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Enzyme–Albumin Polymers

New Approaches to the Use of Enzymes in Medicine

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ABSTRACT

The widespread use of enzymes as drugs or therapeutic agents has been limited by (a) enzyme availability, (b) biodegradation of administered enzyme, (c) immunogenecity of the enzyme as a foreign protein, and (d) accessibility of the enzyme to the appropriate site of action. It has become obvious that due to these limitations, the administration of free or native enzyme is not likely to be effective. Various mechanisms of protecting or packaging enzymes to offset some of these drawbacks have been described. We have been successful in producing conjugates of a number of different enzymes with a molar excess of homologous albumin. The resulting enzyme–albumin complex is resistant to proteolytic and heat inactivation and is apparently non-immunogenic. Using specific ligands crosslinked to the enzyme–albumin conjugate we have been able to target these conjugates to specific receptor sites and specific tissues. Ligands including cell surface-recognizing antibodies and hormones such as insulin have been used. These approaches offer new possibilities for the increased use of enzymes in medicine.

INTRODUCTION

Enzymes possess characteristics that in principle allow them to be "ideal" drugs. They generally have exquisite specificity and high catalytic efficiencies, while they are usually nontoxic and perform under

physiological conditions. In fact the topical administration of proteolytic enzymes for use as a bacteriocidal agent was first recognized by Purdon more than a century ago (*1*) and for many years now the oral administration of enzymes, especially as digestive aids, has been practiced. More recently the potential of enzyme therapy has been accepted in almost every field of medicine (*2*). Neoplasias have been treated with L-asparaginase based on the assumption that unlike normal cells certain tumor cells have absolute requirements for the amino acid L-asparagine. Fibrinolytic enzymes such as streptokinase and urokinase have been used in the treatment of thromboembolytic vascular disease. Proteolytic enzymes have been used extensively, especially via the oral route for the treatment of digestive disorders such as cystic fibrosis, chronic pancreatitis, and pancreatic recession. The most successful use of enzymes in medicine has been that of clotting factor replacement used to treat a wide range of disorders both inherited and acquired. A recent publication, *Enzymes as Drugs,* details the many recent advances in the use of enzymes in medicine (*3*).

Perhaps the most widely discussed potential for enzyme therapy is in the treatment of those "inborn errors of metabolism" manifested as enzyme deficiency diseases. Garrod (*4*) first suggested the possibility of enzyme replacement at the turn of the century, but only in the past 20 years, as we have begun to understand the nature of these enzyme deficiencies, has the possibility been actively considered. The demonstration by deDuve and others (*5*) of lysosomal storage diseases occurring as a result of deficiencies or defects in specific lysosomal hydrolases spurred further investigation into the possibility of treating many of these disorders by infusion of exogenous enzyme. Table 1 lists a few examples of lysosomal storage diseases that may be amenable to enzyme therapy. The history of enzyme administration for the treatment of some of these diseases has to date been disappointing. Although there has been some limited evidence of lowering of plasma and hepatic substrate levels following enzyme infusion in no case has there been a marked clinical improvement (*6,7*). Nevertheless cautious advances are being made in attempts to treat certain lipid storage diseases such as Tay Sachs, Fabry, and Gaucher (*8*). Limitations and problems associated with this approach will be discussed in the following sections.

Although the clinical potential of certain enzymes as drugs has now been demonstrated a number of serious drawbacks mitigate against their common use.

1. Enzymes, once introduced into an organism are subject to the same and sometimes more severe, processes of biodegradation and protein turnover as endogenous enzymes, and as such will be broken down by appropriate proteolytic enzymes, necessitating repeated administration in most instances.

2. Enzymes, as foreign proteins, may be highly immunogenic, causing moderate to severe hypersensitive reactions with second and subsequent administration. This has appeared to be an important limitation in the use of L-asparaginase in the treatment of acute lymphocytic leukemia and in the use of enzyme replacement therapy for the treatment of a number of the "inborn errors of metabolism" (*2*).
3. The required site of action of the administered enzyme has to be considered. Is the administered enzyme to act within the plasma, within a specific tissue, cell or intracellular organelle? The latter would be the case were the treatment of a lysosomal storage disease by enzyme replacement therapy being contemplated. The specific targeting of enzymes and perhaps of equal importance, the passage of appropriate permeability barriers must be considered.

