CHAPTER I

INTRODUCTION

AND

REVIEW OF LITERATURE
The potential of enzymes as therapeutic agents has long been considered. However, the use of enzymes as drugs was limited by their immunogenicity, short half life in circulation and rapid proteolytic degradation in vivo. In recent years a number of investigators have shown that it is possible to enhance the circulatory life of enzymes by their covalent modification with PEG or with dextran. Such conjugates also exhibit reduced immunogenicity. The therapeutic potential of the dextran-enzyme and PEG-enzyme conjugates is now well characterised. A number of enzyme conjugates such as PEG-adenosine deaminase and PEG-uricase have shown enhanced efficacy in curing disorders such as severe combined immunodeficiency and hyperuricemia respectively. Although the circulatory life of enzymes has been enhanced by this method, not much work has been done on the intracellular and intralysosomal stability of enzymes.

Attempts made to use enzyme therapy in the treatment of lysosomal enzyme deficiency disorders have met with limited success only. Such disorders manifest themselves as storage conditions and the substrate of the deficient enzyme accumulates in the lysosomes of the affected cells. The main drawbacks in the treatment of enzyme deficiency disorders include delivery of the enzyme to the sites of substrate accumulation and the rapid intracellular degradation of the enzyme.

Liposomes are ideal carriers for the delivery of enzymes, due to their biodegradability and their ability to deliver the entrapped enzyme to the lysosomes of the affected cells. The use of liposomes as an enzyme carrier was first suggested by Gregoriadis et al (1971). Liposomes can be targeted to different cell types by changing their composition, size, charge and by tailoring the liposomal surface with ligands specific for different receptors on different cell types.
In this study we have tested the effect of modification of enzymes with PEG and dextran on their intracellular and intralysosomal stability. The efficacy of liposomally delivered enzyme conjugate in degrading the stored material in a model storage condition in mice and in preventing any further accumulation of the substrate has also been studied.

1.1 STORAGE DISORDERS

A number of inherited metabolic disorders lead to the accumulation of metabolic intermediates due to the absence of some key enzyme responsible for their degradation. These disorders may be caused due to the deficiency of either a cytosolic or a lysosomal enzyme. Here we will limit our discussion to lysosomal enzyme deficiency disorders only with particular reference to sphingolipidoses and a lysosomal glycogen storage disorder.

1.1.1 Lysosomal sphingolipid storage disorders

One of the first lysosomal enzyme deficiency in sphingolipidoses was reported from this laboratory in collaboration with Austin in 1963. They showed that metachromatic leukodystrophy was caused by the deficiency of arylsulfatase - A which led to the accumulation of cerebroside-3' sulfate in the lysosomes. Cerebroside-3' sulfate was subsequently found to be the substrate for arylsulfatase - A (Mehl and Jatzkewitz, 1965, Farooqui and Bachhawat, 1973). Since then a number of enzymes are now known to be absent in a variety of sphingolipid storage disorders as shown in Fig 1. A variant form of metachromatic leukodystrophy is now known to be caused by the absence of a factor required for the hydrolysis of sulfatide even when the enzyme is active.

One of the most prevalent sphingolipid storage disorders is Gaucher's disease which is caused by the deficiency of the enzyme β-glucocerebrosidase. It results in the accumulation of glucosylceramide in various
**FIGURE 1**

Cer-β-Glc-β-Gal-α-Gal-β-GalNAc-β-Gal (G₃₁)

↓ NANA

G₃₁ Gangliosidosis ↓ β-Galactosidase

Cer-β-Glc-β-Gal-β-GalNAc (G₃₂)

↓ NANA

Tay-Sach's disease ↓ β-Hexosaminidase A

Cer-β-Glc-β-Gal (G₃₃)

↓ NANA

Neuraminidase

Cer-β-Glc-β-Gal-α-Gal-β-GalNAc (G₃₄)

↓ β-Galactosidase

Cer-β-Glc-β-Gal (G₃₅)

↓ β-Glucosidase

Sphingomyelinase

Cer-P-choline

Niemann-Pick disease

β-Galactosidase

Cer-β-Gal

Krabbe's disease

Arylsulfatase A

Metachromatic leukodystrophy

Farber's disease

Ceramidase

Sphingosine + Fatty acid

Cer-β-Gal-3-SO₃.
organs especially the liver and the spleen. Due to its prevalence, Gaucher's disease has been extensively studied. A naturally occurring canine model of Gaucher's disease has been reported but was not propagated (Hartley and Farrow, 1982). Attempts have also been made to mimic the disease in mice by inhibiting glucocerebrosidase (Kanfer et al, 1982). More recently, Tybulesicz et al (1992) have generated an animal model for Gaucher's disease by creating a null allele in embryonic stem cells through gene targeting and using these genetically modified cells to establish a mouse strain carrying the mutation. Such mice have been shown to store glucocerebroside in lysosome of cells of the reticuloendothelial system.

Fabry's disease is another sphingolipid metabolism disorder. It is caused by the deficiency of $\alpha$-galactosidase. It was first noticed as pain in the joints especially when subjected to extremes of temperature. An accumulation of digalactosylceramide, which is present only in the kidney's of normal individuals, is found in almost all the organs and body fluids.

Krabbe's disease is caused due to the deficiency of galactosylceramide $\beta$-galactosidase (Suzuki and Suzuki, 1983) resulting in the accumulation of galactosylceramide and galactosylsphingosine in the brain (Svennerholm et al, 1980). It causes mental retardation followed by death by the age of two.

Another sphingolipid metabolic disorder Tay Sach's disease is caused due to the deficiency of $\beta$-hexosaminidase A. Early symptoms of this disease include a spot on the eye which causes blindness. It quickly progresses through motor retardation to a vegetative state. A variant form of Tay Sach's disease is caused even when the enzyme is active. Tay Sach's disease AB variant is caused due to the absence of a factor required for the hydrolysis of GM2 ganglioside. Table I lists a variety of sphingolipidoses and the nature of defect in each type of disorder.
# TABLE I

**DEFECTS IN LYSOSOMAL SPHINGOLIPID STORAGE DISORDERS**

(Tager, J.M. 1985)

<table>
<thead>
<tr>
<th>Disease</th>
<th>Deficient hydrolase(s)</th>
<th>Nature of Defects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tay-Sachs disease</td>
<td>β-Hexosaminidase A</td>
<td>1. mRNA for α-precursor absent</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2. Reduced synthesis of α-precursor</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3. Insoluble α-precursor</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4. Labile α-precursor</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5. Mature α-chain larger than normal and yields inactive enzyme</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6. Defective association of α-precursor with β-precursor</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7. Reduced α-precursor synthesis and defective association with β-precursor</td>
</tr>
<tr>
<td>Tay-Sachs disease</td>
<td></td>
<td>Absence of factor required for hydrolysis of GM2 ganglioside</td>
</tr>
<tr>
<td>Metachromatic leukodystrophy</td>
<td>Arylsulfatase A</td>
<td>1. No precursor of arylsulfatase A formed</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2. Arylsulfatase A rapidly degraded in lysosomes</td>
</tr>
<tr>
<td>Metachromatic leukodystrophy</td>
<td>Arylsulfatase A with sulfatide as substrate</td>
<td>Absence of factor required for hydrolysis of sulfatide</td>
</tr>
<tr>
<td>variant form</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Disease</td>
<td>Deficient hydrolase(s)</td>
<td>Nature of Defects</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------</td>
<td>-------------------------------------------------------</td>
</tr>
<tr>
<td>Gaucher's disease</td>
<td>β-Glucocerebrosidase</td>
<td>1. β-Glucocerebrosidase with decreased stability, enhanced degradation in lysosomes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2. Mature β-galactosidase reduced in amount and aggregates poorly</td>
</tr>
</tbody>
</table>

The third column indicates the defects in synthesis, processing or stability of hydrolases, or other proteins, responsible for different forms of the diseases. The table omits a number of suspected lysosomal disorders whose bases are not yet well understood.
As mentioned above, in some cases storage conditions are caused not only due to the deficiency of the enzyme but also due to the deficiency of some other lysosomal proteins which have non-enzymic roles, and function as activators or stabilizers of enzymes. Saposins constitute one such class of proteins, four of which are derived from a single precursor prosaposin by proteolytic processing. Saposins are small heat stable glycoproteins (12-14 kDa) and are required for the lysosomal hydrolysis of a variety of sphingolipids (Table II).

