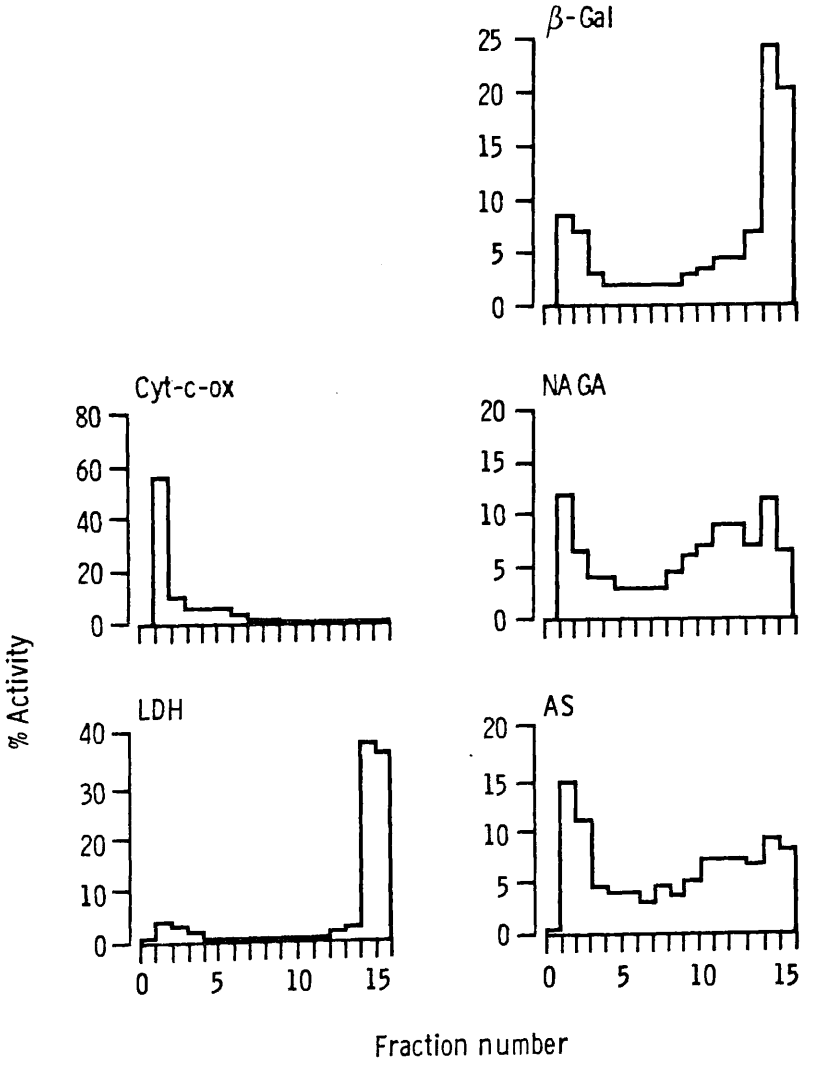

Fig. 16

The subcellular distribution of marker enzymes on a 8.5-40% w/v sucrose sedimentation gradient obtained from a non-loaded rat liver that had been kept in perfusion in the presence of CB.

The profiles of the distribution of cytochrome-c-oxidase in the sedimentation gradients of DMSO-treated livers was more diffuse than in the case of CB-treated livers. This difference was also observed in the lactate dehydrogenase distribution and was possibly an artifact caused by a slightly disturbed gradient.

The differential centrifugation results showed there was no difference in the cell debris and cytosol activities between the CB- and DMSO-treated rat livers (Table 13). However, when the above results were compared to those obtained from non-perfused livers, it was shown that although the cell-debris-associated lactate dehydrogenase and protein were similar in perfused and non-perfused livers, the lysosomal enzymes were higher in the former than in the latter and cytochrome-c-oxidase was lower in the perfused than in the non-perfused livers. There was no difference in the lactate dehydrogenase and cytochrome-c-oxidase activities in the cytosol fractions between perfused and non-perfused livers, whereas the lysosomal enzymes, and to a lesser extent the protein found in these fractions, were higher in the perfused than in the non-perfused livers (Table 13).

In order to investigate to what extent the added CB was taken up by the liver during the perfusion period, a preliminary experiment was carried out using ^{3}H-CB. At the end of the perfusion period 27% of the recovered activity was associated with the liver. Differential fractionation studies of the above liver homogenate showed that 78% and 14% of the liver-associated activity was recovered in the cytosol and cell debris fractions respectively.

Relative specific activities and % activities						
	Cell debris fraction			Cytosol		
	A	B	C	A	B	C
AS	1.8	2.0	1.2	0.2	0.2	0.02
	54.5%	59.4%	38.5%	8.9%	8.3%	0.5%
β-GAL	2.0	1.8	0.9	0.2	0.1	0.05
	58.6%	49.4%	28.5%	10.2%	6.5%	0.9%
NAGA	1.7	2.0	1.0	0.1	0.1	0.02
	50.0%	57.0%	31.5%	6.3%	6.1%	0.4%
LDH	0.2	0.6	0.6	2.0	1.6	2.7
	7.1%	15.8%	18.5%	82.0%	76.0%	78.3%
Cyt-c-ox	0.6	0.4	1.4	0.0	0.0	0.0
	17.4%	12.5%	44.5%	0.0%	0.0%	0.0%
Protein	1.0	1.0	1.0	1.0	1.0	1.0
	29.7%	28.3%	32.0%	41.6%	46.4%	29.0%

Table 13

The relative specific activities and the percentage activities associated with the cell debris and the cytosol fractions obtained from livers kept in perfusion in the absence (A) and the presence of CB (B) and non-perfused livers (C).

3.1.3 Discussion

The subcellular distribution studies gave reproducible results which were in agreement with previous reports using similar techniques (Danpure, 1971). The minor differences observed in the profiles of the distribution of different lysosomal enzymes reflect both the intra- and intercellular heterogeneity of the lysosomal population (Davies, 1975). The latter must be borne in mind when quantitative estimates of particular subcellular fractions, based on the activities of marker enzymes, is made. Apart from a completely homogeneous population, the activity estimates will not be proportional to the number of organelles, but rather to their mass, assuming that the enzyme activity in a particular organelle is proportional to the "size" of the structure. The heterogeneity of lysosomes though makes even this relationship not totally valid. Although the *in vivo* administration of Triton WR-1339 caused a definite shift of all lysosomal markers to lower buoyant densities, as shown by isopycnic gradient centrifugation, the extent of the shift was different for the different lysosomal enzymes. Similar previous observations have been reported (Warburton and Wynn, 1977) and the observed difference in the behaviour of different markers could be the result of both the heterogeneous uptake of Triton WR-1339 by different lysosomal populations in the same cell and/or the heterogeneous uptake of the detergent by the different cell types present in the liver.

The rather low recoveries of cytochrome-c-oxidase activity from the gradient is in agreement with the inhibition of the enzyme by sucrose (Danpure, 1971).

Hepatocyte cultures have been used extensively for the study of a variety of functions characteristic of these cells (Jeejeebhoy et al., 1975; Jeejeebhoy et al., 1977,Crane and Miller, 1977). The results obtained from the present study reflect very well some of the problems involved with the use of this technique. For example, low cell yields from the hepatocyte isolation procedure increase the possibility that a small non-typical population of hepatocytes has been selected. In addition, quantitation of cell death and other manifestations of the functional disorganisation of the cells is difficult to achieve.

For the purposes of the present study (including liver perfusions) cell death has been defined as the bursting open of the cell which would result in the appearance of all intracellular components in the medium. On this basis, the investigation of the release of a cytosolic marker (e.g. lactate dehydrogenase) would be a reasonable choice for the quantitation of the process.

Previous studies have shown that freshly isolated hepatocytes tend to loose a variety of molecules, lactate dehydrogenase included (Pariza et al., 1974). In another series of experiments (Gebhardt et al., 1978), it was shown that although freshly isolated hepatocytes lose a considerable amount of their lactate dehydrogenase activity

into the medium, no further loss of the enzyme was recorded after 4 days in culture. Also the present study has shown that although lactate dehydrogenase is released to a considerable extent during the culture period, its release (nominally giving a viability of 66%) is not reflected either in the Trypan Blue (viability 93%) or the protein remaining in the cell pellet (90%).

Therefore lactate dehydrogenase release during the culture period may be a more sensitive measure of membrane disorganization rather than a marker of overt cell death. As a consequence the real value of monitoring any changes in the lactate dehydrogenase release was in evaluating the CB effect on the release of lysosomal enzymes, rather than clarifying the origin of the latter in the medium in the absence of the microfilament poison.

The increased initial release of lactate dehydrogenase and lysosomal enzymes from non-preincubated hepatocytes as compared to preincubated cells, indicates that during the preincubation period hepatocytes, depending on the degree of their damage, either died or repaired their damage.

Three distinct domains, the sinusoidal, the contiguous and the canalicular, can be recognised in the hepatocyte cell membrane on the basis of their physiological function (Wisher and Evans, 1977). Purification of the above domains and analysis of their lipid, protein and glycoprotein composition showed that, although the functionally differentiated regions of the hepatocyte membrane showed similar compositions, differences in the distribution

of the different membrane components examined occured (Wisher and Evans, 1977). For example, it was shown that the sinusoidal and the canalicular subfractions contained the highest amount of a number of glycoproteins and, in the case of lipids, that the bile canalicular subfraction was enriched in unesterified cholesterol and sphingomyelin. Hepatocytes isolated with the collegenase perfusion method showed a plasma membrane composition similar to that obtained from intact liver, but exhibited lower specific activities of some of the membrane bound enzymes (Wisher and Evans, 1977). Also the possibility that a redistribution of the components of the plasma membrane occured during the isolation procedure cannot be excluded. This, together with the evidence showing an extensive vacuolization of the isolated cells, loss of microtubules (de Brabander et al., 1978) and possible disorganisation of microfilaments (Miettinen et al., 1978) could account for the high base-line release of lysosomal enzymes observed in the present studies in the absence of CB.

The addition of CB brought about a small increase in the release of lysosomal enzymes, which was statistically significant, but had no effect on the release of lactate dehydrogenase. The shapes of the release of lysosomal enzymes were not altered by the presence of the poison and the different patterns of release of the different enzymes, possibly a result of intralysosomal heterogeneity, were still maintained. The results suggest that the increased release of lysosomal enzymes observed in the presence of CB was not due to increased cell death or disorganization of

the plasma membrane, as shown by the lack of an effect on the release of lactate dehydrogenase. It seems likely, therefore, that the observed increased release was the result of the induction of exocytosis.

The effect of the drug appeared a short time after its addition and remained nearly constant for the whole length of the incubation period. Previous studies have shown that CB brings about major morphological changes in hepatocytes as early as 5 min. after its addition (Gravella et al., 1976). The observation that the second dose of CB did not further increase the release of lysosomal enzymes would suggest that the first dose of CB was sufficient for the complete disruption of the CB-sensitive microfilaments.

The small effect of CB on the release of lysosomal enzymes could be due to the already high release of the enzymes in its absence, which could limit the availability of sufficient plasma membrane surface for fusion and/or appropriate lysosome populations. It could also probably interfere with the cellular membrane recycling processes.

The isolated-perfused-liver system has the advantage over hepatocyte cultures of utilizing an intact organ, the structural and presumably functional integrity of which is much better maintained than that of isolated cells. On the other hand, the problem of the evaluation of cell death remains and one also has to account for the heterogeneity of the cells present in the tissue (i.e. Kupffer and epithelial cells in addition to hepatocytes).

Amongst the parameters that have been measured in the quantitation of cell death are the release of cytosolic enzymes and potassium into the perfusate (Bombeck et al., 1968; Bartosek et al., 1973). It has been shown (Bombeck et al., 1968) that the potassium levels in the perfusate increase in response to anoxia and the loss of potassium from the tissue is believed to be the result of the failure of the ATP driven Na^+K^+ pump in response to the cessation of oxidative phosphorylation.

In the present study the release of lactate dehydrogenase (and potassium) in the medium was low as compared to hepatocyte cultures and also CB failed to augment it. The latter is important in evaluating the CB effect on the release of lysosomal enzymes since this drug has been shown to interfere with the glucose uptake and consequently the energy production by cells (Zigmond and Hirsch, 1972; Mizel and Wilson, 1972). The lack of a CB effect on the release of both lactate dehydrogenase and potassium could indicate that the drug does not interfere with the energy production in the isolated perfused rat liver, at least to such an extent that would interfere with the membrane organization.

De Duve and Wattiaux (1966) proposed that "excretion in bile is the major pathway of unloading of the lysosomes of hepatic parenchymal cells". This hypothesis is supported by morpholigical evidence showing that injected colloidal gold particles (Shnitka,1965) and endogenous ferritin particles (Bradford et al., 1969) can be seen within both the lysosomes of hepatocytes and biliary canaliculi, and biochemical evidence showing the presence of lysosomal acid

hydrolases in the bile (LaRusso and Fowler, 1979). In the present study attempts to collect bile during the perfusion period were unsuccessful because the bile flow was very low, probably as a result of the interruption of the enterohepatic circulation (Bombeck et al., 1968). As a result the relative contribution of the release of lysosomal enzymes from Kupffer and parenchymal cells to the amount of the enzymes found in the perfusate is uncertain.

However, even if the attempts to collect bile had been successful, the study of the release of lysosomal enzymes in the bile would have been limited to livers perfused in the absence of CB, since this drug has been shown to cause choleostasis (Phillips et al., 1977).

The addition of CB selectively increased the release of arylsulphatase and β-galactosidase, but had no effect on the release of lactate dehydrogenase.

The finding that DMSO reduced the amounts of the lysosomal enzymes, but not of lactate dehydrogenase, found in the perfusate, as compared to those in its absence, indicated that the effect of CB on the release of lysosomal enzymes was probably masked to a certain extent by the presence of DMSO.

There was no latent period between the addition of the drug and the onset of its effect. The selective increased release of lysosomal enzymes, but not lactate dehydrogenase is in good agreement with previous studies using macrophages (Davies et al., 1973a) and neutrophilic leucocytes (Davies et al., 1973b) and indicates that the

disruption of microfilaments in the isolated perfused rat liver system leads to exocytosis.

The lack of effect of the known inhibitors of the uptake of lysosomal enzymes by the liver on the amounts "released" into the medium could either mean that lysosomal enzymes released by the perfused liver lack the required recognition sites for uptake or that the concentrations of the inhibitors used were too low.

The present investigation of the uptake of β-galactosidase from an exogenously added crude lysosomal preparation showed a very slow uptake of the enzyme by the isolated perfused liver in the absence of CB, as compared to the *in vivo* clearance rates observed for a variety of other lysosomal enzymes (Stahl *et al.*, 1976). Previous studies have shown that the rate of clearance of lysosomal enzymes by isolated perfused livers is much lower than the corresponding *in vivo* rates (Schlesinger *et al.*, 1976) and also that the *in vivo* clearance of purified β-glucuronidase can be inhibited by crude lysosomal extracts (Stahl *et al.*, 1976). These observations could account for the low rate of clearance obtained in the present study. The inhibition of the uptake by CB indicates that microfilaments are involved in the process. However, the results obtained both from the effect of uptake-inhibitors on the levels of lysosomal enzymes released in the medium and the investigation of the uptake of exogenously added β-galactosidase did not give an answer to the question of the relative contribution of uptake and release in the levels of the enzymes found in the perfusate in the exocytosis studies.

The sedimentation-gradient distribution profiles of the lysosomal enzymes of livers after perfusion in the presence and the absence of CB were not significantly different from each other.

There was no difference in the enzyme activities associated with the cell debris and the cytosol fractions obtained from livers that had been kept in perfusion either in the absence or the presence of CB. However, the cell debris fractions of perfused livers contained higher amounts of lysosomal enzymes than the equivalent fraction from non-perfused livers, whereas the lactate dehydrogenase activities and the protein content was similar in both cases. This probably indicates that lysosomes have enlarged during the perfusion, possibly as a result of increased autophagy.

It also appears, as judged by the lysosomal enzyme activities associated both with the cytosol (fractions) and the top fraction of the sedimentation gradients of perfused livers, that the perfusion has made the lysosomes more fragile, so that more are ruptured during the homogenisation procedure.

The preliminary investigation of the uptake of ^{3}H-CB by the perfused liver showed that only 27% of the added label was associated with the liver at the end of the perfusion period. Although the chemical nature of the label found either in the liver or the perfusate was not investigated, the result indicated that there was no significant depletion from the perfusate of added CB during the perfusion period. The high percentage (78%) of the liver-associated

radioactivity found in the cytosol, assuming that the radioactivity was in the form of ^{3}H-CB, indicated either that CB was taken up into the cytosol of liver cells or that CB bound to plasma membrane receptors was released during the homogenization procedure. Although no association constants for CB binding sites on the liver cell membrane are available, the values obtained for other cells (Lin and Lin, 1979) would make the latter explanation unlikely. Since most of the plasma membrane is found in the cell debris fraction the possibility that the radioactivity associated with that fraction (14%) represented membrane bound ^{3}H-CB cannot be excluded.

3.2 PVP-loaded Livers

3.2.1 Introduction

Polyvinylpyrrolidone (PVP) is a synthetic macromolecule that has been used extensively both clinically and experimentally (Regoeczi, 1976). Its use as an additive to various pharmaceutical preparations to modify their pharmacological effects has led to the description of a PVP-storage disease, arising from the accumulation of the macromolecule in the cells of the skin, bone and the liver (Reske-Nielsen *et al.*, 1976). Morphological examination of the affected tissues revealed the accumulation of the undigested macromolecules inside vacuoles. It has been shown that the *in vivo* accumulation of PVP in the epithelial cells of rat egg yolk sacs resulted in changes in the apical vacuolar system of these cells (Roberts *et al*, 1976). In both cases it had been assumed that the vacuoles were lysosomes.

The aim of the work described in this section was to investigate the ability of PVP-loaded lysosomes to exocytose their contents in the presence and the absence of the microfilament poison CB, using the systems of rat-hepatocyte cultures and isolated perfused rat livers.

Although the previously mentioned histological evidence had indicated that PVP accumulated inside lysosomes, no biochemical evidence was available. As a result it was essential to establish the lysosomal localization of the macromolecule and the first section of the following study is concerned with the investigation of the

subcellular distribution of PVP in rat liver. Gel-chromatography studies using Sephadex G-100 were also carried out in order to investigate to what extent the ^{125}I that was injected, accumulated intralysosomally and released during perfusion was still attached to PVP.

3.2.2 Results

3.2.2.1 Uptake and Subcellular Distribution

The uptake and subcellular distribution of ^{125}I-PVP in rat liver has been studied 4, 8 and 15 days after injection.

Over the time periods studied, the amount of radioactivity associated with the liver was constant and higher than that associated with either the spleen, kidneys or the thyroid (Table 14).

The subcellular distribution of ^{125}I-PVP and marker enzymes, in the liver 4 and 8 days after injection, was investigated using the technique of sedimentation-gradient centrifugation. The profiles of the distribution of ^{125}I-PVP were similar at both time points and closely resembled those of the lysosomal enzymes (Fig. 17, 18). However, a considerable part of the ^{125}I-PVP was associated with the top two fractions of the gradient, corresponding to the lactate dehydrogenase peak. When profiles of the distribution of lysosomal enzymes in sedimentation gradients from ^{125}I-PVP-preloaded livers were compared to those obtained from non-preloaded ones (Fig. 7), (Table 15) it was shown that the presence of ^{125}I-PVP had not changed the sedimentation properties of lysosomes.