APPROACHES TO ENZYME THERAPY

A great many strategies have been developed or considered for enzyme administration. It is now well accepted that protection of the enzyme from immunologic reactivity is essential if the use of nonhuman enzymes is to be considered. In fact, even if a human enzyme is available, care must be taken to determine that the protein is not recognized by the deficient recipient as being foreign (*2,9,10*). Similarly, consideration must be given to the site and nature of the clearance of the enzyme from the circulation if that is to be the route of administration. If the primary site of the enzyme deficiency is within the central nervous system (CNS), then consideration has to be given to delivering the enzyme past the blood brain barrier to sites of substrate accumulation (*7*). Alternatively, there is the possibility of creating a sufficient concentration gradient to pull substrate out of the CNS, dependent on the substate permeability, by depleting substrate levels in the plasma. Even within a given accessible organ, care must be taken towards delivering the administered enzyme to an appropriate site. In the case of Gaucher's disease, preliminary clinical trials using purified human placental glucocerebrosidase demonstrated a preferential uptake of the enzyme by hepatocytes rather than the phagocytic Kupffer cells of the reticulendothelial system (RES) the site of glucocerebroside storage in this disease (*8*). It has been demonstrated that this enzyme possesses sugar residues specific for receptors on hepatocytes that function to clear the enzyme from the circulation (*11*). Enzymatic cleavage of these residues has been shown to cause an increase in the delivery of the glucocerebrosidase to nonparenchymal cells in experimental animals (*11*). At the time of this writing there was no reported use of this deglycosylated enzyme in a Gaucher patient, al-

TABLE 1
Representative List of Inherited Lysosomal Storage Disorders Demonstrable as Enzyme Deficiency Diseases

Disease	Defective enzyme	Accumulating substrate	Clinical picture
Lipid Storage			
Gaucher (glucocerebrosidoses)	Glucosylceramidase	Glucosylceramide, lactosylceramide, glucosyl sphingosine	Juvenile or adult onset, hepatosplenomegaly infantile onset, severe retardation, death by 2 yr
Tay-Sachs GM_2 gangliosidoses	β-Acetylhexosaminidase A	GM_2 ganglioside	Infantile onset, severe retardation, death by 5 yr
Fabry (ceremidetrihexosidosis)	α-Galactosidase A	Digalactosyl ceramide galactosyl-galactosyl-glucosyl ceramide	Juvenile onset, renal and cardiovascular insufficiency, death by age 30 to 40 yr
Niemann-Pick sphingomyelinosis, Type A	Sphingomyelin phosphodiesterase	Sphingomyelin	Infantile onset, severe retardation, hepatosplenomegaly, death by 4 yr

Carbohydrate Storage			
Mannosidosis	Mannosidase A and B	Mannose containing polysaccharides and glycopeptides	Infantile to juvenile onset, severe psychomotor retardation
Pompe (Type II Glycogenosis)	α-1,4-Glucosidase	Glycogen	Infantile onset, cardiomegaly and respiratory distress, muscle weakness, death by 2–3 yr
Hurler (MPS I-H)	α-L-Iduronidase	Dermatan sulfate Heparan sulfate	Infantile onset, retardation, hepatosplenomegaly, death by 20 yr
Hunter (MPS II Type A)	Iduronate sulfatase	As in Hurler's	Similar to but milder than Hurler's

though one would have to be concerned with changes in antigenicity of the enzyme following removal of the specific sugar groups.

