ACTIVE ENDOCYTOSIS

OF FIBROBLAST β-GLUCURONIDASE BY CULTURED CELLS

by

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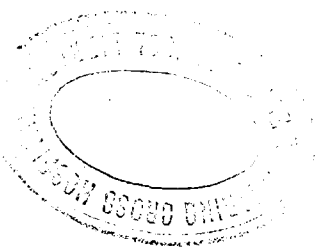

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ABSTRACT

Mouse 3T3 cells were chosen as donors of β-glucuronidase because they secreted more enzyme (25 units per 10^6 cells per day) than any of the other types of fibroblast tested. Medium was collected from log phase fibroblasts cultured in monolayers or on microcarriers and β-glucuronidase precipitated from it in 45% saturated ammonium sulphate. Further purification was achieved by affinity chromatography on Concanavalin A-Sepharose followed by ion-exchange chromatography on DEAE-Sephacel. This resulted in a 250 fold purification with a 39% yield of activity. Further purification of β-glucuronidase by affinity chromatography on immobilised substrates was unsuccessful because of non-specific ion-exchange and hydrophobic binding effects, which interfered with the specific binding of the enzyme to the ligands.

Peritoneal macrophages from AKR mice were used as recipient cells in uptake experiments because they have low endogenous levels of β-glucuronidase which is heat labile. Human fibroblasts (cell line GM151) which were totally deficient in β-glucuronidase were used as additional recipient cells in these experiments. Uptake of the β-glucuronidase secreted by 3T3 fibroblasts into both mouse peritoneal macrophages and human fibroblasts was rapid and saturable, with maximum rates of 14% per hour per mg cell protein (macrophages) and 9.6% per hour per mg cell protein (fibroblasts). The rates of uptake of human platelet and placental β-glucuronidase were less rapid than uptake of β-glucuronidase from 3T3 fibroblasts, which in turn was 140 and 800 times more rapid than fluid phase pinocytosis of horseradish peroxidase into macrophages and fibroblasts.

Mannose 6-phosphate at a concentration of 5mM inhibited uptake of β-glucuronidase from 3T3 fibroblasts into both fibroblasts and macrophages, but fructose 1-phosphate at the same concentration inhibited uptake only into fibroblasts and glucose 6-phosphate had no effect. At higher concentrations, mannose containing molecules, for example mannose (50mM), α-methylmannoside (50mM), and yeast mannan (500μg/ml), inhibited

uptake of 3T3 β-glucuronidase into macrophages but did not inhibit uptake into fibroblasts. Glucose and fructose did not inhibit uptake into macrophages or fibroblasts, whereas N-acetylglucosamine was marginally effective against uptake into both types of cell. Pre-treatment of β-glucuronidase with alkaline phosphatase abolished uptake into fibroblasts but had no effect on uptake into macrophages.

These results show that mouse 3T3 fibroblasts secrete the lysosomal enzyme β-glucuronidase in a form that is recognised as a high uptake ligand not only by the phosphomannosyl receptor present on fibroblasts but also by the mannose/N-acetylglucosamine receptor on macrophages.

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CHAPTER 1

INTRODUCTION

Since the uptake of asialoglycoproteins into liver was first demonstrated by Morell et al. (1968), and endocytosis of lysosomal enzymes into fibroblasts was reported by Hickman and Neufeld (1972), many researchers have published the results of their work on receptor mediated endocytosis of glycoproteins into a variety of cells. Five different types of cell surface receptor for glycoproteins have now been identified, and uptake of molecules isolated from many different cells and tissues has been demonstrated. One of the practical applications for these uptake pathways is the targeting of lysosomal enzymes, many of which are glycoproteins, to the appropriate tissues and thence to the lysosomes of patients with enzyme deficiency diseases. Most of these in vitro experiments which demonstrated that cells could take up lysosomal enzymes by receptor mediated endocytosis, used intracellular enzymes extracted from homogenised organs. Hickman and Neufeld (1972) showed that fibroblasts secreted lysosomal enzymes which were taken up by receptor mediated endocytosis into other fibroblasts, but it is not known whether lysosomal enzymes secreted by one type of cell can be taken up by cells of another type. This knowledge is of crucial importance when enzymes given to patients with lysosomal storage diseases are secreted by donor cells of a different type to the recipient cells in which undegraded material is stored; cells of the reticuloendothelial system, for example.

The main aim of this thesis was to demonstrate that exchange of lysosomal enzymes between different types of cell could take place, and to show that fibroblasts secreted a lysosomal enzyme which could be taken up into cells of the reticuloendothelial system by receptor mediated endocytosis.

1.1 CLINICAL ASPECTS OF ENZYME REPLACEMENT THERAPY IN PATIENTS WITH DEFICIENCIES OF LYSOSOMAL ENZYMES

Among the many inherited disorders of cellular metabolism, much attention has been given to those diseases which result in the storage of partially degraded macromolecules within the lysosomes. Lysosomes are the primary sites for intracellular degradation of molecules with high molecular weights, and contain approximately sixty different enzymes which work in concert to degrade very large molecules to their constituent monosaccharides and amino acids (for a review see Barrett and Heath, 1977). When one of these enzymes is genetically absent or functionally defective, undegraded residues of macromolecules are stored inside the lysosomes (Stanbury et al., 1978), and prolonged storage of these products eventually leads to cellular malfunction followed by progressive deterioration of the tissues and organs primarily responsible for the degradative processes.

The extent of the damage depends largely on the type of stored product: for example, patients with GM1- and GM2-gangliosidoses store gangliosides which are constituents of nerve cell membranes, and severe nervous disorders are major symptoms of the diseases. Patients with mucopolysaccharidoses, on the other hand, store glycosaminoglycans, which are synthesised primarily by connective tissue cells, and consequently this group of patients have severe bone and joint deformities (McKusick, 1978).

1.1.1 The Mucopolysaccharidoses

A large number of lysosomal storage diseases have been classified

on the basis of the type of macromolecule stored within them (Brown, 1957; Matalon and Dorfman, 1969). The mucopolysaccharidoses are one such group of related enzyme deficiency diseases. The first of these diseases to be described was reported at the beginning of this century, when John Thompson studied three patients with Hurler disease in Edinburgh (1900-1913). In 1917 Hunter syndrome was described, followed by Morquio A and B, Sanfilippo syndrome (of which four sub-types are now known: Kresse et al., 1980) and Maroteaux-Lamy syndrome. Scheie syndrome was also characterised as a varient of Hurler syndrome (Weismann and Neufeld, 1970). More recently, Sly and colleagues described a case of β-glucuronidase deficiency which also belongs to this group of disorders (Sly et al., 1973), and since then other cases of this deficiency have been identified (Pfeiffer et al., 1977). The mucopolysaccharidoses as a group occur at a rate of 1 in 20,000 live births (Gibbs et al., 1980) and few sufferers from these diseases live beyond their second decade.

The biochemical manifestations of mucopolysaccharidoses gave these diseases their collective name. Excessive glycosaminoglycan storage and excretion of storage products in urine result from these enzyme deficiencies. It has been only in the last decade or so, however, that the underlying reason for excessive storage of glycosaminoglycans has become apparent. Fratantoni et al. (1968) first demonstrated that fibroblasts from a patient with Hurler syndrome did not degrade glycosaminoglycans as rapidly as did normal fibroblasts. They next reported that abnormal glycosaminoglycan catabolism in fibroblasts from patients with Hunter syndrome could be corrected when they were co-cultured with fibroblasts from normal donors or with fibroblasts from patients with other types of mucopolysaccharidosis (Fratantoni et al.,

1969). This correction was later shown to be due to soluble factors secreted by the cells, cell to cell contact not being required for cross-correction. Similar cross-correction was subsequently demonstrated between normal fibroblasts and those obtained from patients with Sandhoff disease (Reuser et al., 1976; Halley, 1980), and between fibroblasts from four patients suffering from Sanfilippo syndrome (Kresse et al., 1971) showing that each type of patient had a different inherited genetic defect.

The corrective factor for Sanfilippo A disease was the first to be purified from normal human urine, and was identified as the enzyme heparan sulphate sulphatase (Kresse and Neufeld, 1972); since then a number of other corrective factors have also been identified as lysosomal enzymes (Bach et al., 1972; 1973; O'Brien, 1972; Von Figura and Kresse, 1972; Fluharty et al., 1974; Gniot-Szulzycka and Donnelly, 1976). For a summary of the enzymes which are now known to be deficient in the mucopolysaccharidoses group of diseases see Table 1.

Replacement of the deficient enzymes therefore appeared to be the most effective method of treatment for patients with these diseases, rendering it necessary to determine whether missing lysosomal enzymes could enter the deficient cells, and induce breakdown of the glycosaminoglycans within the lysosomes. In vitro experiments have shown that lysosomal enzymes can be taken up by active endocytosis into the lysosomes of deficient fibroblasts (O'Brien et al., 1973; Hall et al., 1973; Von Figura and Kresse, 1974) and that these 'corrected' cells are then able to catabolise their stored glycosaminoglycans. The corrective activity of each enzyme was assessed by measuring its ability to reduce the incorporation of $[^{35}S]$-SO_4 into glycosaminoglycans of deficient cells (Hall et al., 1973), and it was found that only small quantities

Table 1

MUCOPOLYSACCHARIDE STORAGE DISEASES

Mucopolysaccharidosis			Deficient Enzyme	Storage Product
MPS IH	Hurler syndrome		α-iduronidase	Dermatan sulphate Heparan sulphate
MPS IS	Scheie syndrome		α-iduronidase	Dermatan sulphate Heparan sulphate
MPS II	Hunter syndrome (severe & mild)		Iduronate sulphatase	Dermatan sulphate Heparan sulphate
MPS III	Sanfilippo syndrome	A	Heparan N-sulphatase	Heparan sulphate
		B	N-acetyl-α-glucosaminidase	Heparan sulphate
		C	Acetyl-CoA: α-glucosaminidase N-acetyltransferase	Heparan sulphate
		D	N-acetylglucosamine 6-sulphate sulphatase	Heparan sulphate
MPS IV	Morquio syndrome	A	N-acetylgalactosamine 6-sulphate sulphatase	Keratan sulphate Chondroitin sulphate
		B	β-D-galactosidase	Keratan sulphate Chondroitin sulphate
MPS VI	Maroteaux-Lamy		N-acetylgalactosamine 4-sulphatase (Aryl sulphatase B)	Dermatan sulphate
MPS VII	β-glucuronidase deficiency		β-glucuronidase	Chondroitin sulphate Dermatan sulphate

of enzyme were sufficient to induce near normal catabolic activity (Bach et al., 1972; O'Brien et al., 1973; Hall et al., 1973). Lysosomal enzymes from a number of sources have since been shown to correct deficient human fibroblasts in culture (Brot et al., 1974). In addition, in many of these lysosomal deficiency diseases, experiments showed that an enzymically inactive gene product was synthesised which was immunologically cross reactive with antisera to the deficient enzyme (Neuwelt et al., 1971; Mersmann and Buddecke, 1977; Bell et al.; 1977; Von Figura and Voss, 1979). These findings made enzyme replacement therapy a promising method for the treatment of storage diseases, since normal enzymes could be taken up by deficient cells when introduced into patients, and were unlikely to provoke immunological reactions when in the circulation.

1.1.2 Methods of Enzyme Replacement Therapy

Many different methods have been employed to introduce normal enzymes into deficient cells in vivo (Gregoriadis and Dean, 1979: review). Infusion of fresh plasma was the earliest method attempted, and some limited benefits were reported in patients with Fabry's disease (a deficiency of ceramide trihexosidase: Mapes et al., 1970), in patients with Hunter and Hurler syndromes (Di Ferrante et al., 1971) and Sanfilippo disease (Dean et al., 1973). In each case, however, the beneficial effects of these transfusions lasted for less than one month and required repeated administration to produce sustained improvements. Similar, but more marked effects were observed after infusion of leucocytes into a child with Hunter syndrome (Knudson et al., 1971), and into a patient with Fabry syndrome (Brady et al., 1974) (for a direct comparison of treatment of mucopolysaccharidoses by plasma infusion or

by leucocyte transfusion see Nishioka et al., 1979). Later when it had become apparent that each type of storage disease was the consequence of a deficiency in a single lysosomal enzyme, administration of the purified enzymes themselves was attempted. For example, purified human placental ceramide trihexosidase was infused into patients with Fabry's disease (Brady et al., 1973) and glucocerebrosidase from the same source infused into patients suffering from Gaucher's disease (Brady et al., 1974; Beutler et al., 1977); in each case similar results were obtained to those produced by leucocyte infusions.

Unfortunately, infusions of plasma, leucocytes or purified enzymes all had only transient therapeutic effects because of the short half lives of the infused enzymes. For example, β-glucuronidase extracted from human platelets had a half-life after uptake into human deficient fibroblasts of fourteen days (Bach and Liebmann-Eisenberg, 1979), whereas bovine liver β-glucuronidase had a half-life of 3.5 days in these fibroblasts (Hickman and Neufeld, 1972), and human liver β-glucuronidase, a half-life of approximately 21 days (Lagunoff et al., 1973).

In view of this limitation, several other methods have been devised in order to increase the half-life of enzymes in vivo. Encapsulation of β-glucuronidase, β-galactosidase (Ihler et al., 1973) and aryl sulphatase (Fiddler and Desnick, 1976a) in erythrocyte ghosts was suggested as a possible method of delivery of enzyme to liver and spleen cells, although trials have only been carried out in mice; microencapsulation of catalase in polymeric vesicles also appeared to have beneficial results in acatalasaemic mice (Chang and Poznansky, 1968). Liposomes have been used as vehicles for delivery of hexosaminidase A to deficient

leucocytes *in vitro* (Cohen *et al.*, 1976), and invertase has been targeted to the lysosomes of livers of infused rats by this method (Gregoriadis and Ryman, 1972). Infusions of enzymes entrapped in liposomes have not, however, proved beneficial for treatment of lysosomal storage diseases because of the very painful side effects induced (Belchetz *et al.*, 1977; Ryman and Tyrrell, 1980: review).

Chemical modification of lysosomal enzymes in order to extend their half-lives has also been attempted: after cross-linking α-galactosidase with hexamethylene diisocyanate, Snyder *et al.*, (1974) demonstrated that the enzyme had become protease resistant *in vitro*, but the *in vivo* effects of infusing such modified enzymes have not been reported, and the infusion of indestructible lysosomal enzymes might result in storage of lysosomal enzymes themselves, a possibility which has not yet been investigated.

A more radical approach to enzyme replacement therapy was to transplant organs or tissues taken from normal donors into enzyme deficient patients. It was hoped that transplanted tissues would continue to function and provide a continuous supply of normal lysosomal enzymes thus obviating the need for the repeated treatments which were required when plasma or infused enzymes were administered. Spleen (Groth *et al.*, 1971) and kidney (Desnick, R. *et al.*, 1973) transplants have been used to treat patients with Gaucher's disease, but although a slight increase in β-glucosidase activity was detected in the patient who received the kidney transplant, both patients died within a few months. Kidney transplants have been also used to treat patients with Fabry's disease (Desnick, S.J. *et al.*, 1973), and here significant increases in plasma and urinary trihexosylceramide (the deficient

enzyme) sustained over a period of months were detected. The main disadvantage with this type of therapy is that intricate surgery is required and there is a distinct risk of organ rejection if donor and recipient are not fully HLA compatible.

More recently attention has focussed on the nature of the mucopolysaccharidoses at the level of cellular malfunction, and in view of the evidence that skin fibroblasts have the ability to induce cross-correction in vitro (Fratantoni et al., 1969), Dean and coworkers (1975) grafted full thickness skin onto the forearm of a four year old patient with Hunter syndrome. Despite the administration of immunosuppressant drugs, the graft was rejected because each parental donor had only two compatible HLA antigens. During the period over which this patient was monitored, an increase in urinary oligosaccharides of low molecular weight was observed, together with a reduction to normal levels of the sulphate/hexuronic acid molar ratio. These changes persisted, however, for nine months after grafting, long after the apparent rejection of the grafted tissue. There was also an increase in the output of urinary α-L-iduronate sulphate sulphatase (measured as fibroblast corrective factor) which suggested that some of the fibroblasts present in the graft had survived and had continued to supply the deficient enzyme. The success and relative convenience of this in vivo technique compared to that of organ grafts led to the use of transplanted fibroblasts themselves as a modification of this type of treatment. Similar increases in urinary excretion of corrective factors and low molecular weight oligosaccharides have since been demonstrated in patients with Hunter syndrome (Dean et al., 1976), Hurler syndrome (Gibbs et al., 1977; Gibbs et al., 1978) and Sanfilippo A syndrome (Dean et al., 1980) who received transplants of normal fibroblasts. More recently, a bone

marrow transplant in a patient with Hurler disease led to considerable clinical and biochemical benefits (Hobbs et al., 1981b) indicating that cell transplants of this type have a good deal of promise as a long lasting form of enzyme replacement therapy.

With both fibroblast and bone marrow transplants, there are a number of questions concerning the ability of cells to survive and function in vivo which have yet to be answered. It is one of these questions, namely whether transplanted fibroblasts are able to transfer enzymes to other types of deficient recipient cells in patients, which is the main concern of this thesis. The long lasting biochemical changes observed in patients who have received fibroblast transplants suggested that these fibroblasts continued to secrete lysosomal enzymes into the circulation, although consistent clinical improvements were not observed. The reasons for this lack of clinical benefit are not yet clear, but it is not known, for example, whether the transplanted fibroblasts divide, or if they migrate from the site of transplantation. The mechanism of correction has yet to be investigated in order to establish whether transfer of enzymes between donor and host cells takes place or whether recipient cells are stimulated to synthesise their own active enzyme. The precise type and location of the recipient cells responsible for breakdown of stored glycosaminoglycans is as yet also unknown, although cells of the reticuloendothelial system have been suggested as the main target for lysosomal enzymes by Stahl et al. (1976b) following their infusion studies in rats. Treatment of patients with mucopolysaccharidoses or other storage diseases is still experimental and has been only used in advanced cases in which patients already showed symptoms of mental retardation. As a result, positive evidence for improvement of mental faculties after transplant has not

yet been produced. This may be a consequence of the inability of corrective factors to cross the blood-brain barrier, or of failure of the cells in damaged nervous tissue to regenerate. In addition, competition for uptake of donor enzyme by other organs such as the liver may reduce the amount available to cells of the central nervous system. The amount of enzyme supplied by transplanted fibroblasts has not been determined, and consequently the optimum number of fibroblasts required for complete correction of the symptoms of these diseases *in vivo* is also unknown.

In order, therefore, to improve the techniques used for enzyme replacement therapy with transplanted donor cells, it is necessary to understand more fully the mechanism by which cells take up lysosomal enzymes. Among the main parameters which are examined in this thesis are, firstly, whether cells like fibroblasts can secrete lysosomal enzymes bearing recognition markers for uptake into different types of cell; and secondly, whether the uptake markers on secreted lysosomal enzymes can be recognised by different types of cell surface receptor.

1.2 RECEPTOR MEDIATED ENDOCYTOSIS OF LYSOSOMAL ENZYMES INTO CELLS

Five different receptors for glycoproteins have now been identified which are present on the surfaces of cells (for a review see Neufeld and Ashwell, 1980): galactose and fucose receptors are found on mammalian hepatocytes, an N-acetylglucosamine receptor on avian hepatocytes, a mannose/N-acetylglucosamine receptor on reticuloendothelial cells and a mannose 6-phosphate receptor on fibroblasts. Lysosomal enzymes are almost all glycoproteins (Barrett, 1969; Tulsiani et al., 1978; Bainton, 1981) and so studies on the uptake of neoglycoproteins and modified plasma glycoproteins into cells provide additional information about the pathways by which lysosomal enzymes may enter cells, in addition to studies on the enzymes themselves.

The galactose receptor was the first to be identified and it was the work of Ashwell and coworkers which first indicated that plasma glycoproteins could be cleared from the circulation by receptor mediated endocytosis into cells. In rabbits it was observed that intravenously infused asialoceruloplasmin was rapidly cleared from the circulation by the parenchymal cells of the liver (Morell et al., 1968). This glycoprotein was taken up specifically into hepatocytes, and the exposed galactose residues on the molecule were rapidly cleaved in the lysosomes of the hepatocytes (Gregoriadis et al., 1970). These results were used by Morell et al., (1971) as evidence for their proposal that a general route operated for uptake of denatured glycoproteins into hepatocytes, where these molecules would then be broken down within the lysosomes. The survival of glycoproteins in the plasma depended on the presence of terminal sialic acid residues present on their oligosaccharide chains.

Neuraminidase treatment of a range of glycoproteins in order to remove sialic acid groups resulted in a rapid, saturable clearance of these molecules from the plasma into hepatocytes. This pathway has since been shown to operate for the clearance of rabbit antihapten antibody in infused rabbits (Windelhake and Nicolson, 1976), which increased from 0.05%/hr to 0.2%/hr after neuraminidase treatment. Uptake of lysosomal enzymes via galactose receptors has, however, only recently been reported by Ullrich and Von Figura (1982).

Galactose receptors have been isolated from rabbit liver by affinity chromatography on columns of asialoorosomucoid-Sepharose (Hudgin et al., 1974). These receptors are transmembrane proteins (Harford and Ashwell, 1981), have a requirement for calcium ions (Pricer and Ashwell, 1976) and cannot distinguish between galactose- or glucose-bearing glycoproteins (Stowell and Lee, 1978; Schlesinger et al., 1980) although some differential uptake of galactosyl- and glucosyl-bovine serum albumin into hepatocytes has been observed by Kawaguchi et al., (1981). Receptors which recognise fucose residues were identified on hepatocytes when human lactoferrin was infused into rats (Prieels et al., 1978). Similar infusion studies have shown that avian hepatocytes have receptors which recognise N-acetylglucosamine residues, but have no galactose receptors (Lunney and Ashwell, 1974).

Receptors which recognise mannose or N-acetylglucosamine residues have been identified by many researchers as being present on the surfaces of non-parenchymal cells in the liver. Agalactoglycoproteins, which have terminal N-acetylglucosamine residues, were cleared from the circulation of infused rats by this pathway (Stockert et al., 1976; Stahl et al., 1976b). β-Glucuronidase and N-acetylglucosaminidase

prepared from liver lysosomes were also cleared by this pathway and competed with each other for uptake by this receptor (Stahl et al., 1976a) while the enzyme ribonuclease B, whose side chains contain mannose, was rapidly taken up by the livers of infused rats (Baynes and Wold, 1976). Asialoglycoproteins, however, did not inhibit the uptake of β-glucuronidase into the livers of infused rats (Stahl et al., 1975), suggesting that the receptors which recognised galactose-containing glycoproteins were specific for them, and distinct from those receptors which recognised N-acetylglucosamine- or mannose-containing glycoproteins. Receptors which recognise mannose/N-acetylglucosamine residues have been purified from rabbit liver by affinity chromatography (Kawasaki et al., 1978) from rat liver (Townsend and Stahl, 1981) and also from rabbit serum (Kozutsumi et al., 1980). Like the galactose receptor, these receptors require calcium for binding, and mannose-containing glycoproteins compete with those bearing N-acetylglucosamine residues for binding to the receptor.

Mannose/N-acetylglucosamine receptors have also been identified on the plasma membrane of rat alveolar macrophages (Achord et al., 1977a; Stahl et al., 1978; Warr, 1980; Stahl et al., 1980) and on mouse peritoneal macrophages (Ezekowitz et al., 1981). The receptors present on alveolar macrophages were similar to those found in the liver in their ability to recognise mannose bearing glycoproteins, such as human placental β-glucuronidase (Achord et al., 1978; Schlesinger et al., 1980), and in their affinity for α1-2 linked mannose residues (Achord et al., 1977b; Stahl et al., 1978). The rapidity of uptake of human placental β-glucuronidase into alveolar macrophages has been correlated with its avidity of binding to Concanavalin A-Sepharose (Achord et al., 1977a) thus providing additional evidence that mannose residues are

required for recognition of glycoproteins by this receptor. From these results it was concluded that the mannose/N-acetylglucosamine receptor provided a common pathway for uptake of glycoproteins, including many lysosomal enzymes, into fixed tissue macrophages in vivo, that is, cells of the reticuloendothelial system.

Mannose phosphate receptors have been identified on fibroblasts (Kaplan et al., 1977), on chondrocytes (Rome and Miller, 1980) and also on hepatocytes (Ullrich et al., 1978, 1979a, b; Sahagian et al., 1981). Most of the evidence for the existence of phosphomannosyl receptors has come from the study of endocytosis of lysosomal enzyme by human deficient fibroblasts in culture. Hickman and Neufeld (1972) observed that normal human fibroblasts secreted lysosomal enzymes that could be taken up by other fibroblasts; fibroblasts from patients with I-cell disease (Mucolipidosis II) secreted very large amounts of a number of lysosomal enzymes, and these secreted enzymes were not taken up by other fibroblasts. Enzymes secreted by normal fibroblasts were, however, taken up by I-cell fibroblasts (Wiesmann et al., 1971). From these experiments, Hickman and Neufeld formed an hypothesis which stated that secretion of lysosomal enzymes by fibroblasts was obligatory before they were recaptured and packaged internally into lysosomes. This explanation presupposed that a 'recognition marker' existed on normal enzymes and that this marker ensured they were recaptured safely and internalised. Enzymes secreted by I-cells were assumed to have defective recognition markers. Further evidence for this hypothesis was obtained when goat-anti-human α-L-iduronidase antibody was added to normal human fibroblasts and was shown to deplete the activity of intracellular α-L-iduronidase (Neufeld et al., 1977).

Since the 'secretion-recapture' hypothesis was first published, many researchers have challenged the concept that secretion of lysosomal enzymes is an obligatory step before these enzymes can be packaged (Lloyd, 1977; Von Figura and Weber, 1978; Fischer *et al.*, 1980), but receptor mediated endocytosis of lysosomal enzymes by fibroblasts in culture has been well characterised. Some of the earliest kinetic studies on uptake of lysosomal enzymes into deficient fibroblasts were published by Von Figura and Kresse (1974), who showed that the rate of uptake of urinary N-acetyl-α-D-glucosaminidase into fibroblasts from a patient with Sanfilippo B syndrome increased with increasing enzyme concentration until it reached a steady state, thus implicating a receptor mediated step in endocytosis. These results supplemented those obtained earlier by Lagunoff et al. (1973) who had shown that the β-glucuronidase isolated from human liver was taken up ten times more rapidly than the comparable rate for non-specific pinocytosis.

In 1976 Hieber *et al.* examined the uptake of bovine testicular β-galactosidase *in vitro* into skin fibroblasts taken from patients with generalised gangliosidosis and the effect of a range of inhibitors on the rate of uptake of this enzyme. They concluded that mannosyl residues were an essential part of the recognition marker necessary for assimilation of β-galactosidase. Pretreatment of the enzyme with α-mannosidase inhibited its uptake, as did competition with methyl α- and methyl β-mannopyranosides and mannose containing testicular glycoproteins. Further experiments by Kaplan *et al.* (1977) suggested that these mannosyl recognition markers were phosphorylated, and that the receptors present on fibroblast cell surfaces recognised mannose 6-phosphate residues. D-Mannose inhibited uptake of platelet β-glucuronidase by deficient fibroblasts *in vitro*, but the addition of

a phosphate group at the 6-position improved its inhibitory potency, with mannose 1-phosphate being much less effective as an inhibitor. Pretreatment of the β-glucuronidase with alkaline phosphatase reduced its rate of uptake into fibroblasts, and phosphorylated yeast mannans were very potent inhibitors of receptor mediated endocytosis of this enzyme. A similar set of results were subsequently obtained for the uptake of bovine testicular β-galactosidase (Distler et al., 1979; Hieber et al., 1980) and α-L-iduronidase (Sando and Neufeld, 1977) into fibroblasts deficient in these respective enzymes.

1.3 PROCESSING OF LYSOSOMAL ENZYMES

1.3.1 The Concept of High and Low Uptake Forms of Lysosomal Enzymes

Brot and co-workers (1974) made the first important discovery that β-glucuronidase from different sources had widely varying rates of uptake into deficient human fibroblasts *in vitro*. Human platelet enzyme was taken up more rapidly than β-glucuronidases prepared from liver, placenta or urine. Polyacrylamide gel electrophoresis resolved platelet β-glucuronidase into three bands, suggesting that more than one population of enzyme molecules might be present with similar catalytic activities but different electrophoretic mobilities, and that receptors for uptake of β-glucuronidase into fibroblasts could distinguish between these different forms. Similar results were obtained for uptake of N-acetylglucosaminidase from fibroblasts, placenta or urine into fibroblasts from a patient with Sandhoff disease (Hickman *et al.*, 1974).

The heterogeneity in the rate of uptake of different preparations of β-glucuronidase into fibroblasts was investigated by Nicol *et al.* (1974) who separated two types of spleen β-glucuronidase by chromatography on DEAE-Cellulose, and correlated the high uptake properties of the more acidic fraction with the slower migrating band of activity separated by electrophoresis on polyacrylamide gel. This fraction was taken up as rapidly as human platelet β-glucuronidase (Brot *et al.*, 1974). The low uptake fraction of spleen β-glucuronidase was less acidic, and more mobile during electrophoresis on polyacrylamide gel than the high uptake fraction, and corresponded in its uptake properties to liver β-glucuronidase (Brot *et al.*, 1974).

Antibody raised to the low uptake fraction of spleen enzyme did not cross-react with the high uptake fraction of spleen β-glucuronidase (Nicol et al., 1974) illustrating the structural difference that existed between these isoenzymes. The heterogeneity of β-glucuronidase purified from bovine liver has also been shown by chromatography on DEAE-Cellulose (Plapp and Cole, 1967), at least nine different isoenzymes being identified, which differed only in their carbohydrate content. These isoenzymes were later separated into lysosomal and microsomal fractions by isoelectric focussing (Owens et al., 1975), and once again the multiplicity of forms revealed was attributed to variations in post-translational addition of carbohydrate residues. The heterogeneity shown by β-glucuronidase isolated from liver was related to its uptake properties by Glaser et al. (1975) who showed that the most acidic fractions of enzyme separated by isoelectric focussing were those most readily taken up by fibroblasts, and that human platelet β-glucuronidase had a greater proportion of the more acidic fractions than enzyme isolated from other sources. The variation in acidity of different isoenzymes was partially related to their degree of phosphorylation (Kaplan et al., 1977). Intracellular enzymes extracted from different sources have different populations of molecules each showing a degree of variation in their amounts of phosphorylated carbohydrate side chains. Enzymes from different organs have greater or lesser quantities of these phosphorylated chains, and are thus recognised as high uptake forms by fibroblasts to different degrees. For example, the β-galactosidase isolated from bovine testis has 2% of its mannose residues phosphorylated and is recognised as a high uptake form for fibroblasts (Distler et al., 1979). Recent evidence has shown that fibroblasts from patients with I-cell disease

are unable to phosphorylate their lysosomal enzymes (Hasilik et al., 1981; Ben Yoseph et al., 1981; Reitman et al., 1981), explaining why enzymes secreted by them are not taken up by fibroblasts.

α-L-Iduronidase is another lysosomal enzyme for which isoenzymes with different uptake properties have been identified. Two forms of the enzyme with different molecular weights were isolated by affinity chromatography on heparin-Sepharose (Shapiro et al., 1976), but only the larger molecular weight from was recognised and taken up by fibroblasts from a patient with Hurler syndrome.

In vivo studies carried out in rats have confirmed the in vitro uptake experiments described by Brot et al. (1974) and Glaser et al. (1975). The β-glucuronidase prepared from rat preputial glands, or liver lysosomes, and the N-acetylglucosaminidase isolated from liver lysosomes were all cleared rapidly from the circulation of rats. Their rates of uptake showed saturation kinetics at high concentrations of infused enzyme, whereas the uptake of β-glucuronidase from rat serum or epididymal N-acetylglucosaminidase was less rapid and entirely concentration dependent (Stahl et al., 1976a). Similar experiments by Ullrich et al. (1978) showed that even low uptake forms of lysosomal enzymes entered fibroblasts by a pathway involving sugar phosphates and supported a previous suggestion made by Sando and Neufeld (1977) that low uptake forms were either contaminated with small amounts of high uptake enzyme, or were being taken up by phosphomannosyl receptors at a much slower rate than high uptake enzyme but at a faster rate than that of simple passive endocytosis.

The use of organ transplants for enzyme replacement therapy must therefore be examined critically in view of the above evidence showing

that some organs produce greater proportions of low uptake enzymes than others. Treatment of storage diseases would not be effective if the enzymes secreted by transplanted organs were not recognised by or taken up from the circulation into deficient cells.

1.3.2 Targeting of Lysosomal Enzymes to Specific Types of Cell

In order for replacement therapy to be effective, lysosomal enzymes must be taken up by cells in which undegraded macromolecules are stored; provision of normal lysosomal enzymes which have appropriate recognition markers is therefore of major importance. All *in vivo* evidence has suggested that liver is the major site in the body for uptake of glycoproteins including lysosomal enzymes (Stahl *et al.*, 1975, 1976a; Schlesinger, 1975, 1980; Stockert *et al.*, 1976; Baynes and Wold, 1976). Rabbit antihapten antibodies are also taken up from circulation into the liver after treatment of the molecules with neuraminidase (Windelhake and Nicolson, 1976). Asialoglycoproteins, however, enter the hepatocytes via galactose receptors, whereas most lysosomal enzymes enter the non-parenchymal cells via mannose/N-acetylglucosamine receptors. An exception to this rule is the uptake of urinary α-N-acetylglucosaminidase into isolated hepatocytes, which seems to occur via phosphomannosyl receptors (Ullrich *et al.*, 1978, 1979a, b). Lysosomal enzymes can also be taken up into fibroblasts via phosphomannosyl receptors (Kaplan *et al.*, 1977), but some enzymes, for example placental β-glucuronidase, are poorly phosphorylated and are consequently only taken up by cells with mannose receptors (Achord *et al.*, 1978).

When uptake of asialoglycoproteins into hepatocytes was reduced by pretreatment with β-galactosidase, these agalactoglycoproteins were

found to be targeted to non-parenchymal liver cells via their freshly exposed N-acetylglucosamine residues (Stockert et al., 1976; Stahl et al., 1976b). Treatment of agalacto-antibodies with N-acetyl-glucosaminidase resulted in their rapid clearance into the kidneys (Windelhake and Nicolson, 1976). This evidence suggests that only the terminal sugar or sugar phosphates present on lysosomal enzymes determine their fate in vivo. Contrary evidence has now, however, indicated that the recognition markers for uptake of lysosomal enzymes might be much more complex than was at first thought. When measuring the rate of uptake of agalactoorosomucoid into liver, Achord et al. (1977b) showed that α1-6 linked mannose residues were more potent inhibitors than α1-2 or α1-3 linked mannose residues. Similar results were obtained for inhibition of the uptake of glycoproteins into alveolar macrophages (Stahl et al., 1978). Baynes and Wold (1976) also suggested that mannose receptors recognised complex mannose configurations. They showed that non-glycosylated ribonuclease A had a long half life in circulation of 550 minutes in infused rats, but ribonuclease B which had oligosaccharide side chains of the form N-acetylglucosamine$_2$ mannose$_{4-5}$ had a half life of only 15 minutes. On the other hand, ribonucleases C and D, which had even more complex mannose configurations than ribonuclease B, had half lives similar to that measured for ribonuclease A. The way in which the mannose residues present on ribonuclease B were linked was shown later to be recognised specifically by the non-parenchymal cells of rat liver (Brown et al., 1978).

Fibroblast phosphomannosyl receptors also appear to recognise specific oligosaccharide configurations. Kaplan et al. (1977) showed that mannose 6-phosphate was a more potent inhibitor of β-glucuronidase

uptake into fibroblasts than mannose 1-phosphate, but the inhibition of uptake of enzyme by very low concentrations of phosphorylated yeast mannans showed that several mannose phosphate residues could be recognised by the receptors (Kaplan et al., 1977; Fischer et al., 1980c), suggesting that binding of enzymes to these receptors was a multivalent process. Evidence that the protein part of lysosomal enzymes might also influence the affinity of the oligosaccharide for these receptors has been presented firstly by Rome and Miller (1980) who modified the arginine residues on high uptake urinary α-L-iduronidase by pretreatment with butanedione and thereby reduced its uptake into fibroblasts, and secondly by Fischer et al. (1980c) who demonstrated that endocytosis of labelled polyphosphomonoesters by fibroblasts was more effectively inhibited by high uptake spleen β-glucuronidase than by the oligosaccharide side chains of this enzyme after they had been removed from the protein with endoglycosidase H.

Although chemical modifications of lysosomal enzymes which would specifically target them to one receptor rather than another have not yet been reported, the addition of mannose residues to albumin has rendered this protein a high uptake ligand for non-parenchymal liver cells (Schlesinger et al., 1980), alveolar macrophages (Stahl et al., 1978, 1980) and peritoneal macrophages (Ezekowitz et al., 1981). Moreover, the lectin ricin from Ricinis Communis was made specifically toxic to fibroblasts by coupling it to a monophosphopentamannose (Youle et al., 1979), suggesting that coupling of appropriate recognition markers might be a promising method for facilitating the endocytosis of low uptake forms of lysosomal enzymes.