In order to further investigate the subcellular distribution of ^{125}I-PVP, the technique of isopycnic gradient centrifugation was used. Mitochondrial-lysosomal pellets were prepared from rat livers preloaded with the

% Injected radioactivity									
	4 days			8 days			15 days		
	Mean	Range	No	Mean	Range	No	Mean	Range	No
Liver	30.5	26-35.8	5	31.3	29.3-33.3	2	37.9	-	1
Spleen	3.6	-	1	5.6	-	1	7.7	-	1
Thyroid	9.5	-	1	10.6	-	1	0.2	-	1
Kidneys	8.6	-	1	11.5	-	1	11.0	-	1

A

	10^3 cpm/g wet liver weight		
	Mean	Range	No
4 days	33.0	23.0-39.0	5
8 days	35.5	32.0-39.0	2
15 days	35.0	-	1

B

Table 14

A: The distribution of label in various organs 4, 8 and 15 days after the injection of ^{125}I-PVP.

B: Amount of radioactivity associated with rat livers 4, 8 and 15 days after the injection of ^{125}I-PVP.

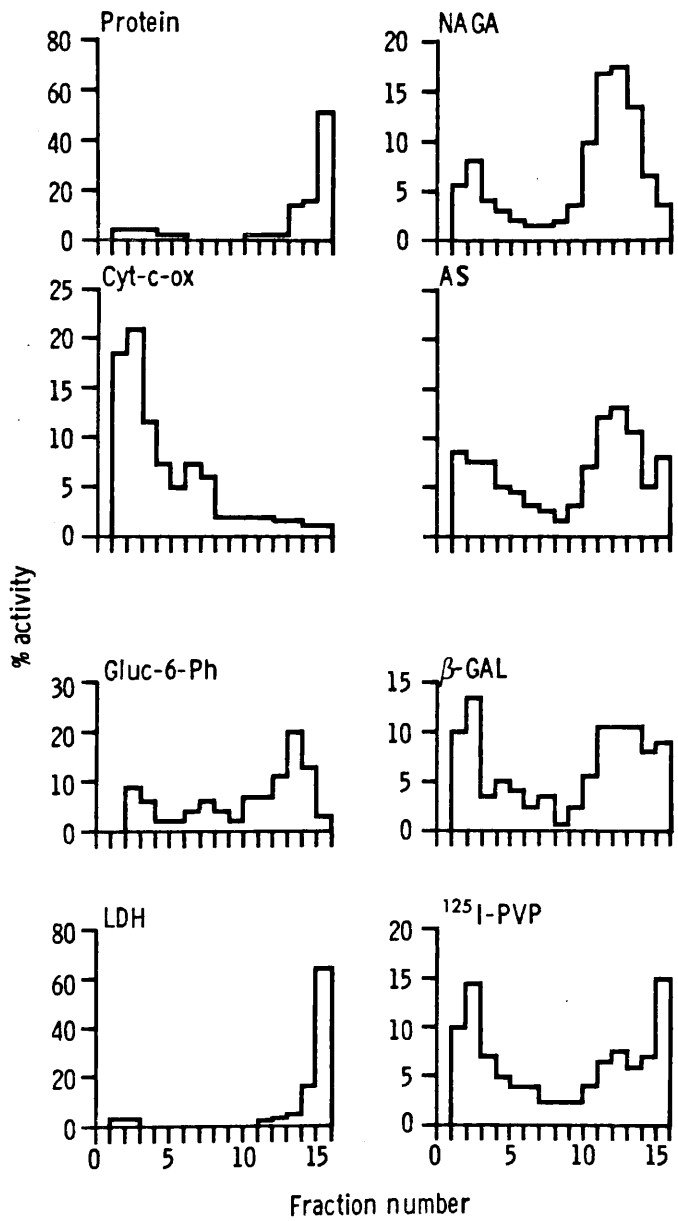

Fig. 17

The subcellular distribution of marker enzymes, protein and ^{125}I-PVP on a representative sedimentation gradient (8.5-40% (w/v) sucrose), 4 days after the injection of ^{125}I-PVP.

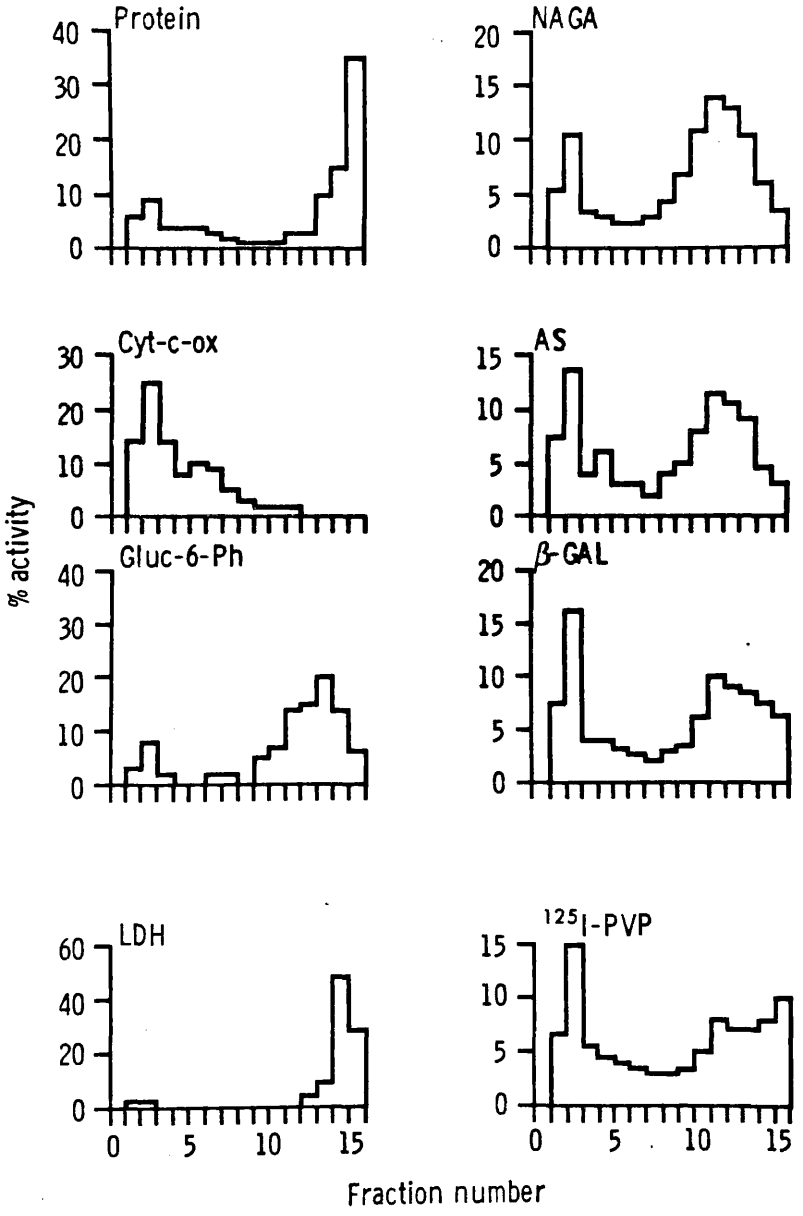

Fig. 18

The subcellular distribution of marker enzymes, protein and ^{125}I-PVP on a representative sedimentation gradient (8.5-40% (w/v) sucrose), 8 days after injection of ^{125}I-PVP.

	% Activity								
	4 days			8 days			Controls		
	Mean	Range	No	Mean	Range	No	Mean	Range	No
AS	42.8	39.0-42.4	3	40.2	-	1	38.9	37.6-40.3	2
β-GAL	41.8	33.8-46.5	3	38.6	-	1	36.3	34.6-38.0	2
NAGA	31.1	25.5-36.1	3	31.5	-	1	28.2	25.0-31.4	2
^{125}I-PVP	42.1	33.0-47.6	3	41.7	-	1	-	-	-

Table 15

The % recovered activity associated with sedimentation gradient fractions heavier than 23% (w/w sucrose) obtained from rat livers injected with ^{125}I-PVP 4 and 8 days earlier, as well as control i.e. non-preinjected rat livers.

macromolecule 4, 8 and 15 days earlier. The percentages of enzymic markers associated with the mitochondrial-lysosomal pellets are shown in Table 16. The profiles of the distribution of enzymic markers and ^{125}I-PVP in isopycnic gradients 4, 8 and 15 days after the injection are shown in Fig. 19, 20 and 21 respectively. The profile of the distribution of ^{125}I-PVP coincided with the profiles of the distribution of lysosomal enzymes in isopycnic gradients. It appeared that from 4 to 8 days, a shift of the ^{125}I label and the lysosomal enzymes β-galactosidase and β-N-acetyl-glucosaminidase to lower buoyant densities occured. So, at 4 days the peak of ^{125}I-PVP and the three lysosomal enzymes equilibrated between 48%-39% (w/w sucrose). At 8 days the peaks of ^{125}I-PVP, β-galactosidase and β-N-acetyl-glucosaminidase had shifted to less dense fractions equilibrating between 45%-37%, 45%-36% and 47%-37% (w/w sucrose) respectively, while arylsulphatase equilibrated between 47%-41% (w/w sucrose). The distribution of lysosomal enzymes at 15 days was similar to that at 8 days, but it appeared that the label had shifted to higher fractions equilibrating between 46%-34% (w/w sucrose).

It has been shown (Wattiaux et al., 1963) that in vivo administered Triton WR-1339 accumulated in the lysosomes causing a decrease in their buoyant density. In the present study in order to obtain further confirmatory evidence of the intralysosomal localization of in vivo administered ^{125}I-PVP, a mitochondrial-lysosomal pellet was prepared from the liver of a rat that had been injected simultaneously with Triton WR-1339 and ^{125}I-PVP 4 days

	% Activity associated with mitochondrial-lysosomal pellet		
	Mean	Range	Number
^{125}I-PVP	42.6	41.5-44.5	3
β-GAL	44.1	42.6-45.7	2
AS	39.8	30.5-49.0	2
NAGA	36.6	36.2-37.1	2
LDH	6.9	5.0-8.8	2
Cyt-c-ox	15.5	14.0-17.0	2
Gluc-6-P	14.5	13.8-15.3	2

Table 16

The activity, as the % of the homogenate activity, associated with the mitochondrial-lysosomal pellet obtained from rat livers preloaded with ^{125}I-PVP.

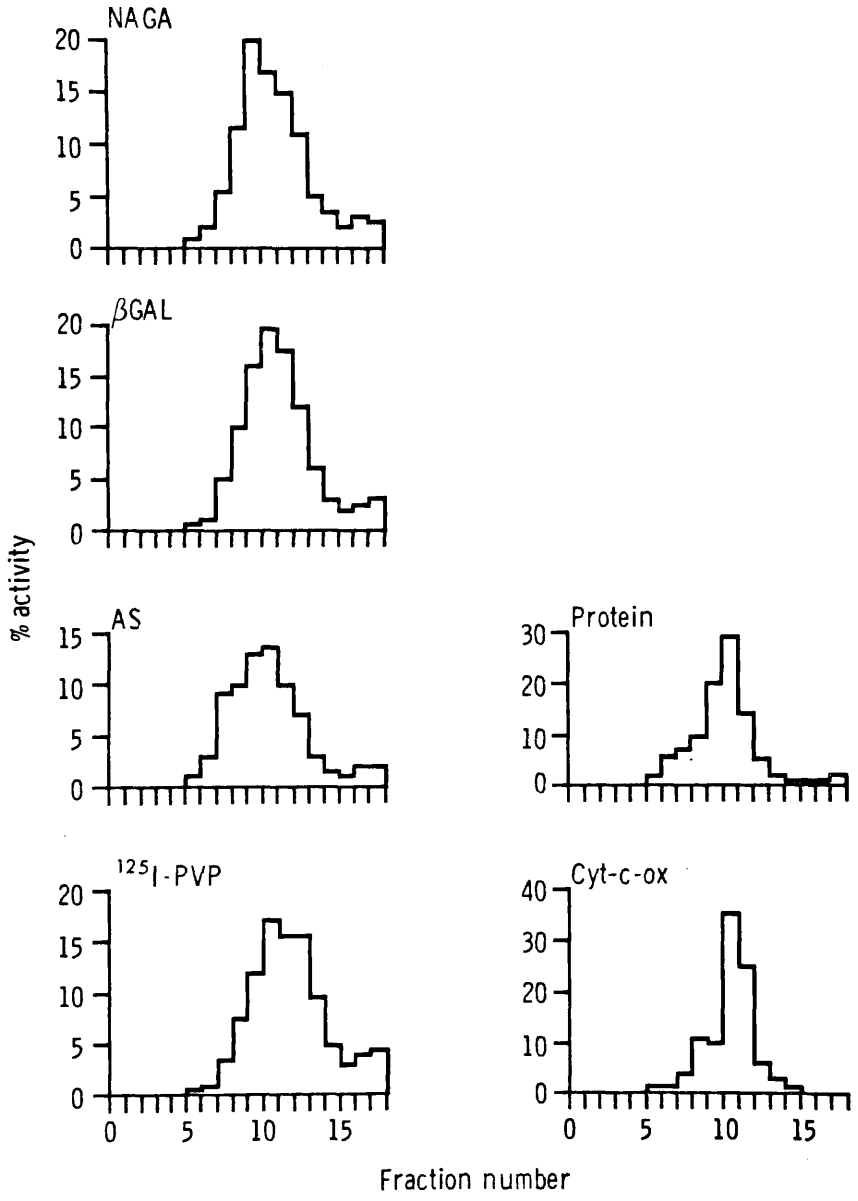

Fig. 19

The distribution profiles of marker enzymes, protein and ^{125}I-PVP on a representative 26.7-82% (w/v) sucrose isopycnic gradient, 4 days after injection of ^{125}I-PVP.

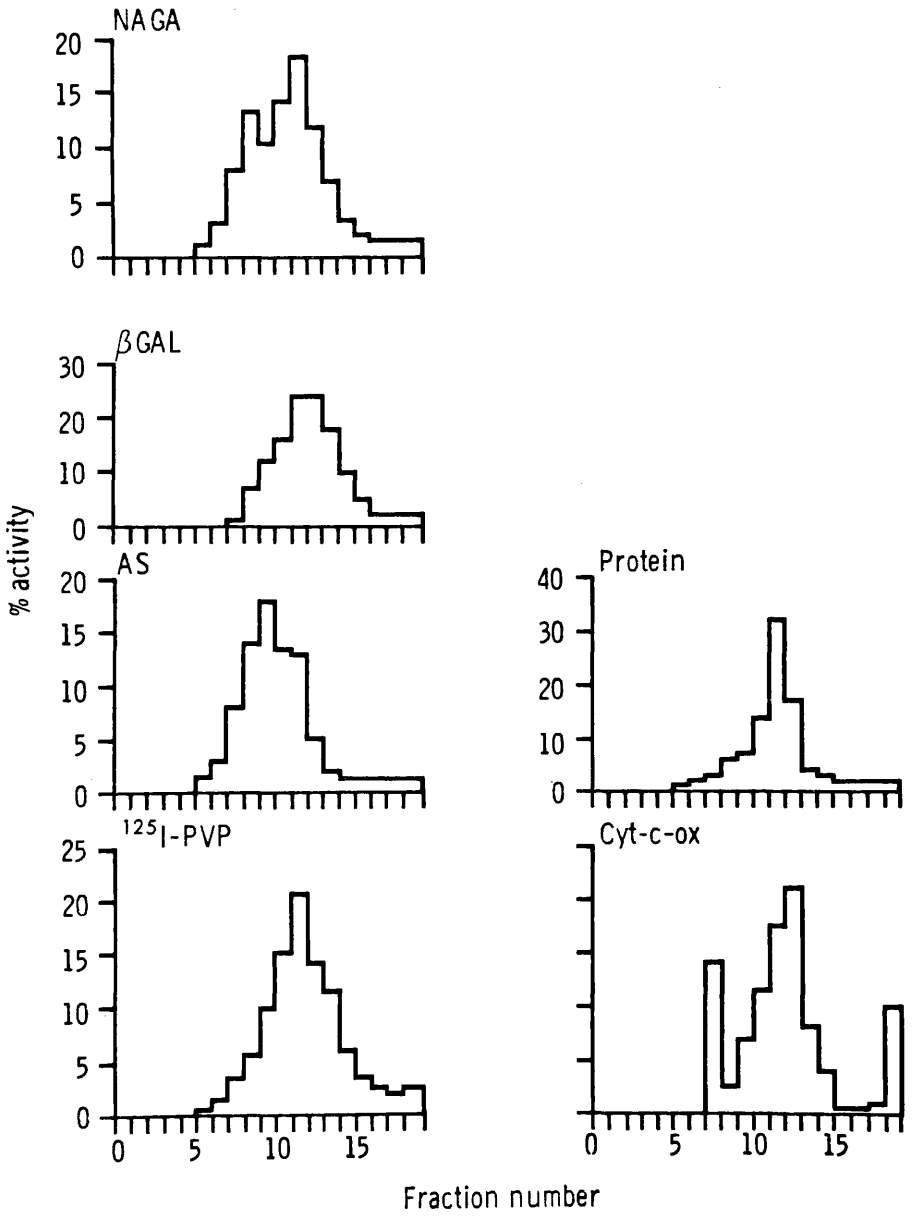

Fig. 20

The distribution profiles of marker enzymes, protein and ^{125}I-PVP on a 26.7-82% (w/v) sucrose isopycnic gradient, 8 days after injection of ^{125}I-PVP.

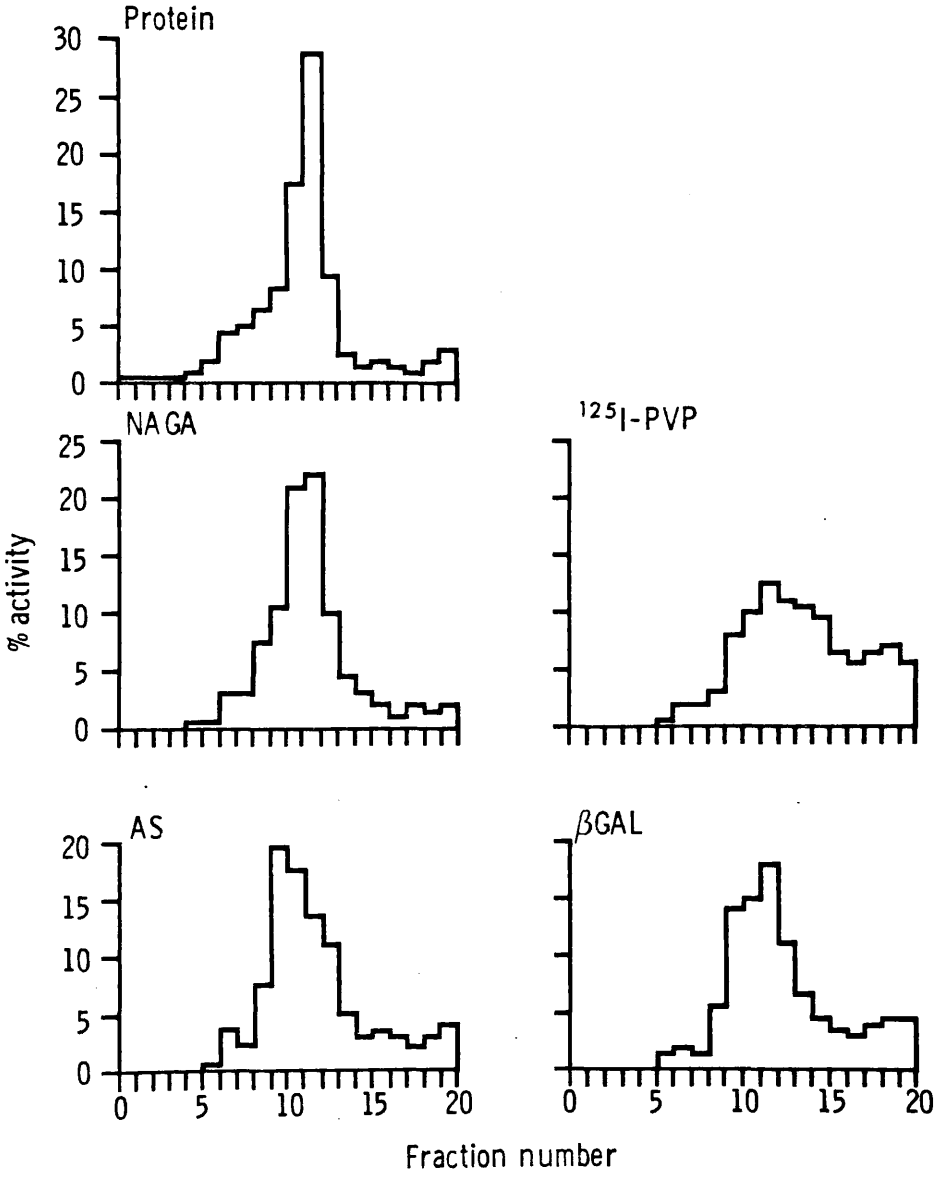

Fig. 21

The distribution profiles of enzymes, protein and ^{125}I-PVP on a 26.7-82% (w/v) sucrose isopycnic gradient 15 days after injection of ^{125}I-PVP.

previously and the distribution of radioactivity and enzymic markers in an isopycnic gradient was investigated. The results (Fig. 22) (Table 17) showed that Triton WR-1339 floated both lysosomal enzymes and ^{125}I-PVP.