Saposin B (previously known as sphingolipid activator protein-1) was found to activate the hydrolysis of cerebroside sulfate by arylsulfatase A and was later shown to activate the hydrolysis of GM1 ganglioside by acid β-galactosidase and of globotriaosylceramide by α-galactosidase (reviewed by Li et al, 1988). Saposin B has a broad specificity and it acts as a detergent solubilizing multiple lipid substrates for enzyme hydrolysis. Deficiency of Saposin B results in tissue accumulation of cerebroside sulfate. The clinical picture resembles that of metachromatic leukodystrophy (Werger et al, 1989).

Saposin C (previously called sphingolipid activator protein 2) was discovered in 1971 by Ho and O'Brien (reviewed by O'Brien and Kishimoto, 1991), when it was shown to activate the hydrolysis of glucocerebrosides by glucosylceramide β-glucosidase. Later, the activation of hydrolysis of galactocerebrosides by Saposin C was reported (reviewed by Glew et al, 1988). Saposin C raises the maximal velocity of these enzymes by as much as 10-fold (Radin, 1984). Saposin A was also found to stimulate the hydrolysis by the same enzymes, but to a lesser extent than Saposin C (Morimoto et al, 1989). Very little hydrolysis occurred in the absence of Saposin A or C. The deficiency of Saposin C has been reported in a patient suffering from a variant form of Gaucher's disease (Christomanou et al, 1986).
<table>
<thead>
<tr>
<th>Previous Names</th>
<th>Enzyme Activated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saposin A</td>
<td>β-glucosylceramidase</td>
</tr>
<tr>
<td></td>
<td>β-galactosylceramidase</td>
</tr>
<tr>
<td>Saposin B</td>
<td>SAP-1</td>
</tr>
<tr>
<td></td>
<td>Salfatide/GM1-activator</td>
</tr>
<tr>
<td></td>
<td>Dispersin</td>
</tr>
<tr>
<td></td>
<td>Non-specific activator</td>
</tr>
<tr>
<td></td>
<td>Arylsulfatase A</td>
</tr>
<tr>
<td></td>
<td>α-galactosidase A</td>
</tr>
<tr>
<td></td>
<td>GM1-β-galactosidase</td>
</tr>
<tr>
<td></td>
<td>many others</td>
</tr>
<tr>
<td>Saposin C</td>
<td>Factor P</td>
</tr>
<tr>
<td></td>
<td>SAP2</td>
</tr>
<tr>
<td></td>
<td>Coglucosidase</td>
</tr>
<tr>
<td></td>
<td>A1-activator</td>
</tr>
<tr>
<td></td>
<td>Heat Stable factor</td>
</tr>
<tr>
<td></td>
<td>β-glucosylceramidase</td>
</tr>
<tr>
<td></td>
<td>β-galactosylceramidase</td>
</tr>
<tr>
<td>Saposin D</td>
<td>Component C</td>
</tr>
<tr>
<td></td>
<td>Sphingomyelinase</td>
</tr>
</tbody>
</table>

(from O'Brien and Kishimoto, 1991)
Saposin D acts as a specific activator of sphingomyelinase activity with no effect on other lysosomal hydrolases (Morimoto et al, 1988). Detailed studies on kinetics have not been carried out, but in crude preparations it increases the rate of hydrolysis 3- to 5-fold.

Another sphingolipid activator protein, known as the GM2 activator protein was shown to be a specific activator for the hydrolysis of ganglioside GM2 by β-N-acetylgalactosaminidase (hexosaminidase A). This protein is genetically distinct from the saposin proteins, and its deficiency has been reported in a variant form of GM2 gangliosidosis (Conzelmann et al, 1978).

1.1.2 Lysosomal glycogen storage disorder

Pompe's disease or Type II glycogenosis is a glycogen storage disorder and is caused by the deficiency of lysosomal α-glucosidase. It results in the accumulation of glycogen in various organs especially the liver, spleen and the cardiac tissues. Although no animal models have been propagated to study this disorder, various investigators have tried to create artificial carbohydrate storage conditions both in vivo and in vitro. Exposure to sucrose of mouse peritoneal macrophages (Cohn and Ehrenreich, 1969) and Chinese hamster fibroblasts (Nyberg and Dingle, 1970) both lacking in invertase activity led to the accumulation of sucrose within their lysosomes. The lysosomes of these cells then appear as phaselucent vacuoles in the perinuclear region. Colley and Ryman (1976) created an in vivo model storage condition by intraperitoneal administration of dextran. It is known that intraperitoneal administration of dextran produces an increase in the density of liver lysosomes (Baudhuin et al, 1965). The artificial storage conditions were very useful for developing various therapeutic approaches for the treatment of such disorders. Animals which have been made deficient in an enzyme in order to mimic a naturally occurring storage condition do not survive long enough for any therapeutic approaches to be tried.
1.2 **THERAPEUTIC APPROACHES** :

1.2.1 **Genetic engineering**

A number of approaches have been tried to cure storage disorders. These include genetic engineering. The idea was to introduce the gene of the deficient enzyme and express it inside the cell in order to alleviate the disorder.

Nontargetted delivery of genes into cells can be accomplished by chemical or physical means (transfection). In this approach many copies of the DNA carrying the healthy gene are mixed with a charged substance typically calcium phosphate, DEAE-dextran or certain lipids. When exposed to these chemicals, the cell membrane is disturbed and the DNA is transported into the cell interior (Sabrook *et al*, 1989). Although the procedure is simple, the efficiency of gene delivery is only 1 in 1000 to 100,000 and this reduces its clinical potential.

Physical methods such as microinjection are very efficient, but are tedious as only one cell can be injected at a time. Electroporation or exposure of cell to electric shock although efficient, can severely damage the cell.

A number of investigators have suggested retroviruses as vectors (Hamer and Leder, 1979, Mulligan *et al*, 1979). Retroviruses, however are not without drawbacks. They can merge only with the cells capable of actively dividing. Most cells, such as mature neurons, do not divide and are not readily amenable to being genetically altered. The cancer causing ability of retroviruses is another drawback.

Fraley *et al* (1979) recognised the potential of liposomes for gene transfer and showed that it is possible to entrap a bacterial plasmid into liposomes. Subsequently they introduced liposomally encapsulated SV40 DNA into plant protoplasts (Fraley *et al*, 1980). Expression in the host cell

Encouraged by the promising potential of liposomes for gene delivery, several people attempted to improve the delivery by exploiting the knowledge of targeting to different receptors. Stavridis et al (1986) used transferin grafted liposomes for in vivo delivery of DNA to bone marrow erythroblasts in rabbits. pH-sensitive immunoliposomes have been used to deliver liposomal DNA to the cell cytoplasm (Wang and Huang, 1989). Recently, Legendre and Szoka (1992) showed that cationic liposomes are superior to pH-sensitive liposomes for delivery of plasmid DNA into mammalian cell lines. After delivery into the cells, DNA should reach the nucleus for expression. Working towards this goal, Kaneda et al (1989) encapsulated human insulin gene in liposomes containing gangliosides and incubated these liposomes with RBC membranes containing non-histone chromosomal protein (HMG1). The vesicles were cointernalised with HMG1 and transported to the liver nucleus, where the gene was expressed. The amount of human insulin in rat serum gradually increased to a maximum after 7-8 days and then decreased rapidly. The therapy was therefore short lived.