1.3.3 Synthesis and Intracellular Modification of Lysosomal Enzymes

Most of the experiments described above have shown that artificial glycoproteins, or intracellular lysosomal enzymes isolated from homogenised organs, could be taken up into cells by receptor mediated endocytosis. Recent evidence has shown, however, that the lysosomal enzymes secreted by cells are processed differently from those sequestered within lysosomes (Gibson et al., 1980; Reitman et al., 1981). Secreted enzymes contain predominantly complex type oligosaccharide side chains which terminate in sialic acid residues, whereas lysosomal enzymes have high mannose side chains with terminal phosphomannosyl residues. This fact is of major importance because if cell or organ transplants are used for replacement therapy, only secreted enzymes would be available for uptake into deficient cells. The current evidence suggests that such secreted enzymes would be recognised by the galactose receptors present on hepatocytes if desialated, but not by mannose or phosphomannosyl receptors present on reticuloendothelial cells or by fibroblasts. Contrasting evidence has shown, however, that normal fibroblasts secrete enzymes which can be taken up in vitro by deficient fibroblasts (Neufeld et al., 1972; Reuser et al., 1976; Halley, 1980). In order to reconcile these conflicting results it is necessary to examine the information which is now available concerning intracellular processing and transport of glycoproteins.

When the 'secretion-recapture' hypothesis was first proposed (Hickman and Neufeld, 1972), it was thought that receptors which recognised glycoproteins were only situated on cell surfaces and might thus facilitate transport of extracellular glycoproteins only as far as the lysosomes. This hypothesis was, however, challenged when

Lloyd (1977) proposed that lysosomal enzymes were bound to lysosomal membranes throughout the process of membrane recycling and that leakage of these enzymes only occurred to a small extent during endocytosis. Von Figura and Weber (1978) also tested Neufeld's hypothesis and produced contradictory evidence which suggested that secretion of lysosomal enzymes by fibroblasts was a minor phenomenon, and that most of these enzymes were transported to the lysosomes without leaving the cells. They showed that an extracellular environment containing 0.5mM mannose 6-phosphate did not decrease the intracellular levels of β-N-acetylglucosaminidase in fibroblasts, even though this concentration was sufficient to inhibit endocytosis of externally added enzyme by 90%. Antibodies to the β-N-acetylglucosaminidase had no effect on intracellular enzyme levels, in direct contrast to the results reported previously by Neufeld et al. (1977).

The work of Fischer et al. (1980a) also provided evidence against the 'secretion-recapture' hypothesis. Following subcellular fractionation of rat liver and other organs and extraction of β-glucuronidase in detergent containing 10mM mannose 6-phosphate, these workers showed that all tissues contained phosphomannosyl receptors in their endoplasmic reticulum. They proposed that lysosomal hydrolases were transported from the GERL region of the Golgi apparatus to the lysosomes without leaving the cell, the enzymes being anchored to intracellular membrane receptors by their phosphomannosyl groups. The presence of intracellular receptors for other recognition markers has also been demonstrated: 70-80% of all mannose receptors were shown to be present within alveolar macrophages (Stahl et al., 1980) and similar percentages of galactose receptors were found inside hepatocytes (Pricer and Ashwell, 1976). These results again suggested that receptor

mediated endocytosis of lysosomal enzymes was not the primary function of glycoprotein receptors. It has been calculated, however, that fibroblasts always have a large number of cell surface receptor sites which are not occupied by bound ligands. Receptors are therefore always available to bind extracellular material having the correct recognition marker, and this might help to explain the results obtained by Hickman and Neufeld (1972).

The function of intracellular glycoprotein receptors appears to be to anchor glycoproteins to membranes throughout their post-translational processing within the endoplasmic reticulum and Golgi apparatus and during their transport to the plasma membrane or to the lysosomes. The precise conditions under which glycoproteins become bound to receptors or remain free is, however, not yet clear.

All proteins destined for glycosylation are synthesised with an additional 'signal peptide' of some sixteen amino acids at the amino terminus of the nascent protein (Lingappa et al., 1978). The signal peptide is necessary for insertion of the protein through the microsomal membrane, and is cleaved immediately after passage of the nascent polypeptide chain through the membrane and before protein synthesis is complete. This process is common to all secretory proteins as well as to transmembrane proteins, such as hepatocyte galactose receptors (Harford and Ashwell, 1981), which are never fully discharged into the lumen of the endoplasmic reticulum.

Some lysosomal enzymes are synthesised as precursor forms. Skudlarek and Swank (1979) showed, for example, that β-galactosidase from mouse macrophages was synthesised as monomers of 82,000 Daltons which were cleaved to chains of 63,000 Daltons. β-glucuronidase from

the same cells was synthesised as monomers of 75,000 Daltons which were clipped to a final form of 73,000 Daltons during synthesis (Skudlarek and Swank, 1981). In fibroblasts, β-hexosaminidase, α-glucosidase and Cathepsin D were shown by Hasilik and Neufeld (1980) to be synthesised in higher molecular weight forms than the mature enzymes, and these workers suggested that the final forms were only eventually attained in the lysosomes.

The addition of oligosaccharide recognition markers to proteins occurs during their transit through the endoplasmic reticulum and Glabe et al. (1980) calculated from their studies on the synthesis of ovalbumin that at least thirty amino acids must be translated as a peptide before glycosylation can begin. The oligosaccharide recognition markers are probably N-glycosidically linked to asparagine residues present on the protein core of lysosomal enzymes, since platelet and spleen β-glucuronidases are both susceptible to the action of endoglycosidase H, an enzyme which hydrolyses the chitobiose units linked to asparagine residues in glycoproteins (Natowicz et al., 1979; Fischer et al., 1980c; Von Figura and Klein, 1979). Glycosylation proceeds by transfer of oligosaccharide chains from the lipid carrier molecule dolichol pyrophosphate, to asparagine residues (Kornfeld and Kornfeld, 1980; Gibson et al., 1980; Filopovic and Von Figura, 1980). The transferred oligosaccharides have a common core structure: $(glucose)_3$ $(mannose)_9$ $(N\text{-acetylglucosamine})_2$ (Gibson et al., 1980). Tabas and Kornfeld (1980) reported that the oligosaccharide side chains synthesised by mouse lymphoma cells were modified during their passage through the Golgi; glucose residues being first removed by glycosidases and N-acetyl-glucosamine residues then being linked to mannose residues via phosphodiester bonds. Phosphodiester bonds have been identified in

β-hexosaminidase and Cathepsin D isolated from human fibroblasts (Hasilik et al., 1980) and mouse $P388D_1$ macrophages (Goldberg and Kornfeld, 1981). These terminal glucosamine phosphate residues rendered the oligosaccharides resistant to the action of both alkaline phosphatase and α-mannosidase (Tabas and Kornfeld, 1980). The transferase enzyme responsible for the addition of N-acetylglucosamine 1-phosphate to terminal mannose residues was shown to be present in the microsomes of rat liver, human placenta, and human fibroblasts by Hasilik and coworkers (1981). They also showed that this N-acetylglucosaminylphosphotransferase was deficient in fibroblasts cultured from patients with I-cell disease. Similar results were described by Reitman et al. (1981) who suggested that in normal cells the penultimate mannose residues on these oligosaccharide side chains were phosphorylated, and that between one and three phosphodiester bonds were added to each oligosaccharide chain. Mouse $P388D_1$ macrophages also synthesised β-glucuronidase with one or two phosphodiesters on each oligosaccharide (Goldberg and Kornfeld, 1981). The N-acetylglucosamine residues were subsequently removed by an α-N-acetylglucosaminylphosphodiesterase (identified in rat liver and human fibroblasts by Varki and Kornfeld, 1980), thereby exposing mannose 6-phosphate residues which might then bind to phosphomannosyl receptors (Fischer et al., 1980a). Enzyme glycoproteins could thus be transported from the GERL region of the Golgi to lysosomes, still bound to membranes via their phosphomannosyl receptors (Burnside and Schneider, 1980). Binding to lysosomal membranes has been shown, however, to be pH dependent (Fischer et al., 1980a) and β-glucuronidase, for example, dissociates from its receptors at a pH below 6.0 leaving the free receptors to recycle. The presence of these 'high mannose'

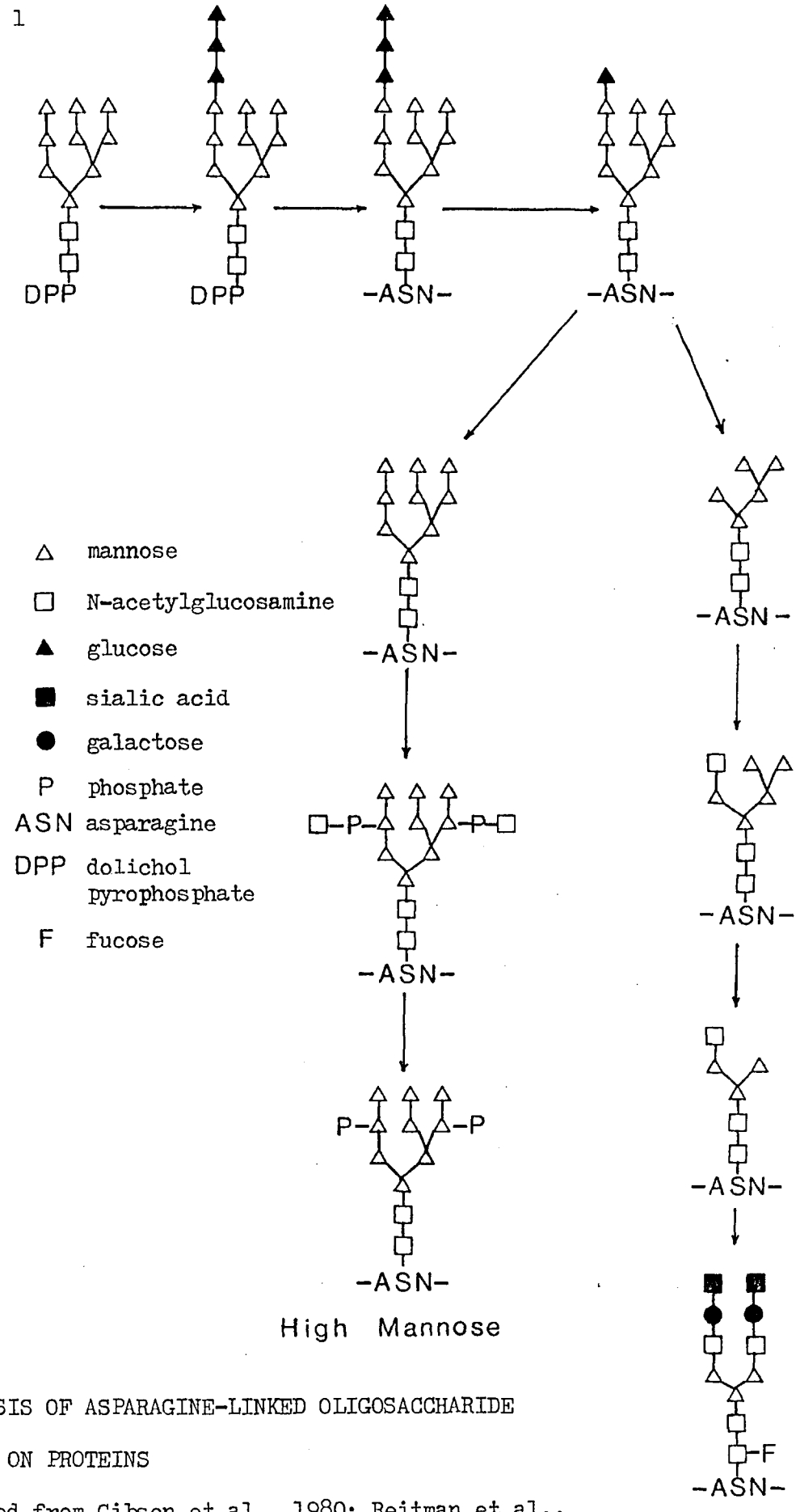
oligosaccharides on lysosomal enzymes can thus act as a signal to direct them to the lysosomes, secretion and recapture being unnecessary for correct lysosomal packaging (Sly et al., 1981).

An alternative glycosylation pathway can operate if N-acetylglucosaminylphosphotransferase does not add phosphate groups to high mannose chains. In such cases, Tabas and Kornfeld (1979) showed that mannose residues were removed by an α-mannosidase present in the Golgi region (Tulsiani et al., 1977), and further N-acetylglucosamine, galactose, fucose and sialic acid residues were then added (Gibson et al., 1980). Fractionation of Golgi membranes in sucrose density gradients has shown that glycosyltransferases are preferentially distributed in the trans region of the Golgi (Rothman, 1981), in a region corresponding to that which was termed the GERL region by Novikoff (1976) and which has been isolated from human fibroblasts by Rome et al. (1979).

Lysosomal enzymes which have been modified in the manner described above are termed 'complex', and enzymes bearing such complex oligosaccharide chains are normally secreted. Hasilik et al. (1981) have shown that the Cathepsin D and β-hexosaminidase secreted by fibroblasts contained predominantly complex type oligosaccharide side chains but that populations of these enzymes sequestered in the lysosomes contained side chains in which the high mannose type predominated. Fibroblasts from patients with I-cell disease secreted enzymes which carried complex oligosaccharide side chains (Miller et al., 1981) because they were deficient in the enzyme which added N-acetylglucosamine 1-phosphate to their mannose residues. The enzymes were therefore diverted into the secretion pathway after being processed to glycoproteins bearing oligosaccharides of the complex type.

The sequence of modifications of N-linked sugar side chains which takes place within the Golgi is summarised in Figure 1. The addition of N-acetylglucosamine 1-phosphate to the oligosaccharide core is the critical step which determines whether an enzyme will be targeted to the lysosomes or secreted. A variant form of pseudo-Hurler dystrophy (Mucolipidosis III) has been recently described by Varki et al. (1981) in which N-acetylglucosaminylphosphotransferase was active against artificial substrates but could not recognise endogenous lysosomal enzymes as suitable substrates. The lysosomal enzymes were thus not phosphorylated and consequently were secreted as in other classical types of Mucolipidosis.

Figure 1



SYNTHESIS OF ASPARAGINE-LINKED OLIGOSACCHARIDE CHAINS ON PROTEINS

(adapted from Gibson et al., 1980; Reitman et al., 1981)

1.4 INTRACELLULAR TRANSPORT OF LYSOSOMAL ENZYMES

The precise pathway through which lysosomal enzymes bound to receptors are transported depends on the type of membrane to which the receptors are attached, and this is true of both newly synthesised endogenous enzymes and those taken up from the extracellular space. Receptors and ligands are enclosed within specialised vesicles, some of which appear to be coated with clathrin, a non-glycosylated protein of 180,000 Daltons, and which have a diameter of 50-150mμ (Pearse and Bretscher, 1981). These coated vesicles are thought to be responsible for the transport of a large number of biological macromolecules. The G protein of vesicular stomatitis virus, for example, is transported from the endoplasmic reticulum to the Golgi region, and from the Golgi region to the plasma membrane in coated vesicles (Rothman and Fine, 1980). Clathrin coated vesicles have also been shown to transport thyroglobulin from the Golgi region to the plasma membrane of thyroid follicle cells (Herzog, 1981). Fluorescent anticlathrin antibodies have been used to determine the precise intracellular location of clathrin by Wehland *et al.* (1981) who showed that this protein was present in plasma membranes and in the GERL region of the Golgi as predicted by Rothman and Fine (1980) from their studies on the transport of G protein from vesicular stomatitis virus.

Receptor mediated endocytosis of extracellular macromolecules into cells takes place after the receptors present on the plasma membrane have clustered into clathrin coated pits (Pearse, 1976; Goldstein *et al.*, 1979). Whether coated vesicles arise from coated pits is not yet clear. Brown and Goldstein (1979) suggested from their studies on patients

with defective low density lipoprotein (LDL) receptors that binding and internalisation were two separate steps, and that although LDL receptors interact with clathrin in coated pits (Pearse, 1976), internalisation of the receptors into coated vesicles occurred even in the absence of LDL (Anderson et al., 1976). Similar conclusions were drawn from the study of asialoglycoprotein receptor internalisation in isolated rat hepatocytes (Warren and Doyle, 1981). The formation of coated vesicles from coated pits is a phenomenon which has itself been challenged by recent evidence from Willingham and Pastan (1980). These researchers observed that α_2 macroglobulin was concentrated on the cell surface into coated pits, and then sequestered into fluorescence-labelled clathrin-free vesicles in which intracellular transport occurred. These vesicles were distinguished from lysosomes as they were not visible when examined under phase contrast microscopy, whereas lysosomes were clearly visible as phase dense bodies. These clathrin-free vesicles were termed 'receptosomes' by Willingham and Pastan (1980). Other recent evidence from Willingham et al. (1981) has shown that β-galactosidase was taken up into Chinese hamster ovary cells after it had bound to mannose 6-phosphate receptors followed by its internalisation through coated pits and encapsulation into receptosomes. While coated vesicles transported LDL receptors to lysosomes (Goldstein et al., 1979), receptosomes transported β-galactosidase firstly to the Golgi region within 8 minutes after binding and from there the enzyme was rapidly transferred to lysosomes, appearing there within 15-30 minutes (Willingham et al., 1981). Pastan and Willingham (1981) have suggested more recently that coated vesicles were artifacts produced when coated pits were sectioned tangentially prior to electron microscopy. These workers supported their argument with evidence from

experiments by Wehland et al. (1981) who introduced anti-clathrin antibodies into the cytoplasm of 3T3 fibroblasts by microinjection. The results of these experiments showed that antibodies to clathrin did not aggregate into coated vesicles indicating that none was freely recycling in the cytoplasm, and that coated pits were stable elements permanently attached to the plasma membrane. The clustering of receptors into coated pits was explained by Pastan and Willingham (1981) from experiments where internalisation of α_2 macroglobulin was inhibited using primary amines, they suggested that receptor-ligand complexes might be anchored in coated pits by cross links between amino groups on the complex chains with amino groups present on proteins in the coated pits. A transglutaminase was thought to be responsible for the cross-linking required for this process, and the activity of transglutaminoses is markedly inhibited by primary amines. These workers also showed that different degrees of inhibition by amines were obtained for different classes of ligand, and thus provided a clue to the manner in which a degree of selectivity could be exercised by coated pits for different ligands. Other workers have also observed that coated pits act as molecular filters. Bretscher et al. (1980), for example, showed that coated pits present on the plasma membranes of mouse fibroblasts actively excluded two membrane proteins, while simultaneously accumulating LDL receptors. Montanesco et al. (1979) also observed that cholesterol was absent from coated pits present on 3T3 fibroblasts although the plasma membrane itself was relatively rich in this lipid (Wall et al., 1980). It is therefore possible that ligand receptor complexes are sorted and segregated at the cell surface before their internalisation.

It remains unclear whether the transient passage of exogenously

added glycoproteins through the GERL region after endocytosis (Willingham et al., 1981) results in any further processing of the oligosaccharide side chains. Indirect evidence from Glaser et al. (1975) suggested that the 'high uptake' fraction of platelet β-glucuronidase was processed to a 'low uptake' from after its endocytosis by deficient fibroblasts. Miller and coworkers (1981), however, suggested that N-acetylglucosaminidase from fibroblasts was still present in a high uptake form after receptor mediated uptake into fibroblasts from a patient with Sandhoff's disease. The apparent contradiction in these observations might be resolved if lysosomal enzymes secreted by fibroblasts were recognised and internalised by a different pathway from that observed for the uptake of intracellular enzymes isolated from other types of cell, such as platelets. Further studies on the fate of infused splenic β-glucuronidase in mice suggested that the liver was not only processing internalised enzyme molecules but also exchanging subunits of the exogenously added enzyme with subunits of endogenous enzyme (Fiddler and Desnick, 1976). Evidence from studies on hybrids of mouse and human fibroblasts has also shown that heteropolymeric β-glucuronidase was produced when the two types of cell were fused (Chern, 1977).

Receptors are recycled after delivery of their bound ligands to the extracellular space, to Golgi or to lysosomes. Such recycling has been demonstrated in fibroblasts (Rome et al., 1979; Rothman and Fine, 1980), alveolar macrophages (Stahl et al., 1980) and hepatocytes (Warren and Doyle, 1981). Receptors for viral G protein were recycled every 1-5 minutes in Chinese hamster ovary fibroblasts (Rothman and Fine, 1980), and the speed of recycling in other cells is such that Muller et al. (1980) calculated that macrophages internalised 186% of

their total plasma membrane per hour, while each of the 500,000 asialoglycoprotein receptors present on hepatocytes (Schwartz et al., 1980) delivered 1000 molecules of ligand per hour to the lysosomes. Thus most types of cell have a tremendous potential for the rapid delivery of enzymes to lysosomes if the enzymes have the correct recognition markers and can bind to these recycling receptors.

From the evidence described above, lysosomal enzymes appear to be sorted, selectively transported through the cell and segregated from the cytoplasm by vesicles into which they are anchored by specific receptors. Contradictory evidence has suggested, however, that lysosomal enzymes secreted by fibroblasts bear the phosphomannosyl recognition markers which should have signalled their inclusion in lysosomes rather than their secretion (Hickman and Neufeld, 1972). This apparent contradiction may be due to a degree of variation in the number of oligosaccharide side chains present on each lysosomal enzyme. Hasilik et al. (1981) showed that the Cathepsin D and β-hexosaminidase secreted by fibroblasts contained oligosaccharide side chains which were predominantly of the complex type and in addition a smaller proportion of molecules with high mannose chains. Bovine testicular β-galactosidase has sixteen oligosaccharide side chains (four per monomer) with a varying number of mannose residues in each chain (G.W. Jourdian, personal communication), and phosphorylation of only 2% of these residues is sufficient to render the enzyme a high uptake form recognised by fibroblasts (Distler et al., 1979). Again, only one mannose phosphate residue per β-glucuronidase subunit was necessary in order to make platelet enzyme a high uptake form (Natowicz et al., 1979) for fibroblasts. Each enzyme may therefore have more than one recognition marker and be able to bind to different membrane receptors.

Although receptors for different recognition markers have been identified on both intracellular and plasma membranes, the strength of binding of each ligand may depend on its local environment. Binding to phosphomannosyl receptors is pH dependent, for example, and β-glucuronidase is dissociated from lysosomal membranes below pH 6.0 (Fischer et al., 1980a); at neutral pH such as that prevailing on plasma membranes, binding of β-glucuronidase to the receptors is much stronger. In addition, the ability of the asialoglycoprotein receptors to bind ligands has been shown to be altered by the phospholipid composition of their environment (Klausner et al., 1980). The intracellular fate of lysosomal enzymes may therefore depend on the strength of their binding to the receptors in each transporting vesicle.

There is now a considerable amount of information describing the synthesis, secretion and uptake of lysosomal enzymes by different types of cell. However, it is not yet known if cells can secrete lysosomal enzymes bearing the correct recognition markers for targeting them to other types of cells with different cell surface receptors. Unless such events can occur in vivo, the use of cell or organ transplants for the treatment of lysosmal storage diseases will only ever be marginally effective. In this thesis I have attempted to investigate this problem by using a model system. This system involves the in vitro culture of mouse fibroblasts, the collection of lysosmal enzymes secreted by these cells, and the measurement of the accumulation of a single enzyme, β-glucuronidase, by recipient mouse macrophages.

1.5 THE MOUSE MODEL FOR STUDY OF RELEASE AND UPTAKE OF LYSOSOMAL ENZYMES IN VITRO

A model system was set up in order to determine the ability of reticuloendothelial cells to take up by receptor mediated endocytosis lysosomal enzymes secreted by fibroblasts. The requirements of this system were firstly a strain of donor fibroblasts which secreted sufficient quantities of lysosomal enzymes to enable their purification to be undertaken, and secondly stable cultures of pure recipient reticuloendothelial cells.

A mouse model system was chosen for a number of reasons. Mouse peritoneal macrophages were chosen as recipient reticulendothelial cells because their isolation and methods of culture have been described frequently (Lee, 1969; Mauel and Defendi, 1971; Cifone and Defendi, 1971; Cifone et al., 1975; Stanley et al., 1976; Stanley, 1979; Van der Zeijst et al., 1978), and because laboratory mice were readily available in large numbers. Mouse peritoneal macrophages have been used as recipient cells for uptake of labelled rat preputial gland β-glucuronidase (Ezekowitz et al., 1981), and have also been shown to take up yeast invertase in an artificially induced lysosomal storage disease induced with sucrose (Cohn and Ehrenreich, 1969). Only a few animal models of lysosomal storage diseases have been described. These include arylsulphatase deficient cats (Desnick, 1981), β-hexosaminidase deficient cats (Ratazzi et al., 1979) and α-mannosidase deficient Angus cattle (Hocking et al., 1972) (for a review see Hommes, 1979). There are, however, strains of mice (AKR and C3H) which have genetically inherited low levels of β-glucuronidase and these have been investigated

extensively (for a review see Paigen, 1979). The low level of β-glucuronidase synthesised by these mice is heat labile (Paigen, 1971; Frankel et al., 1977), an additional advantage of this system, since macrophages can be easily stimulated to synthesise lysosomal enzymes and any such endogenous activity can be inactivated by heating. In addition, fibroblasts from AKR mice have been used as recipient cells for endocytosis of β-glucuronidase from human platelets (Frankel et al., 1977), suggesting that transfer of this enzyme would not be species specific. The transfer of β-glucuronidase between cells from normal strains of mice and cells derived from C3H or AKR mice has been demonstrated previously in several organs of tetraparental mice (Feder, 1976). A strain of human fibroblasts (cell line GM151) derived from a patient totally deficient in β-glucuronidase (Mucopolysaccharidosis type VII) was available as an acceptor cell with which to compare receptor mediated endocytosis of the β-glucuronidase secreted by fibroblasts.

Normal human fibroblasts secrete very small amounts of most lysosomal enzymes in vitro (Miller et al., 1980) but it was hoped that a strain of mouse fibroblasts might be found which would secrete sufficient quantities of β-glucuronidase in vitro to enable the uptake characteristics of the enzyme into both macrophages and deficient fibroblasts to be defined.

The aims of this project were, therefore, to find a strain of mouse fibroblasts which would secrete relatively large amounts of β-glucuronidase, to purify this enzyme, and to determine whether it was taken up into reticuloendothelial cells by receptor mediated endocytosis. In addition, it was hoped to determine the type of receptor responsible

for uptake of the β-glucuronidase into macrophages, and to ascertain whether this receptor differed from that present on the plasma membranes of fibroblasts.

CHAPTER II

EXPERIMENTAL PROCEDURES

2.1 MATERIALS

2.1.1 Biochemicals

Materials obtained from Flow Laboratories (Irvine, Scotland):

Complement (from Guinea Pig Serum)
Culture Media: 199, with Earles Salts; RPMI 1640
Mouse Fibroblasts: 3T3, A9 and Balb 3T12-3
L-Glutamine
Hepes (4-[2-hydroxyethyl]-1-piperazine-ethane) sulphonic acid
Penicillin/Streptomycin
Sera: Foetal Calf, New Born and Donor Horse
Trypsin (E.C. 3.4.21.4)

Materials obtained from Sigma Chemical Co. Ltd. (Dorset, U.K.):

N-Acetyl-D-glucosamine
Albumin (bovine fraction V)
Alkaline Phosphatase (E.C. 3.1.3.1) bovine intestine
Carbodiimide (1-ethyl-3-[3-dimethylaminopropylcarbodiimide]HCl)
Diaminobenzidine HCl
EDTA (ethylenediaminetetraacetic acid)
Fast Blue BB (diazotised 4-amino 2,5-diethoxybenzanilide)
Fast Red TR (4-chloro-o-toluidine diazotate)
D-Fructose
D-Fructose 1-phosphate (disodium salt)
D-Glucose
D-Glucose 6-phosphate (disodium salt)
D-Glucuronic Acid
β-Glucuronidase (E.C. 3.2.1.31) bovine liver
L-Glycine
Horseradish Peroxidase (E.C. 1.11.1.7)
Mannan from bakers yeast
D-Mannose
D-Mannose 6-phosphate (disodium salt)
α-Methyl-D-mannoside
Naphthol AS D Chloroacetate

Naphthol AS BI β-D-glucuronide
Naphthyl acetate
p-Nitrophenyl-β-D-glucuronide
Quinine sulphate
D-Saccharic acid 1,4-lactone
Saccharomyces Cerevisae (bakers yeast type II)
Starch (hydrolysed potato)
Tris (Tris-[hydroxymethyl]-aminomethane)

Materials obtained from Pharmacia Fine Chemicals (Uppsala, Sweden):

CNBr activated Sepharose
Concanavalin A-Sepharose
Cytodex microcarriers
DEAE Sephacel
Sephadex G-200
Sepharose 4B

Materials obtained from Koch-Light (Colnbrook, Bucks):

3'3'-Diaminodipropylamine
4-Methylumbelliferyl 2-acetamido-2-deoxy-β-D-glucopyranoside
4-Methylumbelliferyl β-D-galactopyranoside
4-Methylumbelliferyl β-D-glucuronide
Methylumbelliferone
Sodium dithionite
Triton X-100

Insulin B.P. was obtained from Wellcome (Beckenham, Kent) and Heparin B.P. from Paines and Byrne Ltd. (Greenford, Middlesex). Phosphate buffered saline (PBS) tablets were supplied by Oxoid Ltd. (Basingstoke, Hants), cyanogen bromide by Eastman Kodak Co. (Rochester, N.Y., U.S.A.) and $MgCl_2$ by Hopkins and Williams (Chadwell Heath, Essex).

All other reagents were Analar/Aristar grade and obtained from B.D.H. (Poole, Dorset).

GM151 Human fibroblasts were obtained from the Human Genetic Mutant Cell Repository (Camdem, N.J., U.S.A.); 3T3 fibroblasts and SV40 transformed 3T3 fibroblasts were gifts from Dr. S. Chang (Imperial

Cancer Research Fund, London), and cholera toxin was a kind gift from Dr. Fiona Watt (Kennedy Institute, London).

2.1.2 Laboratory Equipment

Fluorimetric readings were made on a Locarte Model 8-9 fluorimeter (London, U.K.); colourimetric readings on a Cecil CE272 spectrophotometer (Cecil Instruments Ltd., Cambridge, U.K.) and NaCl concentrations were measured with a CDM 83 conductivity meter supplied by Radiometer (Copenhagen, Denmark).

All columns were packed in barrels of syringes supplied by Gillette (Isleworth, U.K.). Samples were dialysed in tubing from Medicell International Ltd. (London, U.K.) which was prepared by boiling firstly in 0.01M EDTA/0.1M Na_2CO_3, and then twice in distilled water prior to use. Measurement of volumes of less than 1ml was made with Finn pipettes (Jencons, Hemel Hempstead, U.K.). Ultrafiltration apparatus and filters were obtained from Amicon Corporation (High Wycombe, Bucks, U.K.).

All tissue culture equipment was supplied by Becton-Dickinson and Co. (California, U.S.A.), with the exception of microcarrier vessels and stirrers which were obtained from Techne Ltd. (Cambridge, U.K.); samples were sterilised by passage through 0.45μm filters from Millipore (London, U.K.). Asceptic procedures were carried out in a Vertical Flow, Laminar tissue culture hood (Envair (U.K.) Ltd., Rossendale, Lancs., U.K.), and cell cultures were maintained at 37°C in a 5% CO_2/95% air, humidified incubator (L.E.E.C. Ltd., Nottingham, U.K.). Photographs of cells were taken on a Diavert microscope (Leitz, Wetzlar, Germany).

2.2 BIOCHEMICAL ASSAYS

2.2.1 Fluorimetric Assays

All of the procedures described below were adapted from the methods of Kolodny and Mumford (1976).

Reagents

4-Methylumbelliferone stock standard: 17.6mg 4-methylumbelliferone were dissolved in 1ml ethanol, and then made up to 100ml in distilled water. From this 1mM stock, a two hundred times dilution was made in distilled water to give a 5μM stock solution which was stored in the dark at 4°C.

Glycine Buffer: 30g glycine, 23g sodium chloride and 16g sodium hydroxide were dissolved in 800ml distilled water and adjusted to pH 10.4; the volume was then made up to 1 litre and the buffer stored at 4°C.

25μM Quinine Sulphate: stock quinine sulphate solution was made by dissolving 4.7mg quinine sulphate in 500ml 0.05M sulphuric acid. A working solution of 2.5μM quinine sulphate was made by diluting the stock ten times in 0.05M sulphuric acid. Both quinine sulphate solutions were kept in brown glass bottles at 4°C.

Fluorimeter Readings

All readings were made on a fluorimeter set with an excitation filter of 362nm wavelength and an emission filter of 440nm wavelength; the scale was set to zero with glycine buffer, and the fluorimeter then set to an arbitrary standard reading of 20 units with 2.5μM quinine sulphate.

A calibration line of fluorescence against methylumbelliferone concentration was constructed using methylumbelliferone standards from 1nM to 5nM. The standards were made by diluting the 5μM methylumbelliferone stock with glycine buffer as follows:

concentration of standard (nM)	volume of stock (ml)	volume of glycine buffer (ml)
1	0.2	1.15
2	0.4	0.85
3	0.6	0.65
4	0.8	0.45
5	1.0	0.25

The fluorescence of standards and samples was measured immediately after termination of the reaction, and enzyme activity in the samples expressed as units/ml of sample, where 1 unit of activity was that amount of enzyme which hydrolysed 1nmol substrate in 1 hour at 37°C.

2.2.1(a) β-Glucuronidase Assay

Substrate: 81mg of 4-methylumbelliferyl β-D-glucuronide were dissolved in 100ml 0.1M sodium acetate buffer pH 4.5. The 2mM substrate was stored at -20°C.

Assay procedure: 50μl of sample or distilled water as a substrate blank was pre-incubated in a water bath at 37°C for ten minutes and then 200μl of substrate was added. The reaction was allowed to proceed for one hour at 37°C and then terminated by adding 1ml of the glycine buffer.

2.2.1(b) β-Galactosidase Assay

Substrate: 16.9mg of 4-methylumbelliferyl β-D-galactopyranoside

was dissolved in 100ml 0.1M sodium citrate buffer at pH 4.5. The 0.5mM substrate was stored at -20°C.

Other reagents and the assay procedure were as described above for β-glucuronidase.

2.2.1(c) N-acetylglucosaminidase Assay

Substrate: 75.8mg of 4-methylumbelliferyl 2-acetamido-2-deoxy-β-D-glucopyranoside were dissolved in 100ml 0.1M sodium citrate buffer pH 4.5. The 2mM substrate was stored at -20°C.

Other reagents and procedures were as described for the assay of β-glucuronidase, except that 15 minute incubation times were regularly used for N-acetylglucosaminidase assay because of the much higher activity of this enzyme in most of the samples assayed.

2.2.2 Horseradish Peroxidase Assay

This was adapted from the method of Steinman and Cohn (1972).

Reagents

Substrate: 50mg diaminobenzidine HCl and 5µl of 30% (v.v.) hydrogen peroxide were dissolved in 50µl 0.1M citrate/phosphate buffer pH 6.0. The substrate was made up freshly and used immediately.

Standard Horseradish Peroxidase (HRP): a range of dilutions of commercial HRP in 0.1% Triton X-100 containing between 1 and 7µg/ml (determined by the method of Lowry et al., 1951) were assayed in the same manner as the unknown samples.

Assay Procedure

500µl of substrate was placed in a silica cuvette (1ml volume) and

the absorbance at 450nm adjusted to zero on a spectrophotometer. 50µl of sample was then added to the cuvette and the increase in absorbance of the mixture recorded at 30 second intervals for 2.5 to 3 minutes. The increase in absorbance when 50µl of 0.1% Triton X-100 only was added to the substrate was measured, and the increase in absorbance at 30 second intervals was recorded for the HRP standards.

The absorbance readings for HRP were plotted against time, the substrate blank values subtracted from them, and the average rate of increase in absorbance/minute/µg HRP/ml calculated. A calibration line was plotted of increase in absorbance per minute against HRP concentration, and the HRP concentration per sample calculated using the standard curve.

2.2.3 Protein Estimation

Protein contents were determined using an automated assay procedure (Heinegård, 1973) based on the method of Lowry et al., (1951). 400µl of sample was mixed with a continuous flow of 5% $CuSO_4$: 10% potassium tartrate : 10% Na_2CO_3 in 1M NaOH in a volume ratio of 1:1:100. The mixture was incubated at 60°C for ten minutes, and then mixed with a continuous flow of 50% (v.v.) Folin and Ciocalteu's reagent in distilled water. The absorbance at 590nm was plotted on a pen recorder, and the protein content of the samples compared with standards containing 10µg - 50µg per ml of bovine serum albumin.