The recoveries obtained from the sedimentation and the isopycnic gradients are shown in Table 18.

In order to investigate the distribution of label and lysosomal enzymes between the lumen and the membrane of lysosomes, the above parameters were measured in the supernatant and the pellet obtained from the high-speed centrifugation of a lysed mitochondrial-lysosomal pellet (see 2.2.1.1) prepared from a rat liver that had been injected with ^{125}I-PVP 4 days earlier. The results (Table 19) showed that both the label and β-galactosidase were mainly recovered in the high-speed supernatant, whereas the majority of arylsulphatase and practically all of β-N-acetyl-glucosaminidase was recovered in the pellet fraction. Also on comparing the above results to those obtained from non-loaded livers, it was shown that the distribution of lysosomal enzymes was similar in both cases (Table 19).

In order to investigate to what extent the injected and the intralysosomally-accumulated ^{125}I was attached to PVP, samples of the injected material and of the supernatant of a lysed mitochondrial-lysosomal pellet suspension (see 2.2.1.1) were passed down a Sephadex G-100 column. The results (Fig. 23) showed that the intralysosomally accumulated label corresponded to the high molecular weight components of the injected material, indicating that the intralysosomally accumulated material was undegraded.

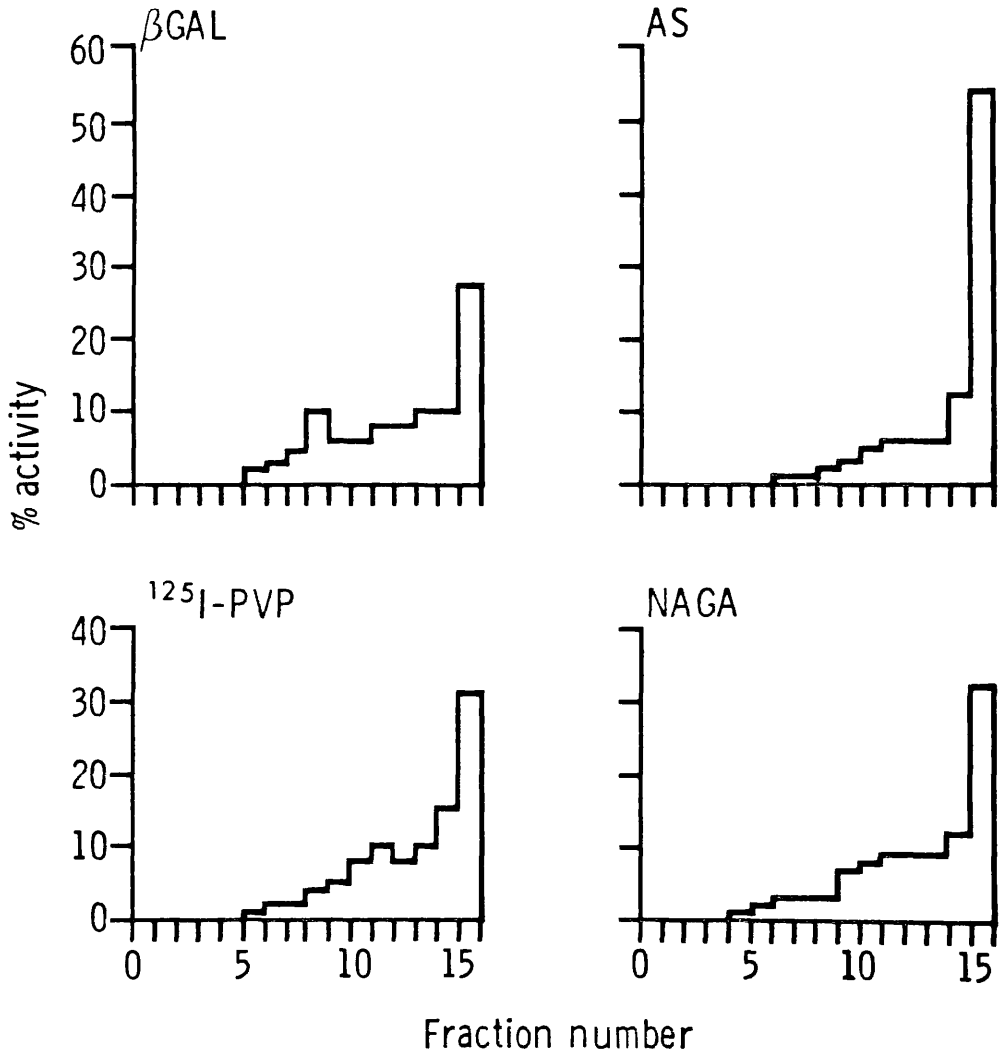

Fig. 22

The effect of Triton WR-1339 on the distribution profiles of lysosomal enzymes and ^{125}I-PVP on a 26.7-82% (w/v) sucrose isopycnic gradient. The detergent and ^{125}I-PVP were injected 4 days earlier.

	% Activity		
	A	B	C
AS	4.2	76.5	47.1
β-GAL	4.4	38.0	43.5
NAGA	3.5	44.2	54.3
^{125}I-PVP	5.7	46.0	-

Table 17

The % activity associated with the top 2 fractions of isopycnic gradients (26.7-82.0% (w/v sucrose)) of rat livers injected:

A: with ^{125}I-PVP

B: with ^{125}I-PVP and Triton WR-1339

C: with Triton WR-1339.

	% Activity recovered from gradients					
	Sedimentation			Isopycnic		
	Mean	Range	No	Mean	Range	No
^{125}I-PVP	102.6	93.1-116.7	4	100.8	98.4-105.0	3
β-GAL	102.6	94.8-109.3	3	95.5	84.8-108.5	3
AS	92.8	86.9-101.7	4	78.0	72.0-83.0	3
NAGA	100.0	75.2-113.5	4	76.0	71.7-83.0	3
Protein	112.9	98.6-131.0	3	91.9	80.0-105.1	3

Table 18

The % activity recovered from the sedimentation and isopycnic gradients (8.5-40.0% (w/v sucrose) and 26.7-82.0% (w/v sucrose) respectively) of rat livers preinjected with ^{125}I-PVP.

	A	B
AS	40%	38%
β-GAL	73%	70%
NAGA	8%	6%
^{125}I-PVP	92%	-

Table 19

The activities, expressed as the percentage of total recovered, associated with the high-speed supernatant of a lysed mitochondrial-lysosomal pellet, obtained from livers loaded with ^{125}I-PVP 4 days earlier (A) and non-loaded livers.

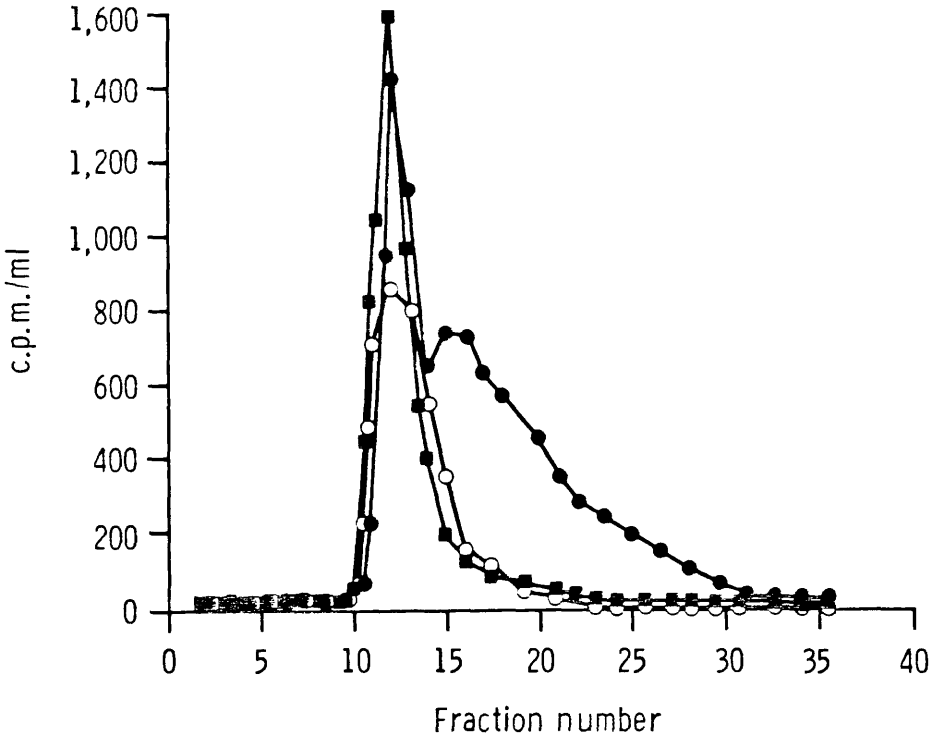

Fig. 23

The elution profile obtained by passing down a Sephadex G-100 column of sample (20 µl) of the ^{125}I-PVP injection solution (● - ●), a sample (1.0 ml) of the high-speed supernatant of a lysed mitochondrial-lysosomal pellet preparation obtained from a rat liver injected with ^{125}I-PVP 4 days earlier (■ - ■) and a sample (1 ml) of the perfusate of an isolated rat liver kept in perfusion in the absence of CB that was loaded with ^{125}I-PVP 4 days earlier (o - o).

3.2.2.2 Hepatocyte Cultures

Hepatocyte cultures were set up using rat livers that had been loaded with ^{125}I-PVP 4 days earlier. The release of label and lysosomal enzymes were investigated both in the presence and the absence of CB.

The number of cells present at each step in the isolation procedure in the present study was not significantly different to the corresponding number of cells isolated from non-loaded livers (Table 20). The activities of lysosomal enzymes, lactate dehydrogenase and label associated with the final cell pellet were in good agreement with the cell-counting results (Table 21). The final cell pellet preparation did not appear to be specifically enriched compared to the liver homogenates, in either lysosomal enzymes or label and the activities of all the enzymes associated with it were comparable to those obtained in the case of non-loaded liver (Table 22).

The presence of ^{125}I-PVP inside the lysosomes did not seem to change the overall shape of the release of either lysosomal enzymes or lactate dehydrogenase as compared to that obtained in the case of non-loaded cells (Fig. 24A and B). The addition of CB brought about a small but consistent increase in the amount of lysosomal enzymes and label released which was statistically significant, as judged by paired t test analysis, but did not affect the release of lactate dehydrogenase (Table 23, Fig. 24 A and B).

	^{125}I-PVP loaded			Non-loaded		
	Mean	Range	No	Mean	Range	No
A	245×10^6	200×10^6 – 270×10^6	3	327×10^6	100×10^6 – 623×10^6	5
B	107×10^6	97×10^6 – 125×10^6	3	74×10^6	33×10^6 – 128×10^6	5
C	44×10^6	35×10^6 – 50×10^6	3	43×10^6	23×10^6 – 53×10^6	5

Table 20

The number of cells present at each step in the isolation procedure in the case of ^{125}I-PVP preloaded and non-preloaded livers:

A: No of cells after the mesh

B: No of cells after the washing step

C: No of cells after the preincubation step.

% activity associated with cells after the preincubation step			
	Mean	Range	No
^{125}I-PVP	55	33-70	5
AS	53	36-65	5
NAGA	46	35-60	5
β-GAL	51	40-70	5
LDH	39	32-52	5

Table 21

The amounts of lysosomal enzymes, lactate dehydrogenase and ^{125}I-PVP associated with the cells after the preincubation step, expressed as the percentage of the activities after the washing step.

	A			B			C
	Mean	Range	No	Mean	Range	No	
AS	271000	23200-32200	5	16260	7000-24000	5	6.0
β-GAL	81800	70000-97000	4	4500	2700-5700	5	5.5
NAGA	462000	310000-600000	4	32340	17000-56000	5	7.0
^{125}I-PVP	622800	450000-775000	5	46700	20000-70000	5	7.5
LDH	13000	10900-14000	4	494	80-900	5	3.8

Table 22

The activities of lysosomal enzymes, lactate dehydrogenase and the radioactivity associated with liver homogenates (A) and hepatocytes after the preincubation step, expressed as absolute activities (B) and (C) as the percentage of (A).

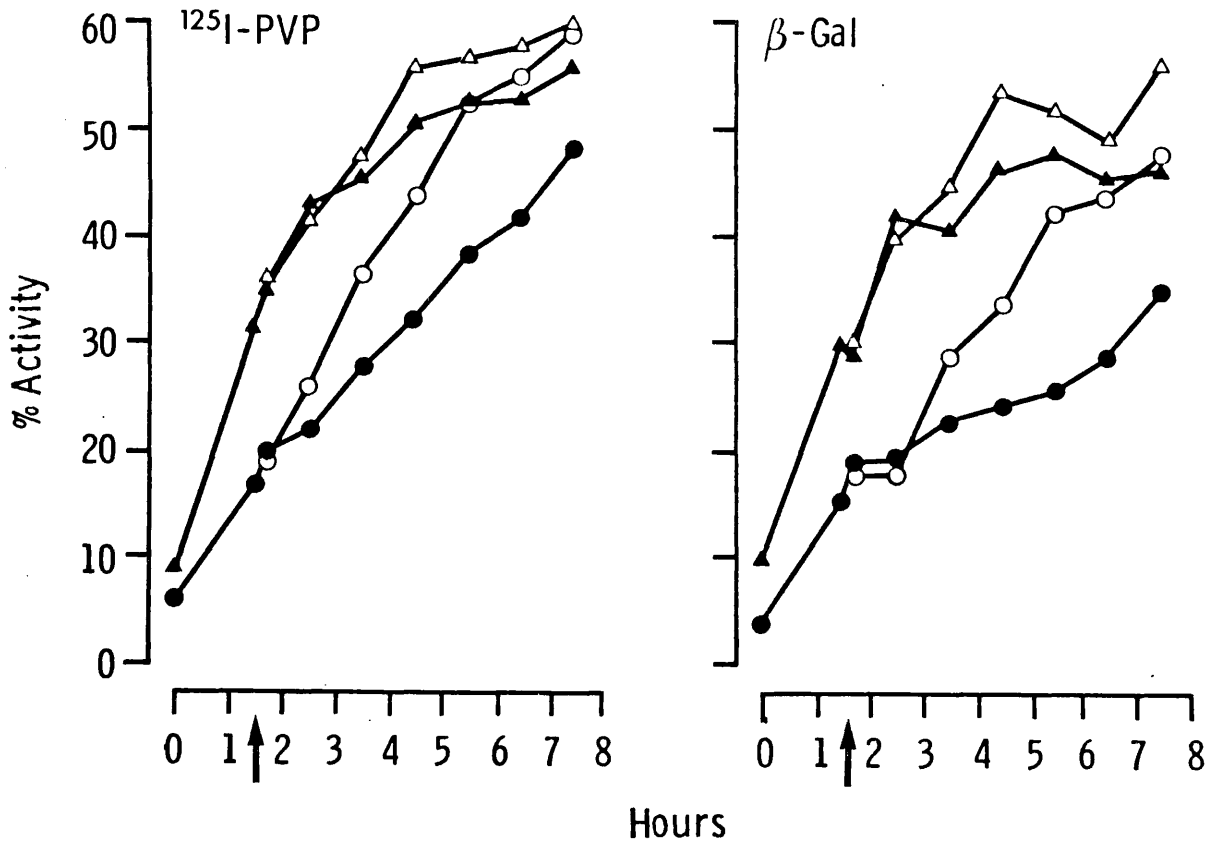

Fig. 24A - see page 132

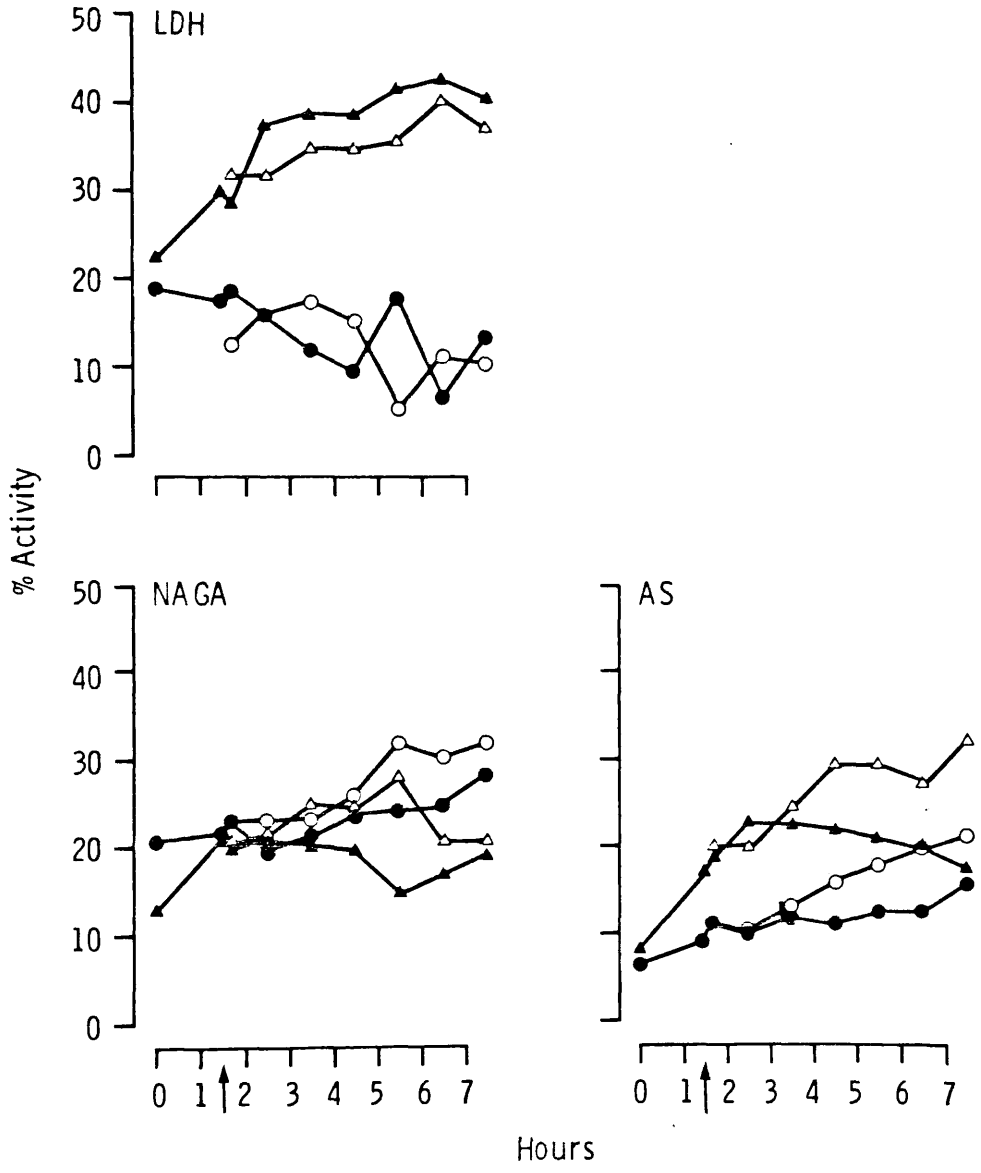

Fig. 24B - see next page

Fig. 24 (A and B)

The activity of β-galactosidase, ^{125}I-PVP (24A), aryl-sulphatase, β-N-acetyl-glucosaminidase and lactate dehydrogenase (24B) released into the culture medium by hepatocytes in short-term cultures, expressed as the percentage of the recovered activities at each time point. Two representative experiments are shown (▲-▲ / △-△ , ●-● / o-o). Open symbols represent CB treated cultures and closed symbols represent DMSO (control) cultures. Both CB and DMSO were added 90 min. after the beginning of the culture period (↑).