Two groups (LeMeur et al, 1985, Yamamura et al, 1985) almost simultaneously treated mice incapable of producing antibodies when exposed to certain synthetic molecules. The deficiency was due to the alteration of a certain gene belonging to the histocompatibility complex (known as H-2 in mice). They injected the cloned gene into the embryos of deficient mice. The resulting mice appeared to be completely cured and were capable of producing antibodies.
The first clinical trial of gene therapy for genetic diseases was conducted at NIH, (for review Verma, 1990), where H.Blaese, W.F. Anderson and their colleagues introduced the gene for the enzyme adenosine deaminase into children suffering from severe combined immunodeficiency disorder.

Although great strides have been made in the expression of exogenous genetic material in animals, however genetic engineering is still in its infancy. The various drawbacks of gene therapy include the difficulty in controlling the fate of DNA inside the cell. For every gene spliced in the correct place, more than 1000 fit randomly into the genome. The other problems to gene therapy include the ethical question of whether gene therapy should be limited to somatic cells or also be applied in the germ cells. Will gene therapy in germ line cells be tampering with human evolution? Till questions such as these can be discussed and answered it would be safer to think of gene therapy in somatic cells only.

1.2.2 Organ transplant

Another approach has been organ transplant as in the case of Fabry's disease. An accumulation of digalactosylglucosylceramide was seen in almost all organs and body fluids of patients suffering from this disorder. Digalactosylceramide is found only in the kidneys of normal individuals were α-galactosidase cleaves the non-reducing terminal galactose residues of the trisaccharide group. The absence of α-galactosidase from the kidney leads to the accumulation of the trisaccharide. Transplantation of a normal kidney in such patients has shown considerable success in alleviating the disorder (Clarke et al, 1972).
1.2.3 Enzyme replacement therapy

The therapeutic approach to storage disorders which has received the most serious consideration is the possibility of replacing the missing enzyme. This approach is based on the premise that the lysosomal apparatus of the cell can take up the glycoprotein enzymes from the extracellular fluid by means of receptor mediated endocytosis. Most of the lysosomal hydrolases are glycoproteins. The cell surface is known to express a number of sugar specific receptors, such as the galactose specific receptors on hepatocytes, (Ashwell and Morell, 1974) and the mannose receptors on kupffer cells (Stahl et al, 1978). Several experimental models have been presented to support this idea. Cohn and Ehrenreich (1969) showed that cultured mouse peritoneal macrophage vacuoles distended by a sucrose load were normalised upon addition of invertase.

Human trials of enzyme replacement therapy were also conducted in a number of patients. Di Ferrante et al (1971) and Erickson, et al (1972) treated patients suffering from Hunter's syndrome with normal plasma. Plasma contains the expected normal enzyme, and as such serves as its source. Plasma infusion, however, met with limited success as the concentration of the missing enzyme is very low and even large quantities of plasma were not sufficient. It must also be pointed out that such patients often suffer from defective cardiovascular apparatus and therefore cannot be subjected to regular plasma infusions (Schieken et al, 1975).

In another trial normal leukocytes were infused in a patient suffering from Hunter's disease. Such patients are deficient in leukocyte iduronate-sulfate sulfatase (Knudson, 1971). No long term improvement was observed in the clinical or biochemical manifestations of the disease. There was however a short term improvement in the α-L-iduronidase levels and a clinical improvement in the stiffness of joints.
The reversal of storage disorders has also been approached by means of direct enzyme replacement. Several trials have been performed with purified enzymes from human sources. Sly et al (1973) described the first patient suffering from mucopolysaccharidosis type VII. Such patients are deficient in β-glucuronidase and they do not degrade sulfated mucopolysaccharides. Clinical symptoms include urinary excretion of elevated amounts of chondroitin 4-sulfate, hepato-splenomegaly and skeletal abnormalities. In 1974, Sly et al treated the patient with highly purified β-glucuronidase from human thrombocytes. In another trial Johnson et al (1973) intravenously administered purified hexosaminidase-A into a patient suffering from Tay-Sachs disease in order to mobilise the GM2-ganglioside stores. Both these investigators met with limited success only, due to the rapid removal of the enzyme from the circulation and its accumulation in the liver. The uptake of the enzyme by the liver limited its therapeutic efficacy in this case as liver was not the only organ involved in the storage of the substrate.

It was gradually realised that enzyme therapy is hampered by many drawbacks which must be overcome before such a therapy can become viable. They include a) adverse immunological reactions against the administered enzyme and b) removal and intracellular degradation of the intravenously administered enzyme by the RES, which may limit the exposure time of the enzyme to the accumulated substrate.

1.3 LIPOSOME AS AN ENZYME CARRIER

In the search for an appropriate carrier for therapeutically active enzymes and proteins it was felt that the various problems of enzyme therapy might be circumvented by the entrapment of enzymes in liposomes.

Liposomes are biodegradable lipid vesicles with concentric lipid bilayers alternating with aqueous compartments. Water soluble substances
like enzymes can be entrapped in these aqueous compartments. Liposomes were first described by Bangham and coworkers in 1963 and by 1968 Sessa and Weissmann had coined the term "liposome" for them.

Over the years, various methods of liposome preparation have been perfected and newer methods developed. They have been comprehensively reviewed by Szoka and Papahadjopoulos (1980). Depending upon their shape and size, liposomes can be classified into three types namely multilamellar, small unilamellar and large unilamellar (Fig. 2). Multilamellar vesicles are spontaneously formed upon hydration of the phospholipid film. They are made up of a number of lipid bilayers surrounding the aqueous compartments. SUVs and LUVs are made up of just one bilayer enclosing one aqueous compartment. They differ from each other only in size.

Liposomes can be formed from many different phospholipids, and the composition most commonly used is egg phosphatidylcholine, with or without cholesterol and with or without anionic lipids such as phosphatidic acid and cationic lipid such as stearylamine. The distance between the bilayers in MLVs is determined by a balance between the repulsive forces (mainly electrostatic interactions between headgroups and hydration forces of the head groups) and by the attractive Van der Waal's forces (LeNeveu et al, 1976). It is possible to increase this distance and thus the aqueous phase entrapped by the addition of upto 10 mol% of charged lipid (either cationic or anionic) into the phospholipid (Bangham et al, 1967).

Liposomes and other particulate ligands are more avidly taken up by the reticuloendothelial cells in the liver and the spleen due to the sinusoidal (discontinuous) epithelia of the capillaries. The endothelia of these vessels has gaps upto 100-200 nm in diameter (Motta and Makable, 1980). This epithelia allows small particulate substances to pass through the vasculature into the surrounding tissue. Most other organs possess the continuous epi-
FIGURE 2

LIPOSOME CLASSIFICATION
CUT AWAY VIEWS

Presented by Dr. Bruce Gabor, USA
thelia which does not allow liposomes to pass through it. The multilamellar liposomes are taken up by cells of the RES, lining the sinusoids in the liver and spleen. Juliano and Stamp (1975) have shown that MLVs are taken up by the RES more rapidly than the SUVs. It was also observed that the negatively charged liposomes were taken up by the liver more rapidly than the positively charged or neutral liposomes (Gregoriadis and Neerunjun, 1974).