2.2.4 Measurement of Sodium Chloride Gradients

The concentration of sodium chloride in every fifth fraction collected from DEAE-Sephacel was measured by comparing the conductivity of the fraction with a range of dilutions of sodium chloride in 5mM Tris/HCl pH 8.0 containing between 50mM and 300mM sodium chloride.

2.3 CELL CULTURE

2.3.1 Isolation of Mouse Embryo Fibroblasts

CBA and AKR mouse fibroblasts were obtained from 19 day old embryos. The embryos were removed under sterile conditions, the foetal eyes, guts and livers discarded, and the remains minced in sterile PBS, passed through a metal sieve, and incubated at 37°C with an equal volume of 0.25% trypsin in PBS for 15-30 minutes. The suspended cells were mixed with foetal calf serum to inhibit the trypsin and collected by centrifugation for 10 minutes at room temperature at 1000rpm in an MSE bench centrifuge. After resuspension in RPMI 1640 medium containing 10% (v.v.) FCS, the cells were counted in a haemocytometer and seeded at approximately 2 - 5 $x10^6$ cells per 75cm^2 flask in 10ml medium. After six hours, the medium was changed to remove non-adherent erythrocytes, and subsequently the cells were treated as normal fibroblast lines.

2.3.2 Screening of Fibroblasts as Enzyme Donors

Balb 3T12-3, A9, and 3T3 mouse fibroblast lines, and CBA mouse embryo fibroblasts were each seeded into duplicate 75cm^2 tissue culture flasks in 15ml Medium 199 containing 10% (v.v.) foetal calf serum (FCS) which had been heated at 65°C for 1 hour to inactivate bovine β-glucuronidase. The medium was supplemented with 2mM L-glutamine, 20mM Hepes, 750mg/l sodium bicarbonate and 50IU/50µg/ml penicillin/streptomycin. The fibroblasts were cultured in an atmosphere of 5% CO_2 95% air at 37°C and 0.5ml samples of medium withdrawn at 2-3 day intervals and assayed for β-glucuronidase activity.

2.3.3 Release of β-Glucuronidase at Varying Concentrations of Foetal Calf Serum

In order to find the lowest concentration of FCS which would support growth of 3T3 fibroblasts and β-glucuronidase release, 1 x10^5 3T3 cells were seeded into 25cm^2 tissue culture flasks in 10ml of Medium 199 containing increasing concentrations of heat inactivated FCS. The cells were incubated for several days at 37°C, and at 48 hour intervals 0.5ml of the medium withdrawn and assayed for β-glucuronidase activity. The ability of 3T3 cells to grow in 5% (v.v.) FCS or 5% (v.v.) heat inactivated FCS was compared. The medium from the cells which were cultured in 5% (v.v.) FCS was heated at 65°C for 1 hour prior to β-glucuronidase assay.

2.3.4 Effectiveness of Serum Substitutes

10% (v.v.) donor horse serum, 5% (v.v.) new born calf serum, 2% (v.v.) mouse plasma, 0.2% (w.v.) serum albumin, 0.08IU/ml insulin or 10^{-10}M cholera toxin (Green, 1978) were tested as medium supplements in place of foetal calf serum, in an attempt to reduce the levels of non-β-glucuronidase protein present in 3T3 fibroblast conditioned medium. FCS which had been passed first through a column of Concanavalin A-Sepharose, was also tested at a concentration of 5% (v.v.) in Medium 199 on 3T3 fibroblasts.

2.3.5 Output of β-Glucuronidase by SV40 Transformed 3T3 Fibroblasts

3T3 fibroblasts transformed by SV40 virus were seeded in duplicate cultures at 5 x10^5 cells per 75cm^2 tissue culture flask and grown in 15ml medium containing 5% (v.v.) of either heat inactivated foetal calf serum or 0.08IU/ml of insulin, or serum free Medium 199. The

activity of the β-glucuronidase they released into the medium was assayed in the same manner as for previous experiments, and the output from transformed cells compared with the output of normal 3T3 fibroblasts.

2.3.6 Growth of 3T3 Fibroblasts on Microcarriers

3T3 fibroblasts were cultured in monolayers in $75cm^2$ flasks until confluent (2×10^6 cells per flask), passaged by the method described in section 2.3.10 and seeded at 1.5×10^7 cells per 10^6 (0.2g) Cytodex beads in 30ml Medium 199 containing 10% (v.v.) FCS. After adhesion and spreading of the fibroblasts had taken place (2-3 hours), the volume of the medium was adjusted to 60ml by adding serum free Medium 199. The cells were incubated on a rocking platform in a dry incubator at 37°C. After 6 days, the medium was harvested and the cells removed from the beads by washing them in PBS followed by incubation in 0.25% trypsin (20ml per 0.2g Cytodex) for 30 minutes. After addition of an equal volume of medium containing 5% (v.v.) FCS the cells and microcarriers were centrifuged at 2000rpm for five minutes, and resuspended in medium containing 5% (v.v.) FCS and an equal volume of microcarriers. The cells were allowed to readhere to the beads and cultured in medium containing 5% (v.v.) FCS. Bulk cultures of 3T3 fibroblasts were set up by pooling seeded microcarriers in microcarrier culture flasks. 7.5×10^6 beads containing approximately 1.25×10^8 3T3 fibroblasts in 500ml of medium were cultured in each flask.

2.3.7 Isolation and Culture of AKR and C3H Peritoneal Macrophages

AKR or C3H mice of either sex were each injected intraperitoneally with 2ml of 1% starch suspended in sterile PBS (Lee, 1969). After

three days, when granulocyte infiltration has waned (Beelen et al., 1978), the mice were killed by dislocation of the neck and the peritoneal cavities washed with 5ml sterile PBS containing 50IU/50µg/ml penicillin/streptomycin and 200U/ml heparin. The harvested peritoneal cells were centrifuged at 2000rpm in an MSE minor bench centrifuge and resuspended in Medium 199 containing 5% (v.v.) FCS and 10% (v.v.) A9 conditioned medium (CM). (The preparation of A9 fibroblast conditioned medium is described below). The peritoneal cells were then counted in a haemocytometer and seeded at approximately 10^6 leucocytes per 35mm petri dish. After incubation for 1 hour at 37°C, the cells were washed three times with 3ml PBS to remove non-adherent leucocytes and erythrocytes, and the adherent peritoneal cells were incubated in the medium containing the growth factor. The endogenous β-glucuronidase activity of the AKR adherent cells was measured during the culture period by freezing and thawing the cells in 0.5ml 0.1% (v.v.) Triton X-100 per plate, followed by centrifugation at 2000rpm for 10 minutes and assay of the soluble cell extracts.

A9 fibroblasts were grown in monolayers or on Cytodex microcarriers for six days in Medium 199 containing 5% (v.v.) FCS, 20mM Hepes buffer, 750mg/l sodium bicarbonate, 2mM glutamine and 50IU/50µg/ml penicillin/streptomycin. The medium was harvested, filtered through a 0.45µm Millipore filter, and stored frozen before being used as a supplement in the medium used for culture of peritoneal macrophages. A9 fibroblasts are a mouse cell line derived from L929 cells which secrete a macrophage growth factor/colony stimulating factor (Lee, 1969; Mauel and Defendi, 1971; Stanley et al., 1976; Cifone and Defendi, 1974; Stanley, 1979).

2.3.8 Characterisation of Adherent Peritoneal Mouse Macrophages

Phagocytosis Assay

Heat killed bakers yeast (5×10^7 cells in 50µl of sterile PBS) and 60µl of guinea pig serum complement were added in 2ml culture medium to AKR macrophages in 35mm dishes (Van der Zeijst et al., 1978). The adherent cells were incubated at 37°C for 30 minutes, washed in warm PBS until washings were yeast free, and fixed for 30 minutes in ice cold methanol. The cells were then stained in 0.5% (v.v.) methylene blue for 5-10 minutes, washed in distilled water and air dried. Macrophages possess receptors for the third component of serum complement (C3) (Kaplan et al., 1978), and will consequently ingest particles which are coated by this opsonising factor (Shurin and Stossel, 1978). The phagocytosis of complement coated yeast is therefore a visual marker for C3 receptors on cells.

Histochemical Stains for Non-Specific Esterase and Chloroacetate Esterase

This was adapted from the method of Stuart et al. (1978).

Reagents

Non-Specific Esterase

stock solution stored at 4°C:	α-Naphthylacetate	1g
	Acetone	50ml
	distilled H_2O	50ml
working solution, made up freshly and filtered before use:	stock	2ml
	0.1M phosphate buffer pH 7.3	15ml
	distilled H_2O	15ml
	Fast Red TR	20mg

Chloroacetate Esterase

working solution, made up freshly and filtered prior to staining:	Naphthol AS D chloroacetate	20mg in 1ml acetone
	0.1M phosphate buffer pH 7.3	10ml
	Fast Blue BB salt	20mg
	distilled H_2O	10ml

Method

Adherent cells on 35mm petri dishes were washed in warm PBS, and then incubated for 30-40 minutes in the non-specific esterase reagent. They were then washed in tap water and incubated for 5-20 minutes with the substrate for chloroacetate esterase. After staining, the cells were washed in distilled water and air dried. Under these conditions macrophages stain red because of their non-specific esterase activity, and granulocytes, which have a high chloroacetate esterase activity, stain blue.

2.3.9 Histochemical Stain for β-Glucuronidase

This was adapted from the method of Fishman and Goldstein (1965).

Reagents

stock substrate stored at 4°C:	Naphthol AS-Bl β-D glucuronide	11mg
	0.05M sodium bicarbonate	1ml
	0.1M sodium acetate pH 4.5	99ml
working substrate, freshly prepared:	stock substrate	5ml
	0.1M sodium acetate buffer pH 4.5	5ml

stain, freshly prepared and filtered:	Fast Blue RR	10mg
	0.01M potassium phosphate buffer pH 7.4	10ml

Method

1ml of substrate was added to cells in 35mm petri dishes and incubated for 3 hours at 37°C. The dishes were then washed with PBS and 1ml of Fast Blue RR added for 10 minutes at 4°C. The dishes were finally washed in PBS, fixed in ice cold methanol and air dried.

2.3.10 Heat Stability of β-Glucuronidase

Samples containing β-glucuronidase prepared from different sources were heated at 65°C in a water bath. At timed intervals, two aliquots of 50µl were withdrawn from each sample, cooled to 4°C, and their β-glucuronidase activity then determined. The thermal stability of β-glucuronidase extracted from AKR macrophages and 3T3 fibroblasts was measured directly by heating samples extracted in 0.1% Triton X-100. The stability of β-glucuronidase secreted by 3T3 cells was determined after concentration of the culture medium in an Amicon ultrafiltration apparatus fitted with a Diaflo XM100 A filter.

2.3.11 Culture of Human β-Glucuronidase Deficient Fibroblasts

GM151 fibroblasts were cultured in 75cm^2 flasks in Medium 199 containing 5% (v.v.) FCS, 2mM L-glutamine, 20mM Hepes buffer, 750mg/l sodium bicarbonate and 50IU/50µg/ml of penicillin/streptomycin. The cells were passaged by washing in 10ml PBS followed by incubation for 10 minutes in 5ml PBS containing 0.25% (v.v.) trypsin. Trypsinisation was terminated by the addition of 5ml complete medium, the cells collected by centrifugation at 2000rpm at room temperature in an MSE

bench centrifuge, and resuspended in an equal volume of medium. The fibroblasts were seeded into fresh tissue culture flasks at a split ratio of 1:5, or on 35mm petri dishes in 2ml culture medium for β-glucuronidase uptake experiments. Assays for uptake of β-glucuronidase were always carried out when the fibroblasts were 50% confluent (approximately 10^5 cells per dish).

2.3.12 Comparison of the β-Glucuronidase Activity of Different Strains of Mouse

The livers and spleens of CBA, Balb/c and AKR mice were homogenised in 0.1% (v.v.) Triton X-100 in distilled water at a wet weight/volume ratio of 50mg/ml. The homogenates were centrifuged in an MSE 18 centrifuge for 20 minutes at 10,000rpm at 4°C, and the supernatant solutions assayed for their β-glucuronidase activities and protein contents. Trypsinised fibroblast pellets from these strains of mice were freeze-thawed six times in 1ml 0.1% Triton X-100 in distilled water, centrifuged at 3000rpm in an MSE bench centrifuge and the supernatant solutions assayed for their enzyme activities and protein contents.

2.4 PURIFICATION OF 3T3 FIBROBLAST β-GLUCURONIDASE

2.4.1 Initial Purification

Medium harvested from 3T3 cells was passed through a 0.45μm Millipore filter to remove any cell debris, and concentrated by ultrafiltration through an Amicon XM100 A filter. The concentrate was then heated for 1 hour at 65°C, and centrifuged to remove precipitated protein.

Concentrated medium was brought to 20% saturation by adding solid ammonium sulphate, the precipitate removed by centrifugation at 10,000rpm in an MSE 18 centrifuge, and the supernatant solution then brought to 35% saturation with further addition of ammonium sulphate. Precipitates were collected in this way at 40%, 45%, 50% and 55% ammonium sulphate saturation, redissolved in Tris/saline pH 7.0, dialysed against the same buffer, then assayed for β-glucuronidase, N-acetylglucosaminidase, β-galactosidase and protein. At this stage of purification, the suitability of various forms of affinity chromatography for further purification of β-glucuronidase were compared.

2.4.2 Purification of Mouse 3T3 Fibroblast β-Glucuronidase with Concanavalin A-Sepharose

A 45% saturated ammonium sulphate precipitate of 3T3 β-glucuronidase was freeze dried after dialysis against distilled water and redissolved in 2ml 25mM sodium acetate pH 5.5 containing 1mM each of $MgCl_2$, $MnCl_2$ and $CaCl_2$ before loading on a 2ml column of Concanavalin A-Sepharose packed in a 5ml syringe barrel (Natowicz *et al.*, 1979). The gel was washed with three bed volumes of loading buffer and the β-glucuronidase

was eluted with increasing concentrations (0.2 to 1.0M) of α-methyl-D-mannoside dissolved in the loading buffer. After dialysis against three changes of 1 litre of 0.15M NaCl at 4°C, the fractions eluted were assayed for their β-glucuronidase activities and protein contents.

In order to increase the recovery of β-glucuronidase and to eliminate non-specific ligand binding, the above method was repeated with the addition of either 0.5M or 1.0M sodium chloride to the loading and eluting buffers. Each fraction eluted was also assayed for N-acetylglucosaminidase activity. Before they were used for uptake experiments, preparations of β-glucuronidase purified by affinity chromatography on Concanavalin A-Sepharose were dialysed against three changes of 1 litre of 1M NaCl, mixed with Sephadex G-200 (25:1 v.v.), and centrifuged at 2000rpm for 10 minutes to remove the Sephadex and any contaminating Concanavalin A (Agrawal and Goldstein, 1965).

2.4.3 Chromatography of β-Glucuronidase on DEAE-Sephacel

3T3 fibroblast β-glucuronidase which had been purified by affinity chromatography on Concanavalin A-Sepharose was concentrated by ultrafiltration on an Amicon XM100 A filter to approximately 4ml and dialysed against three changes of 1 litre of 5mM Tris/HCl pH 8.0. The β-glucuronidase was loaded onto a 5ml column of DEAE-Sephacel which had been previously decanted and washed repeatedly to remove finings, packed into a 5ml syringe barrel and equilibrated with the loading buffer as described by Nicol *et al.* (1974). The Sephacel was washed with three bed volumes of loading buffer and the β-glucuronidase eluted in a gradient of 0-0.3M NaCl in the same buffer. Fractions of 1ml were collected and assayed for β-glucuronidase and N-acetylglucosaminidase activities and for their protein contents.

2.4.4 Preparation of β-Glucuronidase for Uptake Experiments

After purification by chromatography on Concanavalin A-Sepharose and DEAE-Sephacel, the β-glucuronidase was concentrated on an Amicon diaflo fitted with an XM100 A filter to a concentration of several hundreds of units/ml, dialysed into serum free Medium 199 at 4°C, then sterilised by filtration through a 0.45μm millipore filter membrane.

2.4.5 Preparation of β-Glucuronidase from Human Platelets and Placenta

Platelets which had been previously isolated from whole blood, lysed by sonic disruption, and centrifuged at 50,000g to remove all debris, were a gift from Dr. Irwin Olsen. The soluble extracts were purified by chromatography on Concanavalin A-Sepharose, in the manner described above for β-glucuronidase from 3T3 fibroblasts, except that 1mg/ml BSA was added to all loading and eluting buffers to prevent loss of activity during dialysis. The enzyme was then processed in the same way as described above for 3T3 fibroblast β-glucuronidase.

Placental β-glucuronidase was purified by chromatography on Concanavalin A-Sepharose and was a kind gift from Christina Östlund. Samples of this enzyme were prepared for uptake experiments as described above.

2.5 AFFINITY CHROMATOGRAPHY

2.5.1 Preparation of Sepharose 4B-p-aminophenyl-β-D-glucuronide

10mg p-Nitrophenyl-β-D-glucuronide was reduced by dissolving it in 5ml 0.5M $NaHCO_3$/0.1M $Na_2S_2O_4$ and shaking for 2 hours at room temperature (Bloch and Burger, 1974). Hydrolysis of the resulting solution in 1M KOH showed that about 89% of the p-nitrophenylglucuronide was reduced by this method. 5ml of CNBr activated Sepharose 4B was added to this solution and the mixture was shaken overnight at 4°C. The gel was then washed with coupling buffer and remaining active sites were blocked by reacting it with ethanolamine at pH 9.0 for 2 hours at room temperature. After washing vigorously three times in alternating solutions of 0.1M sodium acetate pH 4.0, and 0.1M sodium borate pH 8.0, the gel was stored at 4°C in PBS containing 0.01% sodium azide. Less than 0.2% of the ligand was lost during this washing procedure. Coupling by this method gave a ligand concentration of 35μM/ml of gel, determined by measuring the uronic acid concentration of the reduced solution before and after coupling to the gel. Uronic acid was assayed by an automated procedure (Heinegård, 1973) of the method of Bitter and Muir (1962); the assay was calibrated with standards of 5 to 50μg glucuronolactone.

2.5.2 Affinity Chromatography of β-Glucuronidase on Sepharose 4B-p-aminophenylglucuronide

(a) Using the method of Harris et al. (1973) approximately 2mg bovine liver β-glucuronidase (Sigma) was loaded onto 3ml gel in 2mM Tris/HCl pH 7.3 containing 5mM NaCl. Enzyme activity was eluted with 25ml of 0.1M acetic acid.

(b) 1ml of a crude preparation of β-glucuronidase prepared from CBA mouse liver by precipitation with 30% saturated ammonium sulphate was loaded in 2mM Tris/HCl pH 7.3 containing 5mM NaCl, onto 3ml Sepharose 4B-p-aminophenylglucuronide (Harris et al., 1973). The gel was washed with 10ml loading buffer, then with 10ml each of 0.1M sodium acetate pH 4.5 containing 0.15M NaCl and 2mM Tris/HCl pH 8.0 containing 30mM NaCl. The gel was then eluted with a gradient of acetic acid (0-0.2M). All eluted fractions were assayed for β-glucuronidase, β-galactosidase, N-acetylglucosaminidase activities and protein contents.

2.5.3 Preparation of Sepharose 4B-diaminodipropylamine-saccharolactone

Sepharose 4B was activated with CNBr by the method of Cuatrecasas (1970), and coupled to 3'3'-diaminodipropylamine as described by Harris et al. (1973). Saccharolactone was subsequently coupled to the gel in the presence of 1-ethyl-3-(3-dimethyl-amino-propyl)-carbodiimide HCl at pH 4.8. Unreacted sites on the gel were reacted with ethanolamine at pH 9.0, and the gel washed as described above for the p-aminophenyl-glucuronide-Sepharose preparation. The amount of bovine liver β-glucuronidase (Sigma) bound in 2mM Tris 5mM NaCl pH 7.3, and the amount of activity eluted in 0.1M acetic acid from 2ml of gel was measured. A control gel, with diaminodipropylamine bound to the Sepharose, but without the saccharolactone, was prepared, and the binding of bovine liver β-glucuronidase (Sigma) to it was also assessed.

2.5.4 Affinity Chromatography of β-Glucuronidase on Sepharose 4B-diaminodipropylamine-saccharolactone

(a) 0.5ml of 2mM Tris pH 8.0 containing 1.3mg of bovine liver β-glucuronidase (Sigma) was loaded onto 2ml gel, unbound material washed

through with 9ml of starting buffer, and enzyme eluted with a gradient from 0-2M NaCl. Forty fractions of 0.9ml were collected and assayed for β-glucuronidase and hexosaminidase activities and for protein content.

(b) 0.5mg bovine liver β-glucuronidase (Sigma) was loaded onto 2ml gel in 2mM Tris pH 8.0, washed with 10ml of loading buffer, and then eluted in a gradient of 0-75% ethylene glycol. Forty fractions of 1ml were collected and assayed for β-glucuronidase and N-acetylglucosaminidase activities and for protein content.

(c) 0.8mg bovine liver β-glucuronidase (Sigma) was loaded onto 2ml of the Sepharose-diaminodipropylamine-saccharolactone gel in 2mM Tris/HCl pH 8.0 containing 15% ethylene glycol, and 0.15M NaCl. The gel was washed in starting buffer, and then the β-glucuronidase activity was eluted in a gradient of 0.15M NaCl to 1.5M NaCl in 2mM Tris/HCl 15% ethylene glycol pH 8.0. Fractions of 1ml were collected and assayed for β-glucuronidase and N-acetylglucosaminidase activities, and for protein content.

2.5.5 Binding of β-Glucuronidase to Control Gels

Bovine liver β-glucuronidase (Sigma) was loaded in 2mM Tris 0.15M NaCl pH 8.0 onto Sepharose coupled to p-aminophenylglucuronide or diaminodipropylamine-saccharolactone, or to control gels where unreacted sites had been blocked by coupling to 1M ethanolamine or 1M Tris pH 8.0. The activity which eluted from each column was subtracted from the total loaded and a value for the total β-glucuronidase activity bound was thus calculated.

2.5.6 Specific Elution of β-Glucuronidase from p-aminophenylglucuronide-Sepharose 4B

5mg of a commercial preparation of bovine liver β-glucuronidase (Sigma) was loaded in PBS onto 2.5ml Sepharose 4B-p-aminophenyl-glucuronide using the method described by Lisman and Overdijk (1978). The gel was rinsed with 8 bed volumes of PBS and then eluted with a gradient of glucuronic acid from 0 to 50mg/ml. Fractions of 1.3ml were collected and each was assayed for β-glucuronidase activity and for its protein content by U.V. absorbance at 280nm.

2.5.7 Removal of Glucuronic Acid from β-Glucuronidase after Elution from Affinity Columns

The activity of 10µg bovine liver β-glucuronidase (Sigma) was measured in the presence and absence of 50mg glucuronic acid. Samples of the enzyme-inhibitor mixture and control enzyme were assayed for β-glucuronidase activity during dialysis against two changes of 8 litres of 2mM Tris pH 8.0 or 2mM Tris pH 7.0. The stability of bovine liver β-glucuronidase in 2mM Tris at pH 4-8 at 4°C was also measured.

The stability of bovine liver β-glucuronidase (Sigma) was compared with that of a sample of 3T3 fibroblast β-glucuronidase which had been partially purified by precipitation with 45% saturated ammonium sulphate. Both samples were dissolved in 2mM Tris/HCl pH 8.0 or pH 7.0 for 1 hour at 4°C and then assayed in 0.1M sodium acetate buffers ranging from pH 3.0 to 6.0. Tris/HCl at this concentration did not alter the resultant pH of the mixed buffers.

2.6 UPTAKE OF ENZYMES BY MACROPHAGES AND FIBROBLASTS

2.6.1 Uptake of β-Glucuronidase

Macrophages or fibroblasts were incubated overnight at 37°C in serum free Medium 199 containing 20mM Hepes, 750mg/litre sodium bicarbonate, 2mM glutamine, and 50IU/50µg/ml penicillin and streptomycin. The medium was then removed and replaced with 1ml serum free medium containing appropriate amounts of β-glucuronidase. The macrophages were incubated for 5 hours at 37°C and fibroblasts for 18 hours at 37°C in this medium. The medium was then pipetted into test tubes for assay, the cells washed twice in warm PBS, and β-glucuronidase extracted from the macrophages in 0.5ml 0.1% (v.v.) Triton X-100, and from the fibroblasts in 0.5ml 0.1% Triton X-100 containing 10mM mannose 6-phosphate (Fischer et al., 1980). The cells were frozen and thawed once in the detergent, scraped from the petri dishes with a rubber policeman, and centrifuged at 2000rpm for 10 minutes. The supernatant solutions containing the extracted β-glucuronidase were assayed for enzyme activity and protein content, after heat treatment at 65°C, in the case of macrophages, and after dialysis against 0.15M NaCl in the case of fibroblasts (mannose 6-phosphate strongly interfered with Lowry estimation of protein content). The amount of β-glucuronidase activity in the cells was expressed as units/mg cell protein, and uptake was expressed as units/mg cell protein/hour or %/mg/hour.

In all cases experiments were carried out on duplicate or triplicate plates of cells, and the mean activities given in the Results section. These measurements varied by less than 10% for each point. Control tissue culture plates were incubated with medium containing enzyme, and extracted in the same way in order to determine if there

was any non-specific binding of enzyme to the plates. Other cells were incubated in the absence of enzyme, so that residual activity not attributable to specific endocytosis could be subtracted from the values obtained for cells incubated in the presence of externally added β-glucuronidase.

2.6.2 Horseradish Peroxidase Uptake

Horseradish peroxidase (HRP) was passed through 2ml Concanavalin A-Sepharose in 25mM sodium acetate buffer pH 5.5 containing 1mM each of $MgCl_2$, $MnCl_2$ and $CaCl_2$. HRP which did not bind to the affinity column (81%) was used for uptake experiments, after mixing with Sephadex G200 in 1M NaCl to remove any contaminating Concanavalin A. The HRP was dialysed against serum free medium (two changes of 250ml), and added in a range of concentrations (0.5 - 5.0mg/ml) in 1ml of serum free medium as described for β-glucuronidase uptake. After 3 hours incubation (Steinman and Cohn, 1972; Heiber et al., 1980), the medium was removed and the cells extracted in 0.1% (v.v.) Triton X-100 as described for the β-glucuronidase assay. The HRP activity in the medium and cells was then assayed, and the rate of uptake expressed as ng HRP/mg cell protein/hour. Control plates for intracellular peroxidase activity and non-specific adhesion to plates were included in the experiment as described above for the measurement of β-glucuronidase activity.

2.6.3 Uptake of β-Glucuronidase in the Presence of Inhibitors

When inhibitors of endocytosis were included during uptake experiments they were dissolved in PBS at 20 times the required concentration, sterilised by filtration through a 0.45μm millipore filter and 50μl of the inhibitor solution added to each dish in the presence of 1ml of the enzyme in serum free medium. Control plates

were given 1ml enzyme + 50µl PBS. The effect of the inhibitor on the cells in the absence of extracellular β-glucuronidase was monitored by assaying control plates containing 50µl of inhibitor + 1ml serum free medium. The incubation time was lengthened to 24 hours for both fibroblasts and macrophages in order to increase total enzyme uptake.

2.6.4 Alkaline Phosphatase Treatment of 3T3 β-Glucuronidase

After purification on Concanavalin A-Sepharose and mixing in 1M NaCl with Sephadex G200, 5000 units β-glucuronidase were dialysed against 100mM Tris/HCl pH 7.5 containing 1mM $MgCl_2$, and divided into two equal aliquots. One aliquot of the β-glucuronidase was mixed with 95 units of alkaline phosphatase in the Tris buffer, and both portions were then separately dialysed against two 500ml changes of the Tris buffer overnight at 37°C with gentle rocking (Ullrich et al., 1978). Both aliquots of enzyme were then dialysed against 5mM Tris/HCl pH 8.0, and each loaded onto a 4ml column of DEAE-Sephacel. The gels were washed in loading buffer and then eluted with a gradient of 0 - 0.3M NaCl. 2ml fractions were collected and their β-glucuronidase activity determined. The alkaline phosphatase treated β-glucuronidase peak was pooled, and fractions of the control peak were pooled separately. BSA was added to both control and alkaline phosphatase treated fractions to a final concentration of 0.5mg/ml BSA, the solution concentrated by filtration on a Diaflo XM100 A filter, dialysed against serum free medium, filter sterilised and finally added to the recipient macrophages and fibroblasts as described above.

CHAPTER III

RESULTS

3.1 CELL CULTURE

3.1.1 Release of β-Glucuronidase from Different Strains of Fibroblast

A number of different established mouse fibroblast lines and primary mouse fibroblasts were screened in order to determine which types of cell secreted the largest amounts of lysosomal enzymes without artificial stimulation. It was found that 3T3 fibroblasts, a well established non-transformed cell line, secreted more β-glucuronidase per day than CBA mouse embryo fibroblasts, Balb 3T12-3 or A9 fibroblast lines (Figure 2). The average rate of β-glucuronidase release from 3T3 cells was 25units/10^6 fibroblasts/day. For every unit of β-glucuronidase released, 35 units of N-acetylglucosaminidase and 0.8 units of β-galactosidase were also secreted.

3.1.2 Effect of Serum Concentration and Serum Supplements on Release of β-Glucuronidase from 3T3 Fibroblasts

In an attempt to reduce the level of non-enzyme proteins present in cultures of 3T3 fibroblasts, the cells were grown in decreasing concentrations of FCS and in the presence of serum substitutes. 5% (v.v.) FCS was the lowest concentration which would support adequate growth of 3T3 cells and secretion of β-glucuronidase (Figure 3). The effects of donor horse serum, new born calf serum, mouse plasma, bovine serum albumin, cholera toxin and insulin are summarised in Table 2. None of these substitutes was as effective as the FCS itself in maintaining growth of 3T3 cells. The amount of β-glucuronidase secreted per flask was therefore much lower when any of the substitutes was used instead of FCS in the culture medium. FCS was passed through Concanavalin A-Sepharose in order to remove β-glucuronidase and other

Figure 2

SECRETION OF β-GLUCURONIDASE BY MOUSE FIBROBLASTS IN CULTURE

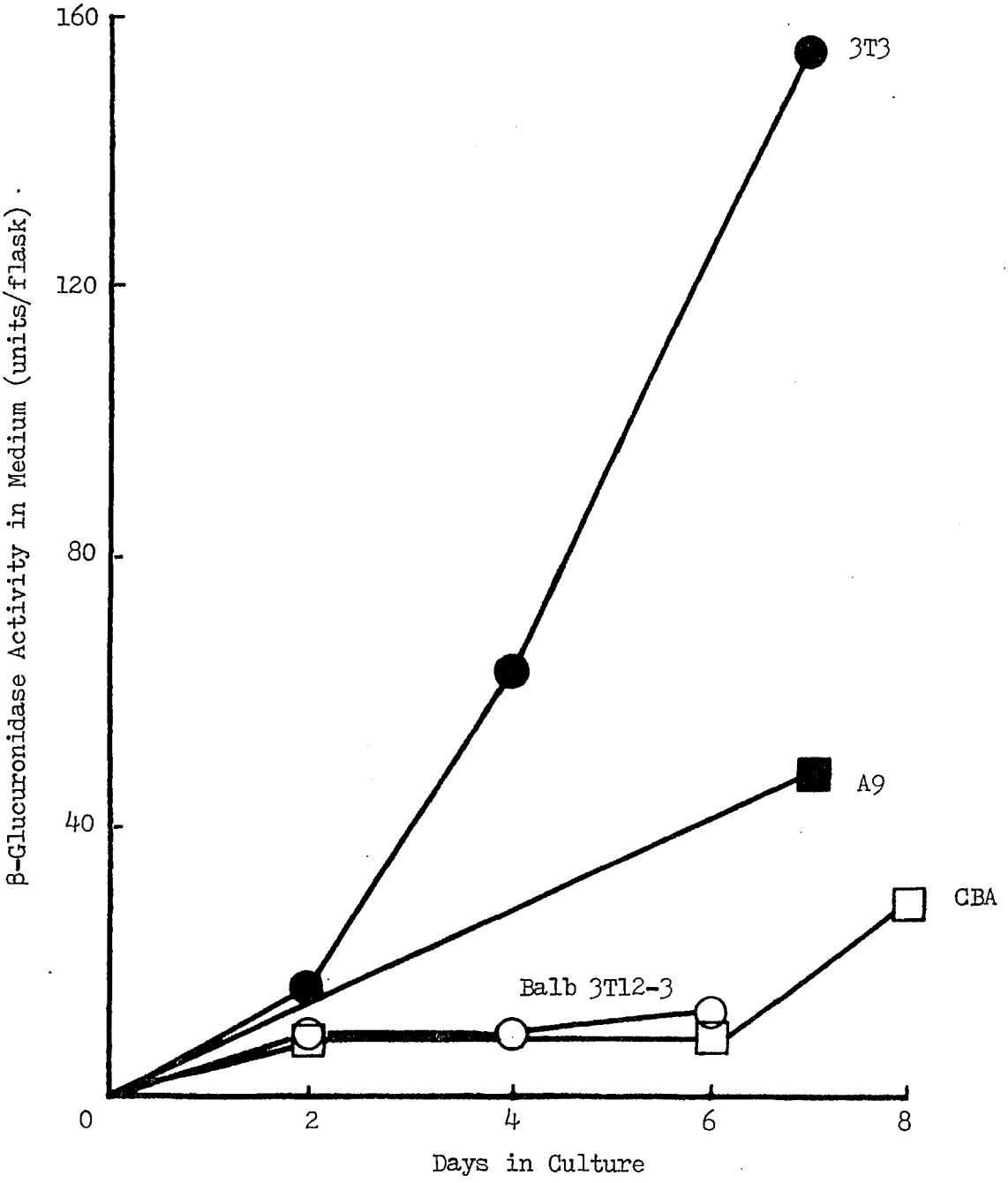


Figure 3

EFFECT OF SERUM CONCENTRATION ON SECRETION OF β-GLUCURONIDASE BY 3T3 FIBROBLASTS

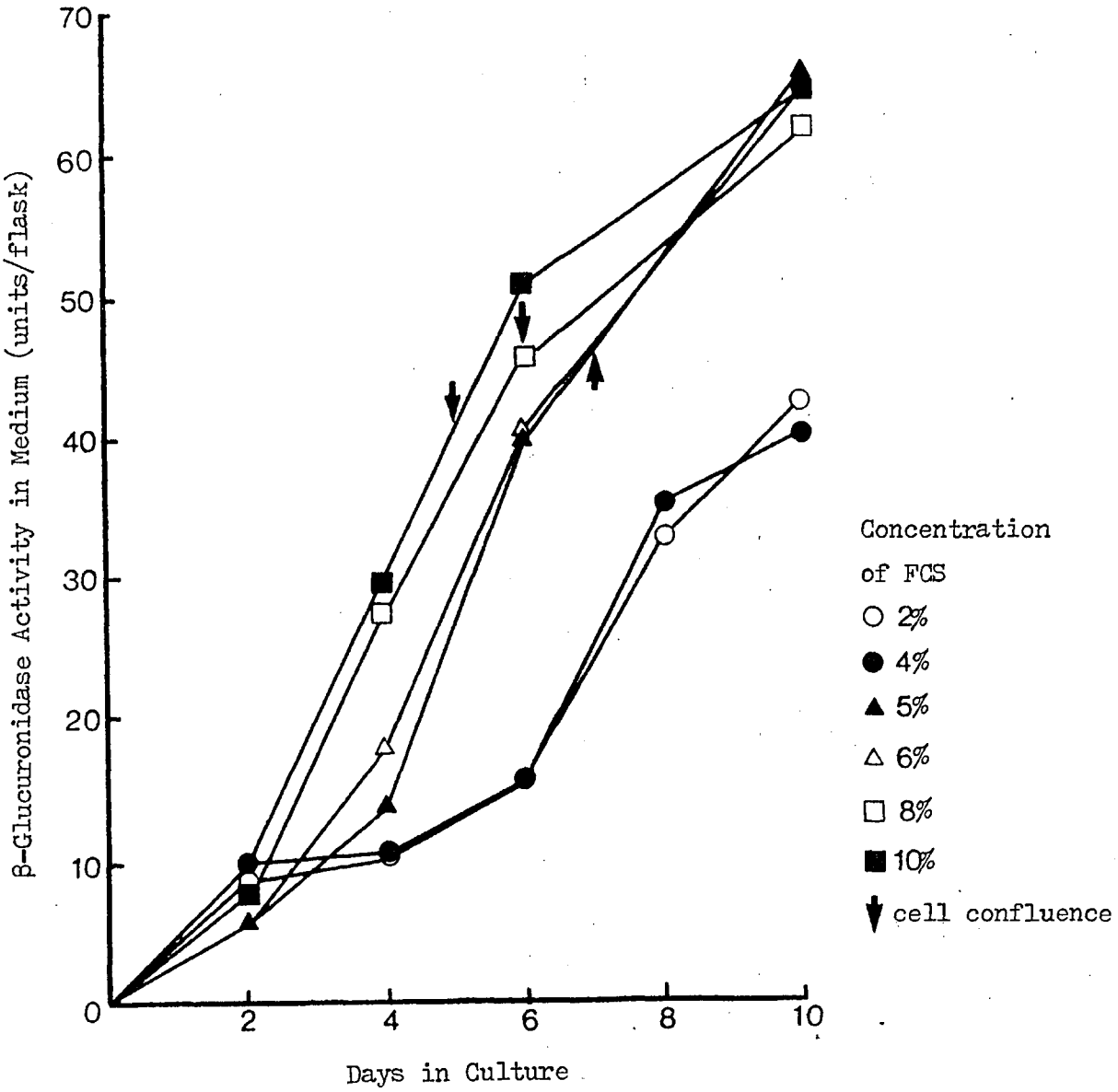


Table 2

THE ABILITY OF SERUM SUBSTITUTES TO SUPPORT GROWTH OF 3T3 FIBROBLASTS

Substitute	Observations
10% donor horse serum	Fibroblasts became full of vacuoles (lipid storage) and did not divide
5% new born calf serum	Growth of fibroblasts slowed
2% mouse plasma (Pruss and Herschman 1979)	Fibroblasts became very granular and division rate slowed
5% FCS after passage through Concanavalin A-Sepharose	Fibroblasts became very granular and stopped dividing
0.2% bovine serum albumin	Fibroblasts did not grow
80mIU insulin/ml	Fibroblasts did not grow
10^{-10}M cholera toxin	Fibroblasts did not grow

mannose containing glycoproteins which might otherwise co-purify with the β-glucuronidase secreted by 3T3 cells. That fraction which did not bind to the affinity gel did not, however, support growth of 3T3 fibroblasts or secretion of β-glucuronidase. The 3T3 fibroblasts would, on the other hand, grow and secrete β-glucuronidase equally well in medium containing 5% (v.v.) FCS or in 5% heat inactivated FCS (Figure 4). 3T3 fibroblasts cultured in 5% (v.v.) FCS are shown in Figure 5: the cells were grown in monolayers (5a) or on cytodex microcarrier beads (5b). In both cases the cells were grown to confluence, trypsinised and reseeded at a split ratio of 1:5. When SV40 transformed 3T3 fibroblasts were grown in culture they divided much faster than the non-transformed cells (Figure 6), and consequently