	Controls			CB treated			p
	Mean	Range	No	Mean	Range	No	Values
^{125}I-PVP	44	37-56	5	55	47-63	5	$p<0.01$
AS	16	12-20	5	23	20-32	5	$p<0.025$
NAGA	20	12-27	5	27	21-31	5	$p<0.01$
β-GAL	33	22-45	5	44	34-56	5	$p<0.01$
LDH	25	3-40	5	25	2-39	5	$p<0.15$

Table 23

The amounts of lysosomal enzymes, lactate dehydrogenase and ^{125}I-PVP, expressed as the percentage of total recovered, associated with the medium of control and CB treated cell cultures at the end of the incubation period(7.5h) and the "p" values obtained from paired "t" test analysis of corresponding control and CB treated cultures.

The shape and magnitude of the release of label closely resembled that of β-galactosidase and it was different to that of both arylsulphatase and β-N-acetyl-glucosaminidase. The above quantitative differences were reflected in the activities of the parameters measured associated with the cell pellet at the end of the incubation period (Table 24). On comparison with the corresponding values obtained from non-loaded cells (Table 7), it was shown that ^{125}I-PVP had not caused any significant change in the amounts of the enzymes released.

The increase in the absolute activities of the enzymes found in the medium over the incubation period was less than the decrease in the cell pellet activities. The difference was the same both in the presence and the absence of CB and was not significantly different from the corresponding difference observed in the case of non-loaded cells (Table 25, 8).

Activity in cell pellet						
	Control			CB treated		
	Mean	Range	No	Mean	Range	No
AS	78.0	67.0-87.0	5	71.0	50.0-83.0	5
β-GAL	61.0	47.5-75.0	5	55.4	39.0-70.0	5
NAGA	82.0	73.6-91.0	5	75.2	65.0-82.0	5
LDH	59.6	44.6-73.0	5	68.7	50.0-86.0	5
^{125}I-PVP	58.4	47.0-66.0	5	49.0	41.0-64.0	5

Table 24

The activities of lysosomal enzymes, lactate dehydrogenase and label associated with the cell pellet at the end of the culture period, expressed as the percentage of the respective activities at zero time.

Changes in the activities associated with the cell pellet and the cell medium during cell culture												
	Cell pellet (decrease)						Cell medium (increase)					
	Control			CB treated			Control			CB treated		
	Mean	Range	No	Mean	Range	No	Mean	Range	No	Mean	Range	No
^{125}I-PVP	1767	1500-2070	5	2000	1400-2500	5	1800	1200-2500	5	2000	1300-2600	5
AS	101	44-207	5	140	36-294	5	41	21-50	5	65	49-84	5
NAGA	140	23-172	5	201	108-291	5	72	52-103	5	110	77-127	5
β-GAL	71	21-96	5	84	43-115	5	52	37-65	5	65	62-68	5
LDH	3	0.4-6.4	5	2.4	0.3-4.8	5	0.24	-0.7-1.5	5	0.4	-0.7-1.6	5

Table 25

The difference in the absolute amounts of enzymes and label, between 0 time and 7.5h , associated with the cell pellet and the cell medium both in the presence and the absence of CB.

3.2.2.3 Isolated liver perfusions

The release of lysosomal enzymes, ^{125}I-PVP and lactate dehydrogenase from isolated perfused rat livers, that had been loaded with the label 4 days earlier, was investigated both in the presence and the absence of CB (Fig. 25, Table 26).

The addition of CB had no effect on the release of any of the parameters measured. Previous experiments (see 3.1.2.3) investigating the release of lysosomal enzymes from isolated perfused non-loaded rat livers showed that the addition of the microfilament poison caused an increased release of the lysosomal enzymes with no change in the levels of lactate dehydrogenase released (Fig. 12).

When the results obtained from ^{125}I-PVP-loaded perfused livers were compared to those obtained from non-loaded perfused livers, it was shown that, in the presence of DMSO (i.e. the controls), the former released their lysosomal enzymes to a greater extent than the latter. It was also shown that although at the end of the perfusion period the lactate dehydrogenase levels found in the perfusate of loaded livers were higher than those in non-loaded livers, the levels of the cytosolic enzyme had started rising only during the last 2 hrs of the perfusion period, whereas the levels of lysosomal enzymes had started doing so earlier (approximately 2 hrs from the beginning of the perfusion).

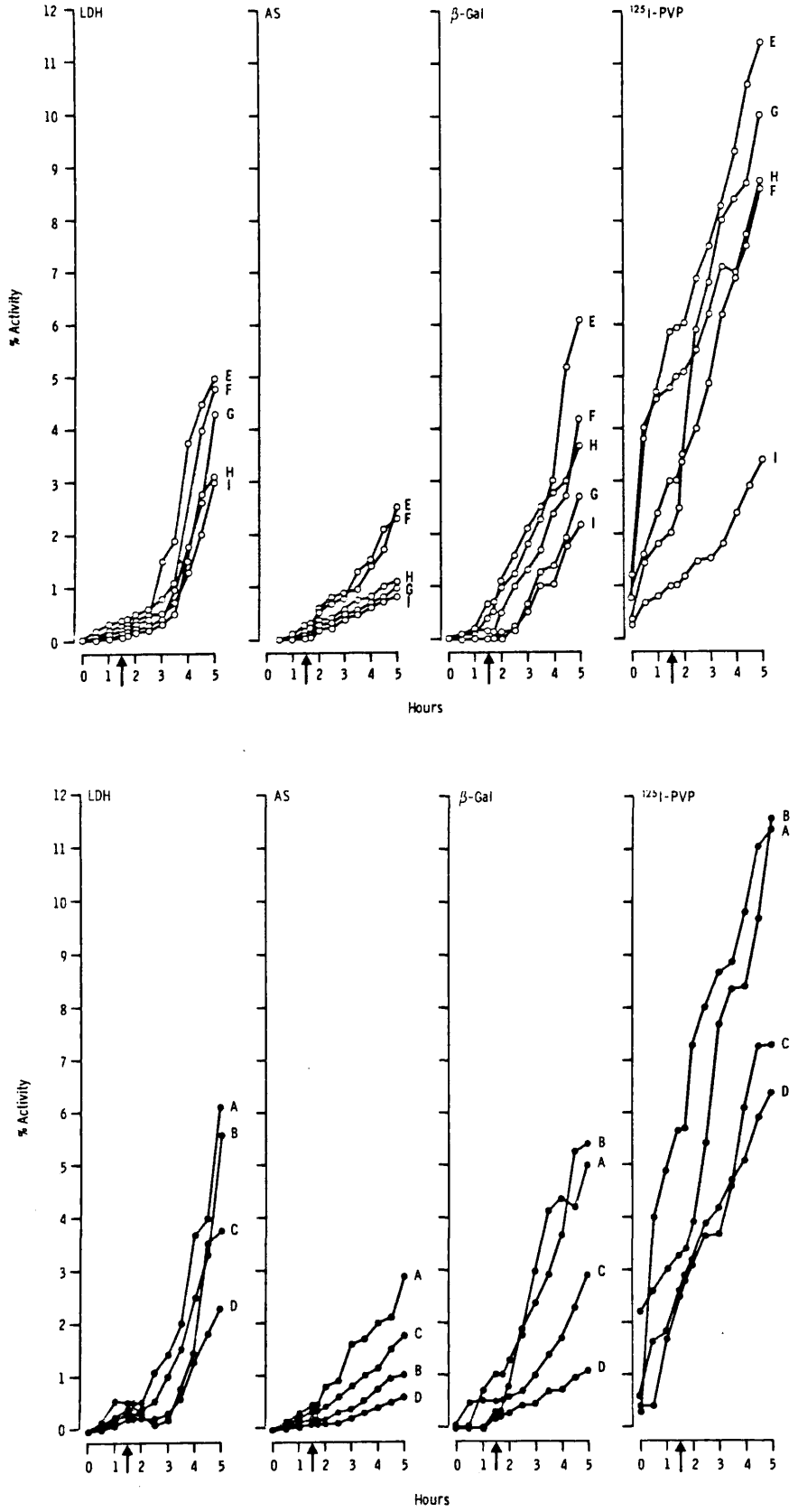

Fig. 25 - see next page

Fig. 25

The release of lactate dehydrogenase, lysosomal enzymes and label from DMSO (control, ●-●) and CB (o-o) treated isolated perfused rat livers, loaded with ^{125}I-PVP 4 days earlier. The arrows represent the time point at which CB or DMSO were added. The letters correspond to different experiments.

	DMSO			CB			P
	Mean	Range	No	Mean	Range	No	Values
AS	1.5	0.6-2.9	4	1.5	0.8-2.5	5	$p<0.9$
β-GAL	3.6	1.1-5.4	4	3.8	2.2-6.1	5	$p<0.8$
^{125}I-PVP	8.4	6.4-11.6	4	9.2	3.4-11.4	5	$p<0.6$
LDH	4.5	2.3-6.1	4	4.0	3.0-5.0	5	$p<0.5$

Table 26

The activities, expressed as the percentage of total recovered, found in the perfusate at the end of the perfusion period in the presence of either DMSO or CB and the statistical significance of their difference, obtained from the students' "t" test analysis of the data.

The total activities recovered at the end of the perfusion are shown in Table 27.

In order to investigate the relative contributions of the release and the re-uptake of ^{125}I-PVP in the amount of label associated with the perfusate, ^{125}I-PVP was added to the perfusate of isolated non-loaded rat livers kept in perfusion and the uptake of label was measured both in the presence and the absence of CB. The uptake was low and there was no significant difference in the amount of radioactivity associated with the liver homogenates at the end of the perfusion between the DMSO and the CB treated livers (Table 28). Also the exogenously added ^{125}I-PVP did not alter the released amounts of either lysosomal enzymes or lactate dehydrogenase (Table 28).

The previous studies (3.2.2.1) investigating the subcellular distribution of ^{125}I-PVP in rat liver showed that the lysosomal enzymes and ^{125}I-PVP exhibited a "bimodal" distribution in sedimentation gradient centrifugation (Fig. 17). In order to investigate whether the lysosomal enzymes and label released during perfusion were preferentially coming from any of the above lysosomal populations, rat livers that had been kept in perfusion were homogenised and the subcellular distribution of lysosomal enzymes and label was investigated. The profiles of the distribution of marker enzymes and label in sedimentation gradient centrifugation were very similar for both DMSO and CB treated rat livers (Fig. 26, 27). When these profiles were compared to those obtained from non-perfused

Activity/wet liver weight								
	A				B			
	Mean	Range	No	SD	Mean	Range	No	SD
AS	8679	5408-12225	4	3611	7014	5052-13063	5	3397
β-GAL	2756	2269-4146	4	926	3350	3142-3783	5	294
^{125}I-PVP	30730	25700-38200	4	5808	23465	2080-29700	5	3791
LDH	510	480-540	4	35	557	296-670	5	225
	C				D			
	Mean	Range	No	SD	Mean	Range	No	SD
AS	11403	9900-13750	5	1429	7838	4950-8800	9	1175
β-GAL	4160	3333-5666	4	1040	2125	1900-2500	4	263
^{125}I-PVP	32151	23000-38387	5	5748				
LDH	565	540-680	4	79	550	380-680	6	1107

Table 27 - see next page

Table 27

A,B: Total activities recovered at the end of perfusions in the absence and the presence of CB respectively.

C: Activities associated with the homogenate of non-perfused livers.

D: Activities associated with the homogenate of non-loaded, non-perfused livers.

	A			B		
	Mean	Range	No	Mean	Range	No
AS	0.7	0.1-1.5	6	0.9	0.8-1.1	3
β-GAL	2.0	0.9-3.3	6	3.0	2.0-3.7	3
LDH	3.9	0.9-8.3	6	4.6	2.1-6.0	3
^{125}I-PVP	2.3	1.0-5.0	6	2.7	1.1-5.0	3

Table 28

The % cumulative activity of enzymes released in the perfusate and the % of the exogenously added ^{125}I-PVP associated with livers kept in perfusion in the absence (A) and the presence of CB (B) at the end of the perfusion period.

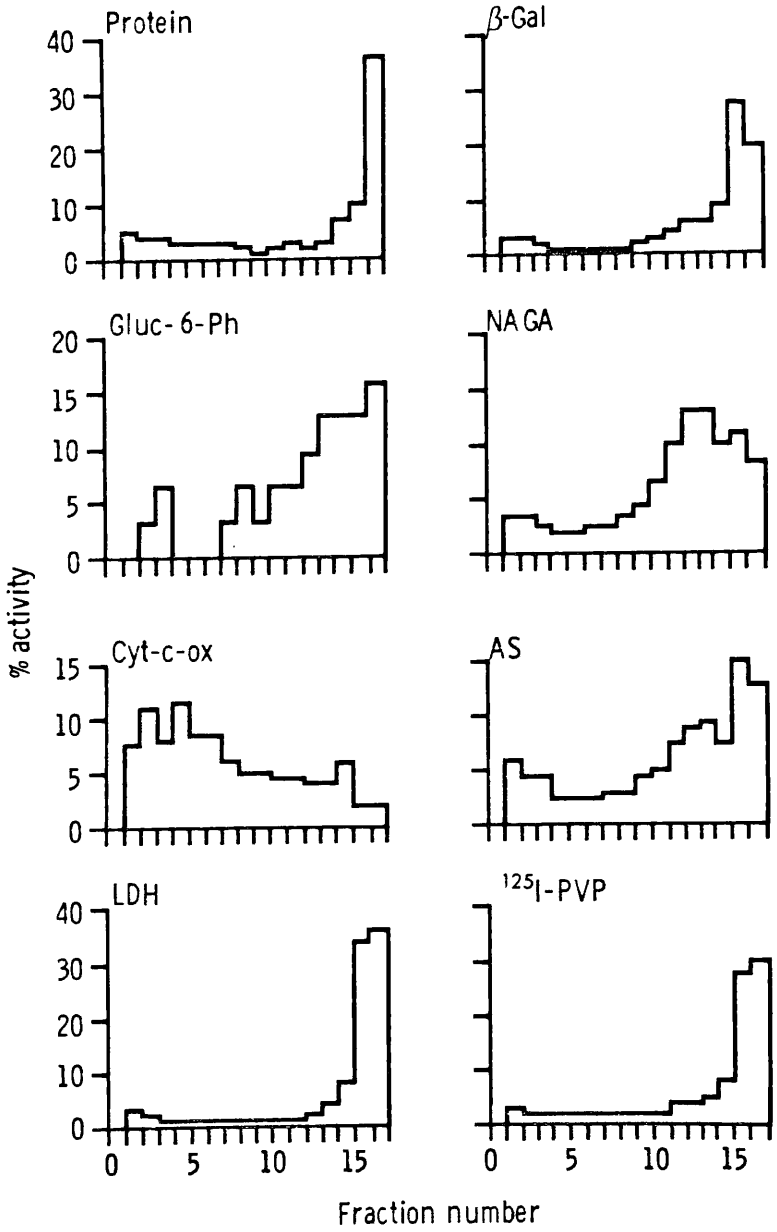

Fig. 26

The distribution profiles of marker enzymes, protein and ^{125}I-PVP obtained from the sedimentation gradient (8.5-40% (w/v) sucrose) centrifugation of liver that had been kept in perfusion for 5.5 hr, in the absence of CB. ^{125}I-PVP had been injected 4 days earlier.

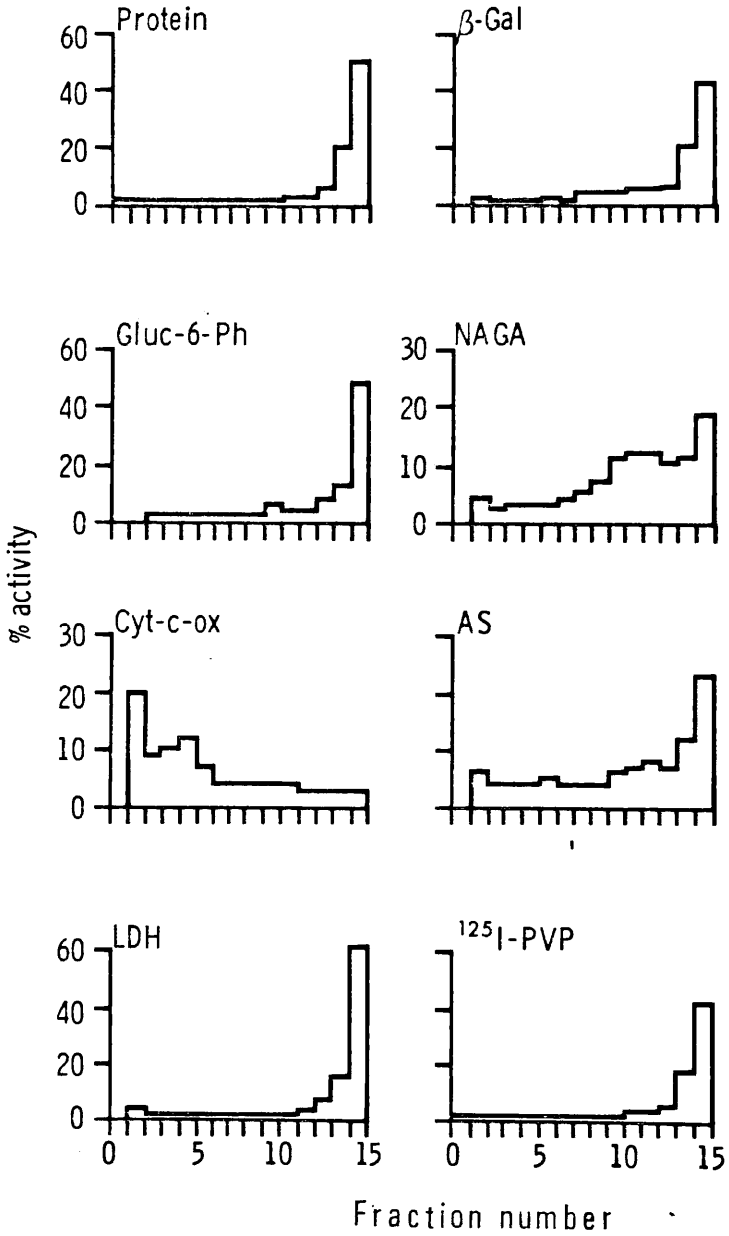

Fig. 27

The distribution profiles of marker enzymes, protein and ^{125}I-PVP obtained from the sedimentation gradient (8.5-40% (w/v) sucrose) centrifugation of liver that had been kept in perfusion for 5.5 hr, in the presence of CB. ^{125}I-PVP had been injected 4 days earlier.

livers (Fig. 17), it was shown that the distribution of label and lysosomal enzymes, but not that of the other marker enzymes, had changed. So the activities of lysosomal enzymes and label associated with the heavier sedimentation gradient sucrose fractions had decreased whereas the activities found at the top of the gradient had increased (Table 29, 30). The most marked differences between perfused and non-perfused livers existed in the distribution of β-galactosidase and ^{125}I-PVP which closely resembled each other. The changes in the distribution of lysosomal enzymes in perfused loaded livers were very similar to those observed in perfused non-loaded livers (Table 29, 30; Fig. 15, 16).