In an address to the German Chemical Society in 1909, Paul Ehrlich said, "We must learn to aim, and to aim in a chemical sense". In predicting the existence of antibodies . . . . "molecules with an affinity for certain tissues," he anticipated their use as carriers for delivery of drugs/enzymes to specific tissue sites (*12*). It took some 50 yr before Mathé and colleagues (*13*) demonstrated that antibodies could be used to direct therapeutic agents to specific cells. Since then the number of carrier systems and "magic bullets" used as homing devices has blossomed to include encapsulating systems such as: lipid vesicles or liposomes (*14,15*), synthetic microcapsules (*16,17*), biologic microcapsules (*18*), red blood cells (*19*), fibroblasts and carrier systems such as plasma proteins, hormones, lectins, antibodies, synthetic peptides, and glycoconjugates (*2,10*). Each system demonstrates specific advantages in terms of reducing immunogenicity, protecting the therapeutic agent from rapid bioinactivation and the potential to deliver enzyme/drug to a specific site of action. It should be pointed out that aside from the now common use of slow-releasing drug depots no enzyme or drug delivery systems using such carriers has been used in extensive clinical trials and it is unlikely that any one system will serve as a panacea to direct and or protect enzymes following in vivo administration.

ENZYME–ALBUMIN POLYMERS

We have chosen to study a system that we felt may have certain advantages. Albumin is a natural and abundant plasma protein and it was our initial objective to examine the possibility of creating a non-immunogenic and non-antigenic soluble polymer or complex of a specific enzyme with an excess of homologous albumin. Thomas and coworkers (*20*) have demonstrated that crosslinking uricase and L-asparaginase to an excess of albumin produced a complex with a molecular weight in the range of 500,000 that was water soluble and had a greatly increased resistance to heat denaturation. Our early studies served primarily to determine if the use of homologous albumin might serve to: (a) reduce the immunogenicity of foreign proteins (enzymes) and thus reduce the risk of hypersensitivity reactions and (b) increase the ability of the enzyme to remain in the circulation following intravenous administration. Both of these objectives were met as polymers of hog liver uricase and albumin were shown to be non-immunogenic and non-antigenic (*21*) when compared to the native enzyme, while the circulation half-life for the enzyme increased from 2 h for the uncomplexed enzyme to 18 h for the enzyme–albumin conjugate (*22*). Similar characteristics have now been demonstrated for a number of other enzyme systems including superoxide dismutase (*23*), α-1,4-glucosidase (*24,25*) and L-asparaginase (*26*). It is

interesting to note that Abuchowski and coworkers (27) have achieved similar results to our own using soluble conjugates of enzyme and polyethylene glycol (PEG).

In this paper we will deal primarily with the use of enzyme–albumin conjugates with an eye towards enzyme therapy for lysosomal storage diseases. We have chosen Pompe's disease or Type II Glycogenosis. It is considered a prototype lysosomal storage disease affecting liver hepatocytes and muscle, death usually occurring within the first 2 yr as a result of cardiac and respiratory dysfunction. Attempts at enzyme therapy using α-1,4-glucosidase from fungal sources yielded no clinical improvement (*6*) and only very little encouragement in terms of lowering hepatic glycogen stores as determined at autopsy.

ENZYME ACTIVITY

Earlier studies showed that crosslinked enzymes: uricase, superoxide dismutase, catalase, and L-asparaginase with a 10–20-fold excess of albumin retained as much as 90% of the original activity of the uncomplexed enzyme, dependent on the conditions and nature of the crosslinking reaction. Crosslinking agents have included glutaraldehyde, water-soluble carbodiimides, sodium periodate, and other bifunctional reagents. Table 2 indicates that human placental α-1,4-glucosidase retains its catalytic activity towards its natural substrate, glycogen even after conjugation with a 20-fold excess of human albumin. The conjugate has a molecular weight of 8.5×10^5 suggesting an average molar compo-

TABLE 2
Enzyme Recovery Rates Following Crosslinking with Albumin

Enzyme[a]	Crosslinking conditions	Substrate	Recovery, %
Uricase (hog liver)	pH 6.5 + uric acid	Uric acid	40
Uricase (hog liver)	pH 9.1 + uric acid	Uric acid	66
Uricase (hog liver)	pH 9.1 − uric acid	Uric acid	4
α-Glucosidase (yeast)	pH 6.8 + PNPG	PNPG[b]	65
α-Glucosidase (yeast)	pH 6.8 + PNPG	Glycogen[c]	2
α-Glucosidase (human)	pH 6.8 + maltose	PNPG[b]	0
α-Glucosidase (human)	pH 6.8 + maltose	Maltose[c]	69
α-Glucosidase (human)	pH 6.8 + maltose	Glycogen[c]	58