Figure 4

SECRETION OF β-GLUCURONIDASE BY 3T3 CELLS GROWN IN HEAT INACTIVATED AND NORMAL FOETAL CALF SERUM

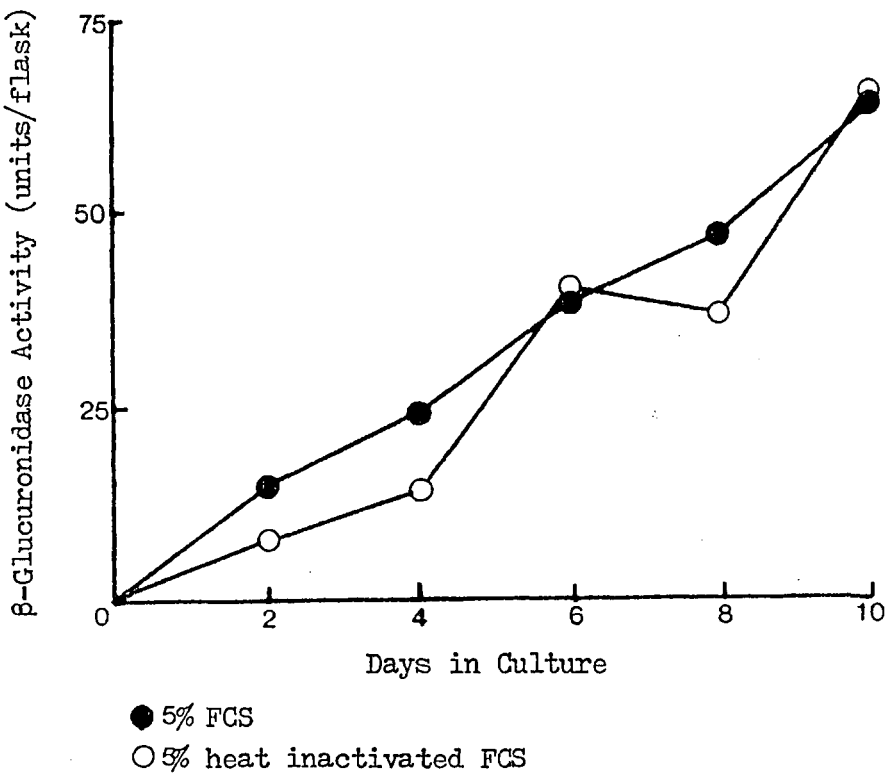
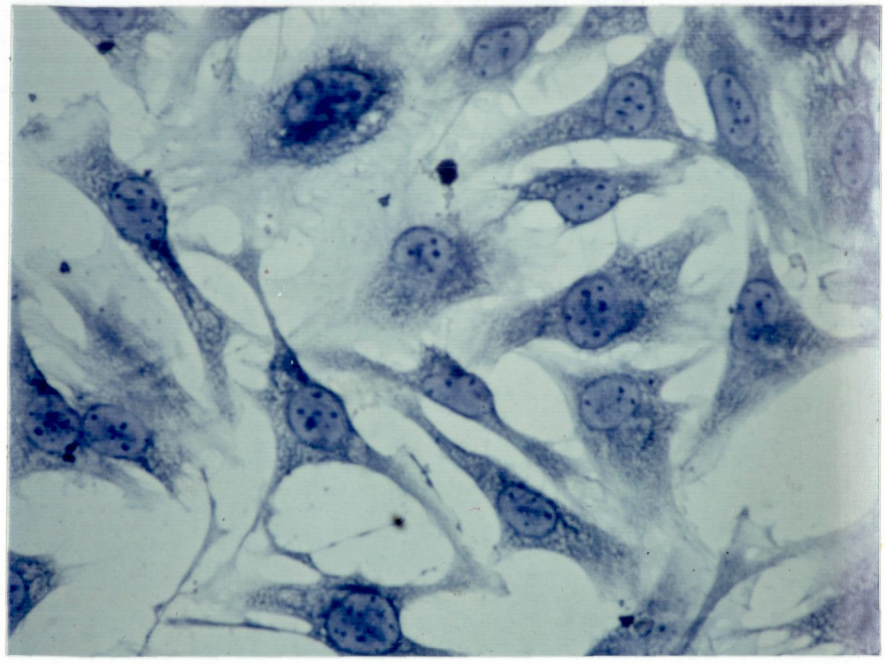


Figure 5

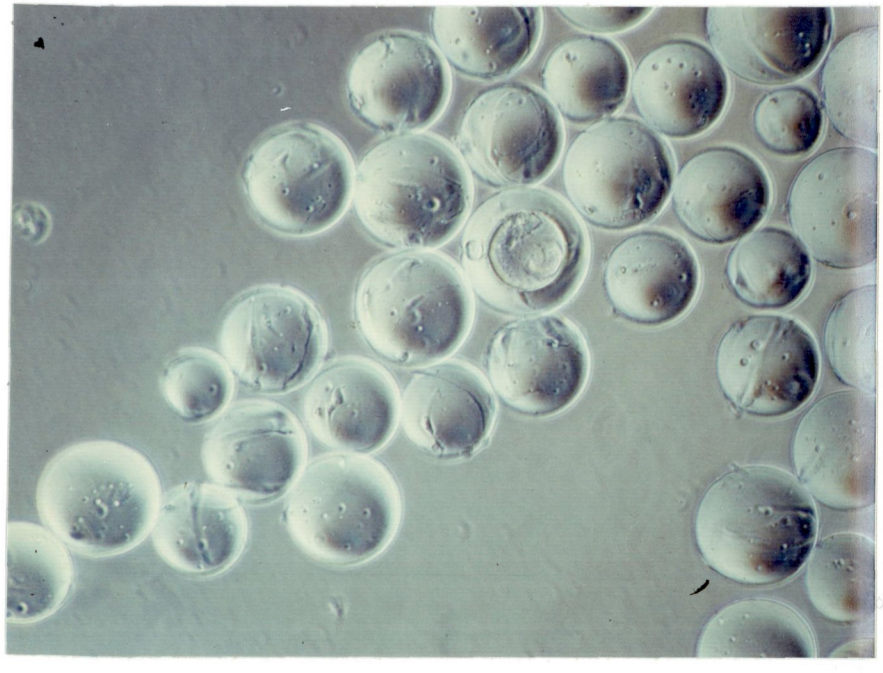
MOUSE 3T3 FIBROBLASTS IN CULTURE

(a) 3T3 Fibroblast Monolayers in Culture



3T3 Fibroblasts were grown to semiconfluence, fixed in ice cold methanol, and stained with 5% methylene blue. 40x magnification.

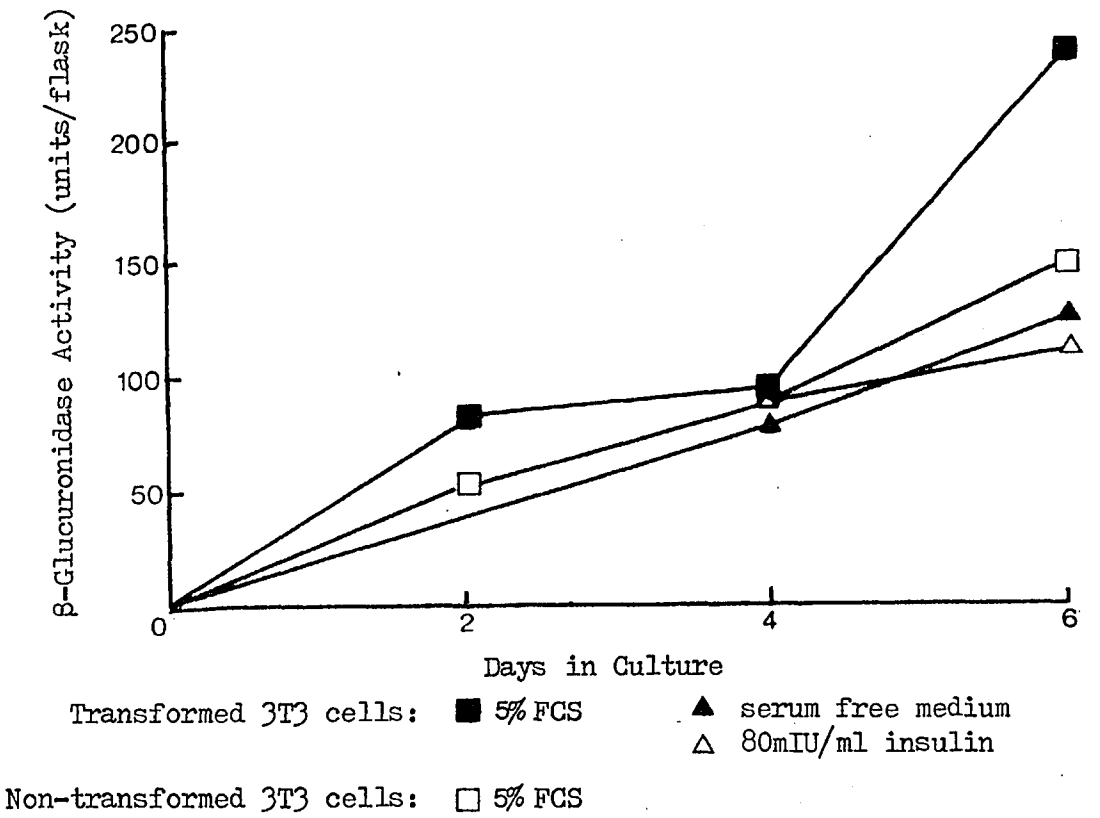
(b) 3T3 Fibroblasts Cultured on Cytodex Microcarriers



Living 3T3 fibroblasts in culture after seeding at low density on microcarriers. 10x magnification.

Figure 6

SECRETION OF β-GLUCURONIDASE BY 3T3 FIBROBLASTS TRANSFORMED WITH SV40 VIRUS



released more β-glucuronidase in a given period of time than non-transformed cells seeded initially at the same density.

3.1.3 β-Glucuronidase Activity of AKR Macrophages in Culture

The specific activity of β-glucuronidase in adherent peritoneal macrophages was determined at different times during their first ten days of culture. The intracellular activity of the harvested exudate cells averaged 168 units/mg cell protein, but after four days in culture the adherent macrophages had an average specific activity of only 53 units/mg (Figure 7). The drop in intracellular activity over the first four days of culture was not due to loss of non-adherent cells since most of these were washed away after the first hour of incubation; moreover, the floating cells had a relatively low specific activity, which averaged 65 units/mg. After four days in culture, the intracellular activity of the β-glucuronidase began to increase gradually, reaching an average of 228 units/mg at day ten. In contrast to the drop in intracellular activity, secretion of enzyme by the peritoneal macrophages increased from 30 units/mg cell protein after one hour in culture to an average of 1205 units/mg after ten days. When macrophages were incubated overnight in serum free medium on day three, prior to uptake experiments on day four, there was a marked drop in their intracellular activity which amounted to almost 50%.

On day four approximately 100% of the adherent peritoneal cells took up complement coated yeast particles (Figure 8a); each cell contained many yeast particles (Figure 8b). These photographs indicate that the cells had cell surface receptors for complement. All of these cells stained positively for non-specific esterase activity, which is a characteristic marker for macrophages (Figure 9), but none of them

Figure 7

β-GLUCURONIDASE ACTIVITY OF AKR MACROPHAGES IN CULTURE

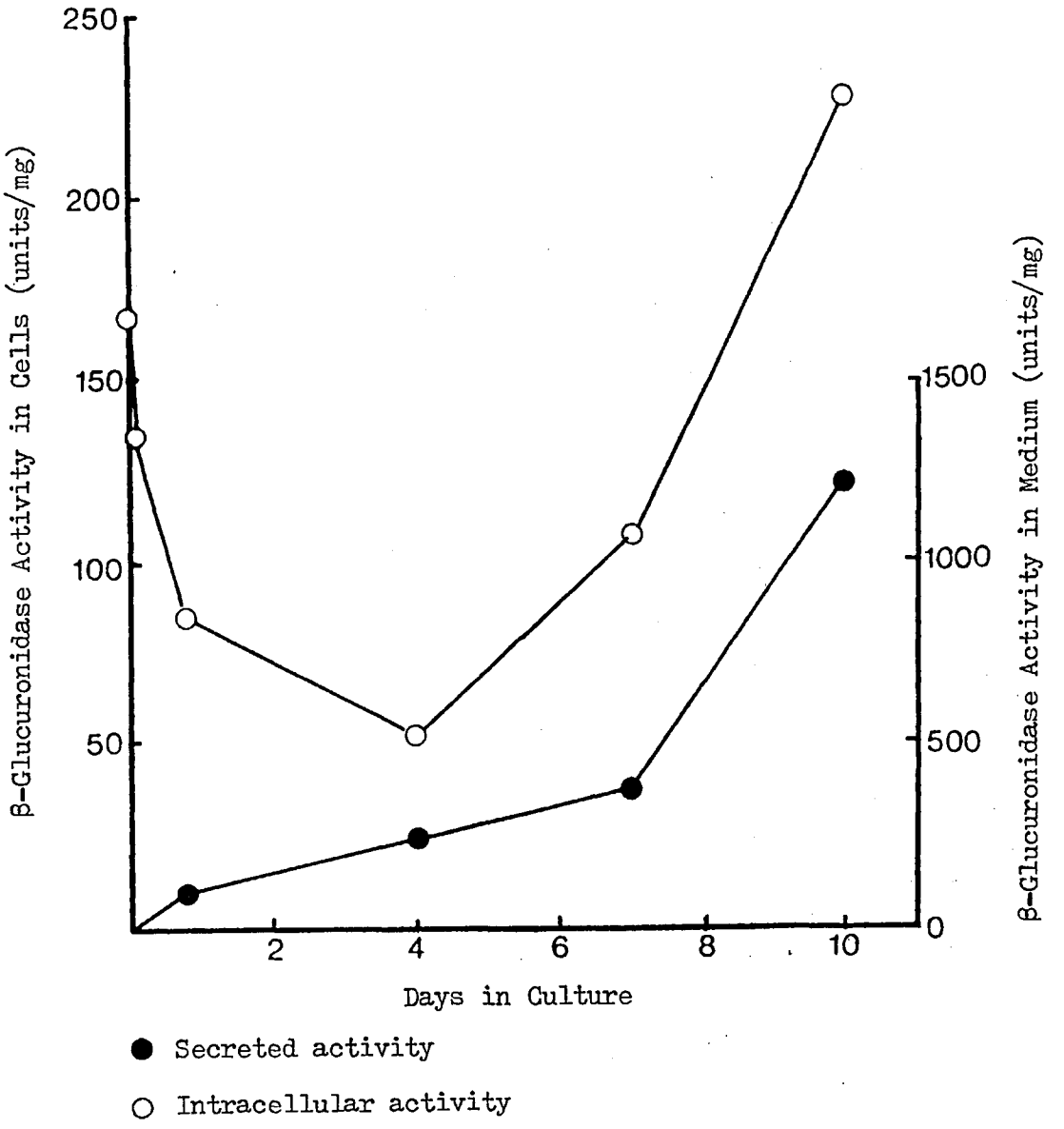
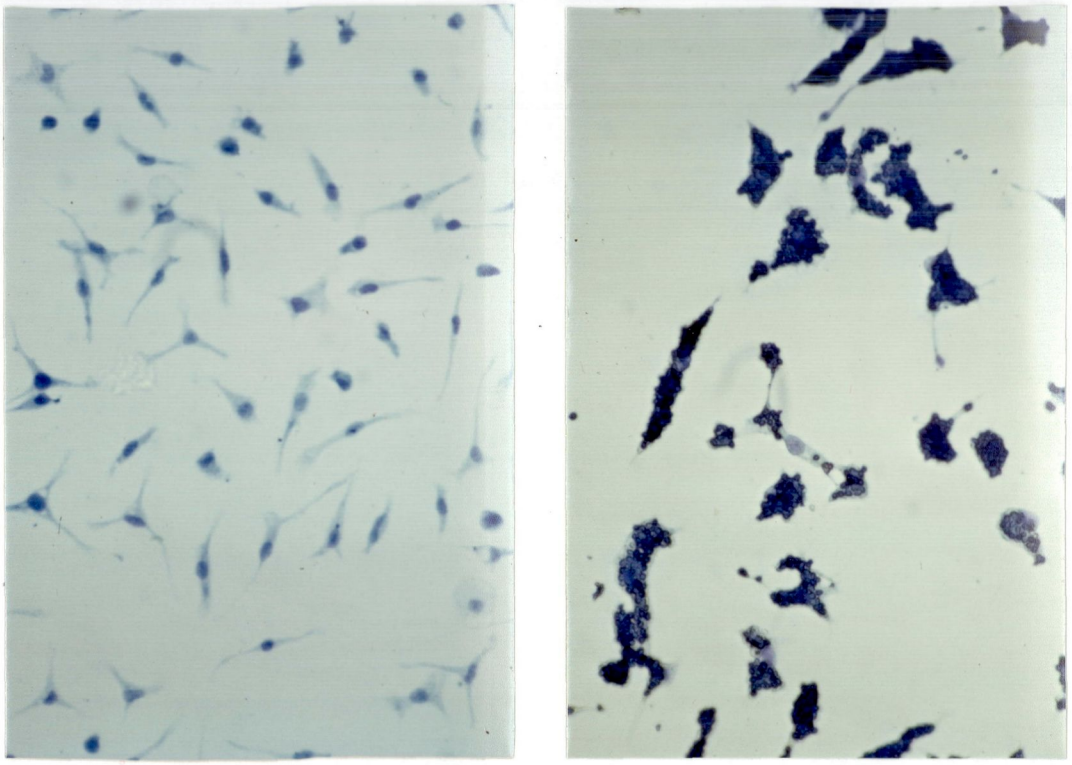


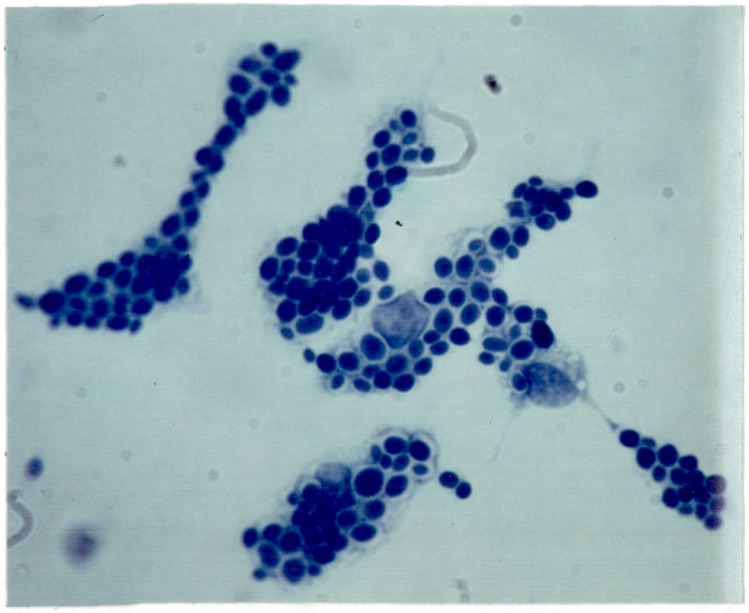
Figure 8

UPTAKE OF COMPLEMENT COATED YEAST PARTICLES BY MOUSE PERITONEAL MACROPHAGES



I II

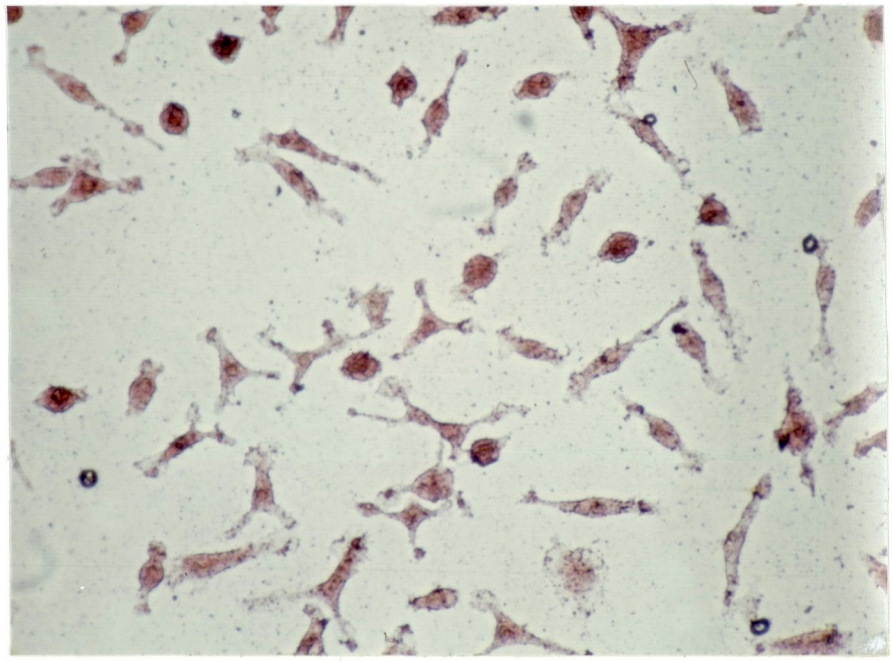
(a) AKR macrophages were fixed with methanol at 4°C and stained with methylene blue either before (I) or after (II) incubation with complement coated yeast. 40x magnification.



(b) AKR macrophages after incubation with complement coated yeast particles. 400x magnification.

Figure 9

NON-SPECIFIC ESTERASE ACTIVITY IN AKR MACROPHAGES



AKR macrophages were stained histochemically for non-specific esterase activity (red stain) and chloroacetate esterase activity (blue stain). 40x magnification.

stained positively for chloroacetate esterase activity indicating that there was no contamination of the cultures by granulocytes.

3.1.4 Heat Stability of β-Glucuronidase from Different Sources

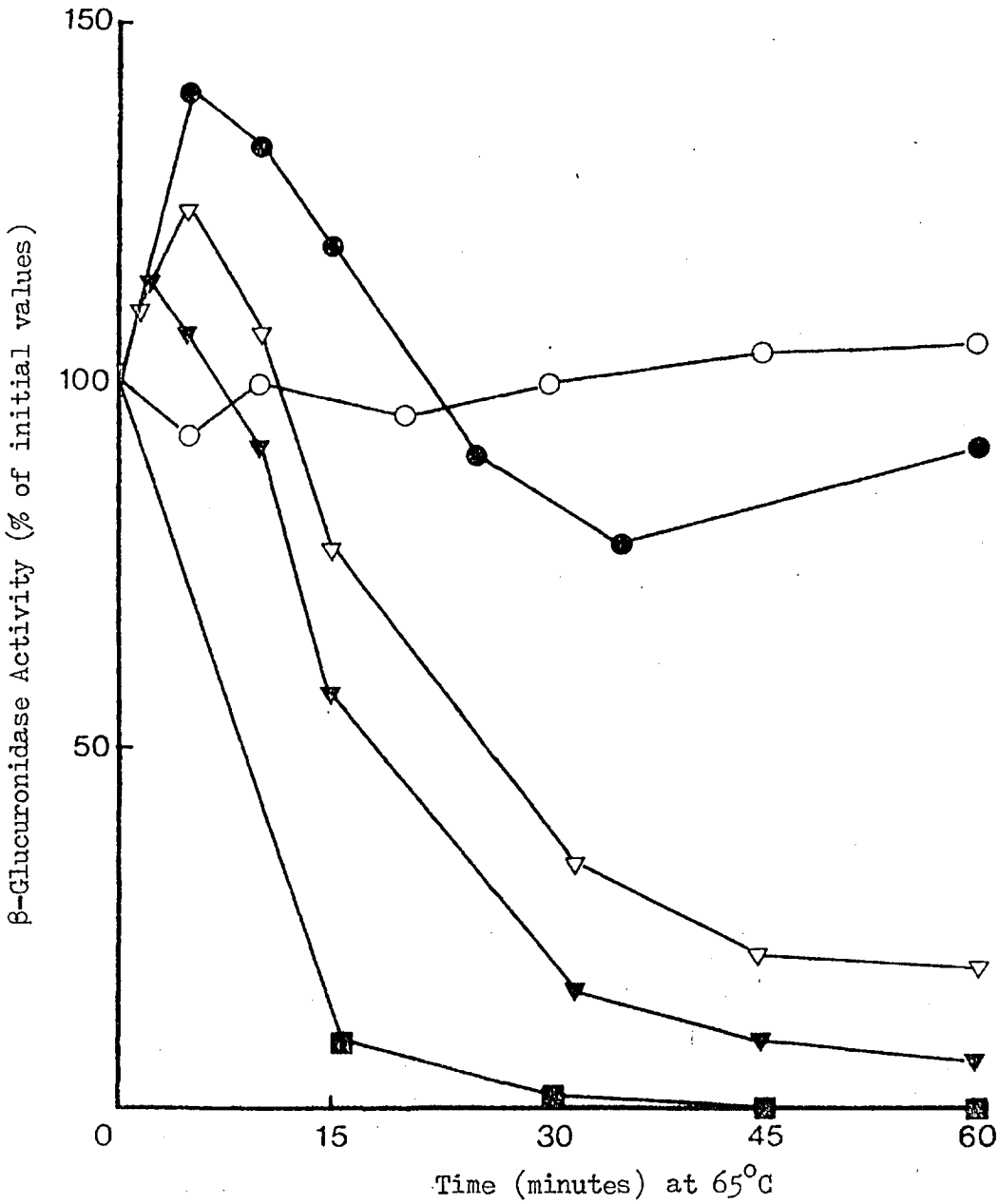
The relative heat stabilities of β-glucuronidase from a number of sources are shown in Figure 10. The β-glucuronidase extracted from the AKR macrophages was heat labile, its specific activity falling to 19% and 6% of the starting values after one hour at 65°C for cells harvested from female and male mice respectively; subsequently cells from both sexes were pooled for experiments. β-Glucuronidase from C3H mouse macrophages (pooled from both sexes) was also heat labile, only 17% of the original activity remaining after heating for one hour at 65°C; this is in agreement with the findings of Ganschow and Paigen (1967). Figure 10 shows that the β-glucuronidase present in FCS was also heat labile, its activity falling to 2% of the original value in only 30 minutes. However, both the intracellular β-glucuronidase extracted from 3T3 cells and that secreted by them retained nearly 100% of original values after one hour at 65°C.

3.1.5 Specific Activity of β-Glucuronidase from Different Strains of Mice

Table 3 shows that the organs of normal mouse strains such as CBA and Balb/c have more β-glucuronidase than the corresponding organs from AKR mice. 3T3 fibroblasts have normal levels of intracellular activity when compared with CBA mouse embryo fibroblasts, and as such were not analogous to fibroblasts from patients with I-cell disease (Mucolipidosis II). A9 fibroblasts also had low levels of β-glucuronidase activity since they were derived originally from C3H mouse embryos

Figure 10

HEAT STABILITY OF β-GLUCURONIDASES FROM DIFFERENT SOURCES



β-Glucuronidase:

- ● intracellular 3T3 fibroblasts
- ▽ intracellular AKR ♀ macrophages
- ▼ intracellular AKR ♂ macrophages
- ■ in FCS
- ○ secreted by 3T3 fibroblasts

(Littlefield, 1964), a strain which has genetically determined low levels of this enzyme (Paigen, 1961).

Table 3

ACTIVITY OF β-GLUCURONIDASE IN MOUSE CELLS FROM DIFFERENT SOURCES

Strain	β-glucuronidase activity (units/mg protein)		
	fibroblasts	liver	spleen
CBA	115.5	111.5	237.5
BALB/C	n.d.	121.5	147.0
3T3	124.3		
BALB 3T12-3	152.2		
A9	33.4		
AKR	44.8	2.7	17.1

n.d. not determined

3.2 PURIFICATION OF β-GLUCURONIDASE

3.2.1 Initial Purification

β-Glucuronidase from 3T3 cells was quite stable when heated for one hour at 65°C (Figure 10), while N-acetylglucosaminidase was almost completely inactivated under the same conditions. Heat treatment of the medium concentrate was therefore carried out prior to ammonium sulphate precipitation to inactivate both FCS β-glucuronidase and most of the N-acetylglucosaminidase secreted by 3T3 cells.

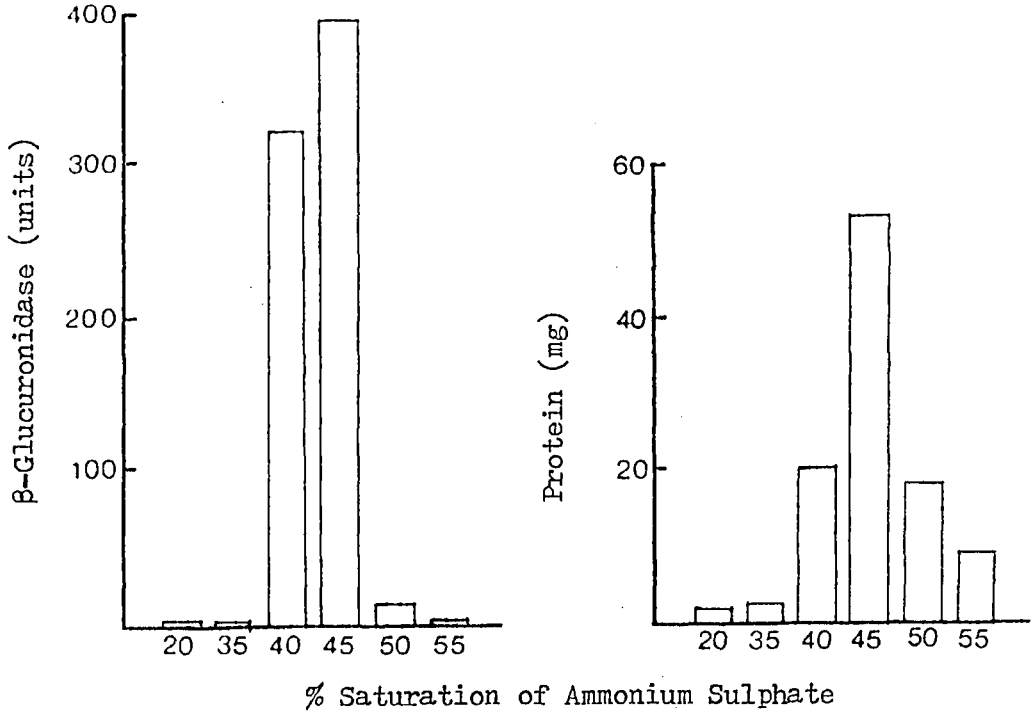
The activities of the β-glucuronidase, β-galactosidase and the remaining N-acetylglucosaminidase secreted by 3T3 fibroblasts, together with the protein contents of fractions precipitated by increasing concentrations of ammonium sulphate are shown in Figure 11. Most of the β-glucuronidase activity precipitated at concentrations between 35% and 45% saturated, and for this reason precipitation in 45% saturated ammonium sulphate was chosen as the initial step in purification of the enzyme. Very little protein precipitated in either the 20% or 35% saturated fractions and these steps were therefore omitted, N-acetylglucosaminidase, β-galactosidase and a large proportion of the remaining protein were left in the 45% supernatant solution, and could be precipitated at higher concentrations of ammonium sulphate. All precipitates were redissolved and dialysed against three changes of one litre of 0.15M NaCl.

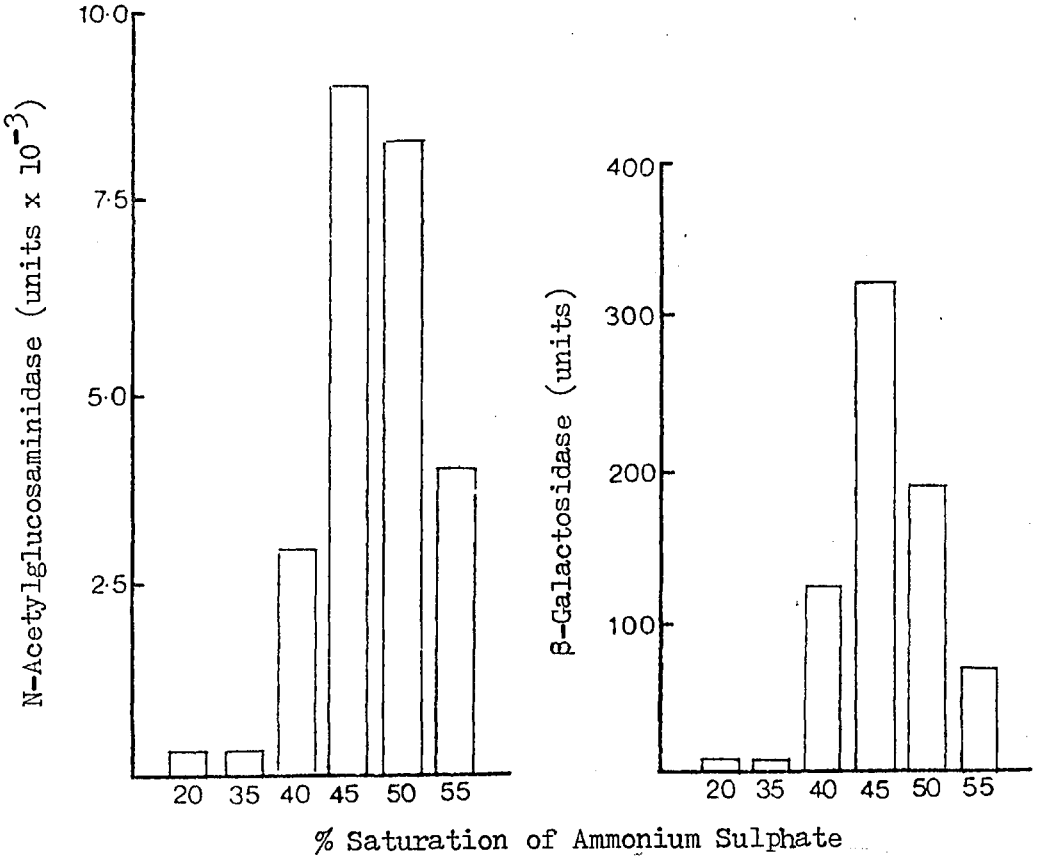
3.2.2 Affinity Chromatography of 3T3 β-Glucuronidase on Concanavalin A-Sepharose

Further purification of β-glucuronidase secreted by 3T3 fibroblasts

Figure 11

PRECIPITATION OF SECRETED LYSOSOMAL ENZYMES WITH INCREASING CONCENTRATIONS OF AMMONIUM SULPHATE





was obtained by loading enzyme which precipitated in ammonium sulphate on Concanavalin A-Sepharose. β-Glucuronidase activity was eluted in varying concentrations of α-methylmannoside and NaCl. The modifications in the eluting conditions that were made in order to obtain a high purification and a high yield of β-glucuronidase activity are described below.