The cytosol fraction obtained from perfused livers exhibited higher specific activities of lysosomal enzymes and ^{125}I-PVP than the corresponding fraction from non-perfused livers, the relative specific activities of the other enzymes being the same. Also the relative specific activites of all the parameters measured associated with the cell debris fraction obtained from perfused livers were, with the exception of cytochrome-c-oxidase, slightly higher than those of non-perfused livers (Table 31).

In order to investigate to what extent the released ^{125}I was associated with PVP, a sample of the perfusate was passed down a Sephadex G-100 column. The results (Fig. 23) showed that the elution profile of the released label coincided with that of the intralysosomally-accumulated label and with the elution profiles of the high molecular weight

components of the injected material, indicating thus that the released label was ^{125}I-PVP rather than free ^{125}I.

	A	B	C	D	E	F
AS	22.0	21.9	42.8	28.4	46.1	38.9
β-GAL	12.5	11.7	41.8	22.5	28.9	36.3
NAGA	16.3	19.4	31.1	25.2	39.2	28.2
^{125}I-PVP	13.2	17.1	42.1	-	-	-
LDH	10.0	8.6	6.4	-	16.9	10.0
Gluc-6-P	9.6	14.0	18.0	-	-	11.0
Cyt-c-ox	62.1	68.1	77.0	68.8	90.8	93.0

Table 29

The % recovered activity associated with sedimentation gradient fractions heavier than 23% (w/w) sucrose obtained from ^{125}I-PVP loaded rat livers that had been kept in perfusion in the absence (A) or the presence (B) of CB, from ^{125}I-PVP loaded non-perfused livers (C) non - loaded livers, perfused in the absence (D) and the presence of CB (E), and non-loaded non-perfused liver (F).

	A	B	C	D	E	F
AS	29	35	13	23	20	6.0
β-GAL	54	63	19	42	45	20.0
NAGA	20	29	10	20	19	13.0
^{125}I-PVP	58	45	22	-	-	-
LDH	70	78	81	60	75	70.0
Glu-6-P	29	59	20	-	-	29.0
Cyt-c-ox	5	7	9	5	2	3.0

Table 30

The % recovered activity associated with the top two fractions of the sedimentation gradients of ^{125}I-PVP loaded livers that had been kept in perfusion in the absence (A) and the presence of CB (B), ^{125}I-PVP loaded non-perfused livers, non-loaded livers that had been kept in perfusion in the absence (D) and the presence (E) of CB and non-loaded non-perfused livers (F).

Relative Specific Activities						
	Cell debris fraction			Cytosol		
	A	B	C	A	B	C
AS	1.10	1.60	1.00	0.40	0.20	0.08
β-GAL	1.30	1.70	0.90	0.18	0.10	0.03
NAGA	1.40	1.80	1.10	0.04	0.07	0.06
^{125}I-PVP	1.00	0.80	0.90	1.00	1.00	0.22
LDH	0.80	0.30	0.40	1.50	2.00	2.70
Gluc-6-P	1.00	1.10	0.90	0.00	0.00	0.00
Cyt-c-ox	1.60	2.00	1.40	0.00	0.00	0.00

Table 31

The relative specific activities of marker enzymes and ^{125}I-PVP associated with the cell debris and the cytosol fractions obtained from livers that were kept in perfusion either in the presence of DMSO (A) or CB (B) and non-perfused control livers (C).

3.2.3 Discussion

The uptake of PVP has been investigated previously both *in vivo* and *in vitro*. *In vitro* studies using rat egg yolk sacs and rat peritoneal macrophages have shown that PVP is taken up slowly, most probably as the result of fluid pinocytosis (Roberts *et al*., 1976; Pratten *et al*., 1977). The results obtained in the present study showed that the *in vitro* uptake of PVP by the isolated perfused rat liver is a slow process. Although such an observation would be in agreement with fluid pinocytosis, the inability of CB to inhibit the uptake would suggest that it occured through micropinocytosis, since CB has been reported to inhibit fluid pinocytosis, but not micro pinocytosis (Wills *et al*., 1972). However, it has also been reported (Jahn, 1976) that CB can induce endocytosis in the isolated perfused rat liver and the possibility that the inability of CB to inhibit the uptake of PVP in the present study is due to two compensatory phenomena i.e. endo- and exocytosis occuring simultaneously cannot be ruled out.

Using low doses of ^{125}I-PVP (10-20 mg/ kg body weight) Recoeczi (1976) showed that low molecular-weight components of the injected material are rapidly excreted whereas the ones with higher molecular weights are taken up by the reticuloendothelial system, the uptake being slow. In the present study PVP was injected in larger quantities than the previously mentioned, i.e. 1 g/kg body weight and although no exact quantitative studies of the intercellular distribution of PVP were carried out, the results obtained from isolated hepatocytes showed that PVP was taken up not

only by Kupffer cells, but also by hepatocytes. This observation is in good agreement with earlier morphological studies which showed the presence of PVP containing vacuoles in rat hepatocytes after the injection of the former in a 1 g/kg body weight dose (Van Hoof as reported by Lloyd 1973). The fact that the liver-associated radioactivity in the present study remained constant over the 4, 8 and 15 day periods examined, could mean either that maximum uptake had already taken place at 4 days and no more material was taken up or that uptake still went on, but was masked by a high rate of turn over of the macromolecules inside the cell. Earlier findings (Ravin et al., 1952) would seem to favour the former hypothesis.

The present subcellular fractionation studies have confirmed the previous indications that PVP is localised inside lysosomes. It has also been shown that its accumulation has no effect on the sedimentation properties of lysosomes, whereas it appeared to shift the bouyant densities of the lysosomal enzymes to lower values at injection periods longer than 4 days.

The accumulation of PVP in the liver though appeared to have caused an increase in the tissue levels of arylsulphatase A + B, β-galactosidase and β-N-acetylglucosaminidase (by 45%, 93% and 33% respectively), but had no effect on the lactate dehydrogenase levels. The increased lysosomal enzyme activities are in good agreement with the previous observation (Meijer and Willighagen, 1963) that PVP accumulation in mouse liver was associated with increased acid phosphatase activity.

The presence of the macromolecule did not affect the number of hepatocytes isolated from loaded livers. Also the similarity between the enzyme activities and the radioactivity associated with the final cell preparation indicates that the isolated hepatocytes did not represent a subpopulation of cells which was selectively enriched in either label or lysosomal enzymes.

In agreement with the results obtained from non-loaded hepatocyte cultures, ^{125}I-PVP loaded hepatocytes released their lysosomal enzymes to different extents, the release of β-galactosidase being the highest and most similar to the release of label. The observed different releases could be due either to ^{125}I-PVP being taken up preferentially by β-galactosidase-rich lysosomes which are preferentially released and/or to the fact that a larger proportion of β-galactosidase and ^{125}I-PVP than arylsulphatase and β-N-acetylglucosaminidase is found in the lumen of lysosomes than in association with the lysosomal membrane. Also the presence of ^{125}I-PVP did not appear either to change the amounts of enzymes released or to interfere with the effect of CB on their release.

^{125}I-PVP-loaded isolated rat livers kept in perfusion appeared to release their lysosomal contents in the absence of CB all through the perfusion period to a higher extent than non-loaded ones, so that by the end of the perfusion period ^{125}I-PVP-loaded livers had released 3 x more β-galactosidase and 2 x more arylsulphatase than non-loaded livers. Also since the increased release of lysosomal enzymes preceded that of lactate dehydrogenase,

it appears that at least a part of this increase in lysosomal enzyme release was not the result of increased cell death. It appears therefore that, in the absence of CB, PVP loading led to the mobilization of lysosomes which were not released from non-preloaded livers under the same conditions.

The fact that ^{125}I-PVP loading did not increase the base line release of lysosomal enzymes from hepatocytes in culture, whereas it did so in the isolated perfused rat liver, could be accounted for by the fact that, assuming that the release observed from non-loaded hepatocytes was mainly due to exocytosis, in isolated hepatocytes all the plasma membrane area available for fusion with lysosomes in the absence of CB, was already engaged in exocytic activity. However, the possibility that the difference between hepatocyte cultures and isolated livers kept in perfusion was due to the presence of more than one cell type in the latter, cannot be excluded. So it is possible that although the presence of ^{125}I-PVP in hepatocyte lysosomes did not alter the base line release of lysosomal enzymes from these cell types, the presence of the macromolecule in Kupffer-cell lysosomes did increase the release of lysosomal enzymes from this cell type in the absence of CB.

The addition of CB did not increase further the release of lysosomal contents from isolated ^{125}I-PVP-loaded livers kept in perfusion. Such a result did not seem to be due to increased uptake of PVP since it was shown that exogenously added PVP was taken up slowly by the isolated

perfused rat livers and at similar rates both in the presence and the absence of CB. Furthermore, since the exogenously added PVP did not change the levels of lysosomal enzymes present in the perfusate either in the presence or the absence of CB, it would appear that the difference between ^{125}I-PVP-loaded and non-loaded perfused livers was due to the intracellular accumulation of the label.

Taking into account the fact that, in the perfusion studies, the lysosomal enzymes in the perfusate will have resulted from the fusion of lysosomes with the sinusoidal domain of the plasma membrane of the cells, the contiguous and canalicular parts of the hepatocyte plasma membrane presumably being inaccessible to the perfusate, it is possible that the inability of CB to further increase the release of the lysosomal contents was due to the preferential fusion of ^{125}I-PVP-loaded lysosomes with areas of the membrane devoid of, rather than covered with, microfilaments.

However, it is also possible, assuming that there exist mechanisms involved in maintaining the plasma membrane area within certain limits, that the lack of a CB effect could be due to such limits having been reached already in the absence of the microfilament poison.

On the other hand, it is possible that in the case of hepatocyte cultures, since possibly exocytosis in this system could have been taking place through the whole disoriented plasma membrane, restrictions like the ones suggested for perfused livers did not apply. So that CB

augmented the release of lysosomal enzymes because there was no depletion of plasma membrane and also by allowing the fusion of lysosomes which did not show a preference for microfilament-devoid areas, with the unmasked plasma membrane areas.

The sedimentation-gradient-centrifugation studies of loaded livers that had been kept in perfusion showed a decrease in the population of large lysosomes, i.e. lysosomes associated with heavier sucrose fractions, when compared to non-perfused loaded livers. The increase in the activities associated with the top sucrose fractions as well as the increased activities found in the cytosol makes it difficult to decide whether the disappearance of the population of large lysosomes was due to their being preferentially released during perfusion or to their increased fragility and subsequent rupture during the homogenisation procedure.

3.3 Dextran-loaded Livers

3.3.1 Introduction

Early studies of the uptake and metabolism of dextran mixtures, that were used as plasma substitutes, revealed that whereas the low-molecular weight components of the mixtures were excreted rapidly in the urine, the higher molecular weight ones were taken up mainly by the liver, where they were slowly metabolised (Terry et al., 1953). Later studies showed that the uptake of dextran by liver was associated with an increase in the acid phosphatase activity of the tissue (Van Duijn et al., 1959).

Since then dextran has been shown to accumulate in the lysosomes of rat liver leading to an increase in their size and density (Baudhuin et al., 1965). The dextran effect was most pronounced 24 hr after injection, but vanished after approximately 1 week. The reversibility of the dextran effect is probably due to the slow digestion of the macromolecule by lysosomal enzyme(s).

Recently a model storage condition has been produced by the administration of ^{3}H-dextran to rats and the effect of the intravenous administration of dextranase, entrapped in liposomes, on the amount of dextran in the liver was examined (Colley and Ryman, 1975).

In the present study the effect of preloading the lysosomes with ^{3}H-dextran on the release of lysosomal contents from isolated perfused liver both in the presence and the absence of CB was investigated. In order to

investigate the subcellular distribution of the *in vivo* administered macromolecule and the changes in the sedimentation and density properties of the lysosomes brought about by its accumulation, under the present experimental conditions, the techniques of differential fractionation and both sedimentation and isopycnic gradient centrifugation were employed.

Gel-chromatography studies using Sephadex G-200 columns were carried out in order to investigate to what extent the ^{3}H that was injected, accumulated intralysosomally and released during perfusion, was associated with dextran.

3.3.2 Results

3.3.2.1 Subcellular Distribution

The subcellular distribution of marker enzymes and ^{3}H-dextran in the livers of rats that had been injected with the macromolecule 4 days earlier, was investigated using the techniques of differential fractionation and both sedimentation and isopycnic density gradient centrifugation.

The differential fractionation studies showed that the cell debris pellet obtained from ^{3}H-dextran-loaded livers exhibited higher lysosomal enzyme activities than the equivalent fraction from non-loaded livers (Table 32), the difference being more prominent for β-galactosidase and β-N-acetyl-glucosaminidase than arylsulphatase. The lactate dehydrogenase activities found in the cell debris pellets from loaded and non-loaded livers were similar.

The distribution profiles of marker enzymes and ^{3}H-dextran in a sedimentation gradient showed that the presence of the macromolecule had changed the sedimentation properties of lysosomes since a pellet rich in radioactivity and lysosomal enzymes was present in the bottom fraction of the sedimentation gradient. Varying amounts of lysosomal enzymes and label were associated with the pellet stuck at the bottom of the sedimentation gradient (7% arylsulphatase, 10% β-N-acetyl-glucosaminidase, 15% β-galactosidase and 20% ^{3}H-dextran) (Fig. 28). There were considerable differences in the distribution profiles of lysosomal enzymes and ^{3}H-dextran, the distribution of label being

	Cell debris fraction		Cytosol	
	A	B	A	B
AS	0.8	1.2	0.1	0.02
	30.0%	38.5%	4.1%	0.5%
β-GAL	1.2	0.9	0.3	0.03
	49.0%	28.5%	10.0%	0.9%
NAGA	1.2	1.0	0.2	0.02
	46.5%	31.5%	5.0%	0.4%
LDH	0.4	0.6	2.1	2.5
	17.0%	18.5%	71.3%	73.5%
^{3}H-Dextran	1.2	-	0.4	-
	46.0%	-	15.0%	-
Protein	1.0	1.0	1.0	1.0
	40.0%	32.0%	34.0%	29.0%

Table 32

The RSA's and % activities associated with the cell debris and the cytosol fractions obtained from livers loaded with ^{3}H-Dextran 4 days earlier (A) and non-loaded ones (B).

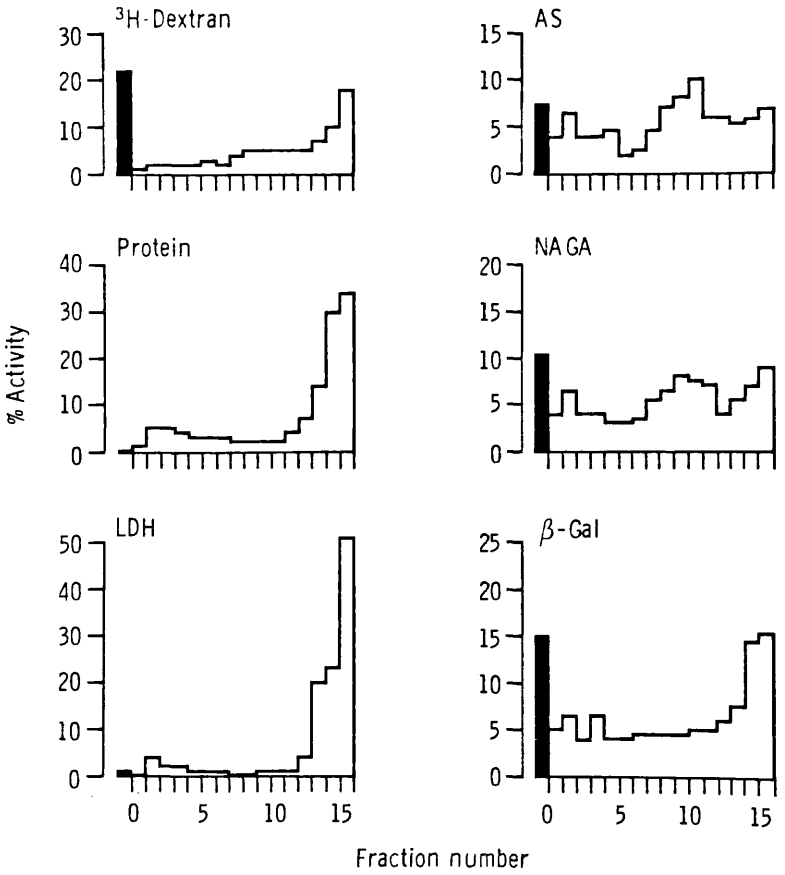

Fig. 28

The subcellular distribution of marker enzymes, protein and ^{3}H-dextran on a representative sedimentation gradient (8.5-40% (w/v) sucrose) 4 days after injection of ^{3}H-dextran.

closer to the distribution of β-galactosidase than that of either arylsulphatase or β-N-acetyl-glucosaminidase (Fig. 28). The top sedimentation gradient fractions as well as the cytosol fractions obtained from ^{3}H-dextran-loaded livers, showed higher lysosomal enzyme activities than the corresponding fractions from non-loaded livers (Table 32; Fig. 28, 7).

The isopycnic gradient centrifugation of the mitochondrial-lysosomal pellet suspension obtained from ^{3}H-dextran-loaded livers showed that as compared to the results obtained from non-loaded livers, there was a shift of all the lysosomal enzymes to higher densities, whereas the protein distribution was unchanged (Fig. 29). However, the extent of the shift was different for each of the enzymes assayed, being more prominent in the case of arylsulphatase. Again, as in the case of sedimentation gradients, the ^{3}H-distribution profile did not correlate closely to the distribution of any of the lysosomal enzymes assayed, the radioactivity peak occuring at denser sucrose fractions than the peaks of lysosomal enzymes (Fig. 29). There was little difference in the activities of lysosomal enzymes and the radioactivity found in the mitochondrial-lysosomal pellet prepared from ^{3}H-dextran loaded livers, the former being similar to the activities in the case of non-loaded livers (Table 33).