[a]The uricase enzyme requires oxygen as a cofactor. Running the crosslinking reaction in the absence of oxygen by bubbling nitrogen through the mixture increased the optimum yield from 10% enzyme recovery to 66% recovery. The yeast α-glucosidase has a pH optimum of 7.0 and almost no activity at pH 4.5, whereas the alpha-glucosidase from human placenta is a lysosomal enzyme with an optimum pH of 4.8 and virtually no activity at normal pH.

[b]Enzyme assay run at pH 6.8.

[c]Enzyme assay run at pH 4.8.

sition of 1 enzyme molecule per 10–12 albumin molecules (*24*). Although the retention of enzyme activity to small natural or artificial substrates such as maltose or PNPG might be expected, it is more surprising that as much as 60% of the enzyme activity is retained to glycogen, a large and bulky substrate. It should be pointed out that great care must be taken to protect the active site of the enzyme during the crosslinking step by carrying out the conjugation in the cold and in the presence of an excess of substrate.

It had earlier been demonstrated that conjugation of uricase or L-asparaginase with albumin increased the heat resistance of the enzymes (*20*). Table 3 demonstrates that the yeast enzyme (α-1,4-glucosidase) is protected from proteolysis by trypsin when in the conjugated form. The human placental form of the enzyme has an endogenous resistance to trypsin. At 37°C, the human placental enzyme has a circulation half-life in rats of approximately 8 h in the native form whereas in the conjugated form the half-life is in excess of 24 h. The increased resistance to heat denaturation and to proteolytic activity is a significant benefit since less enzyme may be administered in order to achieve a significant enzyme replacement. It should be pointed out that any loss of enzyme activity caused in the crosslinking step is quickly made up by the resistance of the conjugated form of the enzyme to bioinactivation.

IN-VIVO TARGETING OF α-1,4-GLUCOSIDASE

In the case of type II glycogenosis, the important site of hepatic glycogen storage are the hepatocytes as opposed to the more phagocytic Kupffer cells. In-vivo administration of native enzyme (from yeast) in rats produces a relatively rapid clearance of the enzyme from the circulation ($t_{1/2}$ of 1 h) and a deposition largely within the Kupffer cell population. Conjugation of an excess of albumin slows the clearance rate significantly ($t_{1/2}$ = 8 h), but no significant shift away from the Kupffer cell

TABLE 3
Trypsin Sensitivity of Enzyme–Albumin Polymers

Enzyme preparation	$t_{1/2}$ at 37°C with 5 U of trypsin
L-Asparaginase (*E. coli*)	30 min
L-Asparaginase–albumin	6 h
α-1,4-Glucosidase (yeast)	10 min
α-1,4-Glucosidase–albumin	180 min
α-1,4-Glucosidase (human placenta)[a]	>> 10 h
α-1,4-Glucosidase–albumin	>> 10 h

[a]Placental α-1,4-glucosidase in both free and polymeric form shows no alteration in activity over a 6 h period at 37°C in the presence of 20% fetal calf serum.

population even in light of the presence of albumin receptors on hepatocytes (Table 4). Antibodies have for some time been considered as possible targeting agents for drugs and enzymes. We therefore prepared polyclonal antibodies against rat hepatocytes. The antisera was adsorbed to rat spleen cells and Kupffer cells to eliminate nonspecific antibodies. The antihepatocyte antibodies were crosslinked to the enzyme–albumin conjugate using glutaraldehyde. Following intravenous injection and clearance of better than 90% of the ^{125}I-labeled enzyme conjugate hepatocyte and Kupffer cell fractions were isolated by collagenase perfusion of the rat liver *in situ* (*28*). Table 4 demonstrates the preferential uptake of enzyme–albumin conjugates to a hepatocyte population when an antihepatocyte antibody preparation has been covalently attached. Attachment of a non-specific antibody yielded no such targeting. Similar experiments have been carried out in mice utilizing RI tumor cells containing the H-2^k antigen and demonstrating a preferential targeting of L-asparaginase-albumin conjugates to the cells when monoclonal antibodies against the H-2^k antigen were covalently attached (*26*).