When aliquots containing 200 units of β-glucuronidase secreted by 3T3 fibroblasts were loaded onto 2ml columns of Concanavalin A-Sepharose in sodium acetate buffer at pH 5.5, 99% of the activity bound, and almost 98% of this activity could be eluted with stepwise increasing concentrations of α-methyl-D-mannoside (Figure 12). The fractions which eluted between 0.4M and 1.0M α-methylmannoside contained 95% of the β-glucuronidase originally loaded and only 4.4% of the total protein, resulting in a purification of 26 fold. When 0.5M NaCl was added to both loading and eluting buffers, β-glucuronidase eluted at lower concentrations of α-methylmannoside together with N-acetyl-glucosaminidase (Figure 13) but the purification fell to only 12.3 fold. The purification of β-glucuronidase was improved to 62 fold by first washing the column with 0.1M α-methylmannoside then eluting with 1.5M α-methylmannoside containing 0.5M NaCl; however the recovery of β-glucuronidase was reduced from 98% to only 37% in this step (Figure 14).

The effect of raising the NaCl concentration to 1M in the loading and eluting buffers is shown in Figure 15a. Under these conditions an improved recovery of the β-glucuronidase was obtained (91% of the activity was eluted with 1.5M α-methylmannoside following a wash with 0.02M α-methylmannoside) but the purification factor of 12 was lower than that achieved in the presence of 0.5M NaCl.

Figure 12

ELUTION OF 3T3 β-GLUCURONIDASE FROM CONCANAVALIN A-SEPHAROSE WITH α-METHYLMANNOSIDE

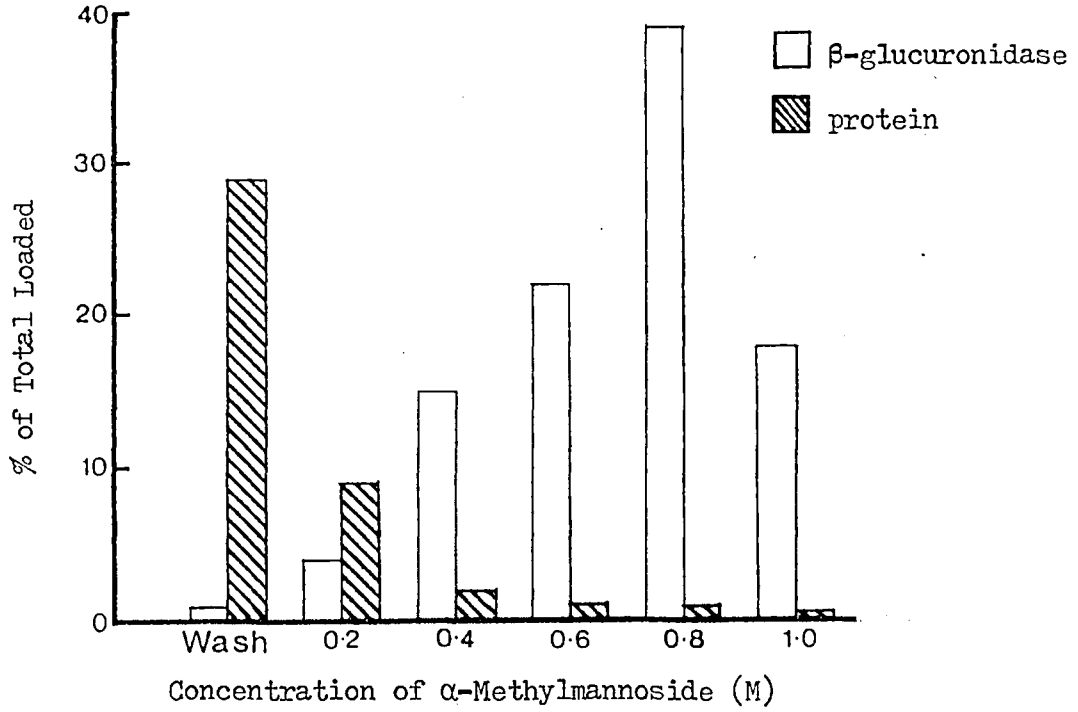


Figure 13

ELUTION OF β-GLUCURONIDASE AND N-ACETYLGLUCOSAMINIDASE FROM CONCANAVALIN A-SEPHAROSE IN α-METHYLMANNOSIDE AND 0.5M NaCl

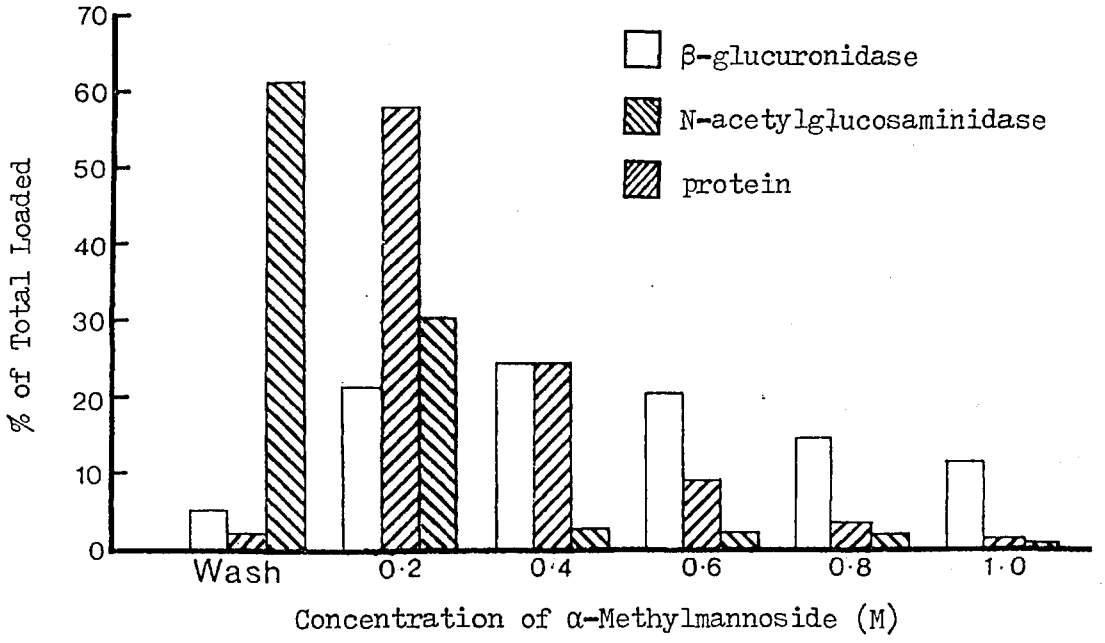


Figure 14

ELUTION OF β-GLUCURONIDASE FROM CONCANAVALIN A-SEPHAROSE IN BUFFERS CONTAINING 0.5M NaCl AND α-METHYLMANNOSIDE

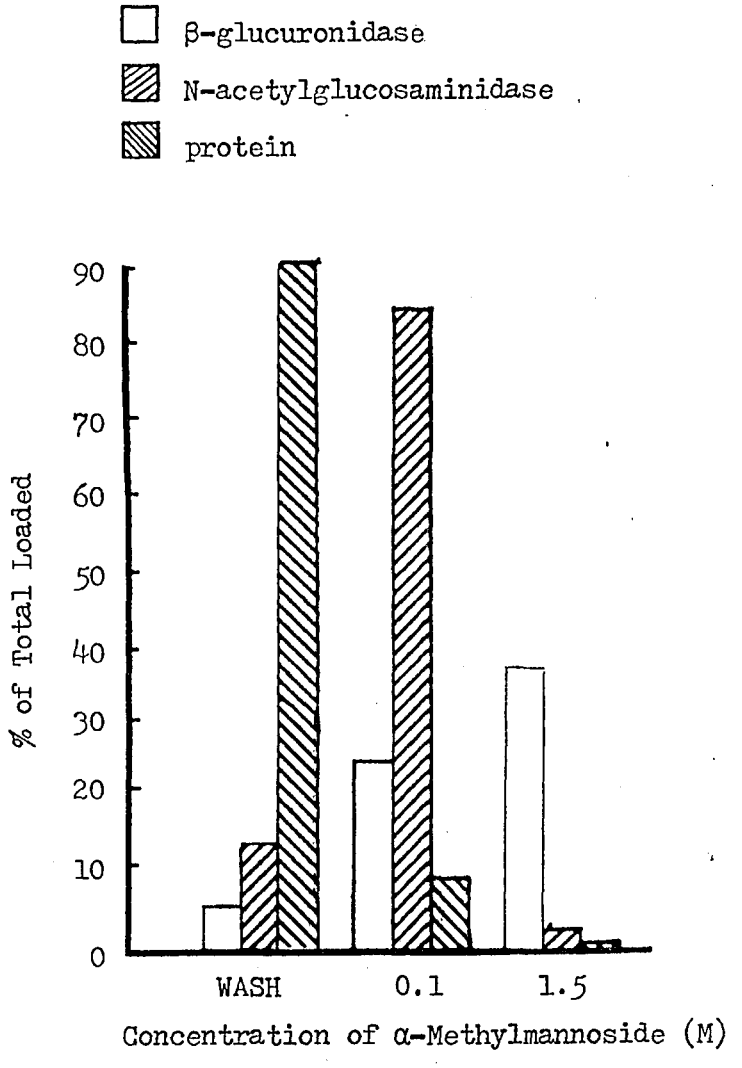
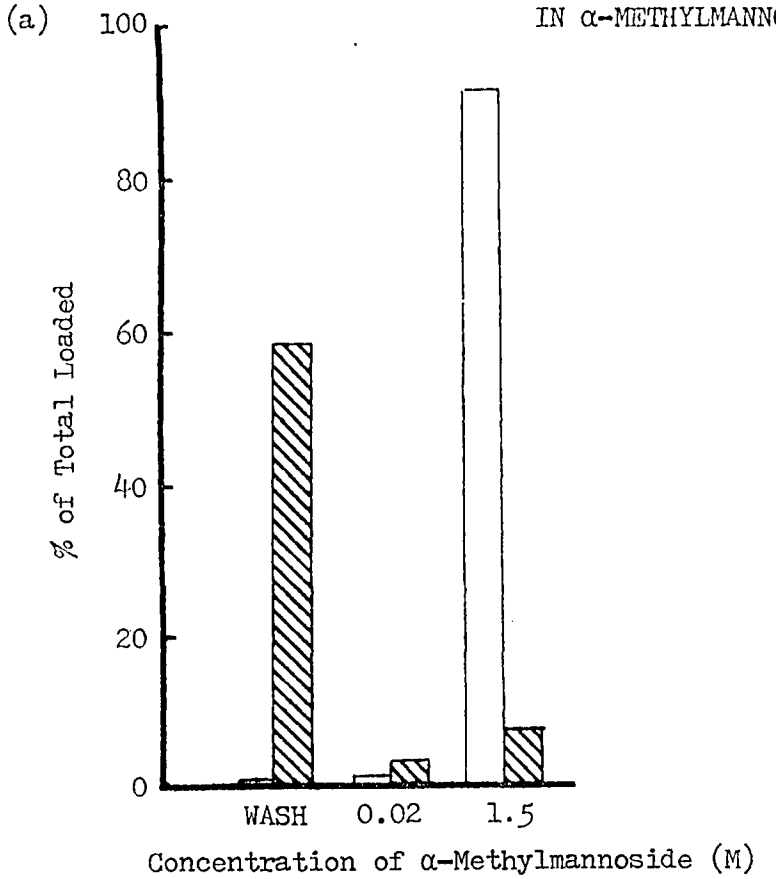
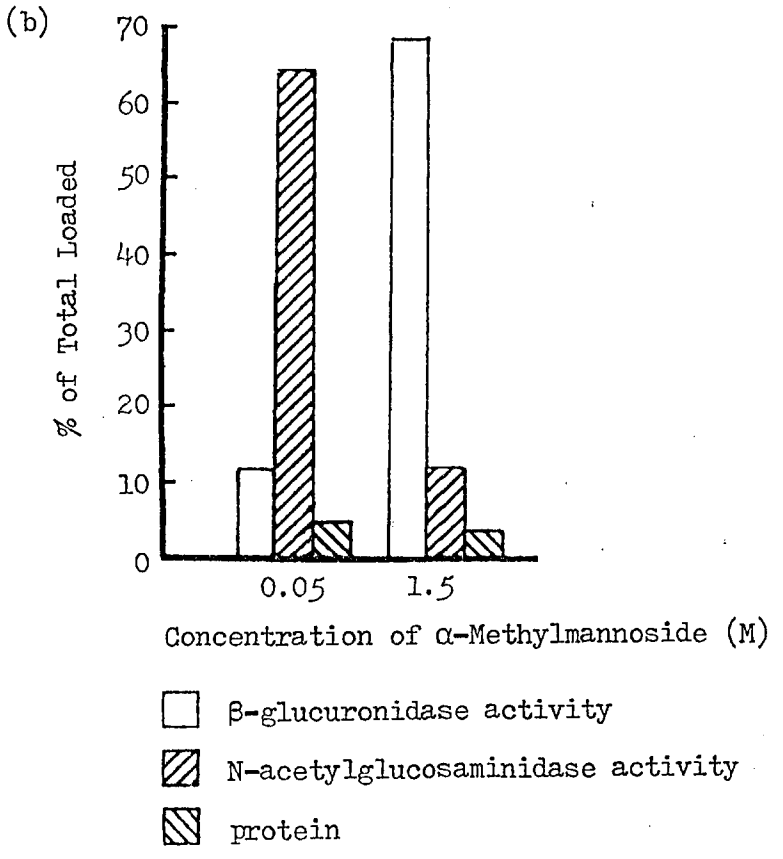


Figure 15 ELUTION OF β-GLUCURONIDASE FROM CONCANAVALIN A-SEPHAROSE IN α-METHYLMANNOSIDE AND 1M NaCl





By altering the concentrations of both α-methylmannoside and NaCl in the eluting buffers, 68% of the β-glucuronidase that was initially loaded could be eluted from the Concanavalin A-Sepharose together with only 3% of the protein. Subsequently β-glucuronidase was routinely eluted in acetate buffer containing 1.5M α-methylmannoside and 1M NaCl, and a purification of 32 fold was obtained (Figure 15b).

3.2.3 Chromatography of 3T3 β-Glucuronidase on DEAE-Sephacel

When 3T3 fibroblast β-glucuronidase was subjected to chromatography on DEAE-Sephacel a small purification (1.7 fold) resulted, with β-glucuronidase eluting in a broad peak between 0.1M and 0.2M NaCl (Figure 16). The N-acetylglucosaminidase secreted by 3T3 cells was slightly less acidic, but could not be entirely separated from the β-glucuronidase by this method. When β-glucuronidase was treated with alkaline phosphatase prior to loading on DEAE-Sephacel, the resulting elution profile was less spread than that of control untreated preparations, and the enzyme was eluted with a lower concentration of NaCl (Figure 17). All of the alkaline phosphatase treated preparations were eluted in 0.15M NaCl, whereas 0.18M NaCl was required to elute the control untreated preparations of β-glucuronidase.

Table 4 shows the degree of purification attained for a typical preparation of β-glucuronidase secreted by 3T3 fibroblasts grown in 5% (v.v.) FCS in Medium 199.

Figure 16

CHROMATOGRAPHY OF β-GLUCURONIDASE AND N-ACETYLGLUCOSAMINIDASE SECRETED BY 3T3 FIBROBLASTS ON DEAE-SEPHACEL

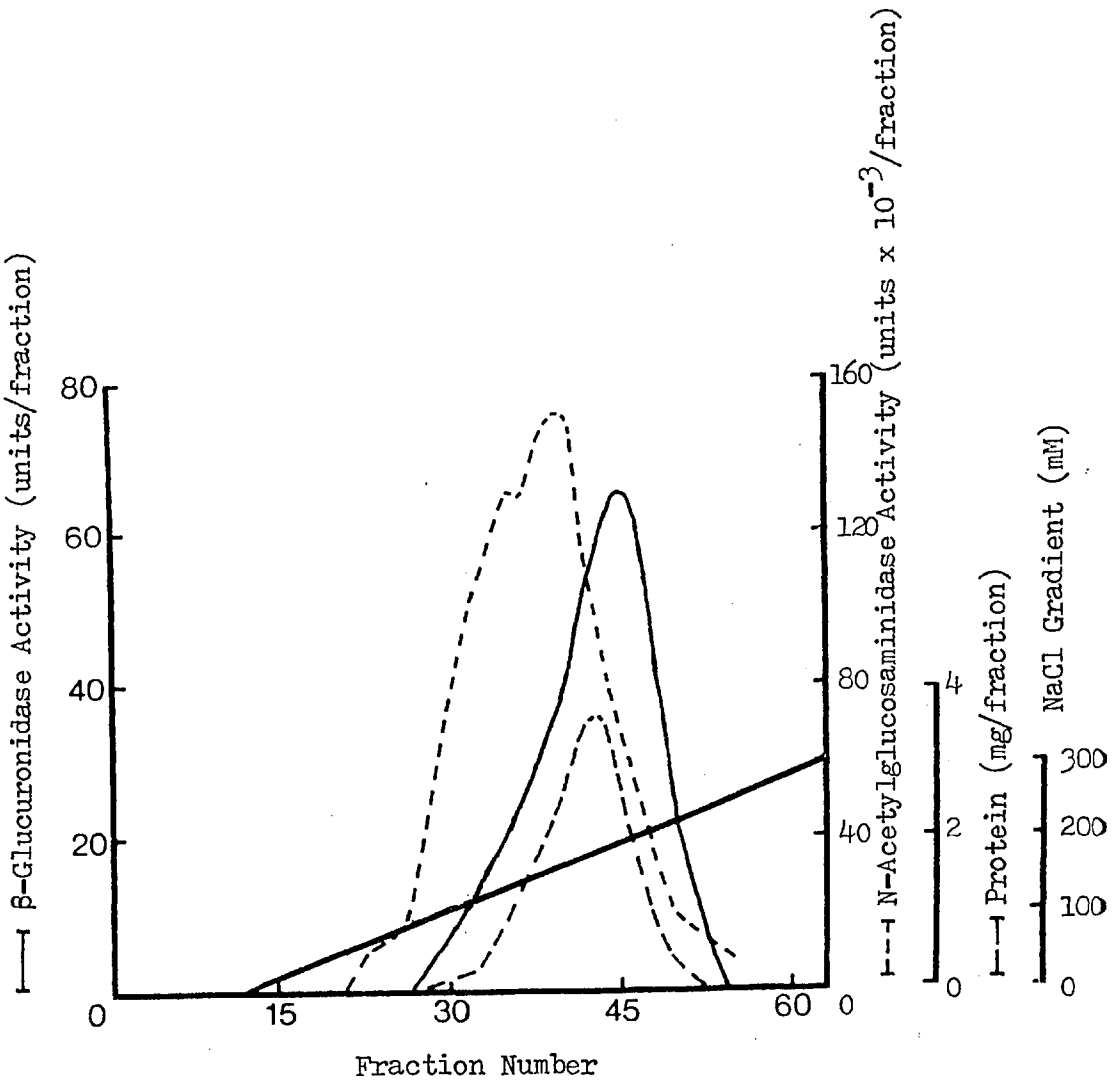
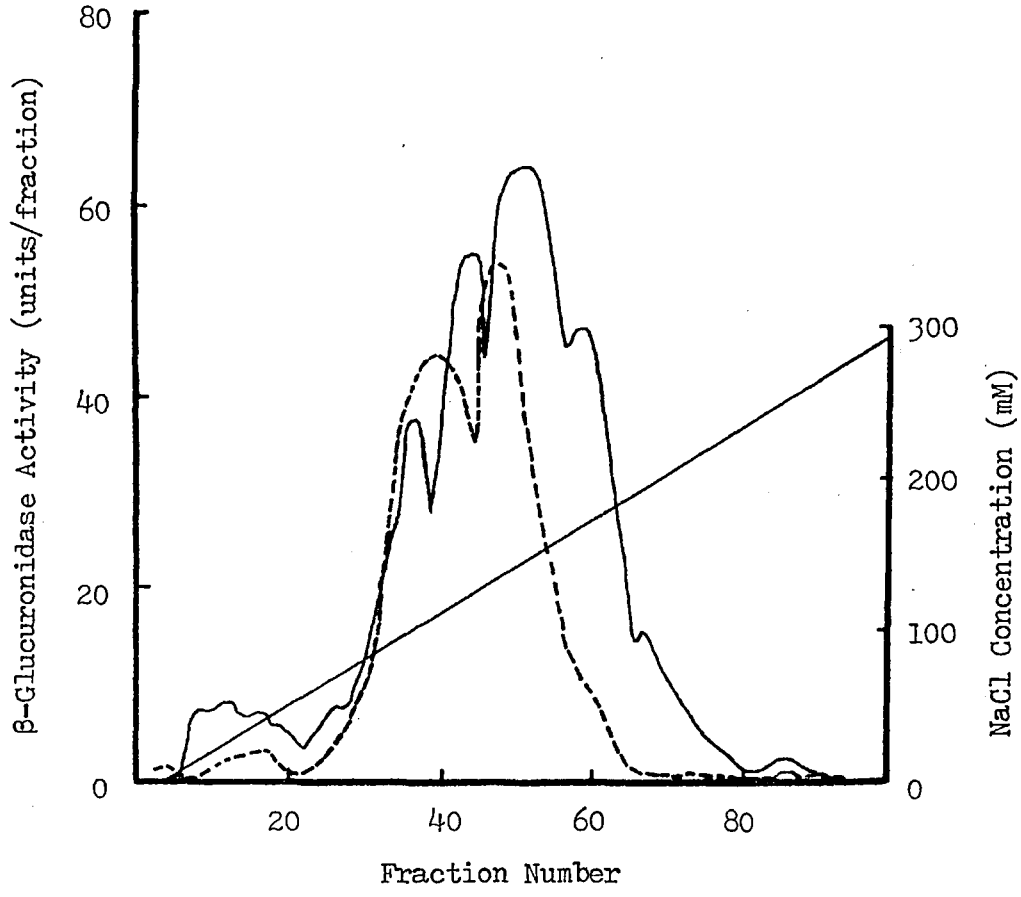


Figure 17

EFFECT OF TREATMENT WITH ALKALINE PHOSPHATASE ON ELUTION OF 3T3 β-GLUCURONIDASE FROM DEAE-SEPHACEL



untreated β-glucuronidase

β-glucuronidase after incubation with alkaline phosphatase

Table 4

PURIFICATION OF β-GLUCURONIDASE SECRETED BY 3T3 FIBROBLASTS

Stage of purification	Activity units	Protein mg	Specific activity	Yield %	Purification factor
Original medium	14,300	4,020	3.6	100	1
Concentrate	12,150	1,940	6.3	85	1.8
Heat treated concentrate	11,190	1,625	6.9	78	1.9
$(NH_4)_2\ SO_4$ ppt	11,830	1,050	11.3	83	3.2
Con A-Sepharose eluate	10,830	23.3	464.8	76	130.7
DEAE-Sephacel eluate	5,594	6.0	924.5	39	256.8

3.2.4 Substrate-specific Affinity Chromatography of β-Glucuronidase

(a) Binding capacity of CNBr activated Sepharose for p-aminophenyl-D-glucuronide

The binding of p-aminophenyl-D-glucuronide to CNBr activated Sepharose 4B (purchased from Pharmacia Fine Chemicals Ltd.) was compared with binding to Sepharose 4B activated by the method of Cuatrecasas (1970).

Table 5

BINDING OF p-AMINOPHENYLGLUCURONIDE TO CNBr ACTIVATED SEPHAROSE 4B

	Amount of ligand in coupling solution (μM)		
	before coupling	after coupling	bound
1ml preactivated Sepharose (Pharmacia)	57	22	35
1ml activated Sepharose (Cuatrecasas, 1970)	52	31	21

Table 5 shows that although the commercial preparation bound more ligand than the laboratory activated Sepharose, both gels bound more than 20μM of ligand per ml of gel. In calculating the theoretical binding of the gel to β-glucuronidase, it was assumed that one molecule of enzyme would bind to one molecule of ligand, and the molecular

weight of β-glucuronidase was assumed to be 280,000 Daltons (Tulsiani et al., 1978).

Calculation:

The binding capacity of 1ml of the laboratory activated gel

= 21μM p-aminophenylglucuronide

= 21μM β-glucuronidase

= $21 \times 10^{-6} \times 280{,}000$g β-glucuronidase

= 5.9g β-glucuronidase

The capacity of the commercial gel, calculated in the same way, was 9.8g of β-glucuronidase per ml of gel and commercial gels were used for all subsequent experiments.

(b) Affinity chromatography of bovine liver β-glucuronidase

Table 6 shows that more than 99% of bovine β-glucuronidase loaded onto the gel bound to p-aminophenylglucuronide-Sepharose 4B, but that only 56% of the activity bound could be eluted with 0.1M acetic acid using the method described by Harris et al. (1973). The degree of purification attained was only 1.1 fold indicating that non-specific binding of other proteins was occurring and that these were being eluted together with the β-glucuronidase.

A 3ml column of commercially activated Sepharose 4B coupled to p-aminophenyl β-D-glucuronide was used. The gel was washed with 20ml of 2mM Tris/5mM NaCl buffer pH 7.3, followed by 25ml of 0.1M acetic acid.

Table 6

AFFINITY CHROMATOGRAPHY OF BOVINE LIVER β-GLUCURONIDASE ON SEPHAROSE 4B-p-AMINOPHENYLGLUCURONIDE

	Total activity units x 10^3	Total protein mg	Specific activity units x 10^3/mg
Enzyme loaded	79.8	2.2	36.2
Buffer wash	0.2	0.4	0.5
Enzyme eluted 0.1M acetic acid	44.5	1.1	40.5

(c) Affinity chromatography of CBA mouse liver β-glucuronidase

The binding specificity of the method of purification described by Harris et al. (1973) was assessed further using a crude preparation of CBA mouse liver β-glucuronidase (for details see section 2.3.12). The activities of β-glucuronidase, β-galactosidase and N-acetylglucosaminidase present in this extract, and the activities of the enzymes eluted in several different buffers from a column of Sepharose 4B-p-aminophenyl-glucuronide are summarised in Table 7. All of the three enzymes bound to the gel but none of them was completely eluted by any of the buffers used. The most effective buffer for purification of β-glucuronidase was in 30mM NaCl, 2mM Tris/HCl pH 8.0, which gave a recovery of only 16% and a purification of 1.4 fold, but which eluted much less of either N-acetylglucosaminidase or β-galactosidase. When the column was eluted with a gradient of acetic acid (0 to 0.2M) all of the enzymes co-eluted in 0.1M acetic acid.

Table 7

BINDING AND ELUTION OF MOUSE CBA LIVER ENZYMES TO p-AMINOPHENYL-GLUCURONIDE-SEPHAROSE

	Total units of enzyme activity			Protein (mg)
	β-glucuronidase	β-galactosidase	N-acetyl-glucosaminidase	
LOADED	5700	940	11600	22.4
WASH I	60	0	390	11.2
WASH II	634	416	77	5.6
WASH III	928	50	28	2.6
GRADIENT IV	493	22	1157	1.0

LOADING BUFFER + WASH I = 2mM Tris/HCl 5mM NaCl pH 7.3

WASH II = 0.1M sodium acetate 150mM NaCl pH 4.5

WASH III = 2mM Tris/HCl 30mM NaCl pH 8.0

GRADIENT IV = 0 - 0.2M acetic acid

(d) Binding of bovine liver β-glucuronidase to saccharolactone-diaminodipropylamine-Sepharose 4B

Saccharolactone (saccharic acid 1,4-lactone) was chosen as an affinity ligand for β-glucuronidase purification because it is a potent competitive inhibitor of the enzyme (Levvy et al., 1952). In order to reduce the effects of steric hindrance which might prevent binding of a large molecule such as β-glucuronidase to the saccharolactone, a spacer arm, diaminodipropylamine, was included between the gel and the sugar ligand, as described by Harris et al. (1973). As a control, a gel with only a spacer arm, but no sugar ligand, was treated in the same manner. The preparation of bovine liver β-glucuronidase (Sigma) used for these experiments contained six times as much N-acetylglucosaminidase as β-glucuronidase and small amounts of β-galactosidase (Table 8). All of these enzymes were bound and eluted in 0.1M acetic acid in a completely non-specific manner, and the degree of purification achieved was thus very low. Furthermore the amount of β-glucuronidase activity bound to and eluted from the control column was similar to that observed with the gel containing saccharolactone indicating that binding of the enzyme to the specific ligand was minimal.

Elution of β-glucuronidase activity from the saccharolactone-diaminodipropylamine-Sepharose 4B was attempted using gradients of sodium chloride (Figure 18), ethylene glycol (Figure 19), or a combination of a sodium chloride gradient run in 15% ethylene glycol (Figure 20) all in Tris/HCl pH 8.0, but none of these procedures succeeded in separating β-glucuronidase from N-acetylglucosaminidase, or other contaminating proteins.

Table 8

BINDING OF BOVINE LIVER β-GLUCURONIDASE TO (A) SEPHAROSE 4B-DIAMINODI-PROPYLAMINE-SACCHAROLACTONE AND (B) SEPHAROSE 4B-DIAMINODIPROPYLAMINE

	β-glucuronidase	β-galactosidase	N-acetyl-glucosaminidase	protein mg
(a)				
Units of activity loaded	48100	2400	293100	0.800
Units eluted in buffer	200	0	7500	0.095
Units eluted in 0.1M acetic acid	11500	200	180900	0.288
	purification of β-glucuronidase = 0.7 fold			
(b)				
Units of activity loaded	48100	2400	293100	0.800
Units eluted in buffer	600	0	15000	0.138
Units eluted in 0.1M acetic acid	27100	2650	227800	0.318
	purification of β-glucuronidase = 1.4 fold			

Figure 18

AFFINITY CHROMATOGRAPHY OF BOVINE LIVER β-GLUCURONIDASE ON SACCHAROLACTONE-DIAMINODIPROPYLAMINE-SEPHAROSE 4B: ELUTION IN A GRADIENT OF SODIUM CHLORIDE

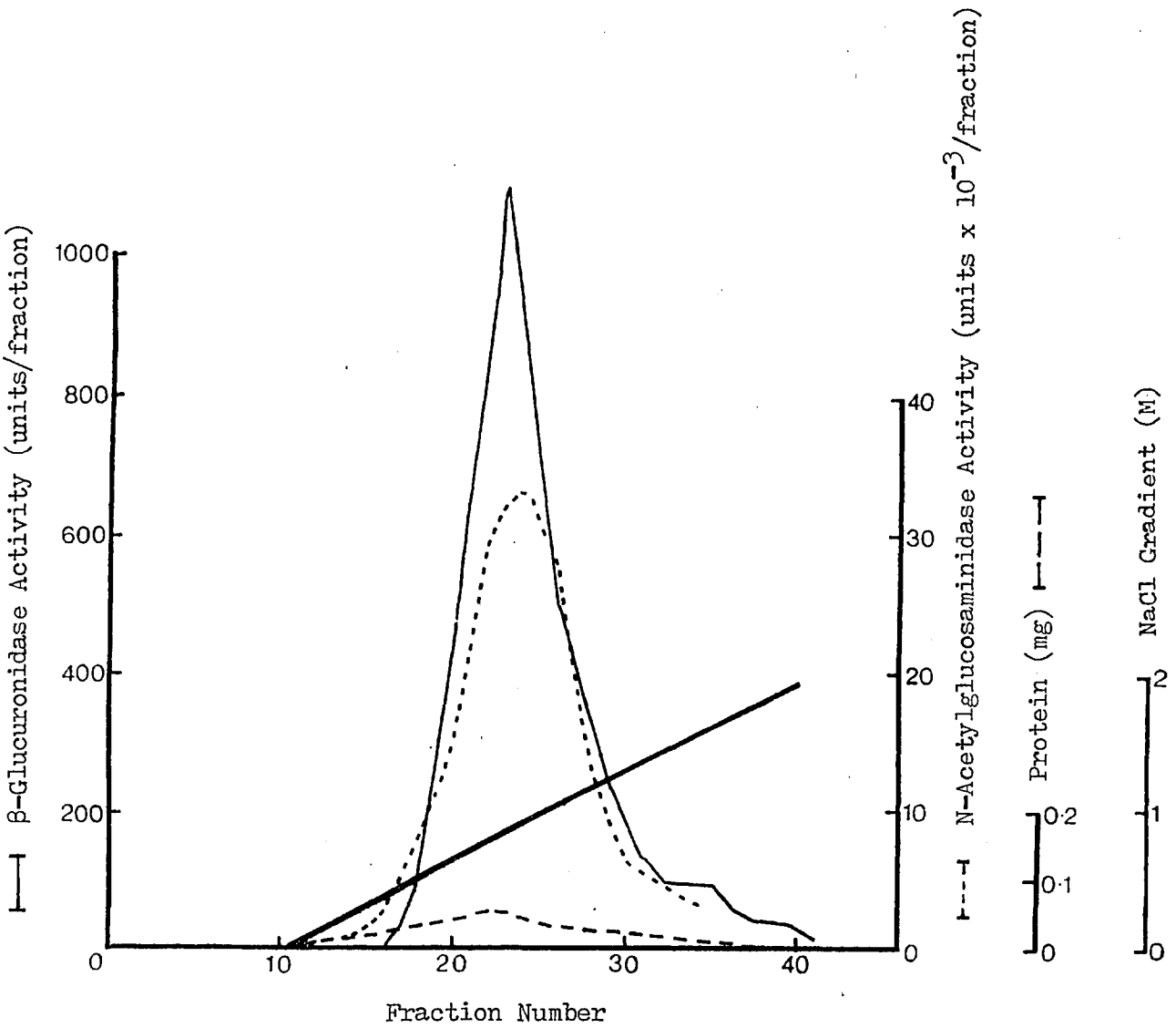


Figure 19

AFFINITY CHROMATOGRAPHY OF BOVINE LIVER β-GLUCURONIDASE ON SACCHAROLACTONE-DIAMINODIPROPYLAMINE-SEPHAROSE 4B: ELUTION IN A GRADIENT OF ETHYLENE GLYCOL

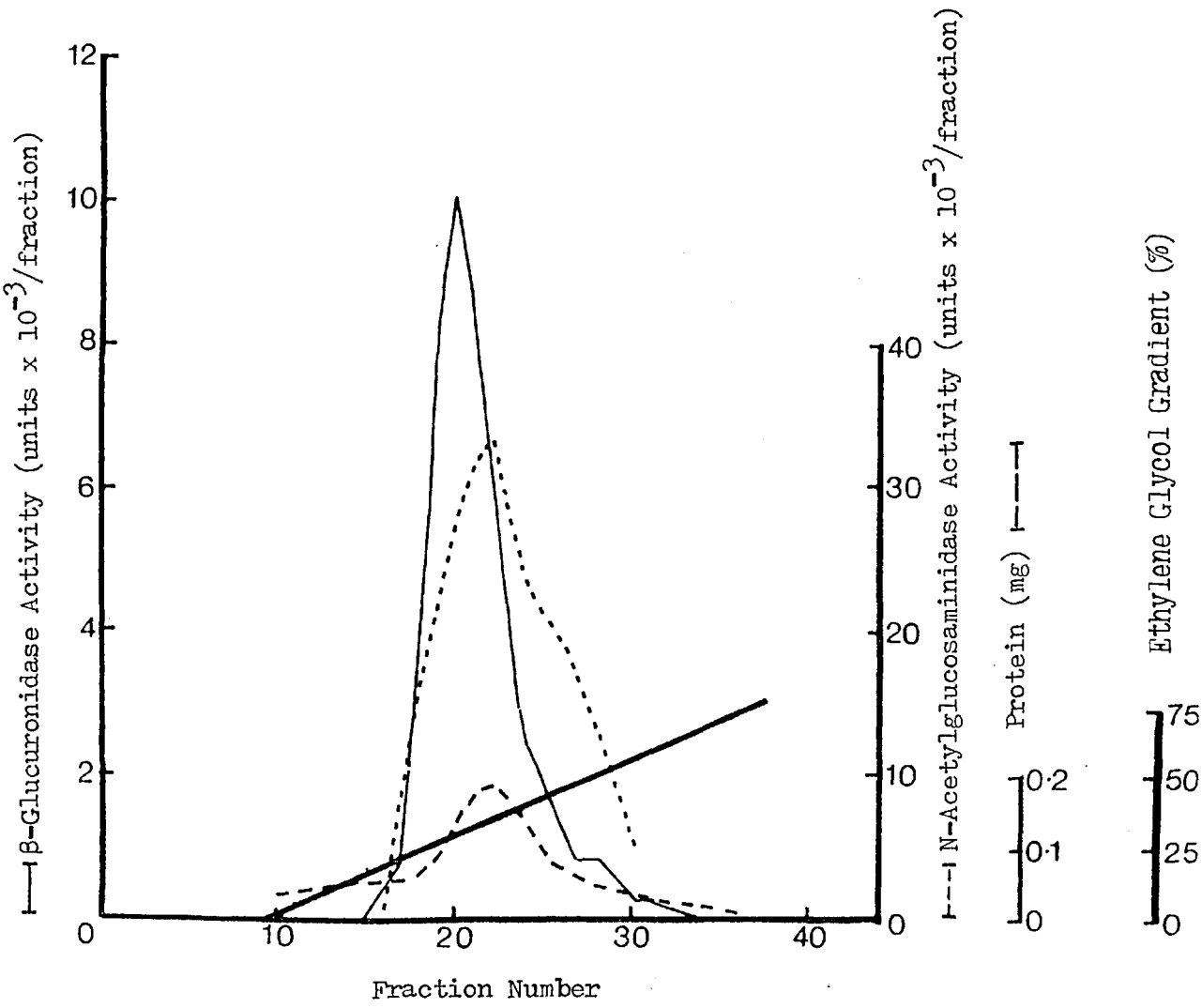
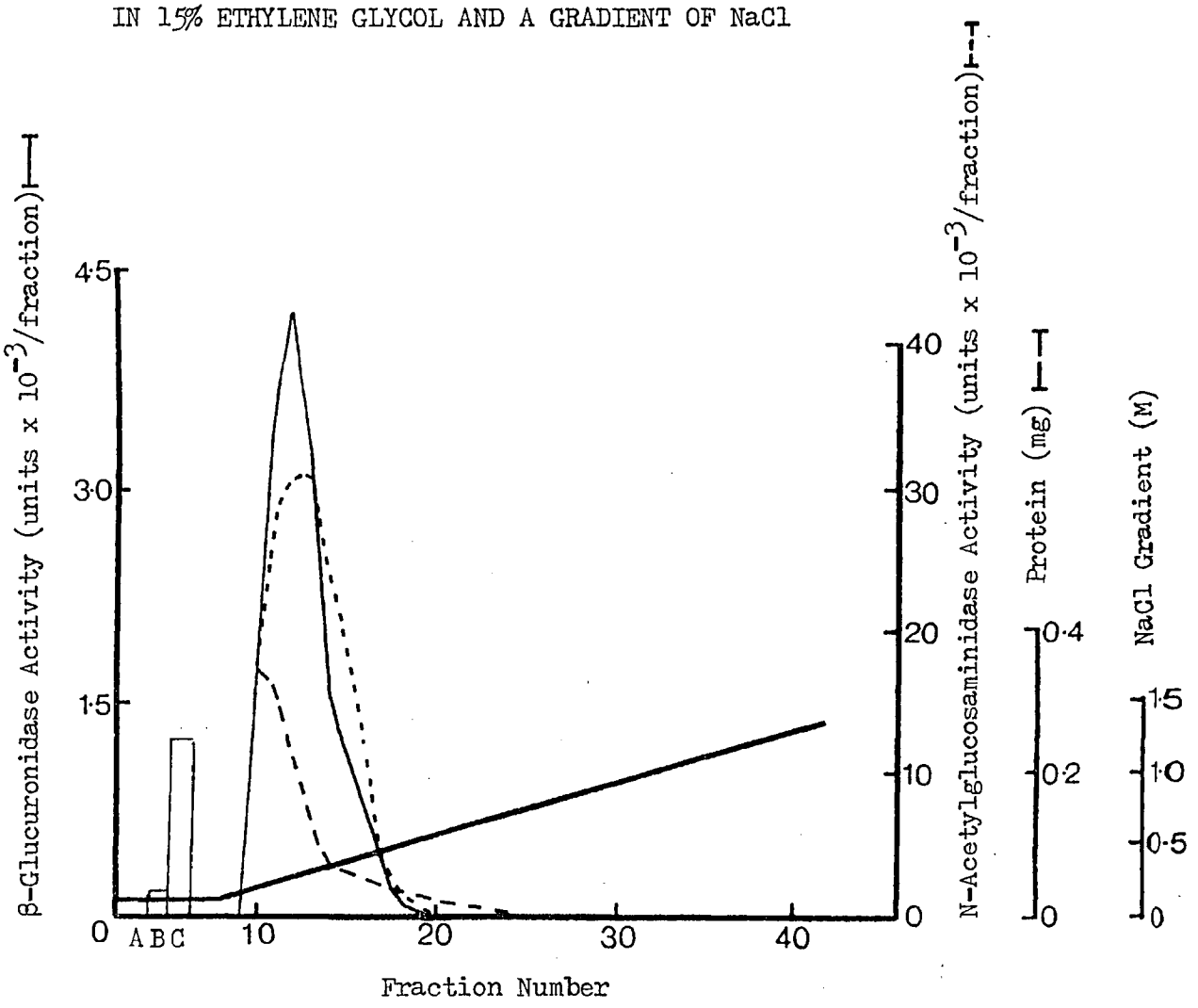


Figure 20

AFFINITY CHROMATOGRAPHY OF BOVINE LIVER β-GLUCURONIDASE ON SACCHAROLACTONE-DIAMINODIPROPYLAMINE-SEPHAROSE 4B: ELUTION IN 15% ETHYLENE GLYCOL AND A GRADIENT OF NaCl



A = β-glucuronidase

B = N-acetylglucosaminidase

C = protein

(c) Binding capacity of affinity gels for bovine liver β-glucuronidase

Table 9 summarises the actual binding capacities determined for control and ligand coupled Sepharose gels. Ethanolamine, which is recommended by Pharmacia ('Affinity Chromatography: principles and methods', p. 14) as an agent for inactivating unbound sites which remain on the Sepharose after ligand coupling, clearly increased the non-specific binding capacity of the uncoupled gel. This effect is probably due to the additional amino groups introduced by this method thereby increasing the ion-exchange capacity of the gel. When the active sites were blocked with 1M Tris/HCl pH 8.0 instead of ethanolamine, non-specific ion-exchange effects were considerably reduced.