The isopycnic gradient centrifugation of a mitochondrial-lysosomal pellet prepared from the liver of a rat that had been injected with Triton WR-1339 and,

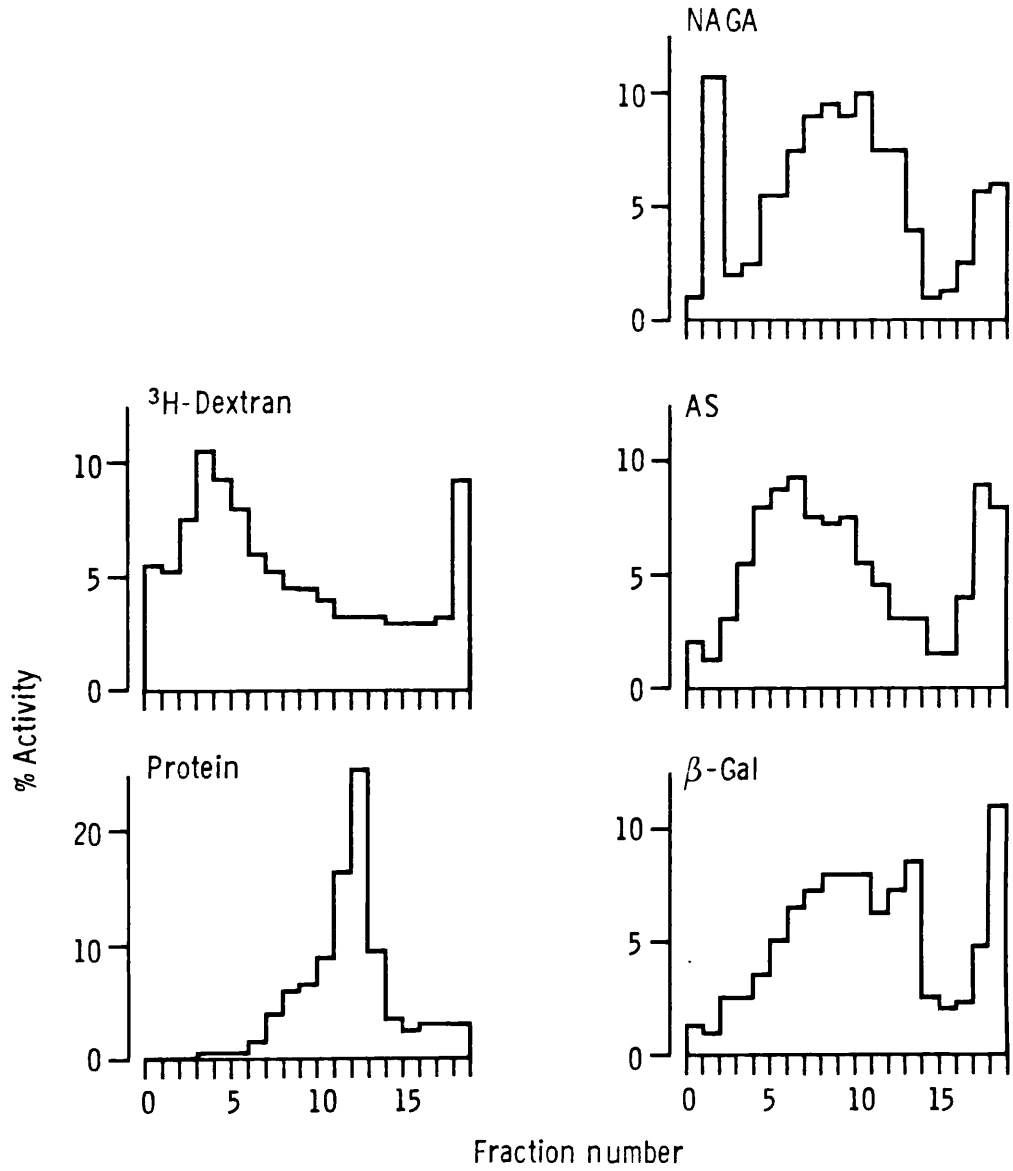

Fig. 29

The distribution profiles of enzymes, protein and ^{3}H-dextran on a 26.7-82% (w/v) sucrose isopycnic gradient 4 days after the injection of ^{3}H-dextran.

	A	B
AS	51.8	43.1
β-GAL	55.0	46.2
NAGA	60.0	51.0
LDH	8.0	7.0
^{3}H-Dextran	-	49.0

Table 33

The activities associated with the mitochondrial-lysosomal pellets, expressed as the % of the homogenate activity, prepared from the homogenates of non-preloaded (A) and ^{3}H-Dextran preloaded (C) rat livers.

10 min. later, ^{3}H-dextran 4 days earlier, showed that the radioactivity and the lysosomal enzymes, but not the protein, had shifted to lower density fractions (Fig. 30). The distribution profiles of the lysosomal enzymes showed minor differences and the arylsulphatase distribution profile was closer to that of the label.

There was no statistically significant difference between the enzyme activities found in ^{3}H-dextran loaded and non-loaded livers (Table 34). Table 35 shows the distribution of label and lysosomal enzymes between the high-speed supernatant and the pellet fractions obtained from a lysed mitochondrial-lysosomal pellet (see 2.2.1.1). It shows that the label and β-galactosidase were found mainly in the supernatant whereas the majority of arylsulphatase and practically all of β-N-acetyl-glucosaminidase were found in the pellet fraction. It appeared that the presence of ^{3}H-dextran did not cause a redistribution of the lysosomal enzymes between the supernatant and the pellet fractions, as compared to non-loaded livers (Table 35).

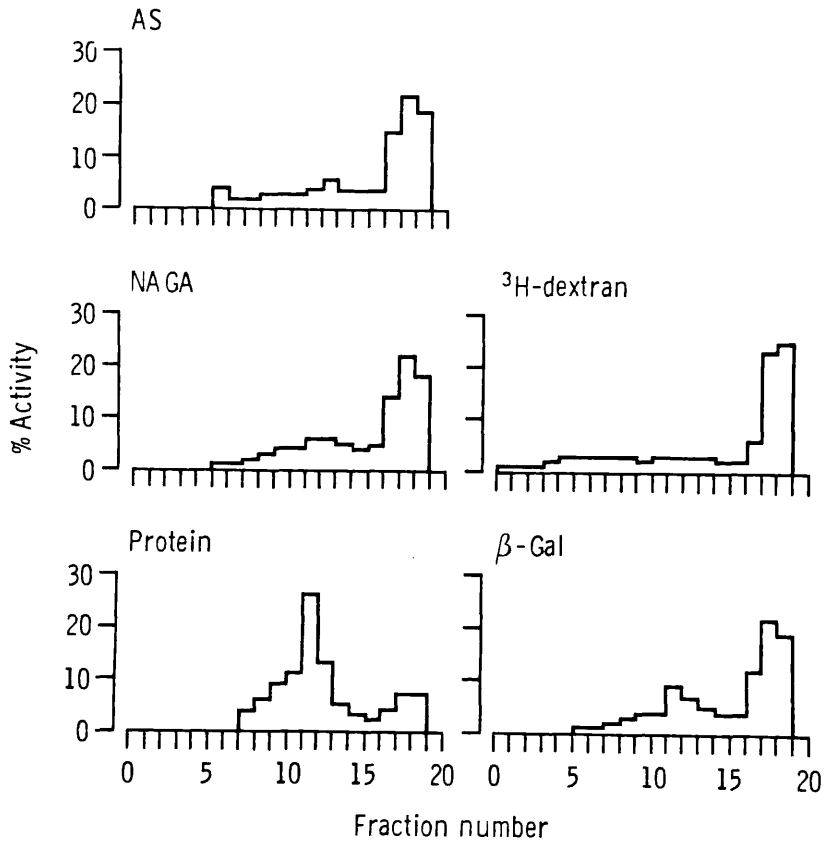

Fig. 30

The effect of Triton WR-1339 in the distribution profiles of lysosomal enzymes and ^{3}H-dextran on a 26.7-82% (w/v) sucrose isopycnic gradient. The detergent and ^{3}H-dextran were injected 4 days earlier.

	A			B		
	Mean	Range	No	Mean	Range	No
AS	8496	7169-9823	3	7838	4950-8800	9
β-GAL	2506	2160-2850	3	2125	1900-4500	4
NAGA	12910	10720-13800	3	11853	10650-13330	4
LDH	481	319-643	3	550	380-680	6

Table 34

The lysosomal enzyme and lactate dehydrogenase activities/g wet weight found in ^{3}H-dextran - loaded (A) and non-loaded (B) livers.

	A	B
AS	41%	38%
β-GAL	71%	70%
NAGA	9%	6%
^{3}H-dextran	94%	-

Table 35

The activities, expressed as the percentage of total recovered, of lysosomal enzymes and label associated with the high-speed supernatant of a lysed mitochondrial-lysosomal pellet prepared from a liver loaded with ^{3}H-dextran 4 days earlier (A) and a non-loaded liver (B).

3.3.2.2 Isolated Liver Perfusions

The release of lysosomal enzymes and label from isolated perfused rat livers loaded with ^{3}H-dextran 4 days earlier, was investigated both in the presence and the absence of CB.

Isolated perfused livers loaded with the macromolecule, released considerable amounts of lactate dehydrogenase, β-galactosidase and label into the perfusate in the absence of CB, the release of arylsulphatase being somewhat lower (Fig. 31). In comparison with non-loaded perfused livers, ^{3}H-dextran-loaded ones had released by the end of the perfusion period approximately 6.6 x lactate dehydrogenase, 4.5 x β-galactosidase and 2.5 x arysulphatase. However, the increase in the release of lactate dehydrogenase occured later than that of lysosomal enzymes.

When CB was added to the perfusate of isolated ^{3}H-dextran-loaded livers kept in perfusion, an increased release of all the enzymes measured was observed (1.4 x lactate dehydrogenase, 2.5 x arylsulphatase, 1.5 x β-galactosidase) whereas the release of the label was only 1.2 x higher. A statistically significant increased release was observed only in the case of arylsulphatase (Table 36). On comparing the shapes of the release of the different parameters measured in the presence and the absence of CB, it appeared that the addition of the drug brought about an immediate increase in the release of arylsulphatase, β-galactosidase, a small increase in the release of label and had no effect on the release of lactate dehydrogenase.

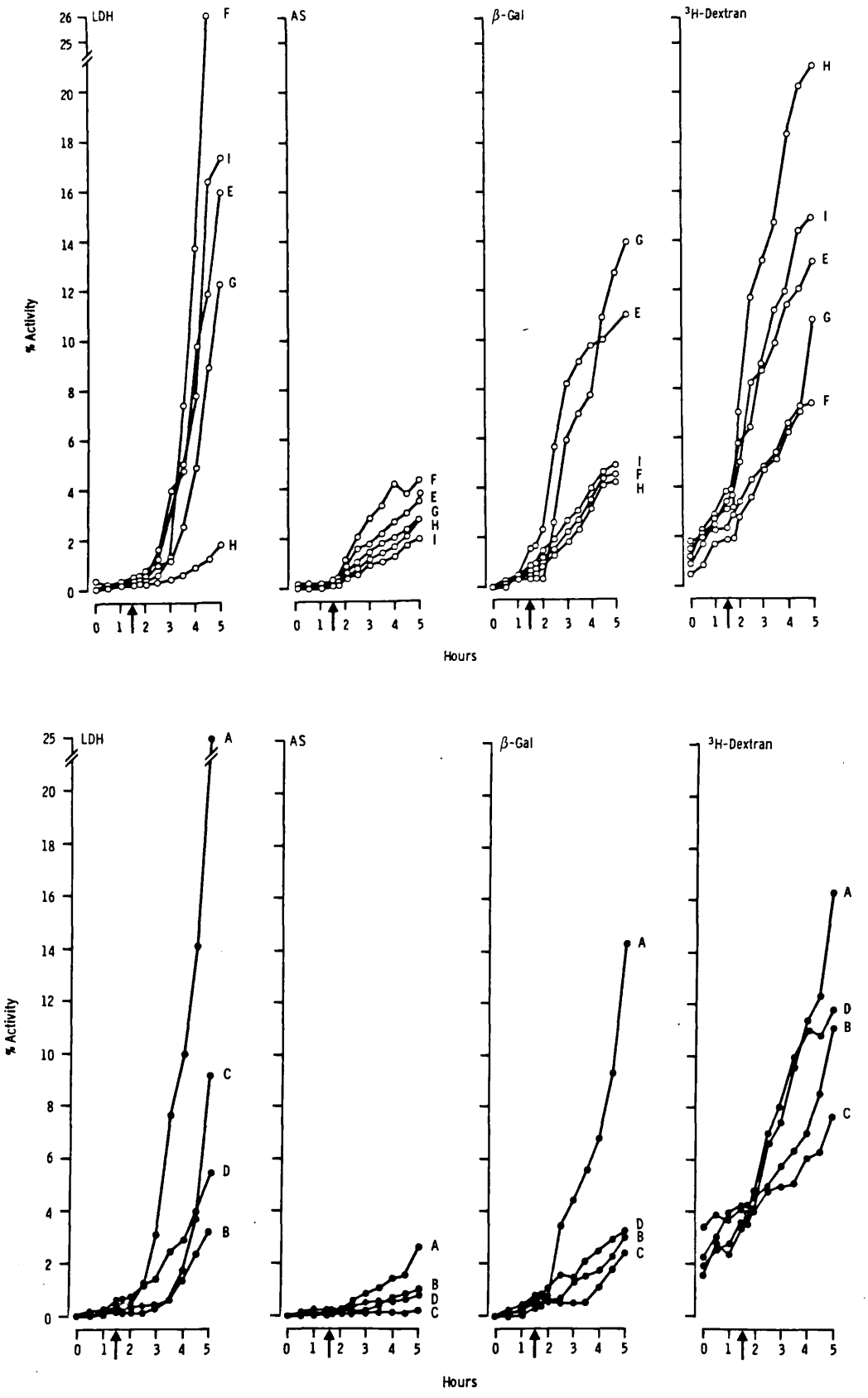

Fig. 31 - legend overleaf

Fig. 31

The release of lactate dehydrogenase, lysosomal enzymes and label from DMSO (●-●) or CB (o-o) treated isolated perfused rat livers, loaded with ^{3}H-dextran 4 days earlier. The arrows represent the time point of the addition of CB or DMSO. The letters correspond to different experiments.

	DMSO			CB			P
	Mean	Range	No	Mean	Range	No	Values
AS	1.2	0.2-2.6	4	3.2	2.0-4.4	5	$p<0.02$
β-GAL	5.8	2.4-14.4	4	8.0	4.2-15.0	5	$p<0.50$
^{3}H-dextran	12.0	7.8-16.3	4	13.2	6.4-21.0	5	$p<0.60$
LDH	10.7	3.0-25.0	4	14.7	1.9-2.6	5	$p<0.60$

Table 36

The activities, expressed as the percentage of total recovered, found in the perfusate at the end of the perfusion period in the presence of either DMSO or CB and the statistical significance of their difference, obtained from the students' "t" test analysis of the data.

For example, 60 min. after the addition of CB the release of lactate dehydrogenase being the same as in the absence of the drug, there was an increased release of the lysosomal enzymes (4 x arylsulphatase, 4 x β-galactosidase) the increase in the release of label being much lower (1.2 x).

In order to investigate to what extent the released label was still associated with dextran, a sample of the perfusate was passed down a Sephadex G-200 column. The elution profile thus obtained was compared to that of the injected dose and also to the elution profile obtained for the material found in the high speed supernatant of a sonicated mitochondrial-lysosomal pellet suspension (Fig. 32). The results showed that the elution profiles of all the tested samples were similar and showed peaks at areas corresponding to high-molecular weights.

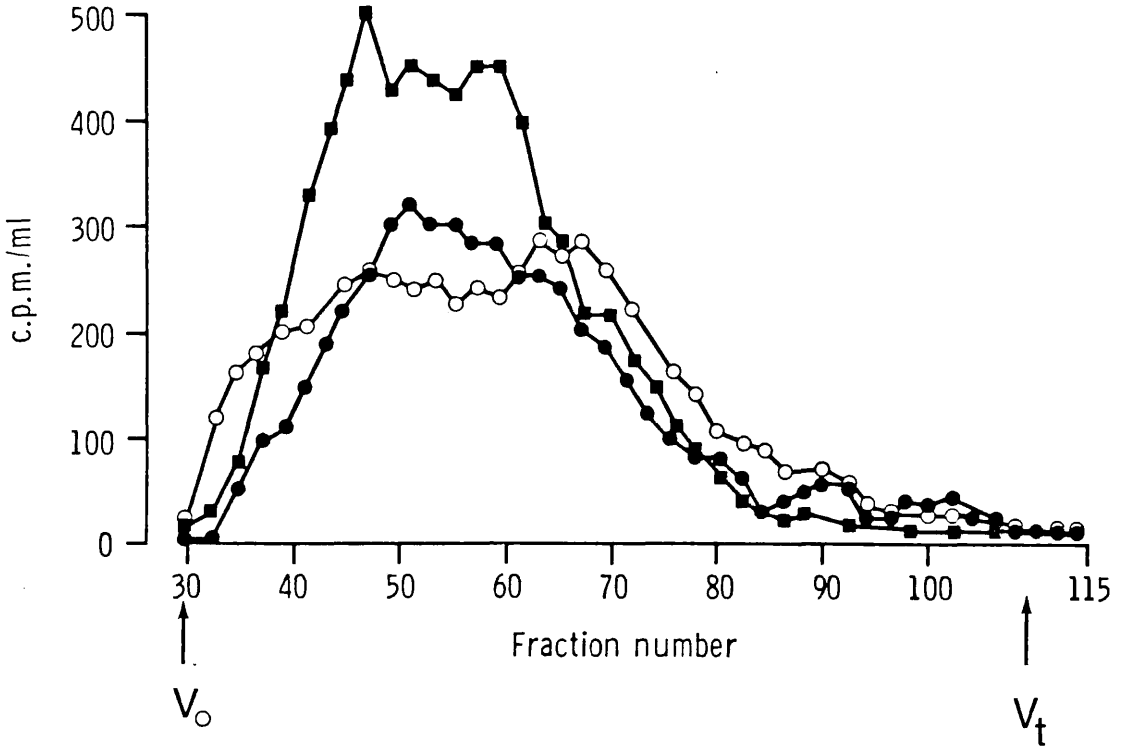

Fig. 32

The elution profiles obtained by passing down a Sephadex G-200 column of a sample (20λ) of the ^{3}H-dextran injection solution (● - ●), a sample (0.5 ml) of the high-speed supernatant of a lysed mitochondrial-lysosomal pellet preparation obtained from a rat liver loaded with ^{3}H-dextran 4 days earlier (■ - ■) and a sample (1 ml) of the perfusate of an isolated rat liver kept in perfusion in the absence of CB that was loaded with ^{3}H-dextran 4 days earlier (o - o).

3.3.4 Discussion

The subcellular distribution study of the *in vivo* administered ^{3}H-dextran is in agreement with previous observations (Baudhuin *et al.*, 1965; Thines-Sempoux, 1968 as quoted by Lloyd, 1973) that intralysosomal accumulation of the material changed both the sedimentation and density properties of lysosomes. The results, however, showed that the subcellular distribution of dextran did not completely parallel that of lysosomal markers. The above observation could mean either that dextran was stored in vacuoles which had never fused with lysosomes or that part of the dextran-containing lysosomes had lost their enzyme activities. The use of Triton WR-1339 did not elucidate the problem, since the results obtained could mean that Trition WR-1339 and ^{3}H-dextran were taken inside the cell in the same endocytic vacuoles which did not necessarily have to fuse with lysosomes or that vacuoles containing dextran but not lysosomal enzymes could fuse with Triton-filled vacuoles.

It would be possible to investigate the first possibility by injecting ^{3}H-dextran and Triton WR-1339 at different time points.

The subcellular distribution studies also showed that the intralysosomal accumulation of ^{3}H-dextran affected the sedimentation and density properties of different lysosomal subpopulations to different extents. In particular, it was observed that the change in the sedimentation properties of lysosomes was mainly manifested in a

β-galactosidase-rich subpopulation, as indicated by both the sedimentation-gradient-distribution profiles of the different lysosomal enzymes and also by the activities found in the cell debris fractions. Supporting evidence based on the assumption that enlarged lysosomes tend to be more fragile to mechanical disruption (Wattiaux et al., 1963) is provided by the fact that the cytosol and the top fractions of the sedimentation gradient contain higher amounts of β-galactosidase and label than either arylsulphatase or β-N-acetylglucosaminidase. On the other hand the isopycnic gradient studies showed that the increased density associated with the intralysosomal localization of ^{3}H-dextran was mainly manifested in a lysosomal population rich in arylsulphatase. The density properties of β-N-acetylglucosaminidase- and β-galactosidase-rich lysosomes were changed to a lesser extent. The observed difference did not appear to be due to the increased fragility of lysosomes rich in β-N-acetylglucosaminidase and β-galactosidase.