The fact that the polymeric complex containing enzyme activity appears to target to the correct cell type does not, however, indicate delivery of enzyme activity to a lysosomal compartment. Figure 1 and Table 5 demonstrate that targeted α-1,4-glucosidase may be localized to a lysosomal fraction within the liver. Figure 1 actually follows the ^{125}I-labeled

TABLE 4
Targeting Enzyme–Albumin Polymers to Hepatocytes[a]

^{125}I-labeled protein preparation[b]	^{125}I radioactivity, cpm/mg of cell protein		
	Hepatocytes	Kupffer cells	Ratio
α-1,4-Glucosidase	108 ± 22	1030 ± 131	0.10 ± 0.01
Albumin	42 ± 20	177 ± 49	0.25 ± 0.05
Enzyme–albumin polymer	95 ± 31	508 ± 133	0.20 ± 0.03
Anti-F IgG	109 ± 15	629 ± 120	0.16 ± 0.03
Anti-H IgG	233 ± 32	280 ± 40	0.85 ± 0.09[c]
Anti-F-IgG-polymer	182 ± 39	1067 ± 189	0.17 ± 0.02
Anti-H-IgG-polymer	317 ± 80	259 ± 11	1.23 ± 0.15[c]

[a]Various enzyme or antibody preparations were injected intravenously. When 80–90% of the injected label had cleared from the circulation, the rats were anesthetized and the livers were perfused with collagenase to separate hepatocytes from Kupffer cells. The ratio represents the counts/mg cell protein in the hepatocytes over that in the Kupffer cells. The anti-F-IgG represents a control antibody derived from the serum of rabbits immunized with human skin fibroblasts. The anti-H-IgG preparation was derived from the serum of a rabbit that had received repeated injections of isolated rat hepatocytes. The antiserum was absorbed with rat spleen cells prior to conjugation using glutaraldehyde.

[b]F = fibroblasts, H = hepatocytes.

[c]Significantly different from all other preparations at $P < 0.001$.