Table 9

BINDING OF BOVINE LIVER β-GLUCURONIDASE TO AFFINITY GELS

Sepharose 4B	units bound/ml gel
No ligand	9970
No ligand (treated with ethanolamine)	32500
No ligand (treated with 1M Tris pH 8.0)	9600
p-aminophenyl-β-D-glucuronide (treated with ethanolamine)	28100
diaminodipropylamine (treated with 1M Tris pH 8.0)	24000
diaminodipropylamine-saccharolactone (treated with 1M Tris pH 8.0)	24000

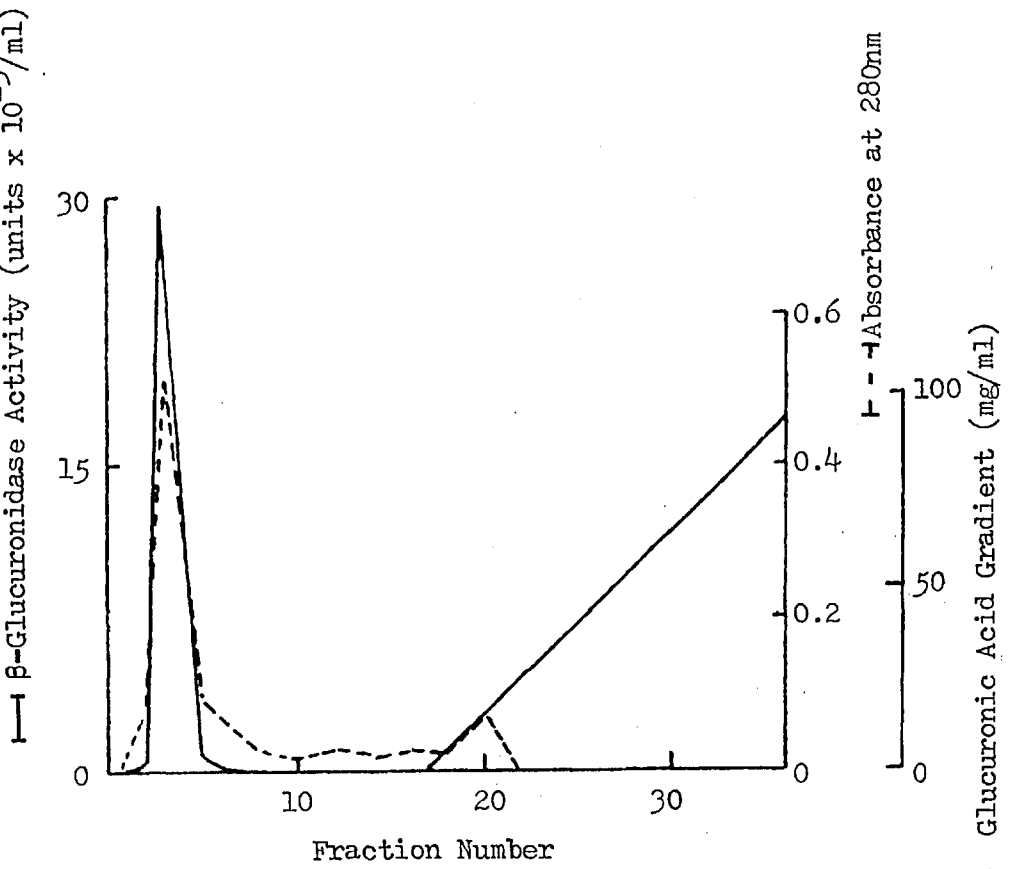
(f) Specific elution of bovine liver β-glucuronidase from p-aminophenylglucuronide-Sepharose

Specific binding of β-glucuronidase to either p-aminophenylglucuronide or saccharolactone ligands was not possible using the method described by Harris et al. (1973), and so affinity chromatography of this enzyme was attempted by adapting the method of Lisman and Overdijk (1978) who relied on specific elution for purifying hexosaminidases A, B and C from bovine brain tissue on CH-Sepharose-p-aminophenyl-2-acetamido-2-deoxy-β-D galactopyranoside.

Figure 21 shows that when 89,000 Units of β-glucuronidase was loaded onto 2.5ml of Sepharose 4B-p-aminophenylglucuronide in PBS, only 35,000 Units bound, and no β-glucuronidase was detected in the fractions eluted with a glucuronic acid gradient. This result may have been due to the inhibition of the β-glucuronidase activity by the eluting buffer. Dialysis of the eluate against control enzyme-inhibitor mixtures showed that while only 0.05% of the original glucuronic acid was present in these mixtures after dialysis against three changes of 8 litres of distilled water, no enzyme activity could be detected in either control mixtures or column eluates. It was thought that dialysis at alkaline pH might improve recovery of β-glucuronidase activity by opening out the glucuronic acid ring structure, thus reducing its affinity for the active site of β-glucuronidase (J. Stirling, personal communication). Bovine β-glucuronidase, however, lost most of its activity during dialysis against 2mM Tris/HCl pH 8.0, or on standing at 4°C in the same buffer, so that only a small recovery of activity from enzyme-inhibitor mixtures was possible. Dialysis of β-glucuronidase-glucuronic acid mixtures in 2mM Tris/HCl pH 7.0 resulted in a smaller loss in activity

Figure 21

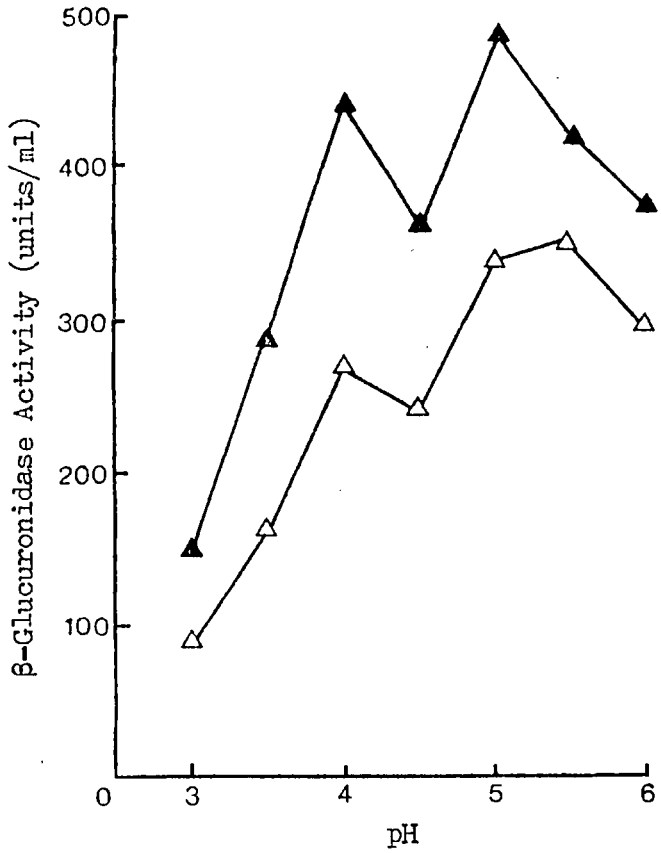
SPECIFIC ELUTION OF BOVINE LIVER β-GLUCURONIDASE FROM p-AMINOPHENYLGLUCURONIDE-SEPHAROSE 4B



of control preparations of β-glucuronidase, but very little recovery of β-glucuronidase activity from the inhibited mixtures. Figure 22 shows the effect of pH on both bovine liver β-glucuronidase and 3T3 mouse β-glucuronidases. Bovine liver β-glucuronidase was partially inactivated by standing in Tris buffer at pH 8.0, but the degree of inactivation observed was similar at all pH values measured. The β-glucuronidase secreted by mouse 3T3 fibroblasts was, however, unaffected by standing at pH 8.0. Bovine liver β-glucuronidase appeared to have two pH optima at pH 4.0 and pH 5.0, whereas 3T3 β-glucuronidase had one broad peak of activity which was maximal at pH 4.0 to 4.5. p-Aminophenylglucuronide was a poor eluting ligand when substituted for glucuronic acid in this system, but did not inhibit β-glucuronidase activity.

Figure 22 EFFECT OF pH ON THE ACTIVITY OF β-GLUCURONIDASE

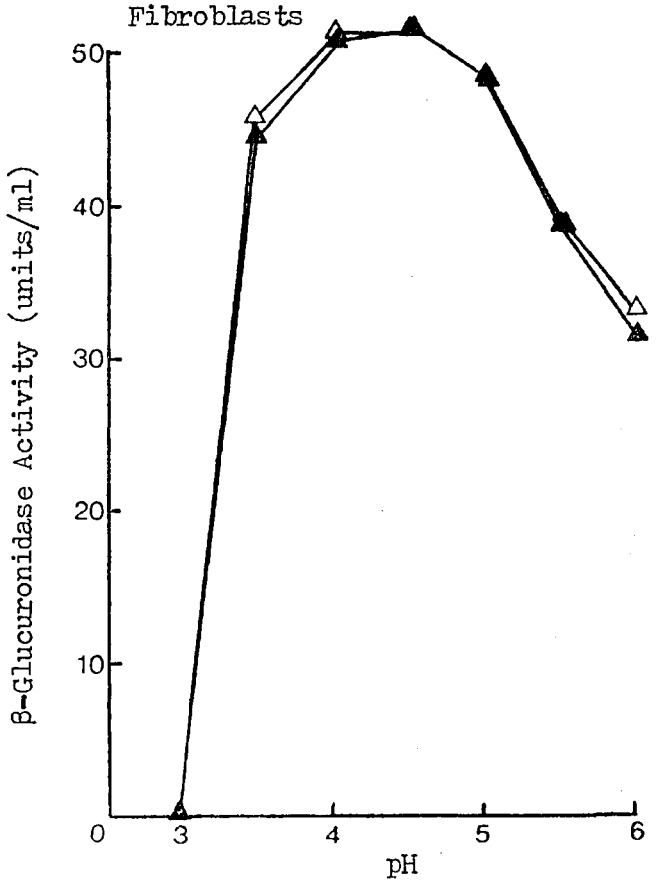
(a) β-Glucuronidase from Bovine Liver



▲ stored at pH 7.0

△ stored at pH 8.0

(b) β-Glucuronidase from Mouse 3T3 Fibroblasts



3.3 UPTAKE EXPERIMENTS

3.3.1 Uptake of 3T3 Fibroblast β-Glucuronidase into AKR Macrophages

The rate of increase in the specific activity of β-glucuronidase present in AKR macrophages after incubation with 290 units/ml of enzyme secreted by 3T3 fibroblasts is shown in Figure 23. The net values were obtained by differential subtraction of endogenous levels of enzyme present in control macrophages. The rate of uptake was linear over an eight hour period of incubation and proceeded at a rate of 24 units/mg cell protein/hour. In subsequent experiments a five hour period of incubation was chosen because readily detectable quantities of enzyme could be measured within the cells after this length of time. With longer periods of incubation than eight hours the rate of uptake of β-glucuronidase began to decrease and between 8 and 24 hours little further accumulation was observed.

The amounts of β-glucuronidase taken up by AKR macrophages when increasing concentrations of enzyme were added to the culture medium is shown in Figure 24. Specific activities were calculated both for samples which had been heat treated to inactivate endogenous β-glucuronidase, and for untreated samples from which control values had been subtracted. The specific activities calculated by both methods were identical, but values obtained after heat treatment were chosen in all subsequent experiments because impurities present in different batches of crude 3T3 β-glucuronidase preparations might stimulate macrophages and thus induce an increased synthesis of endogenous enzyme which would, however, be heat labile. The data presented in Figure 24 show that uptake of 3T3 β-glucuronidase was both

Figure 23

UPTAKE OF β-GLUCURONIDASE FROM 3T3 FIBROBLASTS INTO AKR MACROPHAGES

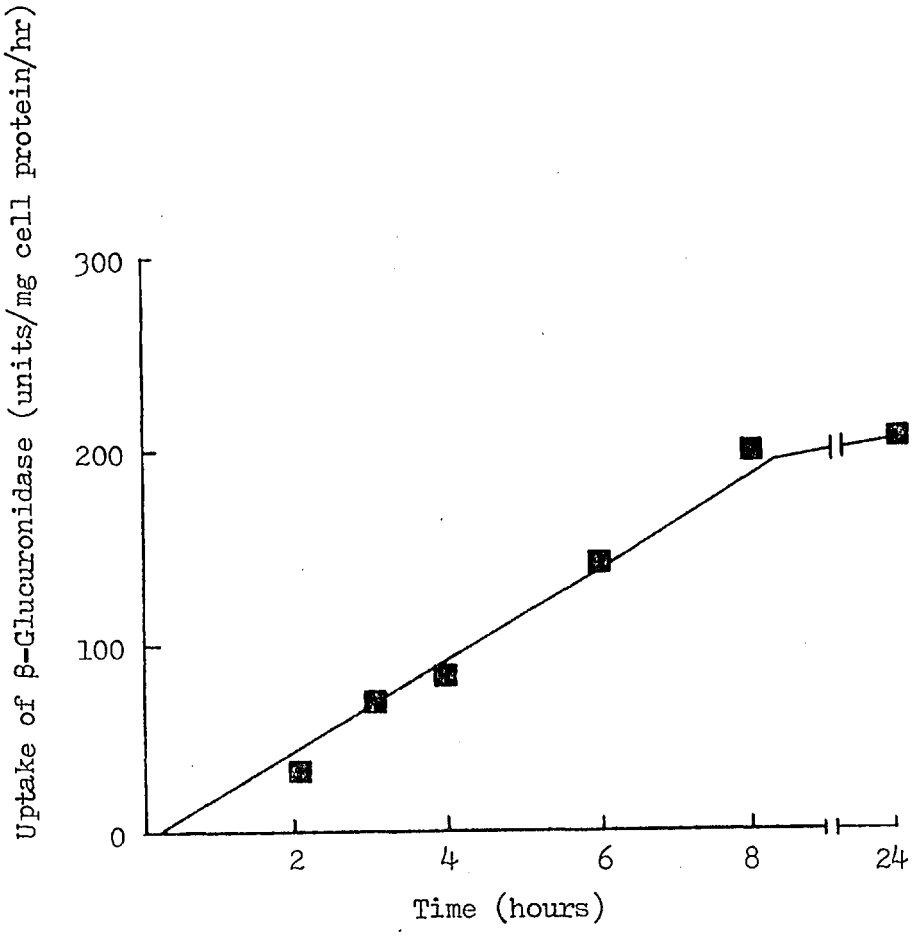
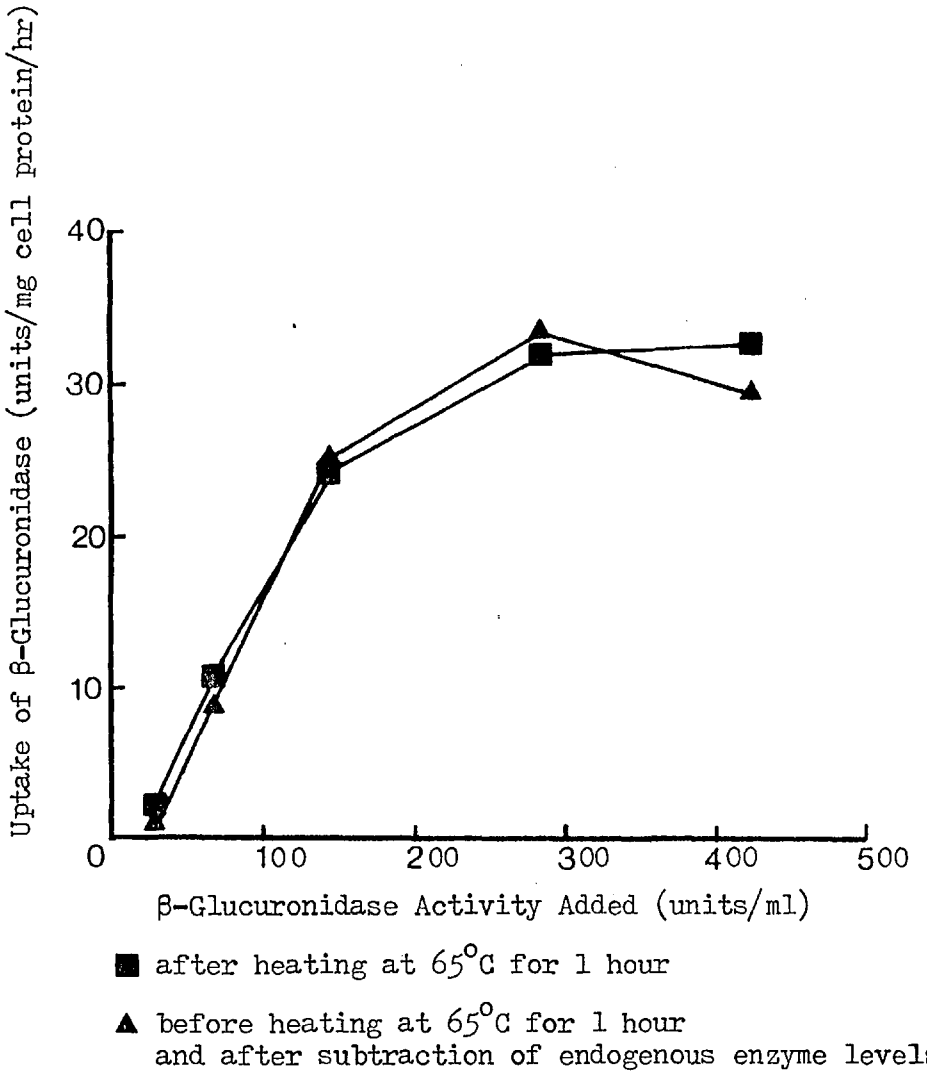


Figure 24

UPTAKE OF INCREASING CONCENTRATIONS OF β-GLUCURONIDASE FROM 3T3 FIBROBLASTS INTO AKR MACROPHAGES



rapid and saturable with a maximum rate of endocytosis of approximately 32 units/mg cell protein/hour at saturating concentrations (above 300 units/ml).

3.3.2 Uptake of 3T3 Fibroblast β-Glucuronidase into C3H Mouse Macrophages

The uptake of β-glucuronidase into C3H macrophages which also have low levels of heat labile enzyme was compared with uptake into AKR macrophages. Figure 25 shows that uptake into C3H macrophages was similar to that observed for AKR macrophages but that the maximum rate of uptake was considerably lower at only 20 units/mg cell protein/hour, and saturation occurred at concentrations above 100 units/ml.

3.3.3 Uptake of 3T3 Fibroblast β-Glucuronidase into GM151 Fibroblasts

Figure 26 shows that when 151 fibroblasts were incubated with 70 units/ml of β-glucuronidase, the increase in specific activity of the enzyme present within the cells was linear over an eighteen hour period of time, with a rate of 5 units/mg cell protein/hour. After 25 hours of incubation, the rate of uptake had slowed to 3.8 units/mg cell protein/hour, and so in subsequent experiments an eighteen hour period of incubation was used to measure the rate of uptake of β-glucuronidase into the cells.

Figure 27 shows human 151 fibroblasts which were stained to show β-glucuronidase activity after uptake from 3T3 fibroblasts. The β-glucuronidase was distributed in discrete packages, which resembled lysosomes. The staining pattern for 151 cells was similar to that observed after direct interaction of mouse lymphocytes with human 151 fibroblasts (Olsen *et al.*, 1981). Control GM151 fibroblasts which had

Figure 25

UPTAKE OF β-GLUCURONIDASE FROM 3T3 FIBROBLASTS INTO C3H MACROPHAGES

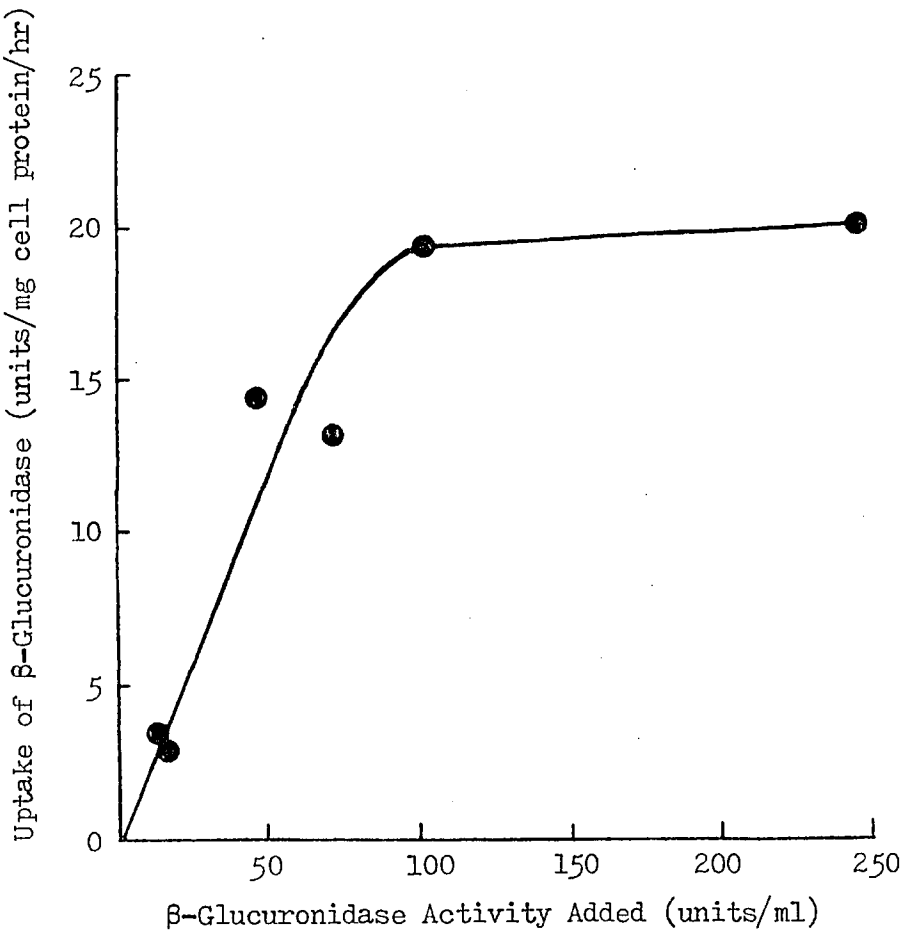


Figure 26

UPTAKE OF β-GLUCURONIDASE FROM 3T3 FIBROBLASTS INTO GM151 FIBROBLASTS

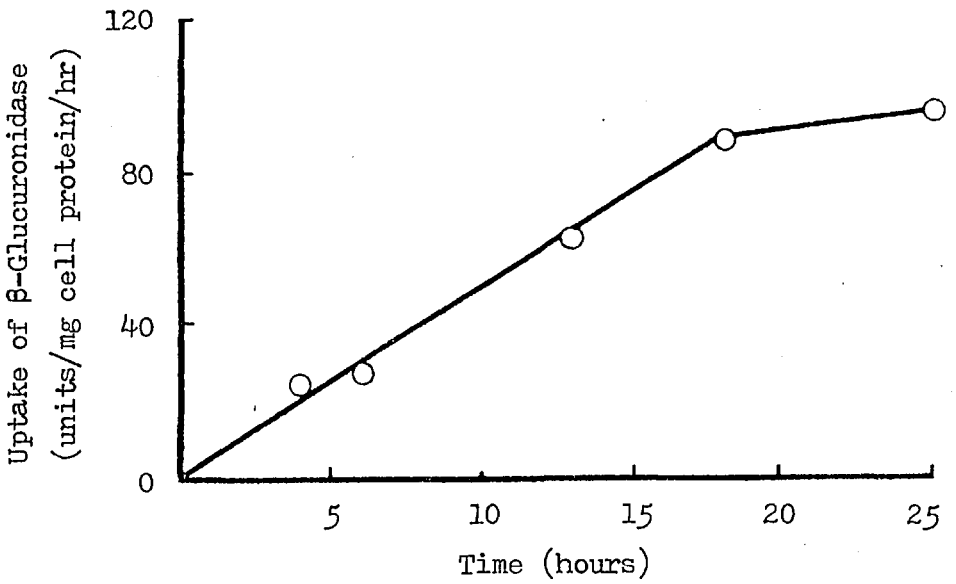
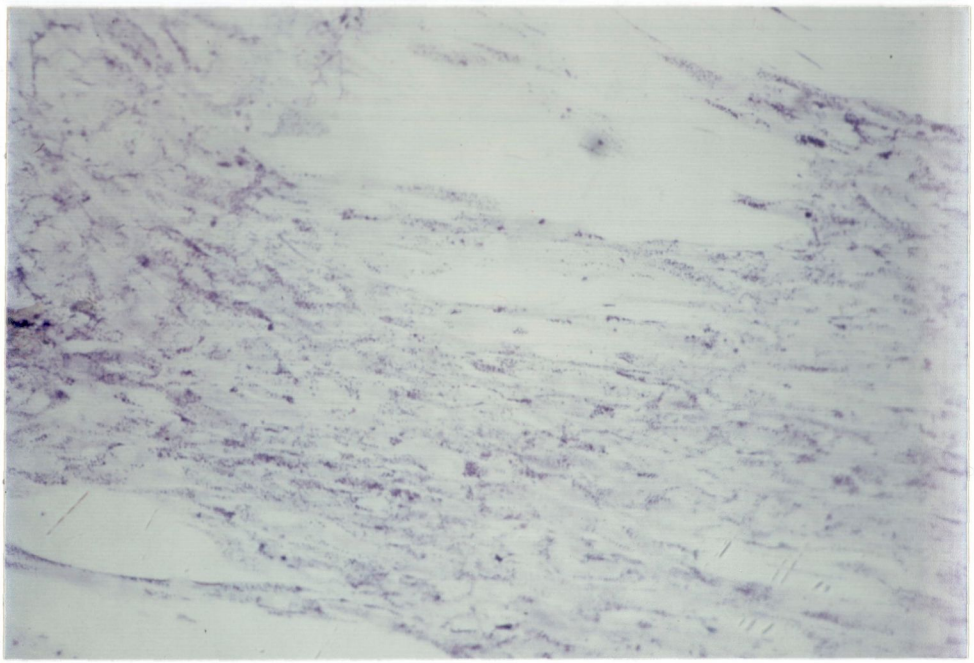
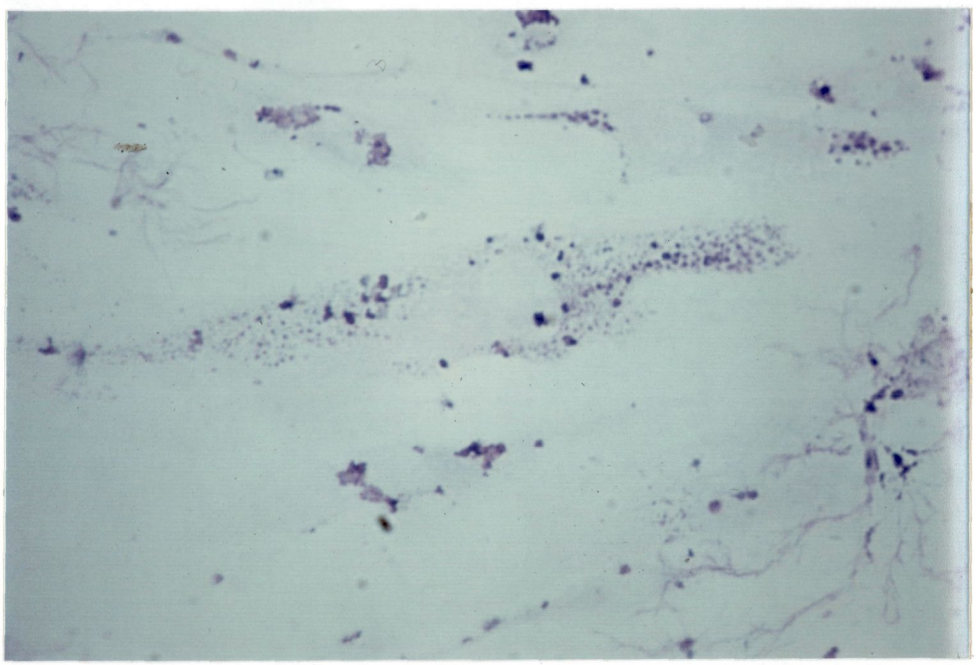


Figure 27

HISTOCHEMICAL LOCALISATION OF β-GLUCURONIDASE ACTIVITY IN GM151 CELLS



(a) After incubation with β-glucuronidase secreted by 3T3 fibroblasts, all GM151 fibroblasts had acquired enzyme activity. 40x magnification.



(b) Each GM151 fibroblast contained small, discrete areas of β-glucuronidase activity surrounding the nucleus of the cell. 400x magnification.

been incubated without externally added β-glucuronidase, were completely colourless when stained in an identical manner.

3.3.4 Uptake of β-Glucuronidases from Different Sources into Macrophages and Fibroblasts

Uptake of 3T3 fibroblast β-glucuronidase into both AKR macrophages and 151 fibroblasts was equally rapid (Figures 28 and 29). Platelet β-glucuronidase was taken up almost as rapidly as 3T3 enzyme into fibroblasts, but less rapidly into macrophages. Placental β-glucuronidase was, however, taken up at a measurable rate only into macrophages. Uptake of β-glucuronidase from all sources into both types of cell was saturable with the exception of uptake of placental enzyme into fibroblasts, indicating that enzymes from all other sources were taken up by receptor mediated endocytosis.

3.3.5 Uptake of Horseradish Peroxidase into Macrophages and Fibroblasts

The rate of uptake of horseradish peroxidase (HRP) into AKR macrophages and 151 fibroblasts increased with increasing concentration, and did not reach saturation (Figure 30) showing that uptake of this enzyme proceeded by passive pinocytosis in agreement with the findings of Walter *et al.* (1980) who used the macrophage cell line J774.2. The amount taken up into each type of recipient cell was directly proportional to the concentration of HRP present in the culture medium. The proportion of HRP accumulated from the extracellular medium by each type of cell was, however, considerably smaller than the proportion of β-glucuronidase taken up by the cells in analogous experiments (Table 10). Uptake of HRP was assumed to be linear over the time period of these experiments as described by Steinman and Cohn (1972).