Gregoriadis and Ryman (1972) have shown that the activity of an enzyme in liposomes remains latent while they are in circulation and much of the enzyme activity is recovered from the hepatic lysosomes indicating that the liposomally encapsulated enzyme is protected from the environment.

There are many advantages in the use of liposomes as enzyme carriers. They include avoidance of the presence of a foreign protein in the circulation, the lack of immunological response and the possibility of directing the enzyme carrier to a particular tissue by changing the lipid composition, size, charge etc. It is also possible to tailor the liposomal surface to target them to the diseased tissue.

1.3.1 Cell-specific targetting of liposomes

In a systematic approach to the problem of designing liposomes that would bypass the RES, Surolia et al (1975) set up a very simple liposome model system to study the receptor-ligand interaction. A 2-fold increase in the GM1 concentration, for example, led to a 20-fold increase in the rate of interaction with the lectin. Further, it was observed that the phase transition temperature of the phospholipid component of liposomes, the length of the surface-bound oligosaccharide chain and cholesterol concentration also affect the binding of the terminal sugar with the lectin (Surolia and Bachhawat, 1978).
In a series of decisive experiments, Ashwell and Morell (1974) characterised a receptor specific for β-gal containing glycoproteins on hepatocytes. Surolia and Bachhawat (1977) then started their studies on in vivo targetting to the hepatocytes using galactosylated liposomes. They observed that following intravenous administration, up to 45% of the administered liposomes containing GM1 ganglioside accumulated rapidly (within 15 minutes) in the liver by a β-gal specific receptor mediated pathway.

Later Ghosh and Bachhawat (1980) showed that intravenously administered liposomes containing asialo GM1 ganglioside, accumulated in the liver. This accumulation was dependent on the density of the galactose residues on the liposomal surface, and was inhibited by competition with asialofetuin. It was observed that β-gal liposomes preferentially accumulated in the hepatocytes as compared to α-gal liposomes (Ghosh et al., 1981) indicating that the hepatic receptors are specific for β-gal containing liposomes. When the livers of mice previously administered aGM1 liposomes were perfused, the hepatocytes were found to be three times more efficient than the nonparenchymal cells in taking up these liposomes (Ghosh et al., 1982, Dasgupta and Bachhawat 1985). These observations that uptake of liposomes by hepatocytes is galactose dependent were subsequently confirmed by a number of other laboratories (Spanger and Scherphof 1983, Szoka and Mayhew 1983, Gregoriadis and Senior 1984).

A variety of other targetting ligands have also been used for this purpose. Weissmann and Coworkers (1977) have shown that liposomes coated with aggregated IgG provide a far better endocytic stimulus than the uncoated liposomes. Aggregated rather than native immunoglobulins preferentially coat and partially insert into liposomes forming lattices in which key Fc regions are exposed on the surface of the liposomes. These Fc regions act as ligands for Fc receptors of the polymorphonuclear leukocytes.
(PMN) and consequently provoke endocytosis. Uptake of HRP encapsulated in aggregated IgM coated liposomes exceeded that of the free enzyme by 120 fold, native IgM coated liposomes by 60-fold and uncoated liposomes by 50-fold (Weissmann et al, 1975). It has been shown that hexosaminidase A is taken up more actively when presented to Tay-Sach's phagocytes as aggregated IgG liposomes, than when presented in liposomes coated with native IgG or in uncoated liposomes (Cohen et al, 1976).

1.3.2 Long circulating liposomes

a) Modification of liposomal surface with dextran

One important prerequisite to efficient targeting of liposomes is to prevent their uptake by the RES and to enhance their circulatory life, so that they can interact with other cell types also. Pain et al (1984) showed that it was possible to do so by conjugating cyanogen bromide-activated dextran (mol wt 70,000) on the surface of PE containing liposomes. The dextran-coated liposomes were more efficient in retaining the entrapped radioactive compounds in circulation, and the concentration of these liposomes in the circulation 2 h after administration was found to be two fold higher than that of uncoated control liposomes. Other investigators have also used polysaccharides to enhance the in vivo stability of liposomes and for bypassing the RES (Sunamoto, 1986).

b) Modification of liposomal surface with GM1 ganglioside

Allen and Chonn (1987) showed that it was possible to enhance the circulatory life of liposomes by incorporating GM1 ganglioside into the liposomes composed of PC and Chol. GM1 acts synergistically with Chol in stabilising the liposomes (Allen et al, 1985). It not only reduces the susceptibility of liposomes to lysis by blood components but also enhances the circulatory levels 3- to 10-fold. Incorporation of Sphingomyelin (SM)
into PC liposomes at a 1:1 molar ratio led to a dramatic increase in the levels of circulating liposomes with a concomitant decrease in uptake by the liver and the spleen. The levels of SM:PC liposomes in RES at optimal GM1 ganglioside concentrations (7-15 mol%) were > 25-fold lower than those observed for PC:Chol liposomes of comparable size. Allen et al (1991) have shown that compounds such as GM1, SM and Chol, which reduce the uptake of liposomes in vivo also led to a decrease in their uptake by bone marrow macrophages in vitro.

Gabizon and Papahadjopoulos (1988) have also shown that there is an increase in the blood residence time, with a concomitant decrease in the uptake by liver and spleen, for liposomes composed of certain glycolipids in combination with solid phase phospholipids and cholesterol. GM1:DSPC:Chol liposomes elicited the longest circulatory life followed by DPPG:DSPC:Chol, GM1:PC:Chol and DPPG:DSPC:Chol liposomes, in that order. They have also observed a strong correlation between the residence time of liposomes in blood and their uptake by tumors implanted in mice. GM1:DSPC:Chol liposomes showed a 25-fold increase in the liposomal concentration in the tumors and there was a 20-fold increase in the tumor to body ratio, indicating that the accumulation of liposomes was preferentially enhanced in the tumors as compared to the rest of the body tissues.

GM1 has also been used for prolonging the residence time of pH-sensitive liposomes in the circulation. This is particularly relevant as the pH-sensitive liposomes are known to provide an efficient delivery system in in vitro studies. However, pH-sensitive liposomes have not been used successfully in animals due to their high susceptibility to degradation by plasma proteins, accompanied by their rapid uptake by the RES. Liu and Huang (1989) have shown that pH-sensitive liposomes composed of dioleoylphosphatidyl ethanolamine and dipalmitoyl succinylglycerol retain 40-
50% of their pH-sensitivity in the presence of 5% GM1. These liposomes were found to be more resistant to the destabilising influence of plasma components and elicited a longer residence time in circulation. This makes pH-sensitive liposomes potentially useful drug carriers in vivo.

Recently Maruyama et al (1990) using a model system, have emphasised that the long life span of liposomes in the circulation constitutes an essential factor for optimal binding of immunoliposomes to the target cells. GM1 coated immunoliposomes have a long life span in circulation and thus offer a potentially useful delivery system for defined targets.

c) Modification of the liposomal surface with PEG

Although GM1 ganglioside was very successful in prolonging the circulatory life of liposomes, however its potential use in the clinics is severely limited by its high cost. Therefore, a search for a cheaper substitute was started. Klibanov et al (1990) in an attempt to design liposomes with a long life span in the circulation and reduced affinity towards RES, modified the liposomal surface by including a lipophilic derivative of PEG, prepared by coupling activated PEG with PE. They observed that PEG liposomes were highly stable and retained 90% of the entrapped fluorescent markers in the presence of human serum over a period of 24 h. The ability of PEG-PE to enhance the circulatory life of liposomes was striking. The half life of liposomal clearance from blood was <0.5, 0.5, 1.5 and 5h for liposomes made up of PC:Chol, PC:Chol:PEG-stearyl ester, PC:Chol:GM1 and PC:Chol:PEG-PE respectively.