On the basis of the above results and the previous suggestion that "old" secondary lysosomes will tend to be dehydrated (Dingle, 1968), it is possible that the arylsulphatase-rich dense lysosomes resulted from the fusion of ^{3}H-dextran-containing vesicles, which may or may not be enriched in lysosomal enzymes, with "old" secondary lysosomes, whereas the enlarged lysosomes resulted from the fusion(s) of ^{3}H-dextran-containing vesicles with primary and/or newly-formed secondary lysosomes. The preferential persistence of arylsulphatase in "old" secondary lysosomes could be the result of the greater stability of the enzyme

compared to other lysosomal enzymes or if primary lysosomes are involved in exocytosis (Dingle, 1968) and the lysosomal membrane is recycled (Lloyd, 1977), of it being largely membrane bound.

However, assuming that the changes brought about by the accumulation of ^{3}H-dextran were a function of the amount accumulated, it is possible that the observed differences are the manifestation of the heterogeneous uptake of ^{3}H-dextran by different lysosomal subpopulations in the same cell or different cell types.

Previous studies have shown that the intralysosomal accumulation of dextran resulted in increased acid phosphatase activity (Meijer and Willighagen, 1963). The results obtained in the present study, however, showed that there was no significant difference in the lysosomal enzymes and lactate dehydrogenase content of ^{3}H-dextran-loaded and non-loaded livers.

^{3}H-dextran-loaded livers kept in perfusion in the absence of CB released lactate dehydrogenase in considerably higher amounts than non-loaded livers. However, the fact that the increase in the release of lysosomal enzymes, more pronounced in the case of β-galactosidase, preceeded that of lactate dehydrogenase would suggest that at least part of the release of the lysosomal enzymes was not due to cells bursting open.

The range of the values obtained both in the presence and the absence of CB for the release of lactate dehydrogenase, β-galactosidase and label, as well as the

fact that the addition of CB increased the release of lactate dehydrogenase as well as of the other parameters, complicates the interpretation of the results. However, the observation that the addition of CB significantly increased the release of arylsulphatase and immediately increased the release of the lysosomal enzymes and to a lesser extent the release of label, but not that of lactate dehydrogenase, indicates that the microfilament poison induced the selective release of lysosomal enzymes, probably through exocytosis.

The gel chromatography studies showed that the released material was of the high molecular weight, indicating that probably little or no degradation had taken place.

3.4. Triton WR-1339-loaded Livers

3.4.1 Introduction

Since the discovery (Wattiaux *et al.*, 1963) that *in vivo* administered Triton WR-1339 accumulated inside the lysosomes causing a decrease in their buoyant density, the non-ionic detergent has been used widely for the preparation of pure lysosomal fractions and also for the study of some aspects of the physiology of the lysosomal system. The administration of the detergent is also associated with profound changes in lipid metabolism which include increased rate of cholesterol synthesis by the liver and also increased cholesterol blood levels (Kellner *et al.*, 1951).

Studies of the accumulation of the detergent in the liver have shown that its uptake by Kupffer cells is rapid, declining after 24 hrs, whereas incorporation in hepatocytes proceeds over several days (Henning and Plattner, 1975). The uptake of Triton WR-1339 is associated with a variety of cellular changes. For example it has been shown that autophagy occurs very soon after the injection of the material (Trout and Villes, 1979) followed by an increased vacuolisation and hypertrophy of the Golgi apparatus (Wattiaux *et al.*, 1963; Trout and Villes, 1979). The morphological changes in the lysosomal population brought about by the accumulation of Triton WR-1339 are similar to those observed in many lysosomal-storage diseases e.g. fucosidosis (Van Hoof, 1973a), GM-1-gangliosidosis (Van Hoof, 1973b) and mucolipidosis (Van Hoof, 1973c).

The fractionation of the commercially available Triton WR-1339 into high and low-molecular weight components (Henning and Plattner, 1975) made it possible to associate the above observed changes with the high-molecular-weight component (Henning and Plattner, 1975). Both morphological and biochemical evidence suggests that Triton-filled lysosomes (tritosomes) are formed both through endocytosis and autophagy (Thines-Sempoux, 1967; Henning et al., 1970; Henning and Heidrich, 1974).

The intralysosomal accumulation of Triton WR-1339 is associated with increased fragility of these organelles to mechanical rupture, as suggested by the increase in the free lysosomal enzyme activity (Wattiaux et al., 1963). At the same time tritosomes are less labile than normal lysosomes during incubation at pH 5 at 37 ^{o}C (Wattiaux et al., 1963).

Investigations have shown that Triton-filled lysosomes can fuse with endocytic vacuoles containing a variety of materials such as caeruloplasmin (Gregoriadis et al., 1970), dextran (Tsung et al., 1975), colloidal gold (Plattner et al., 1975), Trypan-Blue (Davies, 1973), invertase-containing liposomes (Gregoriadis and Ryman, 1972) and inulin (Warburton and Wynn, 1977), but not with albumin-containing vesicles (Davies, 1973). The discrepancy in these results could be explained by the different cellular origin of lysosomes, i.e. lysosomes from both Kupffer and liver parenchymal cells were isolated and it is possible that different materials are endocytosed to different extents by the two cell populations.

In the present study, ^{125}I-Triton WR-1339 was used

in order to futher investigate the effect that the preloading of lysosomes with indigestible materials has on the release of the lysosomal contents from isolated perfused livers in the absence and the presence of CB. Also, since the intralysosomal accumulation of the detergent has been shown to result in the reduction of the bouyant density of lysosomes, subcellular distribution studies were carried out in order to investigate the effect of the in vivo administered Triton WR-1339 on the distribution of lysosomes in the present study.

Gel chromatography studies using Sephadex G-200 columns were carried out in order to investigate to what extent the ^{125}I that was injected, intralysosomally accumulated and released during perfusion, was associated with Triton WR-1339.

3.4.2 Results

3.4.2.1 The Uptake of Triton WR-1339 and its Effect on the Subcellular Distribution of Marker Enzymes

Differential fractionation studies showed that both the cell debris pellet and the cytosol fraction obtained from Triton WR-1339 loaded livers, exhibited relatively higher lysosomal enzyme activities than the corresponding fractions obtained from non-loaded livers (Table 37). The lysosomal enzyme activities found in mitochondrial-lysosomal (ML) pellets prepared from Triton WR-1339 loaded and non-loaded livers were similar (Table 37). Preliminary studies investigating the distribution of ^{125}I-Triton WR-1339 in the cell debris fraction and the ML pellet showed that the radioactivity distribution was similar to that of the lysosomal enzymes (Table 37).

The results obtained from the isopycnic-gradient centrifugation of an ML pellet prepared from non-iodinated-Triton WR-1339 loaded livers showed that the detergent had caused a definite shift of all lysosomal markers to lower densities, but had no effect on the distribution of mitochondria, as judged by the protein profile (Fig. 9). The extent of the observed shift, however as already mentioned earlier (see 3.1.3) was different for different enzymes. Although isopycnic gradient centrifugation of an ML pellet obtained from an ^{125}I-Triton WR-1339 loaded liver was not carried out in the present study, it has previously been reported (Wattiaux et al., 1963) that the iodinated detergent has the same effect on the buoyant densities of lysosomes as the non-iodinated one.

	Cell debris		Cytosol		ML pellet	
	A	B	A	B	A	B
AS	1.2	1.2	0.1	0.02	1.5	1.7
	47.0%	38.5%	3.6%	0.5%	49.4%	51.8%
β-GAL	1.2	0.9	0.1	0.03	1.5	1.8
	46.0%	28.5%	3.0%	0.9%	51.0%	55.0%
NAGA	1.4	1.0	0.1	0.02	1.3	2.0
	52.0%	31.5%	3.3%	0.4%	44.7%	60.0%
LDH	0.4	0.6	2.7	2.4	0.1	0.3
	15.0%	18.5%	81.3%	3.5%	4.0%	8.0%
^{125}I-Triton	1.1	-	-	-	1.5	-
WR-1339	42.0%	-	-	-	49.0%	-
Protein	1.0	1.0	1.0	1.0	1.0	1.0
	38.0%	32.0%	30.0%	29.0%	33.0%	39.0%

Table 37

The relative specific activities and the percentage activities associated with the cell debris, the cytosol and the mitochondrial-lysosomal pellet fractions prepared from livers loaded with non-iodinated Triton WR-1339 and the distribution of label, in the case of ^{125}I-Triton WR-1339 loaded livers, in the cell debris and the mitochondrial-lysosomal pellet fractions (A), as well as the relative specific activities and percentage activities found in all three fractions from non-loaded livers (B).

The total activities of lysosomal enzymes found in livers loaded with Triton WR-1339 were not significantly different from those found in non-loaded livers (Table 38).

The distribution of label and lysosomal enzymes between the high-speed supernatant and the pellet fractions obtained from a lysed mitochondrial-lysosomal pellet (see 2.2.1.1) was tested. The results showed (Table 39) that the majority of the label and β-galactosidase were recovered in the supernatant whereas the majority of arylsulphatase and practically all of β-N-acetyl-glucosaminidase were recovered in the pellet fraction. It appeared that the presence of ^{125}I-Triton WR-1339 did not cause redistribution of the lysosomal enzymes between the supernatant and the pellet fractions, as compared to non-loaded livers (Table 39).

Measurements of the radioactivity associated with different tissues 4 days after the <u>in vivo</u> administration of ^{125}I-Triton WR-1339 showed that among the tissues studied liver had the highest content (Table 40).

	A			B		
	Mean	Range	No	Mean	Range	No
AS	10776	8425-12100	3	7838	4950-8800	9
β-GAL	2402	1967-2830	3	2125	1900-2500	4
NAGA	12193	9500-14890	3	11853	10650-13330	4

Table 38

The lysosomal enzyme activities, expressed as activity/g wet liver weight, found in Triton WR-1339 loaded (A) and non-loaded (B) livers.

	A	B
AS	40%	38%
β-GAL	70%	70%
NAGA	6%	6%
^{125}I-Triton WR-1339	90%	-

Table 39

The activities, expressed as the percentage of total recovered, of lysosomal enzymes and label found in the high-speed supernatant of a lysed mitochondrial-lysosomal pellet prepared from a liver loaded with ^{125}I-Triton WR-1339 4 days earlier (A) and a non-loaded liver (B).

% Injected radioactivity			
	Mean	Range	No
Kidneys	0.45	0.20-0.60	4
Liver	13.50	11.10-15.80	8
Spleen	1.80	1.00-2.30	4
Thyroid	4.40	4.10-5.00	4

Table 40

The radioactivity associated with different tissues, expressed as the percentage of the injected dose, 4 days after the intraperitoneal injection of ^{125}I-Triton WR-1339.

3.4.2.2 Isolated Liver Perfusions

The release of lysosomal enzymes and label from isolated perfused rat livers, loaded with ^{125}I-Triton WR-1339 4 days earlier, was investigated both in the presence and absence of CB.

In the absence of the microfilament poison, the lysosomal enzymes, arylsulphatase and β-galactosidase, and the label started to appear in the perfusate immediately, whereas there was a delay of approximately 90 min. in the appearance of lactate dehydrogenase (Fig. 34). All the parameters continued increasing in the perfusate and both the lysosomal enzymes and lactate dehydrogenase did so at a higher rate than that observed in the case of non-loaded livers (Fig. 12). So that the final levels of all the enzymes measured in the perfusate were higher than those in the perfusate of non-loaded livers (1.7 x for lactate dehydrogenase, 2.6 x for arylsulphatase and 3.5 x for β-galactosidase). The levels of the label released were similar to those of β-galactosidase.

The addition of CB did not bring about any change in the amounts of the parameters measured in the perfusate apart from lactate dehydrogenase which was reduced to about 50% (Fig. 33; Table 41). The levels of both arylsulphatase and lactate dehydrogenase released were similar to those in the case of non-loaded livers, whereas the release of β-galactosidase was 2.2 x greater.

In order to investigate to what extent the released ^{125}I label was still associated with the Triton WR-1339, a

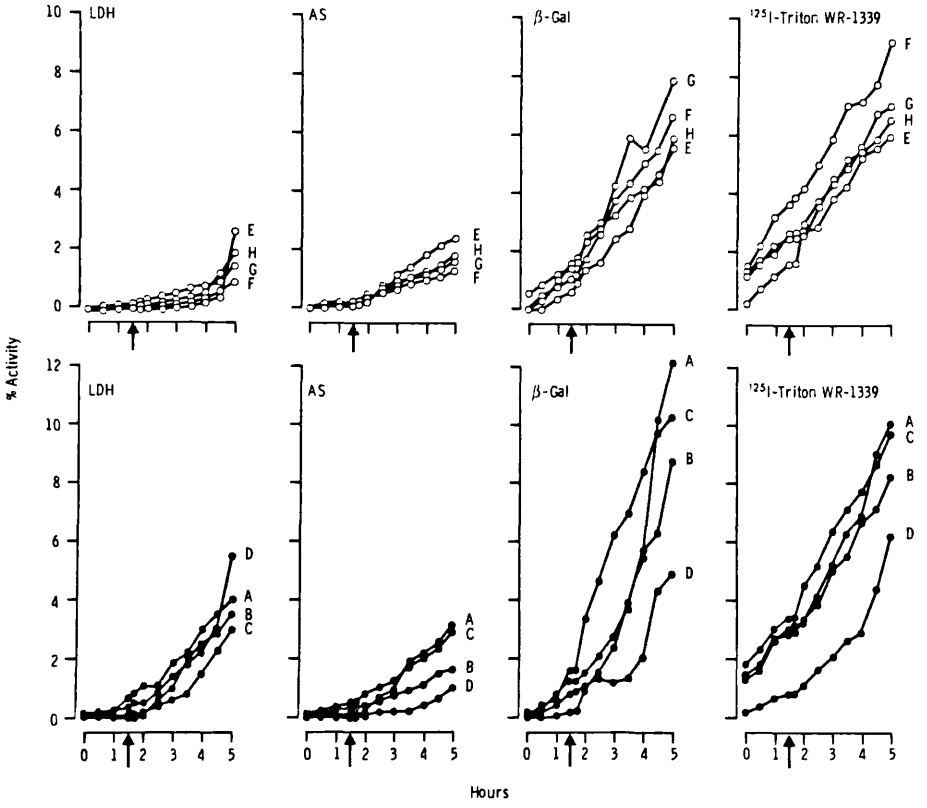

Fig. 33

The release of lactate dehydrogenase, lysosomal enzymes and label from DMSO, controls (●-●) and CB (o-o) treated isolated perfused rat livers loaded with ^{125}I-Triton WR-1339 4 days earlier. The arrows represent the time points at which CB or DMSO were added. The letters correspond to different experiments.

	DMSO			CB			P
	Mean	Range	No	Mean	Range	No	Values
AS	2.2	1.0-3.2	4	1.9	1.3-2.4	4	$p<0.600$
β-GAL	9.1	5.0-12.2	4	6.6	5.6-7.9	4	$p<0.100$
^{125}I-Triton WR-1339	8.6	6.2-10.1	4	7.5	6.0-9.3	4	$p<0.250$
LDH	4.0	3.0-5.5	4	1.8	0.9-2.6	4	$p<0.005$

Table 41

The activities, expressed as the percentage of total . recovered, found in the perfusate at the end of the perfusion period in the presence of either DMSO or CB and the statistical significance of the difference, obtained from the students' "t" test analysis of the data.

sample of the perfusate was passed down a Sephadex G-200 column. The elution profile was compared to that of the injected material and also to the elution profile obtained for the material found in the high speed supernatant of a sonicated mitochondrial-lysosomal pellet (Fig. 34). The elution profile of the injected material showed two peaks of radioactivity, one of high and one of low molecular weight. The profiles of both the label found in the supernatant of the mitochondrial-lysosomal pellet preparation and the label released during perfusion, showed a peak of radioactivity which coincided with the high molecular-weight component of the injected dose. They also showed a peak of higher-molecular weight which coincided with the void volume of the column as determined by Blue Dextran. Furthermore the elution profile of the released label was more diffuse than the profiles of both the injected dose and the mitochondrial-lysosomal associated label, showing a displacement towards the highest molecular-weight region present in the profile of the mitochondrial-lysosomal associated label.

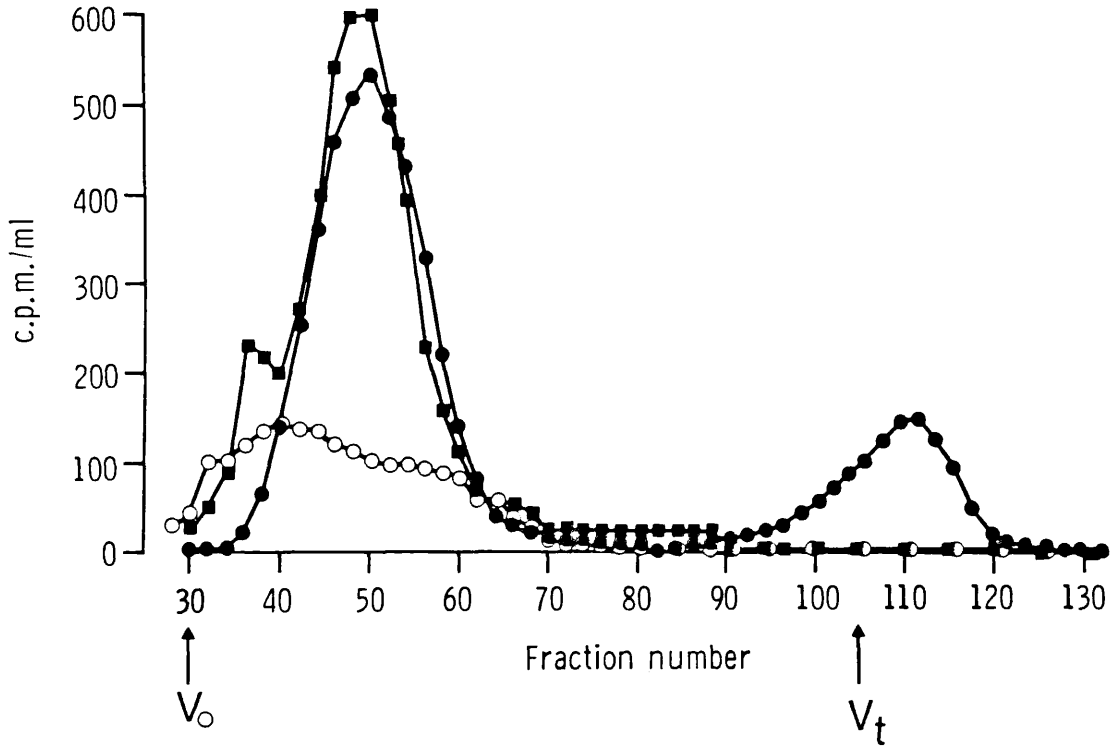

Fig. 34

The elution profiles obtained by passing down a Sephadex G-200 column of a sample (20λ) of the ^{125}I-Triton WR-1339 injection solution (● - ●), a sample (0.5 ml) of the high-speed supernatant of a lysed mitochondrial-lysosomal pellet preparation obtained from a rat liver loaded with ^{125}I-Triton WR-1339 4 days earlier (■ - ■) and a sample (1 ml) of the perfusate of an isolated rat liver kept in perfusion in the absence of CB that was loaded with ^{125}I-Triton WR-1339 4 days earlier (o - o).