Figure 28

UPTAKE OF β-GLUCURONIDASES FROM DIFFERENT SOURCES INTO AKR MACROPHAGES

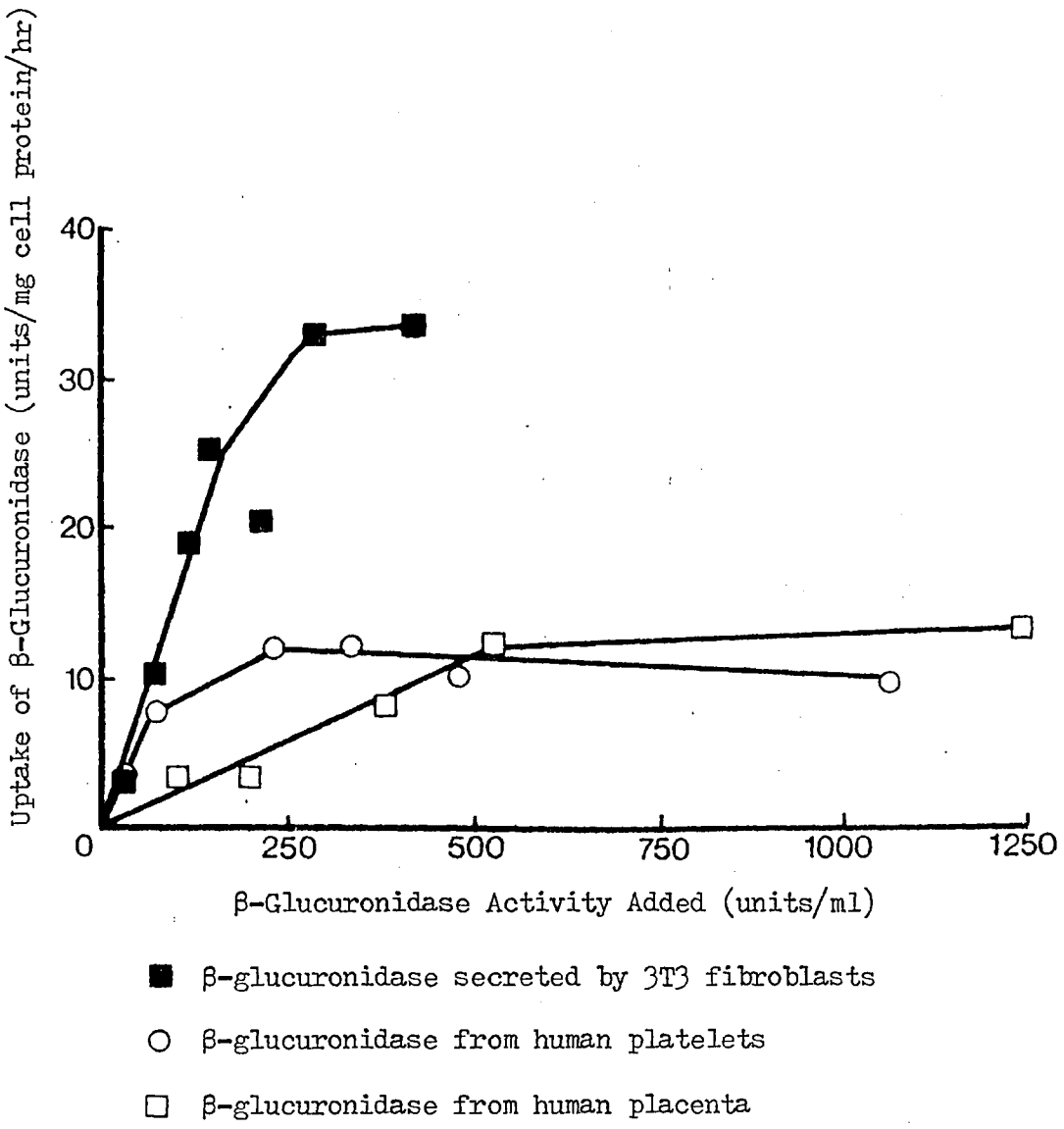
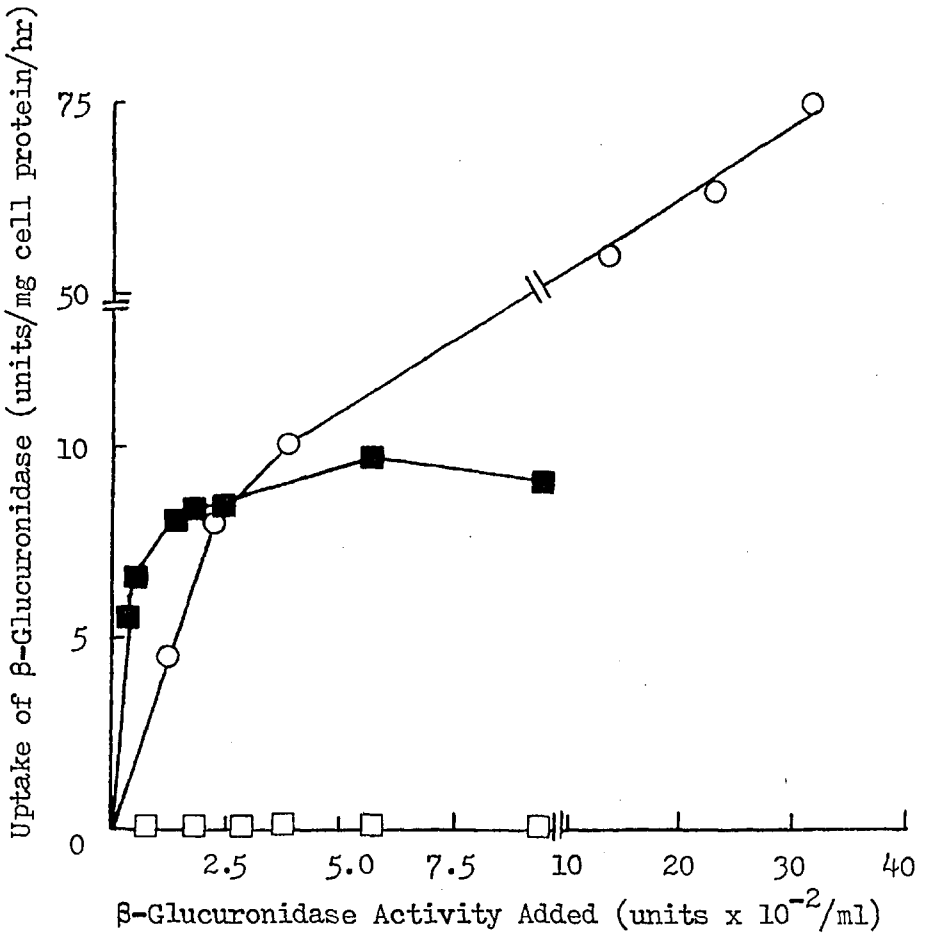


Figure 29

UPTAKE OF β-GLUCURONIDASES FROM DIFFERENT SOURCES INTO GM151 FIBROBLASTS



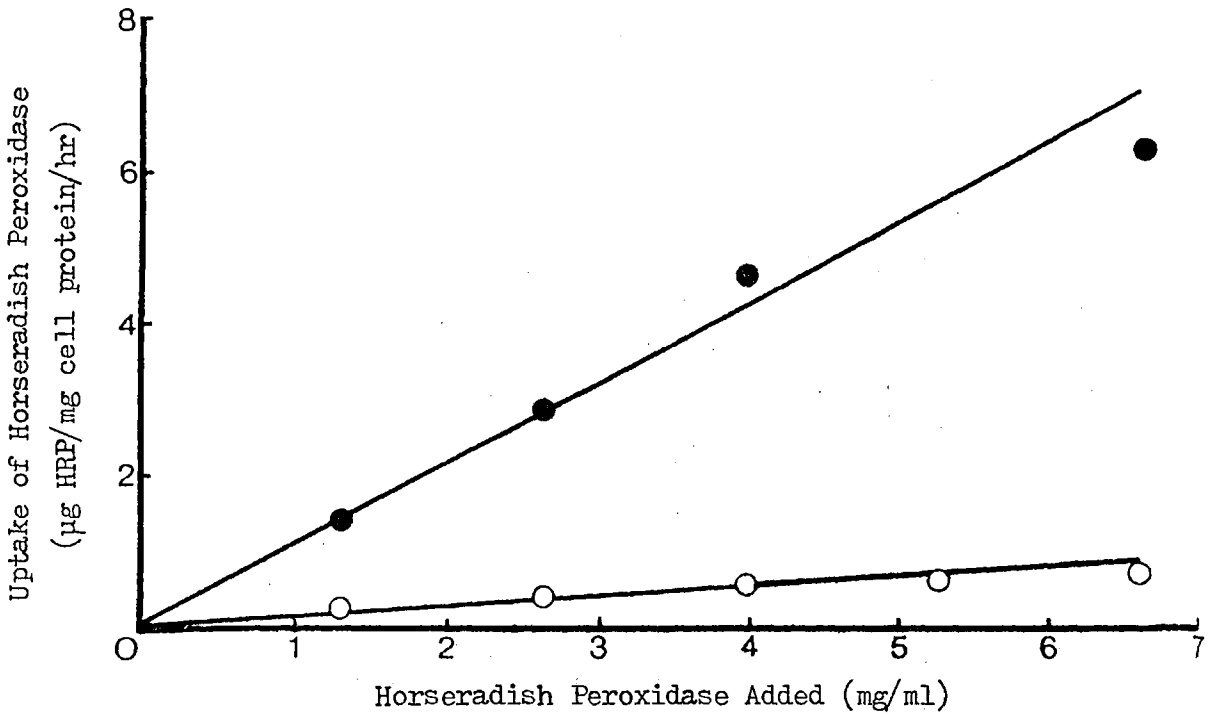
■ β-glucuronidase secreted by 3T3 fibroblasts

○ β-glucuronidase from human platelets

□ β-glucuronidase from human placenta

Figure 30

UPTAKE OF HORSERADISH PEROXIDASE INTO MACROPHAGES AND FIBROBLASTS



● AKR macrophages

○ GM151 fibroblasts

3.3.6 Maximum Rates of Enzyme Uptake into Macrophages and Fibroblasts

In Table 10 the maximum rates of uptake of HRP into both types of cell are compared with the rates of uptake of different types of β-glucuronidase into the same recipient cells. The maximum calculated rates of uptake of 3T3 β-glucuronidase into 151 fibroblasts and AKR and C3H macrophages were similar, being approximately 800 and 140 times the rate of uptake of HRP into fibroblasts and macrophages respectively. Neither β-glucuronidase from SV40 transformed 3T3 fibroblasts, nor that prepared from platelets, was taken up as rapidly as the β-glucuronidase secreted by non-transformed 3T3 cells.

Table 10

MAXIMUM RATES OF ENZYME UPTAKE INTO FIBROBLASTS AND MACROPHAGES

Source of enzymes	Uptake into 151 fibroblasts (%/mg/hr)	Uptake into AKR macrophages (%/mg/hr)
β-glucuronidase:		
mouse 3T3 fibroblasts	9.6	14.0 (18.0)*
human platelets	3.9	10.0
human placenta	0.035	2.3
SV40 3T3 fibroblasts	2.4	not determined
horseradish peroxidase	0.012	0.09

*Indicates maximum rate of uptake into C3H macrophages

3.3.7 Uptake Constants for Endocytosis of β-Glucuronidase

The K_{uptake} values for the saturation curves shown in Figures 28 and 29 were calculated from plots based on the method of Hanes (1932) as follows:

$$\text{Ordinate} = \frac{\text{concentration of β-glucuronidase in medium (units/ml)}}{\text{uptake of β-glucuronidase (units/mg/hr)}}$$

$$\text{Abcissa} = \text{concentration of β-glucuronidase in medium (units/ml)}$$

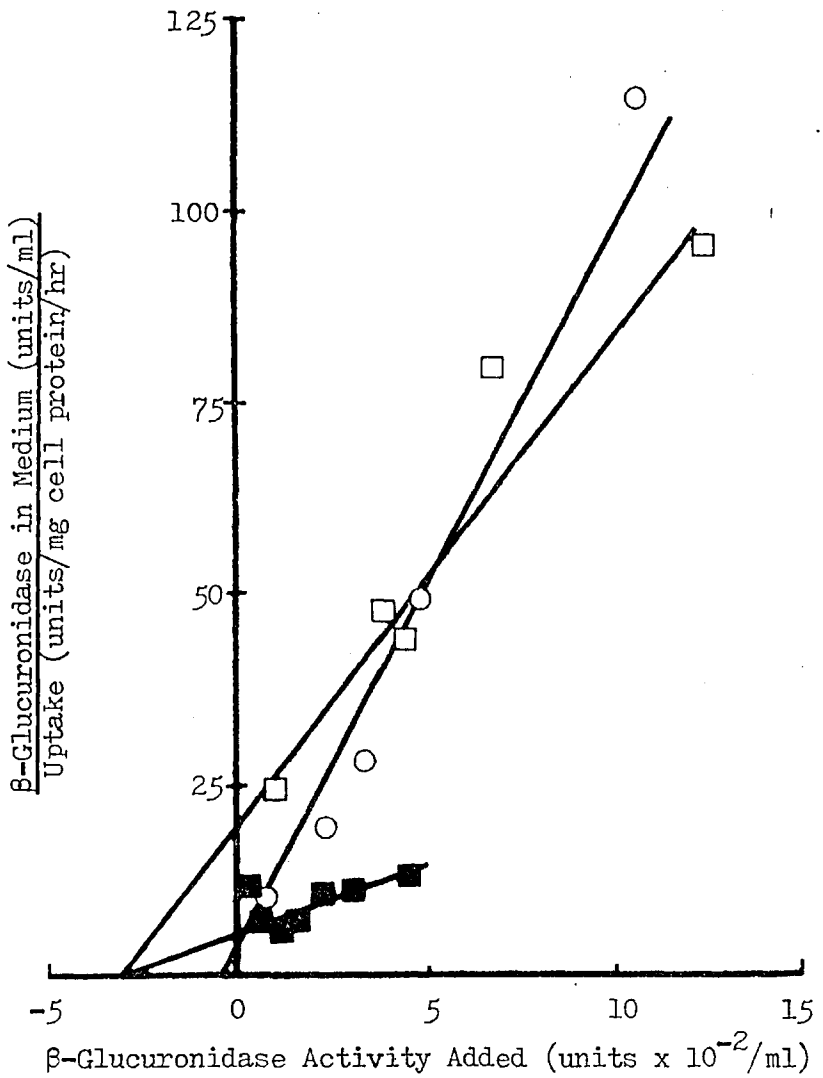
The graphs plotted from these values are shown in Figure 31 (macrophages) and Figure 32 (fibroblasts), and the $K_{uptakes}$ (the points at which the lines intercept the abcissa) are listed in Table 11. In this Table the $K_{uptakes}$ have been converted from units of β-glucuronidase to moles of β-glucuronidase. This calculation assumes that the maximum possible specific activity of β-glucuronidase is 3,900,000 units/mg (Natowicz et al., 1979) and that the molecular weight of lysosomal β-glucuronidase is 280,000 Daltons (Tulsiani et al., 1978). The K_{uptake} in units of activity is converted to moles of β-glucuronidase by the formula

$$\frac{K_{uptake}\ (\text{units})}{3{,}900{,}000 \times 280{,}000} = K_{uptake}\ (\text{M})$$

As can be seen in Table 11, the $K_{uptakes}$ for endocytosis of human placental and 3T3 fibroblast β-glucuronidase into AKR macrophages were the same at 2.8×10^{-10}M. The K_{uptake} for platelet β-glucuronidase into fibroblasts was greater at 3.5×10^{-9}M, but those calculated for uptake of 3T3 fibroblast β-glucuronidase into fibroblasts and platelet β-glucuronidase into macrophages were about ten fold lower at 3×10^{-11}M and 6×10^{-11}M. The rate of uptake of placental β-glucuronidase into fibroblasts was too low to enable a K_{uptake} to be calculated.

Figure 31

HANES PLOTS OF UPTAKE OF β-GLUCURONIDASE INTO AKR MACROPHAGES



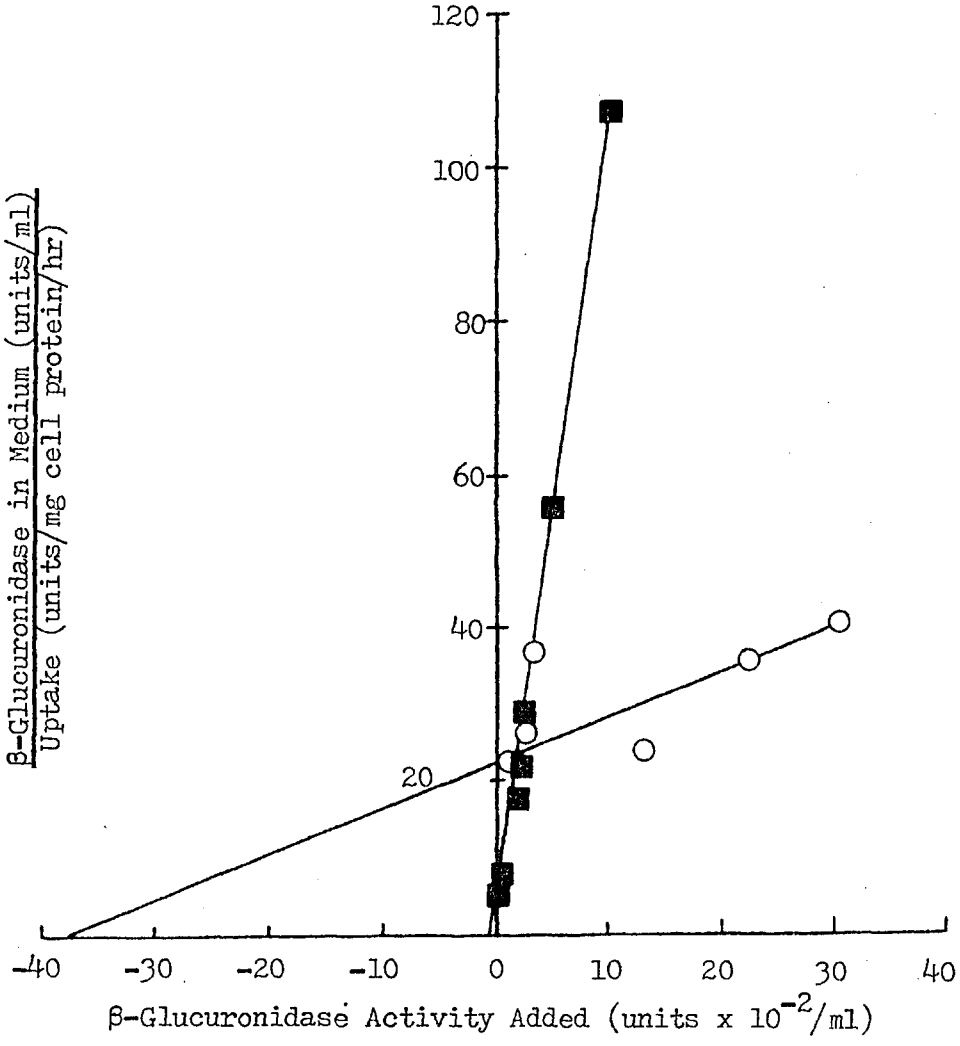
■ β-glucuronidase secreted by 3T3 fibroblasts

□ β-glucuronidase from human placenta

○ β-glucuronidase from human platelets

Figure 32

HANES PLOTS OF UPTAKE OF β-GLUCURONIDASE INTO GM151 FIBROBLASTS



■ β-glucuronidase secreted by 3T3 fibroblasts

○ β-glucuronidase from human platelets

Table 11

$K_{uptakes}$ FOR β-GLUCURONIDASE FROM DIFFERENT SOURCES

β-glucuronidases	AKR macrophages	151 fibroblasts
3T3 fibroblast	2.8×10^{-10}M (6.1×10^{-10}M) *	3×10^{-11}M
3T3 SV40 fibroblast	-	1×10^{-10}M
placenta	2.8×10^{-10}M	-
platelet	6×10^{-11}M	3.5×10^{-9}M

* Indicates value calculated for C3H macrophages

3.3.8 The Effect of Alkaline Phosphatase on Uptake of 3T3 β-Glucuronidase

The profiles of β-glucuronidase eluted from DEAE-Sephacel, before and after treatment with alkaline phosphatase are shown in Figure 17. The more highly negatively charged fraction of the β-glucuronidase was eluted in 0.18M NaCl before reaction with alkaline phosphatase but in only 0.15M NaCl after alkaline phosphatase treatment, which was consistent with the removal of the negatively charged phosphate groups. The control and the alkaline phosphatase treated fractions of β-glucuronidase eluted from the column were each separately pooled and added to macrophages and fibroblasts. The rates of uptake into each type of cell are shown in Table 12.

Table 12

EFFECT OF REACTION WITH ALKALINE PHOSPHATASE ON UPTAKE OF β-GLUCURONIDASE FROM 3T3 CELLS

β-glucuronidase	Rate of uptake (%/mg/hr) into fibroblasts	macrophages
untreated (33)	11.4	10.9
treated with alkaline phosphatase (35)	0	14.4

figures in parentheses indicate amount of β-glucuronidase added in units/ml

Uptake of the alkaline phosphatase treated preparation into fibroblasts was completely abolished, but uptake of this enzyme into macrophages was enhanced, a rate of 14.4%/mg cell protein/hour being calculated compared with a control rate of uptake of only 10.9%/mg/hour. This suggests that the phosphate groups removed by the alkaline phosphatase were essential for uptake of β-glucuronidase into fibroblasts but not necessary, or even slightly inhibitory, of uptake into macrophages.

3.3.9 Effect of Inhibitors on Uptake of 3T3 β-Glucuronidase

A number of different sugars and sugar phosphates at concentrations of 5mM and 50mM were added to the culture medium together with 3T3 fibroblast β-glucuronidase and incubated with fibroblasts and macrophages. The results are summarised in Table 13. This shows that

Table 13

EFFECT OF INHIBITORS ON UPTAKE OF 3T3 β-GLUCURONIDASE INTO MACROPHAGES AND FIBROBLASTS

Inhibitor	% inhibition of uptake into macrophages	fibroblasts
5mM mannose 6-phosphate	34	99
5mM fructose 1-phosphate	n.d.	95
5mM glucose 6-phosphate	n.d.	23
50mM mannose 6-phosphate	73	n.d.
50mM fructose 1-phosphate	0	n.d.
50mM glucose 6-phosphate	0	n.d.
50mM mannose	44	0
50mM α-methylmannoside	63	0
50mM fructose	0	12
50mM glucose	0	10
50mM N-acetylglucosamine	32	24
50mM adenosine monophosphate	n.d.	32
500μg/ml yeast mannan	63	3

all of the phosphorylated sugars inhibited uptake of 3T3 β-glucuronidase into fibroblasts at a concentration of 5mM and that mannose 6-phosphate, which totally inhibited uptake, was more potent than fructose 1-phosphate or glucose 6-phosphate. Fructose 1-phosphate, which is similar in structure to mannose 6-phosphate, was nearly as potent as mannose 6-phosphate, inhibiting uptake into fibroblasts by 95%. Mannose, α-methylmannoside, fructose and glucose at concentrations of 50mM were ineffective as inhibitors of uptake of β-glucuronidase into fibroblasts, illustrating the importance of phosphate groups as essential recognition markers for endocytosis into fibroblasts. 50mM AMP which can increase dissociation of lysosomal enzymes from rat liver lysosomes (Burnside and Schneider, 1980) also inhibited uptake into fibroblasts but was not as effective as the other sugar phosphates, in agreement with the findings of Sando and Neufeld (1977). N-acetylglucosamine slightly inhibited uptake of β-glucuronidase into fibroblasts.

Uptake of β-glucuronidase into macrophages was inhibited by all of the mannose-containing molecules which were tested. Mannose 6-phosphate was the most potent inhibitor, reducing uptake by 34% at a concentration of 5mM and by 73% at a concentration of 50mM. Mannose (50mM), α-methylmannoside (50mM), and yeast mannan (500μg/ml) all inhibited uptake of β-glucuronidase by about 50%. Compounds which did not contain mannose were, however, ineffective as inhibitors with the exception of 50mM N-acetylglucosamine which reduced uptake by 32%. Fructose 1-phosphate, which was almost as potent an inhibitor as mannose 6-phosphate when tested against fibroblasts, was completely ineffective as an inhibitor of uptake into macrophages, showing that mannose residues are of crucial importance for uptake of β-glucuronidase into these cells.

CHAPTER IV

DISCUSSION

Attempts at enzyme replacement therapy using fibroblast transplants have shown that donor fibroblasts appear to secrete lysosomal enzymes *in vivo* after transplant (Dean *et al.*, 1979; 1981). Lysosomal enzymes secreted by fibroblasts *in vitro* can be taken up by other deficient fibroblasts (Neufeld *et al.*, 1977), although *in vivo* studies have shown that most lysosomal enzymes are cleared from circulation by cells of the reticuloendothelial system, particularly Kupffer cells in the liver (Achord *et al.*, 1978).

Successful enzyme replacement therapy using transplant techniques requires that normal enzymes should firstly be secreted into the circulation by transplanted cells and secondly that the enzymes should be taken up by the appropriate target cells. Recent evidence has shown, however, that the lysosomal enzymes normally secreted by fibroblasts carry complex type oligosaccharide recognition markers, while those enzymes which are sequestered in lysosomes contain predominantly high mannose type oligosaccharides (Hasilik *et al.*, 1981). Secreted lysosomal enzymes might not therefore be recognised and internalised by other types of cell, such as cells of the reticulo-endothelial system which have mannose/N-acetylglucosamine receptors on their plasma membranes (Achord *et al.*, 1978; Kawasaki *et al.*, 1978; Townsend and Stahl, 1981).

The first aim of this project was to culture a suitable strain of fibroblasts, and to purify the β-glucuronidase secreted by them. The second aim was to discover whether this enzyme could be taken up by receptor mediated endocytosis into macrophages taken from strains of mice partially deficient in β-glucuronidase. The third aim was to determine whether this secreted β-glucuronidase was recognised and

internalised by the same type of receptor on the plasma membranes of both macrophages and fibroblasts, or whether uptake into each type of cell was mediated by different receptors.

4.1 CULTURE OF MOUSE 3T3 FIBROBLASTS AND SECRETION OF β-GLUCURONIDASE

The results presented in section 3.1.1 show that mouse 3T3 fibroblasts secreted more β-glucuronidase than any other strain of mouse fibroblast tested. Secretion of β-glucuronidase was not specific: 3T3 cells secreted thirty times more hexosaminidase than β-glucuronidase and almost equivalent amounts of β-galactosidase (section 3.2.1). This observation was in direct contrast to the reports of Horvat and Acs (1974) who stated that negligible amounts of lysosomal enzymes were present in medium harvested from 3T3 fibroblasts. The secretion of hexosaminidase and β-glucuronidase by 3T3 cells (900 and 25 units/24 hrs/ 10^6 cells respectively) was at least ten times greater than the amounts secreted by other normal mouse or human fibroblasts and several times more than amounts secreted by fibroblasts from patients with I-cell disease (Wiesmann et al., 1971; Miller et al., 1980). 3T3 cells are not, however, analogous to I-cells: section 3.1.5 shows that 3T3 fibroblasts contained normal levels of intracellular β-glucuronidase activity, in contrast to the internal depletion of enzyme activities observed in I-cells (Hickman and Neufeld, 1972; McKusick et al., 1978; Bach et al., 1979; Hasilik and Neufeld, 1980). It is, therefore, unlikely that 3T3 fibroblasts secreted more lysosomal enzymes than other fibroblasts because of an inability to sequester them in lysosomes. Faulty or weak binding to phosphomannosyl receptors might also result in abnormal secretion of lysosomal enzymes by 3T3 cells. However, since these cells secreted β-glucuronidase in a form recognised as high uptake by other fibroblasts (Table 10), and since 3T3 cells were able themselves to take up externally added platelet β-glucuronidase

rapidly (results not shown), both the degree of glycosylation of their enzymes, and the structure of their phosphomannosyl receptors seemed to be normal.

3T3 fibroblasts have been used by many other workers as normal cells, indeed, they were originally derived from Swiss mouse embryo fibroblasts (Todaro and Green, 1963) and are not virally transformed. Since 1963 3T3 fibroblasts have been used, for example, as feeders for cultures of epithelial cells, because they secrete epidermal growth factors into culture medium (Green, 1978). Whether they secrete growth factors and lysosomal enzymes by similar mechanisms is not known.

Fibroblasts transformed with SV40 virus also secreted high amounts of lysosomal enzymes (Figure 6), but the secretion of lysosomal enzymes by non-transformed 3T3 cells was not a consequence of long term laboratory culture (which might have caused preferential selection of mutant strains), since several batches of 3T3 cells were obtained from both Flow Laboratories (Irvine, Scotland) and the Imperial Cancer Research Fund (London, U.K.) and these different batches secreted β-glucuronidase at the same rate (approximately 25 units/24 hrs/10^6 cells.)

After 3T3 fibroblasts had been chosen as a source of normal secreted β-glucuronidase, systems were investigated in which the cells could be cultured in low concentrations of extracellular proteins as an aid to subsequent purification of β-glucuronidase. Methods of bulk culture were also investigated so that β-glucuronidase could be isolated in sufficient quantities for uptake studies to be carried out on reticuloendothelial cells.

Figure 3 shows that 5% (v.v.) FCS was the minimum concentration

which would support both adequate growth of 3T3 cells and secretion of β-glucuronidase. These results agreed with those of Holley and Kiernan (1974) who showed that DNA synthesis in 3T3 cells was directly controlled by the concentration of serum in which they were grown. Many serum substitutes were tested in an attempt to find a method of growing the cells in medium with lower concentrations of protein (Table 2). Neither new born calf or donor horse serum were as effective at promoting growth of 3T3 cells as FCS when they were used at the same concentrations. Indeed, horse serum stimulated the formation of large phase-lucent vacuoles in the cells, which were identified histochemically as lipid storage vesicles using Oil Red O as a stain (Plaas, 1980). Normal 3T3 fibroblasts are filled with small vacuoles as shown in Figure 5a which contain lipids, but they do not transform into adipocytes unless the cells are cultured for many days at confluence (Green and Kehinde, 1975; Plaas, 1980; Spiegelman and Green, 1980); for this reason, care was always taken to avoid maintaining the cells at confluence.

Autologous mouse plasma was reported to improve the growth of 3T3 cells by Pruss and Herschmann (1979) but at the recommended concentration (2%), 3T3 cells failed to grow successfully. Bovine serum albumin (BSA) was added to medium 199 at 0.2% (w.v.) (approximately the concentration of protein present in serum), but 3T3 cells did not grow in this medium, releasing only very small quantities of β-glucuronidase. Similarly, insulin and cholera toxin either separately or together were also ineffective at promoting growth of 3T3 cells or secretion of β-glucuronidase. The inability of 3T3 cells to grow in medium without FCS may be a result of their apparent requirement for growth factors which are normally present in this serum. They have

been induced to divide in the absence of serum after the addition of Epithelial Growth Factor (EGF) purified from mouse submaxilliary glands or Fibroblast Growth Factor (FGF) purified from brain tissue or pituitary (Nilsen-Hamilton et al., 1980), but the isolation and purification of these factors would have taken too much time to be a practical proposition within the scope of this project.

Removal from serum of all glycoproteins which might subsequently copurify with the β-glucuronidase secreted by fibroblasts was attempted by passing the FCS through an affinity column of Concanavalin A-Sepharose. However, incubation of this Concanavalin A-Sepharose depleted FCS with 3T3 cells at a concentration of 5% (v.v.) in medium resulted in the cells becoming very granular and cell division was arrested. A possible explanation for these effects may have been that trace amounts of Concanavalin A had leaked from the affinity column into the FCS. Similar effects have been described by Dunn and Mallucci (1980) who showed that Concanavalin A inhibited uptake of thymidine, uridine and deoxyglucose into mouse embryo fibroblasts, thereby inhibiting cell division; this lectin has also been shown to bind specifically to 3T3 cells during mitosis (Noonan and Burger, 1973), and to perturb the arrangement of fucosyl glycoproteins on the surfaces of these cells (Doetschman, 1980). It is possible to remove Concanavalin A from serum by mixing it with Sephadex G-200 in 1M NaCl (Agrawal and Goldstein, 1965), but subsequent dialysis used to remove sodium chloride might also have removed low molecular weight factors essential for growth of 3T3 fibroblasts. An alternative explanation for the lack of growth and division of 3T3 fibroblasts in FCS which had been passed through Concanavalin A-Sepharose may have been that vital growth factors were removed from the serum by binding to the Concanavalin A-Sepharose.

An affinity column using anti-β-glucuronidase antibody immobilised on Sepharose might have been used with greater success than the immobilised lectin to remove β-glucuronidase from FCS. Such an antibody was, however, unavailable, so 3T3 fibroblasts were cultured in whole FCS at a concentration of 5% (v.v.) in medium 199. Freshly harvested medium was therefore heated at 65°C to inactivate the contaminating β-glucuronidase present in the FCS before the β-glucuronidase secreted by 3T3 cells was purified (Figures 4 and 10).

Bulk cultures of 3T3 fibroblasts were set up by seeding cells on microcarrier beads in medium containing 5% (v.v.) FCS (Figure 5b). This microcarrier system enabled a much larger number of 3T3 cells to be grown in a similar volume of medium and thereby to concentrate the β-glucuronidase activity secreted by the fibroblasts. A twofold increase in concentration was obtained in this way.

Adding ammonium chloride to the culture medium of fibroblasts was also tried as a method for increasing the rate of secretion of lysosomal enzymes. The effect of ammonium chloride on secretion of lysosomal enzymes by fibroblasts had been described by Sando et al. (1979), and by Gonzalez-Noriega et al. (1980), and similar effects were described for secretion of lysosomal enzymes by macrophages (Tietze et al., 1980; Riches and Stanworth, 1980). When 3T3 cells were cultured for six days in medium containing 10mM ammonium chloride they secreted twice as much β-glucuronidase as controls (results not shown). However, the mechanism by which primary amines increase the release of lysosomal enzymes remains unclear. It has been suggested that ammonium ions might affect delivery of receptor bound enzymes to lysosomes (Sando et al., 1979), or that they inhibit receptor recycling within cells (Tietze et al., 1980;

Gonzalez-Noriega et al., 1980), by raising the intralysosomal pH. Gonzalez-Noriega et al. (1980) showed that binding of β-glucuronidase to phosphomannosyl receptors on fibroblasts was pH dependent, and suggested that enzymes and receptors were dissociated in lysosomes so that the receptors could return to the cell surfaces. If the ligands remained bound, such as might occur when NH_4^+ ions were introduced into the lysosomes, the receptors would become locked into the lysosomal membranes and would not be free to recycle. Contrary evidence from Livesey et al. (1980) suggested, however, that ammonium ions inhibited non-specific fluid phase pinocytosis, normal proteolysis of endogenous proteins and also reduced the ATP/ADP ratio in cells from rat yolk sacs. The evidence of Livesey et al. (1980) together with that of Gonzalez-Noriega et al. (1980), which suggested that phosphorylated enzymes were being released by this mechanism, indicated that the type of enzymes secreted in the presence of ammonium ions might well differ in some important aspect from those which were secreted normally. Such populations of β-glucuronidase might, for example, be enriched with enzyme which is recognised as a high uptake form by fibroblasts. Consequently, the use of these amines in medium used for the culture of 3T3 cells was avoided.

4.2 PURIFICATION OF β-GLUCURONIDASE FROM MEDIUM CONDITIONED BY 3T3 FIBROBLASTS

4.2.1 Initial Purification

The very low specific activity of β-glucuronidase present in the culture medium harvested from 3T3 fibroblasts made purification extremely difficult. No specific antiserum to β-glucuronidase was available for use as a ligand on antibody affinity gels, and so less specific methods of purification had to be used. Moreover, 3T3 fibroblasts secreted relatively small amounts of β-glucuronidase compared with the large amounts readily obtained from homogenised livers or spleens. It was, therefore, only possible to use purification steps which resulted in a high recovery of enzyme.

The β-glucuronidase secreted by 3T3 fibroblasts was first concentrated by ultrafiltration: the Diaflo XM100 A filter used for this had a pore size which allowed molecules of up to 100,000 Daltons to pass through. β-Glucuronidase with an approximate molecular weight of 280,000 (Tulsiani *et al.*, 1978) was thus easily concentrated with negligible loss of activity, while smaller molecules such as serum albumin passed through, effecting a small purification (1.8 fold) of the concentrate (Table 4). Heat treatment of crude β-glucuronidase extracts has been used by Tomino *et al.* (1975), and resulted in a ten fold purification of enzyme from liver homogenates. Heat treatment of concentrated medium, however, resulted in a much lower purification of only 1.1 fold, presumably because the concentrates did not contain the structural proteins which precipitated from organ homogenates during heat treatment. Heating of medium concentrates was a useful

step, however, because it inactivated serum β-glucuronidase (Figure 10). 84% of the N-acetylglucosaminidase activity present in medium concentrates was also inactivated by heating at 65°C for 60 minutes, thereby reducing the β-glucuronidase:N-acetylglucosaminidase ratio from approximately 1:30 before heating to approximately 1:4.8 in the medium concentrates after heating. 62% of the remaining hexosaminidase and 51% of the contaminating β-galactosidase were removed from the concentrated medium by precipitating the β-glucuronidase activity in 45% saturated ammonium sulphate (Figure 11). Ammonium sulphate precipitation has been used as a purification step by many researchers (Stahl and Touster, 1971; Dean, 1974; Tomino et al., 1975; Natowicz et al., 1979) but as with heat treatment, only in the purification of β-glucuronidases from homogenised organs. In each case the purification reported was greater than that obtained for β-glucuronidase secreted by fibroblasts. A small purification of 1.7 fold was however achieved by ammonium sulphate precipitation and the extract was further concentrated by this method, an advantage when loading onto the affinity gels described below.

4.2.2 Purification of β-glucuronidase on Concanavalin A-Sepharose and DEAE-Sephacel

Purification of fibroblast β-glucuronidase by affinity chromatography on Concanavalin A-Sepharose was not the original method chosen. Concanavalin A is a lectin which binds to terminal and subterminal mannose residues on glycoproteins (Hughes and Pena, 1981). At the beginning of this project it was assumed that Concanavalin A might bind only those β-glucuronidase molecules with high mannose chains, and that some of the molecules with complex carbohydrate side chains would thus be discarded during this purification step. The

result of such a purification would be to enrich the populations of high mannose containing molecules which would be recognised as high uptake β-glucuronidase by fibroblasts. This would bias uptake experiments into fibroblasts and macrophages since the preparations used would not be representative of those molecules normally secreted. The possibility that trace amounts of Concanavalin A might leak from the Sepharose columns into the enzyme-containing eluate might also have posed a problem since 'piggy-back' endocytosis into fibroblasts of lysosomal enzymes linked to Concanavalin A has been reported (Gonzalez-Noriega and Sly, 1978).