Blume and Cevc (1990) reported that liposomes coated with PEG-5000 were removed from the blood at only 15% of the rate characteristic of DSPC liposomes. Even 8 h after injection, the level of PEG-coated liposomes in the circulation was higher than that in the liver. Allen et al (1991) have reported a substantial reduction in the uptake of PEG-PE containing liposomes by the bone marrow macrophages in vitro.
Senior et al. (1991) studied the interactions of monomethoxy polyethylene glycol (mPEG)-coated liposomes with plasma by an aqueous two-phase partitioning technique, and then compared these interactions with those obtained for control, i.e., uncoated liposomes. They observed that the distribution of control liposomes in the two-phase partitioning system was rapidly altered with most of the liposomes moving towards the lower phase within 1 minute after incubation with serum. This distribution was similar to that of the plasma itself, indicating that there is some association between the plasma proteins and the liposomal surface. mPEG-coated liposomes, on the other hand, take a much longer time (up to 6 h) to move towards the lower phase. The PEG-coated liposomes, therefore, interact slowly with the plasma proteins in vitro, which may be partly responsible for an increase in their circulatory life in vivo. Senior et al. (1991) have also reported a 30% slower removal of these liposomes from the circulation compared to control liposomes. They further observed that when the serum components associated with the liposomes are removed by gel filtration, mPEG liposomes once again partition as in the absence of serum, indicating that the movement towards the lower phase is not due to the loss of PEG.

The exact mechanism whereby PEG prevents the interaction of opsonins and other serum proteins with liposomes is not known. This may due to a restructuring of water molecules around PEG, which leads to steric exclusion of macromolecules, particularly proteins, from regions of water occupied by the inert PEG (Atha and Ingham, 1981). Blume and Cevc (1990) have calculated the thickness of the surface region inaccessible to water-soluble proteins to be ~2 nm for DSPC liposomes containing 10% DSPE-PEG. PEG chains anchored in a phospholipid may also accentuate repulsion between opposing bilayers (Arnold et al., 1986), thereby preventing excessive aggregation of liposomes and their uptake by the RES. A similar mechanism may be responsible for the enhanced half life of dextran/PEG modified enzymes in circulation.
The ability of liposomes to protect the entrapped enzyme from adverse immunological reactions and to target it to the diseased tissue made them very important tools for enzyme therapy. The efficacy of liposomes in delivering the entrapped enzyme has been studied extensively both in vitro and in in vivo models.

1.3.3 Use of liposomes in enzyme therapy

Gregoriadis and Buckland (1973) exposed Chinese hamster fibroblasts to sucrose. Due to the absence of invertase, there was an accumulation of sucrose in the lysosomes of these cells. Subsequent exposure of these cells to liposomally encapsulated invertase led to the degradation of the stored sucrose indicating that invertase was incorporated into these cells. In another study Colley and Ryman (1976) developed a model storage disorder in rat liver by intraperitoneal administration of dextran. It is known that intraperitoneally administered dextran accumulates in the liver lysosomes thereby increasing their density (Baudhuin et al, 1965). In the absence of the enzyme dextranase, dextran is not degraded in mice. However, administration of liposomal dextranase lead to the degradation of the stored material. Interestingly even the free enzyme was able to reverse the storage condition in these experiments. Although the amount of free dextranase taken up by the liver was found to be considerably less than when it was entrapped in liposomes, it was still enough to degrade all the stored dextran. This study did not show any enhanced efficacy of liposomal dextranase over the free enzyme, due to the small amounts of dextran loaded into the liver and the relatively large amounts of dextranase administered. It however, does serve to show that enzyme entrapped in liposomes can be taken up the liver and released in an active form capable of degrading the stored material.
A number of human trials have also been conducted to test the efficacy of liposomally encapsulated enzyme in the treatment of storage conditions. The first was conducted by Tyrrell, Ryman and coworkers in 1976. A four month old female child with hypotonia, generalised weakness, hepatomegaly and cardiomegaly was diagnosed to be suffering from Pompe's disease caused by the lack of lysosomal α-glucosidase. At eight months the child was treated with liposomes (composition; PC:Chol:DCP, 7:2:1) containing amyloglucosidase for seven days. Though the liver decreased markedly in size during the first four days of treatment, there was no improvement in the heart condition and the child died eight days after the commencement of therapy. In the postmortem tissues examined, only trace amounts of the enzyme could be detected in the liver which was not surprising as most would have been catabolised by the liver cathepsins. The glycogen levels in the muscle and the cardiac tissues were found to be very high. However, those in the liver were lower than expected in a patient suffering from Pompe's disease. The lower levels of glycogen in the liver can be explained, as the liposomes of the composition mentioned would be taken up most avidly by the liver and therefore degrade the material stored there.

In another study, a 25 year old woman suffering from Gaucher's disease was treated with liposomal β-glucosidase for five years by Gregoriadis and co-workers (1980, 1982). The lack of β-glucosidase lead to a rise of the glucocerebroside levels particularly in the cells of the RES. The enzyme deficiency was clinically manifested by the enlargement of the liver and spleen and by bone deformations. After five years of treatment, the patient showed a stabilisation of the clinical condition, an improvement in the function of the RES and absence of toxic effects upon chronic administration of liposomes. Due to the absence of any other patient suffering from Gaucher's disease, it was not possible to compare the efficacy of liposomal
enzyme therapy in the patient with an untreated control. It is therefore not possible to conclusively say that the stabilization of the patients condition was due to the therapy given.

A number of investigators have shown that although liposomally entrapped enzymes could be selectively delivered to the liver lysosomes, but once there they are very rapidly catabolised (Gregoriadis and Neerunjun, 1974). The rapid degradation of the administered enzyme severely limits its therapeutic potential and thereby the efficacy of enzyme therapy in controlling such disorders. Work therefore started in several laboratories to find ways of enhancing the stability of the administered enzyme in the lysosomes. These efforts such as cross-linking of liposome entrapped proteins with glutaraldehyde met with limited success (Gregoriadis and Neerunjun, 1974). In order to successfully improve the stability of the administered enzyme in the lysosomes, it is first important to understand the nature of lysosomal hydrolases. These enzymes, despite being proteinacious in nature are remarkably stable in the acidic milieu of the lysosomes (Pazur and Aronson, 1972, Pazur et al, 1970). Some properties of these enzymes are common such as (a) an acid pH optimum, (b) resistance to autolysis, and (c) their glycoprotein nature. The glycoprotein nature of the lysosomal hydrolases and their resistance to autolysis was very intriguing. Saraswathi and Bachhawat (1970) showed that removal of the terminal sialic acid of the enzyme alkaline phosphatase does not impair its catalytic activity. However, removal of the terminal sugar lead to a markedly reduced survival time in circulation indicating that it had a role to play in the in vivo circulatory life of the enzyme (Baynes and Wold, 1976). Realising the importance of carbohydrates in the in vivo survival of proteins, Bachhawat suggested that it might be possible to prolong the in vivo survival of enzymes by their covalent modification with polysaccharides (1973).
1.4 EFFECT OF COVALENT MODIFICATION OF ENZYMES WITH POLYETHYLENE GLYCOL AND DEXTRAN

Various approaches have been used to enhance the in vivo stability of enzymes and proteins. One of the more successful approaches has been to link proteins to non-immunogenic hydrophilic polymers. Compounds that are to be used for conjugating pharmacologically active proteins and peptides must be able to form stable conjugates, should remain in the circulation for long periods of time and must have low immunogenicity and antigenicity. The two major hydroxyl group containing polymers, polyethylene glycol (PEG), and dextran possess all these qualifications. PEG is a linear, uncharged, non-immunogenic compound with the general structure HO-(CH₂-CH₂)n-OH. It is compatible with blood and is available in various molecular weight ranges. The two hydroxyl groups, which are available for modification caused cross-linking of the conjugates. To overcome this complication, a monofunctional derivative of PEG, monomethoxy PEG (mPEG) is used.