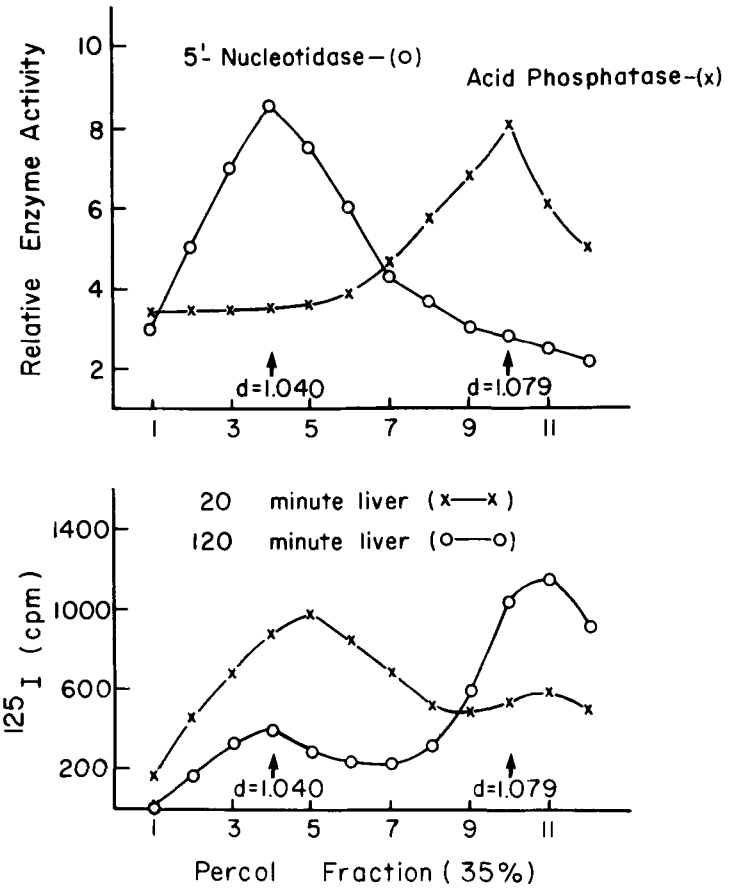

Fig. 1. Lysosomal targeting of enzyme–albumin–antibody polymer. The enzyme conjugate was injected at $t = 0$. At 20 min or at 2 h, rats were sacrificed, the livers excised, and subcellular fractions obtained by standard ultracentrifugation techniques. This fraction was then put on Percoll gradients to isolate lysosomal and membrane fractions that sediment at $d = 1.079$ and 1.040, respectively. This can be seen in the upper panel where fractions (density) are plotted as a function of enzyme activity (5'-nucleotidase for membranes and acid phosphatase for lysosomes). The lower panel demonstrates the distribution of ^{125}I-labeled conjugate between membrane and lysosomal fractions 20 min and 2 h following injection.

polymer (enzyme–albumin–antibody) from a membrane fraction (^{125}I associated with a high 5'-nucleotidase activity) 20 min after injection to a lysosomal fraction (^{125}I associated with an acid phosphatase fraction) at 2 h following injection of the enzyme complex. Table 5 demonstrates that not only can the radioactive label be found associated with a lysosomal fraction with time, but that exogenous enzyme activity can also be found associated with such a fraction. In this case, both the yeast and the human placental enzymes were used. The latter has a pH optimum of 4.8 and we were concerned that it would be difficult to monitor above the

TABLE 5
Lysosomal Targeting of α-Glucosidase[a]

Preparation	Activity in liver lysosomes, U/g	% Injected
Saline (yeast control)	2[a]	
α-1,4-Glucosidase	7[a]	22
α-1,4-Glucosidase–albumin–insulin	10[a]	36
Saline (human placenta control)	9.9[b]	
α-1,4-Glucosidase–albumin	13.5[b]	35
α-1,4-Glucosidase–albumin–insulin	15.1[b]	45

[a]Enzyme was injected intravenously at $t = 0$, and at $t = 2$ h, the liver was excised, and a lysosomal fraction prepared by differential centrifugation.
[b]PNPG as substrate, pH 7.4.
[c]Maltose as substrate, pH 4.8.

activity of the endogenous rat liver α-glucosidase. The yeast enzyme has a pH optimum of 7.4 and little endogenous glucosidase activity was to be expected at this pH. In this case rather than using antihepatocyte antibodies as a targeting agent, we used insulin crosslinked to the enzyme–albumin complex since hepatocytes are known to be rich in insulin receptors. In these experiments we have not differentiated between a Kupffer cell fraction and a hepatocyte fraction, and it may be that a considerable amount of the nontargeted enzyme (i.e., without insulin) is in fact associated with a Kupffer cell fraction.

INSULIN AS A TARGETING AGENT

In the case of Pompe's disease, although there is serious liver involvement and glycogen accumulation causing deficiencies in liver function, the more serious accumulation occurs in muscle of respiratory and cardiac tissue, which invariably leads to organ failure and death usually by the age of two. In an attempt to target enzyme to muscle tissue we sought to determine if we could utilize the insulin receptor as a target since internalization and lysosomal processing of insulin–insulin receptor complexes have been demonstrated (*24*). Table 6 demonstrates attempts at targeting of α-1,4-glucosidase–albumin polymers to two different cell types that possess insulin receptors: (a) chick embryonic muscle cells grown in tissue culture and (b) peripheral human lymphocytes. The insulin was conjugated to the enzyme–albumin complex using either glutaraldehyde or carbodiimide as crosslinking agent. In the described experiments the ratio of enzyme to albumin to insulin is 1:10:60 with a molecular weight averaging 1.2×10^6. Conjugation of albumin to the yeast enzyme appears to decrease binding whereas conjugation of insulin to either yeast or human enzyme–albumin polymers increases binding five- to eightfold.