The β-glucuronidase secreted by 3T3 fibroblasts bound with great avidity to Concanavalin A-Sepharose (section 3.2.2), and for this reason most of the contaminating protein together with some other lysosomal enzymes could be separated from the β-glucuronidase by first washing the gel in sodium acetate buffer pH 5.5 containing 0.05M α-methylmannoside and 1M sodium chloride. β-Glucuronidase activity was recovered by eluting the Concanavalin A-Sepharose with the above buffer containing 1.5M α-methylmannoside. Purification of β-glucuronidase from human spleen has been carried out by affinity chromatography on Concanavalin A-Sepharose (Natowicz et al., 1979) and resulted in a similar degree of purification to that which was attained eventually for fibroblast β-glucuronidase.

The strength of binding of 3T3 fibroblast β-glucuronidase to Concanavalin A may signify that this enzyme has a large number of high mannose side chains on each molecule. Human spleen β-glucuronidase, for instance, which is recognised as a high uptake form by fibroblasts contains approximately 81 mol of mannose per mol of β-glucuronidase,

and can be eluted from Concanavalin A-Sepharose in 1M α-methylmannoside (Natowicz et al., 1979). It is possible that the β-glucuronidase secreted by fibroblasts has a higher number of mannose chains per molecule than spleen β-glucuronidase, or that it has multivalent mannose configurations which mediate this strong attachment to Concanavalin A. It is interesting to note that N-acetylglucosaminidase from 3T3 fibroblasts eluted from the Concanavalin A-Sepharose in lower concentrations of α-methylmannoside than β-glucuronidase from the same source. This observation suggests that the N-acetylglucosaminidase may have fewer mannose residues than β-glucuronidase with which to bind to the lectin.

Any Concanavalin A which may have leaked from the column into the 1.5M α-methylmannoside was removed by mixing the eluate with Sephadex G-200 in 1M NaCl. Under these conditions, Concanavalin A binds to Sephadex (Agrawal and Goldstein, 1965) but the β-glucuronidase remains free in solution and can be collected after centrifugation. Initially the β-glucuronidase collected from fibroblasts was passed through a column of Sephadex G-200 in 1M NaCl in order to remove any contaminating Concanavalin A, but since no further purification was effected by this step (β-glucuronidase eluted in the void volume of the column), simple batchwise mixing was subsequently employed instead. There was no evidence that any contaminating Concanavalin A remained in the fraction containing β-glucuronidase after this treatment since, when 50mM α-methylmannoside was included during endocytosis experiments, it did not reduce uptake of the enzyme by fibroblasts (Table 13). Piggy-back endocytosis of lysosomal enzymes into fibroblasts, mediated by Concanavalin A, is completely inhibited by α-methylmannoside at this concentration (Gonzalez-Noriega and Sly, 1978).

Further purification of β-glucuronidase was effected by ion-exchange chromatography (Table 4). The leading edge of the broad peak of enzyme activity from 3T3 cells eluted in approximately 100mM NaCl, the trailing edge in 180mM NaCl (Figures 16 and 17), much stronger binding than high uptake β-glucuronidase from human spleen (Nicol et al., 1974) which was completely eluted in 100mM NaCl, or β-glucuronidase from human platelets which was also eluted at this concentration (Kaplan et al., 1977). The much greater negative charge on the β-glucuronidase secreted by 3T3 fibroblasts may be a characteristic feature of the species from which the enzyme was derived. Sialic acid residues contribute to this high negative charge, together with phosphate moieties. Alkaline phosphatase, however, induced a shift in the position of elution of the trailing edge of the peak to a less acidic position (Figure 17) suggesting that at least some of the β-glucuronidase molecules were phosphorylated, but that those in the leading edge were not. The overall spread of the elution profile also suggested that this population of β-glucuronidase molecules displayed considerable heterogeneity. This heterogeneity may have been due in part to the action of alkaline phosphatase present in foetal calf serum, or secreted by the cells themselves, under normal culture conditions. Using the method described in Sigma bulletin no. 104, phosphatase activity was measured at pH 10.5, pH 4.8 and pH 7.5 in buffers which were free of phosphate. In each case, the phosphatase activity was well below the levels present in normal adult human serum. At pH 4.8, the acid phosphatase secreted by fibroblasts was equivalent to only 0.67 International Units/litre (IU/l) of medium compared with 2-11 IU/l in normal human male serum and 0.2-10 IU/l in female serum. At pH 10.5, 0.8 IU/l alkaline phosphatase were secreted by 3T3 cells compared with

a range of 13-50 IU/l in adult serum, but at the pH in which the cells were grown (7.5), the phosphatase activity was only 1.2 IU/l. Alkaline phosphatase activity was slightly higher than that of acid phosphatase which may in part have been due to the short half life of the latter (less than 1 hour at 37°C). Since normal culture medium contains phosphate ions which inhibit alkaline phosphatase activity, and since the phosphatases from FCS were also low in culture medium (2.2 IU/l at pH 4.8; 11.7 IU/l at pH 10.5; 2.3 IU/l at pH 7.5), it is unlikely that the β-glucuronidase secreted by fibroblasts was degraded to any significant extent by phosphatases present in culture medium.

The effectiveness of DEAE-Sephacel as a purification step for β-glucuronidase was limited because although N-acetylglucosaminidase activity from crude preparations of 3T3 fibroblast conditioned medium eluted from the gel at a slightly different concentration of sodium chloride to that which eluted β-glucuronidase (Figure 16) most of the remaining protein coeluted with the β-glucuronidase (Table 4). Further steps such as ion-exchange chromatography on CM-Cellulose, or molecular sieve chromatography (Natowicz et al., 1979) might have been used to purify the β-glucuronidase, but each of these methods would have reduced the yield of enzyme activity considerably, and such losses could not be tolerated when the amounts of enzyme secreted by fibroblasts were so small compared with the amounts present in whole organs such as liver.

4.2.3 Substrate Affinity Chromatography of β-Glucuronidase

Affinity chromatography, using immobilised substrates as ligands, was investigated as a method for further purification of the β-glucuronidase secreted by fibroblasts in a high yield. Following the

method described by Harris et al. (1973), p-aminophenylglucuronide or saccharolactone was coupled to Sepharose 4B (via the spacer, diaminodipropylamine). This method had been used to purify commercially prepared bovine liver β-glucuronidase which was eluted from these gels in 0.1M acetic acid, a procedure which was claimed to be specific for β-glucuronidase. In practice, however, the binding of β-glucuronidase to these gels was not specific.

Firstly, samples of similar preparations of β-glucuronidase to those used by Harris et al. (1973) were found to contain both N-acetylglucosaminidase and β-galactosidase in very high amounts (Table 8) although samples of β-glucuronidase were not assayed for the presence of these enzymes by the above workers. Secondly, N-acetylglucosaminidase and β-galactosidase both bound to p-aminophenylglucuronide-Sepharose together with the β-glucuronidase prepared from mouse CBA liver (Table 7). β-Glucuronidase prepared commercially from bovine liver also bound to saccharolactone-diaminodipropylamine-Sepharose (Table 8) together with the same two enzyme contaminants. Thirdly, all of these lysosomal enzymes were eluted together in 0.1M acetic acid (Tables 7 and 8) in a non-specific manner so that the degree of β-glucuronidase purification which resulted was very low.

Several attempts were made to elute β-glucuronidase from these affinity gels by other methods which had not been suggested by Harris et al. (1973). Table 7 shows that small amounts of β-glucuronidase were recovered from the gel when the ionic strength of the eluting buffers was increased or when their pH was raised. Tris/HCl buffer at pH 8.0 released more β-glucuronidase than the other enzymes from the affinity gel, and was therefore used in subsequent experiments. Bovine

β-glucuronidase was eluted from saccharolactone-diaminodipropylamine-Sepharose in gradients of either NaCl, or ethylene glycol, or in combinations of both (Figures 18, 19 and 20), but in each case, the purification achieved was very low and β-glucuronidase could not be separated from N-acetylglucosaminidase. Similar disappointing results were reported by Dean (1974) who tried this method for purifying rabbit liver β-glucuronidase.

Although β-glucuronidase from bovine liver bound equally well to both p-aminophenylglucuronide-Sepharose and to saccharolactone-diaminodipropylamine-Sepharose (Table 9), section 3.2.4 shows that the amount of enzyme bound to each gel was much lower than that predicted by theoretical calculations (Table 5). The steric hindrance encountered when very large molecules such as β-glucuronidase react with relatively small immobilised ligands, may account for the discrepancy observed between actual and calculated binding capacities of the affinity gels. The lack of specificity of the binding of β-glucuronidase to affinity gels was apparent, however, from the results in Table 9, which shows that firstly a considerable amount of enzyme activity bound to uncoupled Sepharose. Secondly, treatment of cyanogen bromide activated Sepharose with 1M Tris pH 8.0 slightly reduced the non-specific binding of β-glucuronidase to Sepharose, but treatment of the gel with ethanolamine increased the affinity of the Sepharose 4B for β-glucuronidase several fold. When p-aminophenylglucuronide was then coupled to the Sepharose prior to ethanolamine treatment, binding of the enzyme to the gel was actually reduced, presumably because the p-aminophenylglucuronide was occupying sites which would otherwise have bound ethanolamine. Ethanolamine can bind to unreacted imidocarbonates present on cyanogen bromide activated Sepharose (see Pharmacia

publication 'Affinity Chromatography: principles and methods', p. 13), and can increase the ion-exchange capacity of the Sepharose by introducing extra amino groups. The ion-exchange properties of affinity columns treated in this manner were apparent when the elution profile of bovine β-glucuronidase in a sodium chloride gradient (Figure 18) was compared with the elution profile obtained for mouse 3T3 β-glucuronidase from DEAE-Sephacel under similar conditions (Figure 16). β-Glucuronidase bound even more strongly to the former than to the ion-exchange column of DEAE-Sephacel. Attempts were made to overcome some of the non-specific binding of β-glucuronidase to Sepharose 4B by repeatedly passing bovine serum albumin through the gel in order to condition it and mop up 'hot spots', however no measurable reduction in binding of β-glucuronidase to the gel was obtained by this method.

The third conclusion to be drawn from Table 9 is that the spacer diaminodipropylamine increased the binding of β-glucuronidase to Sepharose 4B even in the absence of the specific affinity ligand saccharolactone. Previous explanations for the improved binding of enzymes to ligands observed when the latter are attached to gels via such spacers, suggested that these chains reduced the steric hindrance which prevented binding of the enzyme to the ligand (Cuatrecasas et al., 1968; Cuatrecasas and Anfinsen, 1971; Harris et al., 1973). The results described here indicate, however, that this was not the reason for the increased binding of β-glucuronidase to saccharolactone linked via a spacer. An alternative explanation might be that diaminodipropylamine has a number of hydrophobic sites to which β-glucuronidase binds. The diaminodipropylamine-Sepharose thus acted as a hydrophobic chromatography column from which enzyme was dissociated by elution in ethylene glycol (Figure 19).

The apparent lack of specificity of the affinity ligands described above for β-glucuronidase prompted an alternative approach to the use of substrate affinity chromatography. Lisman and Overdijk (1978) had shown that isoenzymes of hexosaminidase could be purified successfully by binding them to Sepharose 4B coupled to p-aminophenyl-2-acetamido-2-deoxy-β-D-galactopyranoside. The enzymes were then specifically eluted in a gradient of 0-50mg/ml N-acetylglucosamine in PBS. When this method was adapted for purification of β-glucuronidase from bovine liver, however, the enzyme eluted in the gradient of glucuronic acid was completely inhibited by the specific eluant (Figure 21). Subsequent attempts to recover the β-glucuronidase activity by dialysis against Tris/HCl buffers at pH 7.0 and 8.0 failed. At pH 7.0, bovine liver β-glucuronidase activity was stable, but the enzyme-inhibitor complex could not be dissociated, whereas at pH 8.0 a small recovery of β-glucuronidase activity was obtained which was offset by an overall reduction in activity due firstly to binding of the enzyme to dialysis tubing and secondly to a partial inactivation of the enzyme at alkaline pH. Interestingly, p-aminophenylglucuronide, an alternative eluant which did not inhibit β-glucuronidase, was also a poor eluting ligand. This suggested that recovery of enzyme activity from affinity gels by elution with such specific ligands depended on the affinity of the eluting ligands for the enzyme: the higher the ligand-enzyme affinity, the lower the concentration of ligand required for elution, but the greater its inhibitory effect on the eluted enzyme.

In view of these findings, the method of affinity chromatography for purification of β-glucuronidase using glucaro-1,4,-lactone-benzidine-Sepharose (Warburton and Wynn, 1977) appears likely to be more successful than the methods investigated in this thesis. It was

reported that this ligand did not bind other glycosidases and was relatively specific for β-glucuronidase. This enabled non-specific elution procedures to be used and resulted in a 650 fold purification of the enzyme in a single step.

An important finding which emerged from the investigation of these different procedures was that β-glucuronidases from different sources behaved very differently during each purification step. Mouse 3T3 fibroblast β-glucuronidase bound more avidly to Concanavalin A-Sepharose than β-glucuronidases from other sources. It bound more strongly than human β-glucuronidases to DEAE-Sephacel (section 4.3.2), it was more stable at alkaline pH than bovine liver β-glucuronidase (Figure 22), and more stable to heating at 65°C than β-glucuronidases from foetal calf serum or AKR macrophages. The pH curves of activity of β-glucuronidases from 3T3 fibroblasts and from bovine liver were also different, the biphasic pH observed for bovine enzyme having been reported by other workers (Barrett, 1972).

The reasons for the marked differences in characteristics of β-glucuronidases from different species and different sources within the same species may in part be attributable to differences in the proportions of the large numbers of isoenzymes which have been shown to exist. β-Glucuronidase is unusual in that a relatively large proportion of it is present in microsomes rather than in lysosomes as is the case for other lysosomal enzymes. When microsomal β-glucuronidase was separated from lysosomal β-glucuronidase by electrophoresis and by ion-exchange chromatography (Owens and Stahl, 1976) there was little difference in the catalytic activities or the molecular weights of the isoenzymes from these two different sources. In rat liver, microsomal

and lysosomal β-glucuronidases were shown to differ in their carbohydrate content (Tulsiani et al., 1978) and in mouse liver, β-glucuronidase was separated into four microsomal isoenzymes which contained between one and four molecules of egasyn. This is a protein of 50,000-55,000 Daltons (Swank and Paigen, 1973; Tomino and Paigen, 1975) which anchors the enzyme into the endoplasmic reticulum. A further contribution to the heterogeneity of β-glucuronidase was the multiplicity of catalytic forms identified by Glaser and Conrad (1980). These workers showed that the β-glucuronidases purified from chick embryo liver, human placenta or rat preputial gland all displayed different K_m forms which could be inhibited or activated by changing the pH or sodium chloride concentrations of the reaction solutions. Many of the differences in the characteristics of β-glucuronidases isolated from these different sources appeared to be related to the carbohydrate side chains present on these molecules (Tulsiani et al., 1975), and these differences in carbohydrate content have been also correlated with their rapidity of uptake into fibroblasts (Glaser et al., 1975). The way in which bovine β-glucuronidase behaved when chromatographed on substrate affinity gels was therefore characteristic of that particular enzyme and consequently it might not have been a good enzyme to use in order to determine precise chromatographic parameters for purification of β-glucuronidase secreted by 3T3 fibroblasts.

4.3 CULTURE OF AKR MACROPHAGES

Peritoneal macrophages which were cultured by the methods described in section 3.1.3, were judged to be free of fibroblasts and granulocytes by their ability to phagocytose complement coated yeast particles (Figure 8) and also by their lack of chloroacetate esterase activity (Figure 9). Attempts to maintain dividing cultures of macrophages by the method described by Lee (1969) were unsuccessful. Cell division and colony formation occasionally occurred after several weeks in culture, but the number of divisions observed in the colonies was limited as might be expected for primary cultures of mouse cells. Subculture of dividing colonies was also only partially successful. Macrophages were completely resistant to the effects of trypsin, but could be removed from culture dishes by incubation in lignocaine, a local anaesthetic (Van der Zeijst et al., 1978; Rabinovitch and DeStefano, 1976). The washed cells were then seeded at lower densities on 35mm dishes, but colony growth in such subcultured cells was rarely observed. The unreliability of macrophage division coupled with the inability to subculture these cells made the setting up of a permanent macrophage line impossible. For the purposes of uptake experiments, therefore, macrophages were seeded at approximately 50% confluence (10^6 cells per 35mm dish) and were used four days after harvesting. The choice of the fourth day of culture for uptake experiments was determined by the levels of intracellular β-glucuronidase activity (Figure 7). The steady reduction in the endogenous activity of AKR macrophages observed during the first four days of culture may have been attributable to the length of time required for them to adjust to culture conditions. Synthesis of β-glucuronidase may have been reduced

over this period, although secretion of the enzyme occurred at a constant rate over much longer periods of time (Figure 7). Intracellular degradation of β-glucuronidase may possibly have been more rapid over the first four days, but the precise contribution of rates of synthesis and degradation to the endogenous β-glucuronidase activity of AKR macrophages was not investigated. Macrophages harvested from AKR mice had much lower endogenous levels of β-glucuronidase than CBA macrophages (M.F. Dean, unpublished data), but the latter cells also showed this steady reduction in activity during their first few days in culture.

Figure 10 shows that the intracellular β-glucuronidase obtained from AKR macrophages, whether derived from male or female mice, was labile to heating at 65°C. β-Glucuronidase secreted from 3T3 fibroblasts was stable under similar conditions, and remained stable after uptake into AKR macrophages. These results meant that after uptake experiments had been carried out, the endogenous enzyme activity present in the macrophages could be differentially inactivated by heating extracts of cells to 65°C, as described for uptake of β-glucuronidase into AKR fibroblasts (Frankel et al., 1977). Any increase in the endogenous β-glucuronidase activity of AKR macrophages which might have been induced by contaminants in the preparation of fibroblast β-glucuronidase would also be accounted for by this method.

4.4 UPTAKE EXPERIMENTS

The results in section 3.3 show that the β-glucuronidase secreted by mouse 3T3 fibroblasts was taken up into mouse macrophages as well as human fibroblasts. The uptake into both types of cell was linear with time (Figures 23 and 26), but uptake into macrophages eventually became non-linear after eight hours' incubation. This suggested that either the extracellular concentration of β-glucuronidase had decreased below the level required to saturate these cell surface receptors, or that the macrophages themselves were inactivating the β-glucuronidase after endocytosis.

The fact that β-glucuronidase secreted by 3T3 fibroblasts was also taken up into human GM 151 fibroblasts (Figure 26) was consistent with the data previously reported by Neufeld *et al.* (1977) and Halley (1980) following their cross-correction experiments. The staining pattern observed in fibroblasts following uptake (Figure 27) showed that the β-glucuronidase was localised in discrete perinuclear packages in a pattern which suggested strongly that these stained inclusions were lysosomes. This pattern of staining was similar to that observed in these cells after uptake of human liver β-glucuronidase (Lagunoff *et al.*, 1973), or after direct interaction with mouse lymphocytes (Olsen *et al.*, 1981).

Uptake of the β-glucuronidase secreted by 3T3 fibroblasts into both macrophages and fibroblasts was rapid and saturable (Table 10, Figures 28 and 29). The rate of uptake into fibroblasts compared favourably with the rate of uptake of platelet β-glucuronidase (Table 10) and with the values obtained for other types of high uptake ligands into

these cells (Brot et al., 1974; Nicol et al., 1974; Kaplan et al., 1977; Sando and Neufeld, 1977; Hieber et al., 1980). The maximum rate of uptake into macrophages was similar to that recorded for uptake of [^{125}I]-mannosyl BSA into peritoneal macrophages by Ezekowitz et al. (1981), but somewhat lower than the values obtained for uptake of placental β-glucuronidase into alveolar macrophages (Achord et al., 1978). The rate of uptake of placental β-glucuronidase into peritoneal macrophages (Table 10) was lower than the reported rates of uptake into alveolar macrophages. This lower rate of uptake might have been a consequence of different culture conditions since alveolar macrophages were cultured in suspension whereas peritoneal macrophages were cultured as monolayers. Suspension cultures might have had higher rates of uptake merely because none of the plasma membranes of the cells was involved in adhesion to the substratum, and therefore a greater number of surface receptors would be available for uptake. The rates of uptake of fibroblast β-glucuronidase into both peritoneal macrophages and fibroblasts were, however, very much greater than the rate of fluid phase pinocytosis, which was determined by measuring the rates of internalisation of horseradish peroxidase (HRP) (Figure 30; Table 10).

Uptake of HRP into GM151 fibroblasts was very low and comparable with values obtained by Hieber et al. (1980) for uptake into fibroblasts from a patient with GM1 gangliosidosis, but uptake into peritoneal macrophages was 7.5 fold greater at 0.09%/mg/hr (Table 10). There are a number of discrepancies in the values reported by other workers for uptake of HRP into these two types of cell. Uptake of HRP into alveolar macrophages has been claimed to occur by receptor mediated endocytosis (Stahl et al., 1978) on the basis of the competition observed between

HRP and β-glucuronidase for uptake into these cells. Contrary evidence suggested that HRP was taken up entirely non-specifically by adherent macrophages (Steinman and Cohn, 1972). The values reported in this thesis for the uptake of HRP into mouse peritoneal macrophages agreed with those of Steinman and Cohn (1972) suggesting that either alveolar macrophages had different receptors on their plasma membranes from those found on peritoneal macrophages, or that different batches of commercially prepared HRP varied in their uptake properties. The latter explanation is more likely, since the HRP purchased from Sigma was taken up initially at a rate of approximately 0.6%/mg/hr into peritoneal macrophages, but after passage through Concanavalin A-Sepharose, that fraction which remained unbound (81% of the total activity) was taken up at only 0.09%/mg/hr. It is possible therefore that this preparation of HRP contained a minor population of molecules bearing high mannose type recognition markers which facilitated their uptake into macrophages by receptor mediated endocytosis. This fraction was removed by binding to Concanavalin A-Sepharose. Alternative methods for measuring fluid phase pinocytosis include the assay of uptake of [^{125}I]-polyvinylpyrrolidone (Williams et al., 1975) which has been used to measure rates of non-specific pinocytosis into cells of the rat yolk sac. The values obtained in such experiments were similar to those measured here for uptake of HRP into macrophages.

Uptake of HRP into both mouse peritoneal macrophages and human GM151 fibroblasts was linear over the range of concentrations chosen for these experiments (Figure 30), further evidence that uptake of this enzyme was indeed an accurate way to measure the rate of non-specific endocytosis into these cells. Uptake of the fibroblast β-glucuronidase into both types of cell was, in contrast to uptake of HRP, saturable

with increasing extracellular concentration of the enzyme (Figures 28, 29 and 30), evidence that the β-glucuronidase was taken up by receptor mediated endocytosis. This was the first demonstration that enzyme secreted by one type of cell could be recognised and taken up by cells of another type, and challenged the proposal put forward by Achord et al. (1978) that the uptake of glycoproteins into fibroblasts and reticulo-endothelial cells was mutually exclusive. Moreover, Table 10 shows that platelet β-glucuronidase, traditionally considered to be a high uptake ligand for fibroblasts (Brot et al., 1974; Glaser et al., 1975), can also be taken up by peritoneal macrophages at a rate far in excess of that recorded for uptake of HRP. Rapid uptake of the β-glucuronidase prepared from placenta was however observed only into macrophages (Figure 28) and not into fibroblasts, a finding which was consistent with previously published results (Brot et al., 1974; Achord et al., 1978). The β-glucuronidase secreted by 3T3 cells transformed with SV40 virus was also taken up into deficient human fibroblasts (Table 10) but the rate of uptake of this enzyme preparation was lower than that obtained for uptake of β-glucuronidase secreted by non-transformed 3T3 cells, and for this reason transformed cells were not used as a source of secreted lysosomal enzymes.

Von Figura and Kresse (1974) were the first researchers to apply the parameters of saturation kinetics to the uptake of lysosomal enzymes by cell surface receptors. They assumed that uptake of ligands bound to receptors on plasma membranes was analogous to the formation of enzyme-substrate complexes, a reaction described by first order kinetics. This model was used to characterise the specificity of the receptor sites present on fibroblasts from patients with Sanfilippo B syndrome for N-acetyl-α-D-glucosaminidase prepared from normal human urine.

The K_{uptake} (the concentration of added enzyme equivalent to half of that required to saturate the surface receptors on the fibroblasts) for their system was 9×10^{-10}M. The K_{uptake} values obtained for receptor mediated endocytosis of β-glucuronidase secreted by 3T3 fibroblasts into both macrophages and fibroblasts (Table 11) show that this enzyme saturated the cell surface receptors at relatively low concentrations compared with the values obtained by Von Figura and Kresse (1974) and by Sando and Neufeld (1977) for other high uptake ligands. There are a number of possible explanations for this observation. Firstly, binding of fibroblast β-glucuronidase might have been a multivalent process, thereby effectively reducing the number of receptors available for uptake on the surfaces of the cells. Secondly, the enzyme might have a particularly high affinity for its receptors, in which case, the reduced rate of dissociation of the enzyme from the receptors after internalisation might result in a reduction in the number of vacant binding sites reappearing on the plasma membranes of the cells. Thirdly, contaminating glycoproteins which copurified with β-glucuronidase on Concanavalin A-Sepharose might have been competing with the enzyme for uptake into the cells by mannose or phosphomannosyl receptors. The correct explanation can only be resolved firstly by measuring the binding of highly purified radiolabelled β-glucuronidase to cell surfaces at $4^{o}C$, followed by Scatchard analysis in order to calculate the number of binding sites available, and secondly by repeating the saturation curves and Hanes plots for uptake into both types of cell with a more highly purified preparation of β-glucuronidase in order to find out if saturation occurs at higher concentrations than those measured for less pure preparations. Preliminary uptake experiments using 3T3 fibroblast

β-glucuronidase purified by antibody affinity chromatography have suggested that the relative impurity of the original preparations was indeed a contributory factor to the low $K_{uptakes}$ obtained in these experiments. Interestingly, platelet β-glucuronidase which had also been purified by Concanavalin A-Sepharose had a similar K_{uptake} value into fibroblasts (Table 11) to that obtained for this enzyme by other workers (Kaplan et al., 1977).

The rapidity of uptake of β-glucuronidase secreted by 3T3 fibroblasts, coupled with the saturation of uptake into both fibroblasts and macrophages, showed that uptake into both types of cell was mediated by specific receptors for the enzyme. Previously published results have suggested that fibroblasts have phosphomannosyl receptors (Kaplan et al., 1977) and macrophages have mannose receptors (Achord et al., 1978; Warr, 1980; Ezekowitz et al., 1981) on their cell surfaces. Evidence from uptake studies indicated that glycoproteins which were recognised as high uptake ligands by mannose receptors were not recognised by phosphomannosyl receptors (Achord et al., 1978). It was therefore important to find out whether the β-glucuronidase secreted by 3T3 fibroblasts was recognised by a previously undescribed mannose 6-phosphate receptor present on macrophages, or if the enzyme contained recognition markers for both phosphomannosyl and mannose receptors. Elicited peritoneal macrophages might possess induced mannose 6-phosphate receptors, but all of the available evidence militates against this possibility. β-Glucuronidase which had been treated with alkaline phosphatase was subsequently not recognised or taken up by human deficient fibroblasts (Table 12), however, phosphatase treated samples of the same enzyme preparation were taken up rapidly into macrophages under the same conditions. Furthermore, uptake of

β-glucuronidase into fibroblasts was inhibited in the presence of phosphorylated sugars, whose effectiveness was in the order: mannose 6-phosphate > fructose 1-phosphate > glucose 6-phosphate. These results were similar to those obtained for the inhibition of α-iduronidase uptake into fibroblasts by Sando and Neufeld (1977), whereas only mannose itself or other mannose containing molecules inhibited uptake into macrophages. Fructose 1-phosphate and glucose 6-phosphate were ineffective inhibitors of endocytosis into macrophages (Table 13), results which were in agreement with the inhibition data obtained for uptake of mannose bearing yeast cells into alveolar macrophages (Warr, 1980).

Uptake of 3T3 fibroblast β-glucuronidase into both macrophages and fibroblasts was inhibited slightly by N-acetylglucosamine at a concentration of 50mM (Table 13). Inhibition of clearance of mannose bearing glycoproteins into alveolar macrophages by N-acetylglucosamine has been reported by other workers (Schlesinger et al., 1980), and seems to be a characteristic of uptake by mannose receptors present on reticuloendothelial cells. The inhibition of β-glucuronidase uptake into fibroblasts by N-acetylglucosamine is more difficult to explain, but has also been observed when uptake of β-glucuronidase from urine was measured in the presence of inhibitors (Ullrich et al., 1978b). Neither glucose nor fructose had any inhibitory effect on uptake into either macrophages or fibroblasts.

Studies with resident, non-elicited AKR macrophages showed identical patterns of uptake to those described above for cells elicited with starch (data not shown), further evidence that in vivo activation of these macrophages did not induce phosphomannosyl receptors on their plasma membranes.

The above data on inhibition together with the results of treatment with alkaline phosphatase and uptake into fibroblasts and macrophages (shown in Tables 10, 12 and 13) clearly indicate that 3T3 fibroblasts secrete β-glucuronidase molecules which have carbohydrate configurations enabling them to be recognised and taken up via mannose 6-phosphate receptors into fibroblasts and via mannose receptors into macrophages. It is not known whether there are two or more populations of enzyme secreted by these mouse fibroblasts, each population bearing different recognition markers, or whether only one population is secreted with oligosaccharide side chains recognised by different types of cells. Recent evidence from the work of Hasilik et al. (1981) has suggested that normal fibroblasts can secrete lysosomal enzymes bearing two or more carbohydrate side chains, and this might also be true of the β-glucuronidase secreted by 3T3 fibroblasts. It has also been indicated that the tertiary configurations of glycoproteins might conceal some oligosaccharide side chains, while exposing others to further processing (Schachter, 27th May 1982, Cell Membranes and Glycoprotein Synthesis, The Royal Society, London. Abs. p. 3) thus producing mature glycoproteins with both 'high mannose' and 'complex' oligosaccharide chains on each protein core. Alternatively, a range of β-glucuronidase isoenzymes might be secreted, some bearing recognition markers for both types of cell. A further possibility is that each oligosaccharide side chain might be recognised by both macrophages and fibroblasts, phosphate groups being essential for recognition by fibroblasts, but non-essential for binding to the mannose receptors present on macrophages. The consistent increase in the rate of uptake of β-glucuronidase into macrophages which was observed after treatment with alkaline phosphatase (Table 11) suggested

that the phosphate groups present on high mannose chains might have reduced slightly the affinity of these oligosaccharides for mannose receptors. Chromatography on DEAE-Sephacel suggested that the enzyme was secreted as a heterogenous population of enzymes which were eluted in a broad peak (Figures 16 and 17). The most acidic portion of the profile of enzyme activity was that which shifted after treatment with alkaline phosphatase, and it was this portion which was taken up most rapidly by fibroblasts (Table 11). The position of elution of the less acidic populations of β-glucuronidase was not altered by treatment with alkaline phosphatase, suggesting that some β-glucuronidase was secreted either without terminal phosphate groups, or with their phosphate groups masked by phosphodiester linkages, such as those which have been observed during the post-translational processing of lysosomal enzymes (Reitman et al., 1981; Tabas and Kornfeld, 1980; Hasilik et al., 1980). While these results confirm that only phosphorylated enzymes can be recognised by fibroblasts, the question of whether phosphorylated β-glucuronidase can be internalised by macrophages remains open. Preliminary experiments in which antibody purified β-glucuronidase secreted by 3T3 cells was depleted of its phosphorylated populations by repeated incubation with fresh β-glucuronidase deficient fibroblasts, followed by incubation of the enzyme remaining in the medium with AKR macrophages, has shown that populations of enzyme which behaved as low uptake ligands for fibroblasts were also recognised only as a low uptake form by macrophages. Consequently, the same population of β-glucuronidase molecules appears to be internalised by both macrophages and fibroblasts.

The fate of fibroblast β-glucuronidase after its internalisation by deficient fibroblasts and macrophages was not fully investigated;

however, subcellular fractionation of deficient fibroblasts following uptake of β-glucuronidase prepared from platelets confirmed that this enzyme was taken up into the lysosomes (Bach and Liebmann-Eisenberg, 1979). Hasilik and Neufeld (1980) have reported that three different lysosomal enzymes were finally processed to the mature form in the lysosomes of fibroblasts, while Glaser et al. (1975) suggested from evidence obtained with isoelectric focussing that the β-glucuronidase internalised by deficient fibroblasts was processed to a less acidic, and therefore possibly less phosphorylated form after endocytosis. The 3T3 β-glucuronidase eluted from DEAE-Sephacel at a less acidic position after uptake by deficient fibroblasts, suggesting that this too may have been dephosphorylated after uptake.

The specific stain used to localise β-glucuronidase could not distinguish between endogenous and freshly internalised β-glucuronidase present in macrophages, and so was not used on these cells. The relatively short length of time during which β-glucuronidase uptake was linear (Figure 23), however, suggested that turnover of this enzyme was rapid. This observation has also been made by Skudlarek and Swank (1981) who showed that the half life to endogenous β-glucuronidase in thioglycollate elicited mouse macrophages was 1.8 days. The uptake of 3T3 fibroblast β-glucuronidase into fibroblasts was linear for a much longer period (Figure 26), indicating that its half life was longer in these cells than in macrophages. Estimates of the half life of β-glucuronidase made by other workers vary from 4-5 days for endogenous hamster fibroblast enzyme (Warburton and Wynn, 1977) to 14 days for exogenously added human platelet β-glucuronidase in human deficient fibroblasts (Bach and Liebmann-Eisenberg, 1979) implying that endogenous, and exogenously added lysosomal enzymes may turn over at different rates.

4.5 CONCLUSIONS

The β-glucuronidase secreted by mouse 3T3 fibroblasts was recognised and taken up by receptor mediated endocytosis into two different types of cell each of which have different surface receptors for glycoproteins. These results suggest that the concept of targeting of lysosomal enzymes to different cells *in vivo* should be re-examined in view of the heterogenous populations of enzyme which can be synthesised and secreted by fibroblasts *in vitro* and which may thus be targeted to more than one type of cell. These findings may help to explain why transplanted fibroblasts when used as donor cells for enzyme replacement therapy have not always produced clinical improvements in treated patients, for although it is possible that transplanted fibroblasts can indeed secrete lysosomal enzymes which may be taken up into cells of the reticuloendothelial system, the human circulation is such that the Kupffer cells of the liver would take up most of any circulating lysosomal enzymes and then promptly degrade them. While the rapid turnover of normal enzymes in the livers of deficient patients can be overcome to some extent by providing a constant supply of freshly synthesised enzymes, in cell and organ transplants for example, the targeting of lysosomal enzymes to cells other than those of the liver may prove more difficult to achieve. If fibroblasts were capable of secreting sufficient quantities of lysosomal enzymes into the circulations of patients, the uptake system of the Kupffer cells could be saturated, and enzymes would then be delivered to other cells of the reticuloendothelial system and connective tissue cells; consequently the treatment of diseases where storage of macromolecules was systemic would become much more effective. The amount of lysosomal enzymes

required to overload the uptake system of Kupffer cells would be, however, beyond the range of fibroblast transplants in their present form, and so this type of enzyme therapy might be better suited to storage diseases such as Gaucher's disease where undegraded molecules are largely localised in the liver. Alternatively, sites of fibroblast transplants might be varied to include areas close to tissues where the bulk of the undegraded macromolecules are stored. In this way optimum transfer of enzyme to deficient cells might be achieved.

In this thesis it has been reported that fibroblasts can secrete different types of β-glucuronidase which are certainly in the high uptake form for two different types of cell. Despite the difficulties attendant upon enzyme replacement therapy using fibroblast transplants it is therefore possible that normal fibroblasts may secrete lysosomal enzymes which can also be taken up by other types of cell such as hepatocytes or nerve cells. If this were found to be the case, the more physiological problems of where to place the transplanted fibroblasts would be well worth solving.

Mouse 3T3 fibroblasts secreted lysosomal enzymes in large amounts compared with other normal fibroblasts. All estimates of the rate of uptake of β-glucuronidase secreted by these cells suggested that the enzyme was similar to that secreted by normal fibroblasts. For these reasons these cells may prove useful tools, firstly for studying the mechanisms of synthesis and secretion of normal lysosomal enzymes by fibroblasts, and secondly for assessing the uptake potential of secreted lysosomal enzymes by many other different types of cells.

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