Dextran is an inert polymer of D-glucose units linked predominantly α-D (1 → 6) and is compatible with blood.

1.4.1 Methods of conjugation

The most common methods of coupling such hydroxyl group containing polymers depend upon the activation of the hydroxyl groups, which are otherwise not sufficiently reactive to form covalent bonds between the polymer and enzyme. Most approaches for the activation involve the introduction of an electrophilic group into the polymer to make it more reactive towards the nucleophiles present in the enzyme. This is followed by coupling with the nucleophilic moieties present on the proteins. The linking reactions should be carried out under conditions that are mild enough to
ensure the chemical integrity of even the most labile enzyme. The method of conjugation to be used should be chosen carefully, so as to result in the minimum loss of activity of the proteins or enzyme molecules.

The E-amino groups of lysine and the N-terminal amino groups of the proteins are most commonly used for coupling them to the activated hydroxyl groups of PEG or dextran. Sometimes the carboxylic groups on proteins are also used for coupling. Some of the more commonly used methods for coupling PEG and dextran to proteins include:

a) CNBr activation

Fig.3a shows that CNBr reacts with the hydroxyl groups of dextran at alkaline pH (10-12) to produce derivatised matrices that react readily with the amino groups on proteins (Axen et al, 1967). Although the use of triethylamine instead of NaOH for maintaining the pH results in extremely high coupling, this method also suffers from several disadvantages. The N-substituted isourea derivatives formed are not completely stable particularly in the presence of other nucleophiles. Another disadvantage is the high toxicity of CNBr.

b) Carbonyldimidazole activation

PEG and dextran may be activated using carbonylating agents such as N,N-carbonyldiimidazole and the coupling efficiency achieved by this method is comparable to that of CNBr (Fig. 3b). This method also results in the formation of peptide-like bonds (Hearn et al, 1981). This reagent is less toxic and the degree of modification can be controlled by the extent of activation of dextran or PEG. The conjugates obtained by this method are extremely stable.
3 a) CNBr activation

\[
\begin{array}{c}
\text{OH} + \text{CNBr} \\
\text{OH} \quad \text{cyanate ester} \\
\end{array}
\]

\[
\begin{array}{c}
\text{OH} \quad \text{(very reactive)} \\
\text{CNBr} \\
\text{interchain rearrangement} \\
\end{array}
\]

\[
\begin{array}{c}
\text{NH} \\
\text{C=NH} \\
\text{isourea derivative} \\
\text{N-substituted imidocarbonate} \\
\text{N-substituted carbamate} \\
\end{array}
\]

3 b) Carbonyldimidazole activation

\[
\begin{array}{c}
\text{OH} + \text{C=O} \\
\end{array}
\]

3 c) Oxirane-based reactions

\[
\begin{array}{c}
\text{OH} + \text{O} \\
\end{array}
\]
c) Oxirane-based reactions

Bis-oxiranes such as 1, 4-butanediol diglycidoxy ether react readily at alkaline pH with hydroxy or amino containing matrix to yield derivatives which possess a long chain hydrophilic reactive oxirane (Fig. 3c). The oxirane-coupled conjugates are extremely stable and are used widely. Other bifunctional reagents that may be used for attachment of proteins to PEG and dextran include divinylsulphone (Porath et al., 1975) and organic sulphonil chlorides (Nilson and Mosbach, 1984).

d) 2,4,6-Trichloro-s-triazine (cyanuric chloride)

Cyanuric chloride reacts with amino, imino and hydroxyl groups to form stable linkages (Fig. 3d). The first chlorine reacts readily at 4°C, the second at 25° and the third at 80°C in aqueous solutions at pH 9.0.

e) Periodate method of oxidation

Periodate oxidation leads to the formation of a Schiff's base linkage between the carbonyl groups on dextran and the free amino groups on the protein (Fig. 3e). This method depends on the oxidation of the cis-vicinal hydroxyl groups of dextran by sodium metaperiodate (NaIO₄) to generate aldehydic functions. The dialdehydes react at a pH between 4 and 6 with primary amines to form Schiff bases, which can be reduced by either NaBH₄ (sodium borohydride) or NaBH₄ CN (sodium cyanoborohydride) to form stable secondary amines. This is a very rapid, simple and safe method which results in the formation of chemically stable dextran-protein bonds.

1.4.2 Circulatory life of the enzyme conjugates

Upon covalent modification with dextran (Table IIIa) or with PEG (Table IIIb), enzymes exhibit enhanced half lives in circulation. Various factors have been evoked to account for this increase in the circulatory life.
3 d) 2,4,6-Trichloro-s-triazine (cyanuric chloride)

\[
\text{CH}_3\text{O-PEG-OH} + \text{Cl-}\begin{array}{c}N=\ N=\ N\end{array}\text{Cl} \rightarrow \begin{array}{c}N=\ N=\ N\end{array}\text{O-PEG-OCH}_3 + \text{Cl}_2
\]

(Activated PEG,)

\[
\begin{array}{c}N=\ N=\ N\end{array}\text{O-PEG-OCH}_3 \rightarrow \text{NH}_2\text{-Prot}
\]

(Activated PEG,)

\[
\begin{array}{c}N=\ N=\ N\end{array}\text{O-PEG-OCH}_3 \rightarrow \text{Prot-NH}
\]

3 e) Periodate method of oxidation

\[
\begin{array}{c}O\end{array}\text{Prot} \rightarrow \text{Prot} \rightarrow \text{Prot} \rightarrow \text{Prot}
\]

\[
\begin{array}{c}H_\text{O-R} \end{array}\text{OH} \rightarrow \text{NH}_2\text{-Modifier} \rightarrow \text{Modifier-N} \rightarrow \text{Modifier-+}
\]

\[
\begin{array}{c}H_\text{O-R} \end{array}\text{OH} \rightarrow \text{NH}_2\text{-Modifier} \rightarrow \text{Modifier-N} \rightarrow \text{Modifier-+} \rightarrow \text{C=+} \rightarrow \text{Cl+}
\]
### TABLE IIIa.