3.4.3 Discussion

The results obtained from the isopycnic gradient centrifugation, in agreement with previous observations (Wattiaux *et al.*, 1963; Henning *et al.*, 1971), showed that under the present experimental conditions Triton WR-1339 injected *in vivo* brought about a decrease in the buoyant density of lysosomes. The differences in the profiles of the different lysosomal enzymes, as discussed previously (see 3.1.5), could be the result of the heterogenious uptake of Triton WR-1339 by different lysosomal populations in the same cell and/or the heterogeneous uptake of the detergent by different cell types present in the liver.

The observed shift of lysosomes to lower densities has been associated with the intralysosomal localization of the detergent (Wattiaux *et al*, 1963). Further indirect evidence for such a localization is provided by the results obtained in the present study which showed that when ^{125}I-Triton WR-1339 was injected *in vivo*, the radioactivity associated with the mitochondiral-lysosomal pellet obtained from the liver of treated animals, was similar to the lysosomal enzyme content of the pellet.

The elution profile of the injected material on Sephadex G200 showed two peaks of radioactivity. Henning and Plattner (1975) managed to fractionate commercially-available Triton WR-1339 into high and low molecular weight components.

It is not clear to what extent the second peak obtained in the present study is microtriton or free iodine since they should both have the same elution volume on Sephadex G-200 and also since at least part of the former should have been removed in the preparative G25 gel chromatography of the labelled material (see 2.2.5.1).

The higher release of lactate dehydrogenase in the absence of CB than in its presence makes the interpretation of the absence of a CB effect on the release of lysosomal enzymes and label difficult. It is not clear to what extent the release of lysosomal enzymes and label in the absence of CB is due to cell death. However, the fact that the β-galactosidase activity and the radioactivity in the perfusate rose before the lactate dehydrogenase activity indicate that at least part of their release was not related to cell death. Supporting evidence for the above notion is provided by the observation that the release of lysosomal enzymes, as compared to non-loaded livers, was increased to a higher extent than that of lactate dehydrogenase. Therefore it seems likely that the presence of the detergent has induced the selective release of lysosomal enzymes through a process other than cell death, i.e. exocytosis.

No evidence is available for a possible stabilising effect of CB on the plasma membrane and the previously obtained results (see 3.1.2.3 and 3.2.2.3) do not suggest a possible role of the drug in the control of the release

of lactate dehydrogenase. However, the approximately 50% reduction in the release of lactate dehydrogenase from Triton WR-1339 loaded livers, brought about by the addition of CB, suggests that the microfilament poison could have such an action in the presence of the detergent. For example, it could counterbalance a destabilizing effect of Triton WR-1339 on the plasma membrane caused by the interaction of the detergent with plasma membrane lipids. The mechanism, however, of such an action is unknown.

The failure of CB to further augment the release of lysosomal enzymes and label from isolated perfused livers, could be a manifestation of the preferential fusion of the tritosomes found in close proximity to the domains of the plasma membrane available for exocytosis i.e. the sinusoidal, with portions of these domains which were devoid of microfilaments.

However, it is also possible, assuming that there exist mechanisms involved in maintaining the plasma membrane area within certain limits, that the lack of a CB effect is due to the exceeding of such limits.

The gel chromatography studies showed that both the released and the mitochondrial-lysosomal-pellet-associated label corresponded with the high molecular weight components of the injected material. The peaks of radioactivity in the elution profiles of the released label and that found in the mitochondrial-lysosomal pellet, coinciding with the void volume of the Sephadex G-200

column, could correspond to Triton WR-1339 associated with the α-lipoprotein suggested by Wattiaux (1966) and Henning and Plattner (1975).

3.5 General Discussion and Proposals for Future Work

3.5.1 General Discussion

The present studies have attempted to gain a greater understanding of some of the factors involved in the extracellular release (exocytosis) of both the enzymic and non-enzymic contents of rat liver lysosomes. The involvement of microfilaments in the process was investigated by examining the release of lysosomal contents from hepatocytes in short-term cultures and isolated perfused livers in the presence and the absence of the microfilament poison cytochalasin B (CB). Furthermore the effect of the alteration of the intralysosomal environment, brought about by the accumulation of indigestible material, i.e. ^{125}I-polyvinylpyrrolidone (^{125}I-PVP), ^{125}I-Triton WR-1339 and ^{3}H-dextran, on the release of lysosomal contents from isolated perfused livers and in one case (^{125}I-PVP) from hepatocytes in short-term culture, both in the presence and the absence of CB, was investigated.

The lysosomal enzymes assayed showed different patterns of release which were maintained under all the experimental conditions employed. In particular the release of β-galactosidase was higher than that of arylsulphatase and β-N-acetylglucosaminidase, in the case of hepatocyte cultures, and higher than that of arylsulphatase in the case of isolated perfused rat livers. Furthermore the release of β-galactosidase was similar to the release of the preloaded labelled material both from hepatocyte cultures and isolated perfused livers. The distribution of the lysosomal

enzymes and label between the high-speed supernatant and the pellet obtained from a lysed mitochondrial-lysosomal preparation showed that β-galactosidase and the labelled material were associated mainly with the supernatant, whereas β-N-acetylglucosaminidase and arylsulphatase were associated mainly with the pellet fraction. This finding, together with previous observations (for a review see Davies, 1975) suggests that the heterogeneous patterns of release of the different lysosomal enzymes were probably due to their being associated with the lysosomal membrane to different extents. Hence, during exocytosis, enzymes that were mainly found in the lumen of the lysosomes were released extracellularly, whereas the enzymes that were mainly membrane-bound remained attached to the lysosomal membrane now incorporated in the plasma membrane, and were recycled back into the cell

The addition of CB selectively increased the net release of lysosomal enzymes from non-loaded and ^{125}I-PVP-loaded rat hepatocytes in short-term cultures and non-loaded isolated perfused livers. Such a finding is in good agreement with the hypothesis that microfilaments, present beneath the plasma membrane, form a barrier which prevents intracellular vesicles from coming in contact with the plasma membrane (Allison, 1973), an event which becomes possible after the microfilament disorganization brought about by CB. However, it is not clear which characteristics of the microfilament organization determine their role in the release of lysosomal contents. For example, it is not clear whether it is the polymerisation of actin into

microfilaments, the polarity of the microfilaments, or the formation of microfilament bundles that is important. Experiments using purified actin and microfilament-associated proteins have shown that CB can interfere with all the above processes (Stossel, 1977; Brenner and Korn, 1979; Brown and Spudich, 1979), but an extrapolation to the situation in whole cells and organs is not easy.

The label released from isolated perfused liver loaded with indigestible material was of macromolecular nature, i.e. ^{125}I-PVP, ^{125}I-Triton WR-1339 or ^{3}H-dextran similar both to that associated with the mitochondrial-lysosomal fractions obtained from livers loaded with any of the above materials and to the high-molecular-weight component of the respective injected material, indicating that the release of label was not due to its slow degradation. Also the subcellular fractionation studies of loaded non-perfused and, in the case of livers loaded with ^{125}I-PVP, perfused livers, showed that the subcellular distribution of labelled material was closer to that of lysosomal than to any other enzymes. The above considerations would suggest that the released material was of lysosomal origin and that there was a fusion step involved in the release of label, since the high-molecular weight material released could not have diffused directly through the plasma or lysosomal membranes.

Isolated perfused livers loaded with indigestible materials, released higher levels of their lysosomal enzymes in the absence of CB than did non-loaded livers. The effect was qualitatively independent of the nature of the

accumulated material, whereas the range of the results made any quantitative difference, dependent on the nature of the stored macromolecules, statistically insignificant. Assuming that it is mainly the lysosomal enzymes associated with the lysosomal lumen that will be released during exocytosis the increased release of lysosomal enzymes brought about by the stored material was not the result of decreased association of the enzymes with the lysosomal membrane (Tables 19, 35 and 39).

The addition of CB into the perfusate of isolated livers loaded with either ^{125}I-PVP or ^{125}I-Triton WR-1339 did not further increase the release of either the lysosomal enzymes or the label. On the other hand, CB appeared to have an effect on the release of the lysosomal contents from ^{3}H-dextran-loaded livers. The observed effect was, however, statistically significant only in the case of arylsulphatase.

On the assumption that there may exist mechanisms for the maintenance of the area of the plasma membrane within certain limits, it has been suggested earlier in this study that the inability of CB to increase further the release of lysosomal contents from loaded livers in perfusion, might be due to the exceeding of the limits.

Nevertheless, the observations that CB increased the release of the lysosomal contents from ^{3}H-dextran-loaded livers kept in perfusion, makes it unlikely that such a control mechanism was responsible for the results obtained in the present study. It is therefore suggested that the

accumulation of indigestible material has changed the relationship between the lysosomes, the plasma membrane and the microfilaments in such a way that lysosomal contents could be released via a microfilament-independent mechanism, as judged by the insensitivity of the process to CB.

This change could have been brought about by the persistence of increased numbers of secondary lysosomes, which resulted from the fusion of endocytic vacuoles containing the indigestible material with lysosomes, near the areas of plasma membrane exposed to the sinusoids, since it is through the latter that exocytosis would occur in the isolated perfused liver system used in the present study. This persistence of secondary lysosomes would increase the probability of the organelles coming into contact and fusing with the plasma membrane. If this were the case, the increased release of lysosomal contents observed in the absence of CB, could conceivably be due to the redistribution of lysosomes intracellularly.

However, such a hypothesis could not account for the lack of a CB effect. Furthermore, previous studies (Roberts et al., 1976) have shown that treatment of rat egg yolk sacs with vacuolating agents (i.e. Triton WR-1339, sucrose, PVP and dextran) did not increase the release of lysosomal enzymes from this system.

It is possible that the release of lysosomal contents from isolated perfused livers loaded with indigestible materials was the result not only of the intracellular

redistribution of lysosomes, but also of the fact that loading had brought about changes in the physicochemical properties of lysosomes, the nature of which influenced their route of exocytosis. Such changes, for example, could determine whether the lysosomes would fuse with areas of plasma membrane devoid of or covered with microfilaments. In this event, the release of the enzymic and non-enzymic lysosomal contents in the absence of CB should be considered as a specific process determined by the properties of the lysosomes, rather than the result of the non-specific effect of the redistribution of lysosomes proposed earlier.

The changes brought about by the loading of lysosomes, resulting in their increased exocytosis via a CB-insensitive mechanism, could be the result of the presence, caused by the loading, of areas of local or generalised membrane instability.

Decreased stability of the membrane, either local or generalised, may result from the interaction of the accumulated material with the lysosomal membrane. In the present study it is possible that such an interaction occured in the livers loaded with ^{125}I-Triton WR-1339, since the macromolecule is a surface-active agent.

On the other hand, the membrane of a vesicle with a potential for exocytosis may exhibit an increased tendency to fuse with the plasma membrane as a result of it having previously participated in a series of fusions with primary lysosomes, the membrane properties of which have been proposed

(Dingle, 1968) to be such as to make them capable of readily fusing with other membranes. The fusion of primary lysosomes with other membranes, in the present case the membranes of other vesicles, will be followed by the intermixing and the redistribution of the components of the two membranes. It is nevertheless probable, that for a certain period areas of local instability, derived from the membranes of the primary lysosomes, will persist which will facilitate the fusion of the newly formed secondary lysosomes with the plasma membrane.

It is also possible that the membranes of the newly-formed secondary lysosomes have acquired from primary lysosomes specific glycoproteins (Schneider et al., 1978; Yamamoto et al., 1980) which would make possible the recognition and the interaction between lysosomal membranes and plasma membrane areas, devoid of microfilaments.

Such a hypothesis may require the increased synthesis of lysosomal enzymes and primary lysosomes which has been shown previously to occur in limb bone rudiment organ cultures in response to sucrose loading (Dingle et al., 1969). However, in the present study increased synthesis of lysosomal enzymes was found only in ^{125}I-PVP-loaded livers. Morphological evidence (Trout and Villes, 1979) showed that the accumulation of Triton WR-1339 in rat liver was associated with hypertrophy of the Golgi apparatus and increased numbers of vesicles budding off its surface, associated with the packaging of lysosomal products. Therefore, it is possible that although the synthesis of lysosomal enzymes is not increased in Triton WR-1339-loaded livers, increased

numbers of Golgi vesicles with membrane properties similar to those of primary lysosomes are formed which could facilitate exocytosis in the way described earlier, i.e. by fusing with the endocytic vesicles containing the indigestible material. Although previous studies on the effect of dextran loading on the rat liver, showed that it resulted in increased levels of acid phosphatase (Mejer and Willighagen, 1963; Thines-Sempoux, 1968, as quoted by Lloyd, 1973), it did not increase the levels of deoxyribonuclease to the same extent (Thines-Sempoux, 1968, as quoted by Davies, 1975). In the present study, ^{3}H-dextran loading did not increase the levels of arylsulphatase, β-galactosidase or β-N-acetylglucosaminidase. The reason for the above discrepancies is not clear, and therefore it is not certain to what extent the increased release of lysosomal contents from ^{3}H-dextran loaded livers could be the result of increased formation of primary lysosomes.

The addition of CB in the perfusate of ^{3}H-dextran-loaded livers, as was also observed in the case of livers loaded with ^{125}I-PVP or ^{125}I-Triton WR-1339, did not increase the release of β-galactosidase and label. On the other hand, unlike ^{125}I-PVP and ^{125}I-Triton WR-1339 loaded livers, the presence of CB did augment the release of arylsulphatase from ^{3}H-dextran-loaded livers. Subcellular distribution studies of ^{3}H-dextran-loaded livers had shown that the accumulation of the macromolecule had made the lysosomes both larger (mainly containing β-galactosidase) and denser (mainly containing arylsulphatase). The mechanisms by which the changes in the sedimentation and

density properties of lysosomes were brought about are not clear; it is possible that the increased density was the result of dehydration of the lysosomes. If that was the case then, as suggested earlier (Dingle, 1968) the membranes of the dense lysosomes would have a decreased surface tension, which could hinder their fusion with the plasma membrane. Although at present no direct evidence is available, relating the changes in the density of the lysosomes to their release, it is possible that it was the release of these dense, presumably dehydrated lysosomes, that was augmented by CB. That then could indicate that microfilaments are involved only in the release of lysosomes, with membrane properties such as are to prevent them from recognising and fusing with plasma membrane areas devoid of microfilaments.

3.5.2 Proposals for Future Work

To test further these hypotheses and also in order to obtain a clearer indication of the cellular origin of the released enzymatic and non-enzymatic lysosomal contents, i.e. whether they are released from hepatocytes or Kupffer cells, and subsequently on the role of microfilaments in the release from different liver cell types, it is essential that the release of the lysosomal contents into the bile, under the conditions tested in the present study, is also studied. On the basis of previous observations (Shnitka, 1965; de Duve and Wattiaux, 1966), it is believed that the lysosomal enzymes released in the bile will come from the lysosomes localised around the bile canaliculus (Norikoff, 1973). In view of the failure to collect bile from isolated perfused livers in the present study, such experiments could either be carried out in the presence of taurocholate, since it has been suggested (Bambeck et al., 1968) that this will maintain bile production from isolated livers kept in perfusion or alternatively they could be conducted in vivo. Also, since CB has been reported to cause choleostasis (Phillips et al., 1977), the possible role of microfilaments in the secretion of lysosomal contents would have to be assessed in a different, perhaps less specific, way. That could possibly be done by using the Ca^{2+} ionophore A23187 which has been shown to increase the release of lysosomal enzymes from leucocytes (Zabucchi and Romeo, 1976). The results thus obtained would provide evidence for the possible involvement of a Ca^{2+} dependent contractile system in the release of lysosomal contents by the liver.

Furthermore, Kupffer cell cultures set up from non-loaded livers and livers loaded with ^{125}I-PVP, ^{125}I-Triton WR-1339 and ^{3}H-dextran, could be used in order to investigate the release of lysosomal contents from this cell type and the involvement of microfilaments in the control of the process.

In the present study the exocytosis of lysosomal contents from hepatocytes in short-term cultures has been investigated only in the case of non-loaded and ^{125}I-PVP-loaded cells. Similar studies, using ^{125}I-Triton WR-1339 and ^{3}H-dextran loaded hepatocytes, would make it possible to compare the effect that the intralysosomal accumulation of different macromolecules has on the release of the lysosomal contents from isolated hepatocytes and also to examine how this effect relates to the results obtained from isolated perfused livers.

Since freshly-isolated hepatocytes in short-term cultures appear to have a disorganised cell membrane and microfilamentous network, (Wisher and Evans, 1977; de Brabander et al., 1978), leading to increased base line release of lysosomal contents, it would be interesting to investigate the release of lysosomal contents from hepatocytes that had been kept in culture for longer periods and therefore had time to recover from the effects of the isolation procedure. However, the value of these studies would be limited by the fact that only a small proportion of the originally isolated hepatocytes would survive and be able to be maintained in longer-term culture (Williams et al., 1977).

In order to investigate further the effect of the accumulation of indigestible material on the release of the lysosomal contents and also, in order to obtain an *in vitro* system more similar to the situation occuring in lysosomal storage diseases, the effect of the intralysosomal storage of more physiological materials should be investigated. The storage of physiologically occuring macromolecules which would normally be digested, could be achieved either by overloading the lysosomes or by inhibiting the lysosomal enzymes. For example, since mucopolysaccharides can lead to the inhibition of lysosomal enzymes (Avila and Convit, 1975), it is suggested that mucopolysaccharidoses could be experimentally induced by the administration of mucopolysaccharides in doses higher than those that the lysosomes could cope with. Alternatively, the storage of mucopolysaccharides as well as of a variety of other materials could be induced by the administration of chloroquine, a lysosomotropic agent which is known to inhibit lysosomal enzymes, possibly as a result of the elevation of the intralysosomal pH (Ohkuma and Poole, 1978).

It has been suggested earlier in this study (see 3.5.1) that the glycoproteins present in the lysosomal membrane may play a role in determining which lysosomal subpopulations fuse with the cell membrane. In order to investigate the above hypothesis, the distribution of the glycoproteins among the different lysosomal subpopulations, as well as their presence in the plasma membrane, before and after exocytosis has occured, could be examined by immunofluorescent studies using antibodies raised against them.

Morphological studies of non-loaded and loaded, perfused and non-perfused livers should be carried out in order to investigate changes in the intracellular topography of lysosomes due to loading and to perfusion and the relation of the intracellular distribution of lysosomes to their exocytosis.

In order to further test the hypothesis that small dense lysosomes from ^{3}H-dextran loaded livers are released via a mechanism involving microfilaments, it is suggested that isopycnic gradient centrifugation of ^{3}H-dextran-loaded livers that had been perfused in the presence and the absence of CB are carried out. Furthermore since the addition of CB to the perfusate of a ^{3}H-dextran-loaded liver significantly increased the release of arylsulphatase, which had been shown to be associated with dense lysosomes, it would be interesting to carry out similar studies using materials, other than ^{3}H-dextran, the intralysosomal accumulation of which would increase the density of lysosomes.

Also in order to further investigate to what extent the differences in the patterns of the release of the lysosomal enzymes observed in the present study were due to their being associated with the lysosomal membrane to different extents, the release of more lysosomal enzymes should be examined in a series of studies similar to the present ones, and compared with their distribution between the pellet and the supernatant fractions obtained from the high-speed centrifugation of a lysed mitochondrial-lysosomal preparation.

Finally, since the disruption of microtubules has been shown to inhibit exocytosis in other systems (for a review see Allison, 1973) and in order to obtain a better idea of the involvement of the cytoskeleton in the phenomenon, in the case of rat liver, it is proposed that the effect of a microtubule poison such as colchicine is examined both in the presence and the absence of CB.

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