TABLE 6
Insulin Mediated Targeting of Enzymes[a]

Enzyme preparation	% Binding	
	Chick muscle cells	Human lymphocytes
^{125}I-α-Glucosidase (yeast)	6.7	4.9
^{125}I-α-Glucosidase (human placenta)	8.1	6.1
^{125}I-α-Glucosidase–albumin (yeast)	4.0	4.2
^{125}I-α-Glucosidase–albumin–insulin (yeast)	30.1	29.1
^{125}I-α-Glucosidase–albumin–insulin (human placenta)	37.1	34.6

[a]Binding conditions were as follows: 0.05 μg of ^{125}I-labeled enzyme preparation was incubated with 2×10^6 chick embryonic pectoral muscle cells or 1×10^6 mouse spleen cells for 30 min at 37°C. The cells were then washed and counted. When the cells were also incubated with chloroquine, binding of insulin-containing polymers increased while internalization of polymers was inhibited. Chloroquine had no effect on binding of free enzyme or enzyme–albumin polymer.

In vivo experiments with insulin conjugates in rats have not been as promising in terms of the targeting of the enzyme to cardiac and respiratory muscle. When α-1,4-glucosidase-albumin polymers were injected intravenously, *no* ^{125}I-labeled enzyme could be detected associated with muscle mass once care was taken to subtract label associated with blood contaminating the tissue. When the enzyme–albumin complex was conjugated with insulin, the amount of enzyme associated with the muscle mass including both cardiac and respiratory muscle rose to 2–3% of the injected dose. The significance of such a value is difficult to ascertain. Although the targeted dose of enzyme is small, considering that the enzyme is probably nontoxic to other targets such as lymphocytes, fat cells, red cells, hepatocytes, etc., it may be significant that using this system we are actually able to deliver enzyme to muscle tissue. Nevertheless the efficiency of the targeting system in terms of the total dose is poor. In the conjugated form with the enzyme–albumin complex, the insulin molecule retains: (a) its ability to bind anti-insulin antibodies and (b) its hypoglycemic properties (*30*). We were able to observe drops in plasma glucose levels following injection of the enzyme–albumin–insulin complexes that were similar in magnitude to injection of similar doses of free insulin. The question of whether the enzyme–albumin–insulin complex actually reaches the lysosomal fraction of muscle cells has still to be determined. Another question that we have yet to address is whether delivery of enzyme to a lysosomal fraction by what can be considered receptor-mediated endocytosis results in the deposition of the enzyme within the same lysosomes where substrate is being accumulated. deDuve (*5*) produced evidence in the early years of study on lysosomes indicating that secondary lysosomes could fuse with one another and with primary

lysosomes. It has, however, still to be shown whether the approach of using lysosomotropic conjugates will result in the delivery of the drug or enzyme to the appropriate lysosomal storage site.

IMMUNOLOGICAL PROPERTIES OF ENZYME–ALBUMIN POLYMERS

The major drawback to the common use of enzymes as therapeutic agents remains the danger of severe hypersensitivity reaction to repeated administration of a foreign protein. The advent of modern cloning techniques and techniques in biotechnology will in time offer the production of human gene products, and thus human enzymes that may offer greatly reduced dangers of immunologic complications. In our first publication (*21*) on the immunologic aspects of enzyme–albumin polymers, we were able to show that polymeric complexes of hog liver uricase and rabbit albumin were non-immunogenic in rabbits (they did not elicit an antibody production) and non-antigenic (antibodies against hog liver uricase did not react with the polymeric structure). As a control we showed that the uricase crosslinked with dog albumin elicited an antibody response in rabbits against the polymer as a whole, against the dog albumin, but not against the hog liver uricase, whose antigenic determinants (it is highly immunogenic in its native form) must therefore have been masked. At that time we stressed that this held for hog liver uricase but we had no evidence to suggest that similar data could be obtained using other enzymes. We have now extended this data to include at least six other enzymes and in each case we were able to produce non-immunogenic complexes of the foreign enzyme along with homologous albumin. These experiments have been performed in both rabbits and mice with similar results. Table 7 presents a summary of this work and a list of enzymes that we have used. We believe that given the ability to perform the conjugation under different conditions we should in each case be able to lower or completely negate the problem of immunogenicity and therefore make the possibilities of enzyme therapy more practicable.