**CIRCULATORY HALF LIFE OF DEXTRAN-ENZYME CONJUGATES**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Half life of Enzyme</th>
<th>Half life of conjugate</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carboxypeptidase</td>
<td>3.1 h</td>
<td>45.6 h</td>
<td>Melton <em>et al</em> (1987)</td>
</tr>
<tr>
<td>Uricase</td>
<td>0.6 h</td>
<td>7.5 h</td>
<td>Yasuda <em>et al</em> (1990)</td>
</tr>
<tr>
<td>Asparaginase</td>
<td>11 h</td>
<td>190 h</td>
<td>Benbough <em>et al</em> (1979)</td>
</tr>
<tr>
<td>Catalase</td>
<td>0.3 h</td>
<td>2.5 h</td>
<td>Marshall <em>et al</em> (1977)</td>
</tr>
</tbody>
</table>

### TABLE IIIb

**CIRCULATORY HALF LIVES OF PEG-ENZYME CONJUGATES**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Half life of Enzyme</th>
<th>Half life of conjugate</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superoxide dismutase (SOD)</td>
<td>0.05 h</td>
<td>16.5 h</td>
<td>Beauchamp <em>et al</em> (1983)</td>
</tr>
<tr>
<td>Adenine deaminase (ADA)</td>
<td>0.5 h</td>
<td>28 h</td>
<td>Davis <em>et al</em> (1981)</td>
</tr>
<tr>
<td>L-Asparaginase</td>
<td>20 h</td>
<td>357 h</td>
<td>Ho <em>et al</em> (1986)</td>
</tr>
<tr>
<td>Uricase</td>
<td>0.6 h</td>
<td>6.6 h</td>
<td>Yasuda <em>et al</em> (1990)</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>0.05 h</td>
<td>1.0 h</td>
<td>Beauchamp <em>et al</em> (1983)</td>
</tr>
</tbody>
</table>
These include steric surface barriers created by PEG or dextran residues attached to the protein. These barriers prevent or reduce the recognition of enzyme with specific receptors on the surface of certain cells. Further, uptake of molecules by the RES is thought to be mediated by some as yet unidentified serum components called "opsonins" (Moghini and Patel, 1989). Opsonins bind to the hydrophobic regions of the molecules. The presence of PEG or dextran makes the enzyme surface hydrophilic which may prevent any interaction with opsonins.

Enhanced circulatory life of enzyme conjugates has also been attributed to the decreased clearance by glomerular filtration by the kidney (Beuchamp et al, 1983). Knauf et al (1988) showed that recombinant interleukin-2 (rIL-2) and some PEG-rIL2 conjugates upto 70 KDa had serum clearance values predicted for small molecular weight proteins by glomerular filtration (Maack et al, 1979, Venkatachalam and Rennke, 1978). The larger the PEG-rIL2 conjugate, slower was the rate of its clearance by kidney due to its progressive exclusion from glomerular filtration.

In vitro studies have shown that the modified proteins exhibit enhanced resistance to proteolytic degradation. A number of studies have described the enhanced stability of dextran or PEG-enzyme conjugates to proteolysis. While trypsin is auto digested within 2 hours, the dextran-trypsin conjugate shows no degradation during the same time period (Marshall and Rabinowitz, 1976). Other enzymes conjugates like PEG-catalase (Abuchowski et al, 1977), dextran-carboxypeptidase G₂ (Melton et al, 1987), PEG-uricase and dextran-uricase (Yasuda et al, 1990) also show enhanced resistance to degradation not only by trypsin but also by chymotrypsin and protease from streptomyces grigeus. The resistance to trypsin may be expected as the substrate amino acids of trypsin namely lysine and arginine are involved in conjugation with PEG and dextran. The reason for enhanced stability against degradation by chymotrypsin is less clear. It may
be due to a steric barrier formed by PEG or dextran to the approaching proteolytic enzyme. Enhanced resistance to proteolytic degradation may also be a contributing factor to the increased circulatory life. In this regard it is interesting to note that PEG-gluconolactone oxidase which is not protected from proteolytic cleavage by trypsin \textit{in vitro}, does not show any increase in its half-life in circulation (Hadley and Sato, 1989). Such modified enzymes are increasingly finding clinical use in the treatment of various deficiency disorders.

1.4.3 Immunogenicity of the conjugates

A large body of work done by various investigators suggests that covalent modification of enzymes with PEG or with dextran renders the protein incapable of eliciting antibodies against itself (Table IV). The freedom from antigenicity is particularly important in the event where retreatment with the enzyme is required at frequent intervals. Streptokinase, for example, is a strongly antigenic bacterial protein. It has the property of clot lysis by activation of the fibrinolytic system of the human blood (Einarsson \textit{et al}, 1979) and its clinical effects have been tried in the treatment of thrombosis. However, the greatest disadvantage associated with the treatment with streptokinase is its antigenicity. Koide \textit{et al} (1982) have shown that it possible to produce a non-immunogenic streptokinase by its modification with PEG under controlled conditions. The degree of modification of streptokinase determined its therapeutic potential. Modification of 8 amino groups resulted in conjugates that showed no binding against its antibody and retained 33% of the streptokinase activity. However, modification of 12 amino groups showed no binding against its antibody, but it also resulted in a complete loss of streptokinase activity.

Lee and Sehon (1977,1978) have shown that coupling of certain allergens to PEG yields conjugates which are non-immunogenic and are
<table>
<thead>
<tr>
<th>Conjugate</th>
<th>Immune reaction of conjugate with antibody raised against free enzyme</th>
<th>Immunogenicity of conjugate</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG-BSA</td>
<td>No interaction</td>
<td>No immune response</td>
<td>Abuchowsk et al, 1977</td>
</tr>
<tr>
<td>PEG-catalase</td>
<td>No interaction</td>
<td>No immune response</td>
<td>Abuchowski et al, 1977</td>
</tr>
<tr>
<td>PEG-rIL2</td>
<td>-</td>
<td>Weak titres of only IgM antibodies</td>
<td>Katre et al, 1990</td>
</tr>
<tr>
<td>Dextran-Uricase</td>
<td>Interacts weakly</td>
<td>Very weak immune response</td>
<td>Yasuda et al, 1990</td>
</tr>
<tr>
<td>PEG-aenosine deaminase</td>
<td>No interaction</td>
<td>No immune response</td>
<td>Davis et al, 1981</td>
</tr>
<tr>
<td>PEG-arginase</td>
<td>No interaction</td>
<td>No immune response</td>
<td>Savoca et al, 1979</td>
</tr>
<tr>
<td>PEG-Streptokinase</td>
<td>No interaction</td>
<td>No immune response</td>
<td>Koide et al 1982</td>
</tr>
</tbody>
</table>
capable of suppressing in an immunologically specific manner, the capacity of animals to mount primary as well as secondary response to sensitising doses of DNP-ovalbumin. To establish the cellular mechanisms underlying the suppression by conjugates, the spleen cells of the tolerised mice as well as their splenic T and B subpopulations were transferred into normal syngenic recipients. They were immunised subsequently with a sensitising dose of DNP-ovalbumin, and it was observed that while the splenic B cells of tolerised donors did not possess any suppressive capacity. The unfractionated spleen cell population as well as the T cells of immunosuppressed mice undermined the capacity of normal recipients vigorous anti-OA responses. It was, therefore concluded that the immuno suppression was primarily if not exclusively due to the activation of T suppressor cells (Lee et al, 1981).

The activation of T-helper cells to most soluble antigens occurs only if the antigen is processed by macrophages and presented to the T cell membrane in association with the appropriate gene product encoded by MHC-I. In contrast, processing of the antigen by the macrophages does not seem to be necessary for the activation of T-suppressor cells. In fact, the very opposite appears to be the case (Takatsu and Ishizaka, 1977; Skidmore and Katz, 1977). It therefore appears that the immuno-suppressive capacity of these conjugates is probably due to their resistance to processing by the macrophages.

Although the PEG or dextran conjugates of enzymes were non-immunogenic in almost all cases, however only in a few cases were they able to suppress in a specific manner the capacity of animals to mount immunological responses to sensitising doses of the unmodified antigen. Savoca et al (1979) have shown that though arginase modified with PEG was rendered both non-immunogenic and non-antigenic, PEG-arginase did not induce tolerance towards the native enzyme. Similarly, mice injected with PEG-catalase remained immune competent for native catalase (Abuchowski et al, 1977).
1.4.4 Intracellular stability

Although enhanced stability, prolonged life span, reduced immuno-genicity and therapeutic efficacy have been demonstrated by a number of investigators, not much work has been done on stabilising enzymes intracellularly with particular reference to the lysosomes. Blomhoff et al (1983) have shown that β-galactosidase is markedly stabilised against degradation in isolated parenchymal and nonparenchymal cells in vitro after its conjugation with dextran. Compared to the native enzyme, degradation was found to be reduced by 35% in the parenchymal and by 43% in the nonparenchymal cells after 80 and 40 minutes of incubation respectively. In another study Melton et al (1987) showed that carboxypeptidase-G2 is stabilised upon modification with dextran. Once again the study was limited to radiolabelling experiments which gave no idea as to the catalytic integrity of the enzyme. The apparent stabilisation of the dextran-CPG2 conjugate in this case may also be due to gradual uptake of the conjugate from the circulation rather than its stability inside the cell. Dextran-CPG 2 conjugate had an extended half life in circulation. No carriers for the intracellular delivery of the enzyme and the conjugate were used, and the uptake of the two by the cells was dissimilar. These studies were based on the presence of the radiolabelled enzyme inside the cell and no attempt was made to study the catalytic and immunological integrity of the enzyme inside the cell. Hence, from these experiments it was not possible to evaluate the intracellular functional integrity of the enzyme.

1.4.5 Therapeutic efficacy of PEG and dextran conjugates

Covalent attachment of PEG or dextran to immunogenic proteins is a general method for reducing, modifying or eliminating the immunogenicity of proteins. It, therefore, finds many applications in immunology and medicine. Human Interleukin-2 (IL-2), obtained from genetically engineered
E. coli as an unglycosylated recombinant protein (rIL-2) has therapeutic potential in treating cancers. However, both the native IL-2 and the genetically engineered rIL-2 are rapidly cleared from the circulation in mice and rats resulting in limited bioavailability of the protein. Katre et al (1987) suggested that increasing the bioavailability of rIL-2 could increase its potency, thus facilitating a more effective use of this protein as a drug. They showed that conjugation with PEG enhanced the solubility of rIL-2, decreased its plasma clearance, and was 60 times more potent than rIL-2 in curing Meth A murine sarcoma in mice.

The possibility of the use of hemoglobin solutions as a blood substitute has long been considered. Studies on the use of stroma-free hemoglobin as a blood substitute have advanced as far as the stage of clinical trials. However, due to its relatively small size, hemoglobin infused into the circulation is rapidly eliminated through the kidney and other metabolic routes, severely limiting its utility. Tam et al (1976) have suggested the use of a soluble dextran-hemoglobin conjugate as a potential blood substitute. They showed that the dextran-hemoglobin conjugate could bind and release oxygen reversibly, and also had a much longer circulating life. It, therefore, increased the functional life span of hemoglobin.

A number of clinical trials have been conducted with PEG-modified enzymes. Davis et al (1981) showed that the circulatory life of adenine deaminase was enhanced 56 times upon its modification with PEG. Hershfield et al (1987) treated two children suffering from severe combined immunodeficiency disease caused by adenosine deaminase deficiency with bovine adenosine deaminase conjugated to PEG. They observed that the principal biochemical consequences of adenosine deaminase deficiency were almost completely reversed. The clinical improvement was indicated by the absence of infection and resumption of weight gain. In another clinical trial, a patient suffering from hyperuricemia was treated with PEG-modified
uricase (Chua et al, 1988). A dose of 2 U/kg body weight every 5 to 6 days was enough to reduce and maintain the plasma urate level at 540 μmole/liter or even lower. Other studies on enzyme deficiency disorders also showed the increased efficacy of enzyme-PEG conjugates in the treatment of such disorders. Lee and Sehon (1977) showed the increased efficacy of PEG conjugates in the treatment of certain allergenic reactions.

The underlying reason for this enhanced therapeutic efficacy is the reduced immunogenicity of the PEG and dextran conjugates compared to that of the free enzyme and their longer circulatory lives. This increases the bioavailability of the enzyme, thereby giving it a better chance to do its job.
AIMS AND OBJECTIVES

The use of enzymes for the treatment of enzyme deficiency disorders has been suggested by a number of investigators. The therapeutic role of enzymes is however limited by a) Immunological response against the administered enzyme, preventing its repeated administration. b) dilution of the enzyme in circulation, so that the amount reaching the sites of substrate accumulation is not sufficient, and c) rapid degradation of the enzyme in lysosomes, which are often the sites of substrate accumulation.

Various investigators therefore decided to use liposomes as carriers of the encapsulated enzymes for the treatment of storage disorders. In vivo studies showed that encapsulation into liposomes prevented dilution of the enzyme in circulation and facilitated delivery of the encapsulated enzyme to the sites of substrate accumulation. Liposomal encapsulation also prevented an immunological response against the administered enzyme, thereby making repeated administration possible. Several clinical trials were also conducted for the treatment of a lysosomal glycogen storage disorder "Pompe's disease" using liposomally encapsulated enzyme β-glucosidase. Although it was possible to destroy the stored substrate using liposomally encapsulated enzyme, the therapeutic effects of the enzyme were short lived due to its degradation in the lysosomes.

Further investigations therefore needed to be carried out to enhance the intracellular particularly the intralysosomal stability of the enzyme in order to reduce the frequency of its administration thereby making enzyme therapy a viable option. With this in view we approached the problem of making intracellularly stable enzymes.

A number of in vitro studies had earlier shown that modification of enzymes with PEG or with dextran enhances the thermal stability of the enzyme and reduces its susceptibility to degradation by trypsin, chymotryp-
sin etc. Work has also been carried out to show that such modified enzymes have a much longer circulatory life than the unmodified enzyme. Long circulating enzymes such as PEG-adenosine deaminase and PEG-uricase have also been used successfully in clinical trials.

Although enzymes have been shown to be stabilized *in vitro*, and their circulatory life enhanced *in vivo*, not much work has been done on their intracellular stability particularly intralysosomal stability. Intralysosomal stability is very important as lysosomes are not only the sites of substrate accumulation in lysosomal storage disorders they are also bags of hydrolytic enzymes which would rapidly degrade the therapeutic enzymes.

Blomhoff *et al* did study intracellular stability of β-galactosidase and they found a marked stabilisation of the enzyme against degradation in isolated parenchymal and nonparenchymal cells, after its modification with dextran. Their studies were however all based upon the presence of the radiolabelled enzyme inside the cell and they made no attempt to study the functional integrity of the enzyme. Although this study served as a pointer, it did not explore the ability of the enzyme in degrading the accumulated substrate or preventing its further accumulation. In another study Melton *et al* showed that carboxypeptidase G2 is stabilised upon modification with dextran. Their conclusions were also based upon the presence of the radioactive marker *in vivo*. The apparent stabilisation of dextan-CPG2 conjugate in this case may also be due to gradual uptake from the circulation rather than actual stabilisation. Both these studies did not show any enhanced stability to denaturation in the acidic milieu of the lysosomes.

With this in view we have approached the problem of modifying enzymes so that their stability inside the cell is enhanced, and delivering them to the macrophage lysosomes. Macrophages are the sites of substrate accumulation in a number of lysosomal enzyme deficiency disorders. It is
known that multilamellar vesicles are rapidly removed from the circulation by the macrophages. MLV's were therefore used as carriers of enzymes throughout the study. Two enzymes, horse radish peroxidase and dextranase were chosen for the study. HRP was used as a model enzyme due to the ease of its detection. It was modified with dextran and the effect of this modification on its circulatory life, intracellular and intralysosomal stability was studied. Liposomes were used as carriers for delivering the native and the modified HRP inside the cell. Next, dextranase was modified with PEG and the effect of modification with PEG and encapsulation into liposomes on the stability of the enzyme was studied. The ability of various formulations of dextranase in degrading the stored substrate in a model storage disorder and in preventing further accumulation of the substrate was also examined.