Using polyethyleneglycol (PEG) conjugated to a number of enzymes including uricase and L-asparaginase, Abuchowski and coworkers (*27*) have also been able to reduce the immunogenicity of otherwise foreign proteins. They suggest that the PEG, rather than simply masking the antigenic determinants of the enzyme, is acting as a toleragen and inducing tolerance in the experimental animal to the attached enzyme that in its native form is highly immunogenic. Although we have yet to explore this possibility utilizing our enzyme–albumin polymer, where we believe that the enzyme is physically masked, we have evidence to indicate that homologous albumin may indeed function as a toleragen. We were concerned that adding insulin or some other targeting agent to

TABLE 7
Immunological Properties of Enzyme–Albumin Polymers[a]

Enzyme preparation	Immune response
Uricase (hog liver)	+++
Uricase–albumin (1:10)	−
α-1,4-Glucosidase (yeast)	+++
α-1,4-Glucosidase–albumin	−
α-1,4-Glucosidase (human placenta)	+++
α-1,4-Glucosidase–albumin (1:10)	−
Superoxide dismutase (bovine)	+++
Superoxide dismutase–albumin (1:5)	+
Superoxide dismutase–albumin (1:10)	−
L-Asparaginase (*E. coli*)	+++
L-Asparaginase–albumin (1:5)	+
L-Asparaginase–albumin (1:10)	−

[a]Antigen preparations were administered either ip (with complete Freund's adjuvant) or iv over a period of 3 months in both mice and rabbits for hog liver uricase, bovine superoxide dismutase, and yeast α-1,4-glucosidase and in mice for L-asparaginase and human placental α-1,4-glucosidase. In each case, homologous albumin (mouse or rabbit) was used. If a heterologous albumin was employed, then antibodies could be detected against the polymer but these antibodies were invariably directed against the heterologous albumin and not against the conjugated enzyme. Yagura et al. (1982) (*30*) have recently reported similar results with respect to the immunogenicity of L-asparaginase–albumin polymers in mice using our preparative procedures.

the albumin–enzyme conjugate in a highly visible form on the outside of the polymer might serve to present the insulin as a hapten and make it highly immunoreactive. Although this is the case for certain polypeptides (both synthetic and natural) and for heterologous albumins, the use of homologous albumin did in fact serve to act as a toleragen and tolerance to the attached insulin was produced (*31*). In a similar fashion we have been able to induce tolerance to the iron-chelating drug desferioxamine by chemically crosslinking it to homologous albumin (*32*). Again the use of a heterologous albumin or Ficoll resulted in a high antibody titer being produced against the attached drug. These results bear some resemblance to previous reports of self-IgE having the ability to function as an effective toleragen (*33*).

SUMMARY

The aim of this work is to modify enzymes in such a manner as to make them more acceptable in terms of bioinactivation and immunologic reactivity. We have chosen albumin as a carrier because it seemed to us that it possessed the "natural" characteristics to allow for prolonged cir-

culation times and reduced immunogenicity. Albumin is in fact a carrier of natural substances such as fatty acids and small polypeptides within the blood stream. Other carrier systems such as polyethylene glycol, synthetic polypeptides, and other plasma proteins may offer similar specific advantages. The question of targeting to specific tissue remains a difficult one. It would appear that while the use of receptor mediated endocytosis to deliver enzymes (or drugs) to lysosomes within cells is attractive, passage across the endothelial permeability barrier to target tissue may prove formidable. This is probably especially true of tissue other than liver and spleen, where the high permeability of the vascular tissue makes access to underlying tissue and cells easier. It is for this reason that delivery of polymeric substances as well as other carrier systems to hepatocytes and Kupffer cells (dependent on the nature of the ligands) would appear at this stage to be very much more promising than delivery across other barriers such as the blood–brain barrier or to tissue such as muscle.

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