EXHIBIT E

PART 2 of 3

muscle enzyme activity. While the evidence from a limited number of infusions in a single patient is difficult to assess, it would certainly appear that this approach to enzyme replacement therapy where muscle tissue is involved deserves further consideration.

5. ENZYME–ALBUMIN POLYMERS

We too have chosen a plasma protein to act as a carrier in attempts at enzyme therapy. In 1974, Thomas and colleagues (Pailott *et al.*, 1974) demonstrated the covalent binding of an excess of albumin with either hog liver uricase or *E. coli* L-asparaginase to form a soluble polymer with a molecular weight of approximately 600,000 (representing approximately eight albumin molecules/enzyme molecule on average). They also observed significantly increased resistance of the conjugate to heat denaturation. We supposed that this might be an ideal carrier to allow for the enzyme molecule to be retained in the circulation. Albumin has a prolonged plasma half-life and we considered the possibility that an excess of a homologous carrier such as albumin might serve to mask or block the antigenic determinants of a foreign enzyme thus circumventing one of the most serious drawbacks to enzyme therapy. This preparation is water soluble and since the cross-linking reaction is relatively gentle (using either glutaraldehyde or water soluble carbodiimide at physiological pH) we anticipated that the albumin molecules in the end product would resemble native albumin. In the following pages we will provide evidence to support this contention. This preparation should not be confused with other albumin preparations such as albumin microspheres (Kramer, 1974; Widder *et al.*, 1982) where intentional denaturation of the albumin is carried out in order to produce the structures which are not water soluble and which form a dispersion. These albumin preparations would be expected to bare little resemblance to the native albumin (nor were they intended to). The topic of magnetically responsive albumin microspheres has recently been reviewed (Widder *et al.*, 1982).

5.1. Preparation

While there is no fixed recipe for the preparation of enzyme–albumin polymers, there are a few basic principles which we follow. We attempt to start with a molar ratio of albumin to enzyme of 10:1. A relatively high concentration of the normal substrate or an artificial substrate is included in the cross-linking medium in order to protect the active sites of the enzyme from reacting. The temperature of the reaction medium is maintained at 4°C in order to slow down the rate of turnover of the enzyme during the conjugation process. Glutaraldehyde is usually our cross-linking agent of choice since it appears to be the most gentle, allowing for the greatest preservation of enzyme activity. Alternatively we have used a water soluble carbodiimide (ECDI cross-links the amino group from one protein to the carboxyl groups of the other) or sodium periodate (which conjugates a sugar residue from one protein to an amino group on the other). The choice of cross-linking agents is dependent on: (a) retention of enzyme activity, (b) resistance of the resultant polymer to biodegradation, (c) immunogenicity and antigenicity of the resultant polymer and (d) ability of the resultant polymer to remain in the circulation. The properties of various cross-linking agents has been extensively reviewed (Means and Feeney, 1971; Wold, 1973; Weetall and Cooney, 1981). The reaction is stopped after a given period of time (dependent on the enzyme and the speed of the cross-linking reaction), by either the addition of a quenching agent (glycine for the glutaraldehyde reaction) by dialysis or by molecular seive chromatography to get rid of excess cross-linking agent (see Table 2, Fig. 1). The resultant polymer is separated from unreacted albumin, enzyme or small polymers by ultrafiltration through Amicon XM300 filters or by gel exclusion chromatography using either Biogel A-50M (Biorad) or Sepharose 4B (Pharmacia). The molecular weight we generally aim for is in the 700,000–900,000 range where the average polymer molecule has one enzyme molecule conjugated with an average of ten albumin molecules. Figure 1 depicts one such separation.

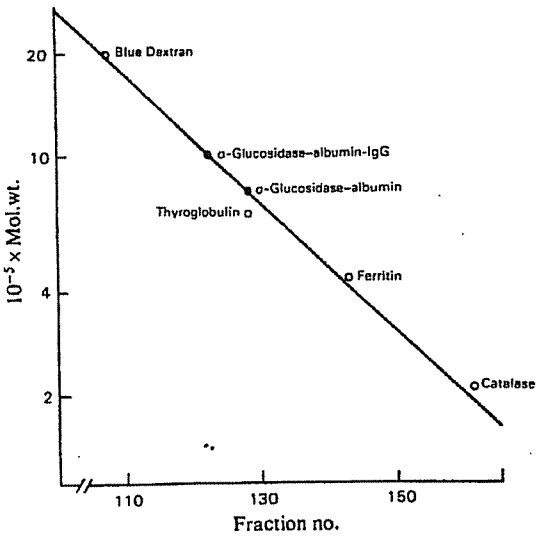

FIG. 1. Molecular weight determined of α-1,4-glucosidase–albumin polymers. Gel chromatography was employed using Bio-Gel A-50M with Pharmacia standards; catalase, ferritin, thyroglobulin and blue dextran 2000. Over 80% of the enzyme–albumin polymer eluted between peaks 124 and 132 indicating a MW range of 7.5×10^5 to 9.5×10^5. The addition of anti-hepatocyte antibodies to the polymer resulted in an average increase in MW of 2×10^5.

The attachment of targeting agents discussed in a following section is performed by attaching the agent to the polymer according to the conjugation procedures described above. Again care is taken to protect the active site of the enzyme during the cross-linking reaction. The cross-linking conditions are chosen so as to retain the biological activity of both the enzyme and the delivery system. For instance, in the use of antibodies to direct polymeric conjugates to specific sites, we attempt to cross-link via the Fc portion of the immunoglobulin as opposed to the $F(ab)_2$ fractions which identifies the cell surface specific antigen. This may be accomplished by utilizing a sugar residue on the Fc portion in order to conjugate the IgG molecule with the enzyme–albumin polymer. In addition to retaining the binding capability of the antibody, it masks the Fc portion, making the new conjugate less likely to bind to Fc receptors thereby avoiding possible activation of the complement system. The Fc portion of immunoglobulins is also known to be the more immunogenic part so that binding this portion to the polymer may in fact decrease the incidence of immune reaction to the IgG molecule. This specific hypothesis has yet to be tested. Another approach which we have used successfully is to incubate the albumin with a ten-fold molar excess of glutaraldehyde, dialyze to get rid of the cross-linking agent and then add the enzyme along with the substrate. This approach avoids extensive albumin–albumin cross-linking while still producing active and protected enzyme–albumin polymers.

5.2. PHYSICAL PROPERTIES

The molecular weight of the resultant polymers is a function of the amount of cross-linking agent used (e.g. glutaraldehyde) and the length of time the conjugation reaction is allowed to proceed. Figure 1 represents a molecular weight determination for α-1,4-glucosidase-albumin polymer and an enzyme–albumin–IgG polymer made as described in Table 2. On average the polymer has a molecular weight of 750,000 with more than 80% of the protein eluting in the range of 600,000–900,000, suggesting an average of one enzyme molecule per twelve albumin molecules close to the molar ratio of the starting reactants. Addition of IgG molecules (in this case an adsorbed heterologous antisera against rat hepatocytes) to the polymer increased the average molecular weight to 10^6 suggesting that between one and two molecules of IgG had been conjugated to each polymer molecule. Smaller polymeric complexes could be made by decreasing the reaction time with glutaraldehyde.

TABLE 2. *Cross-Linking Recipe*

2 mg α-1,4-Glucosidase (from human placenta)
50 mg Human Albumin
6 mg Maltose
3 ml Buffer (PBS at pH 6.8 or 5.0)
50 μl Glutaraldehyde (25%)

The reaction components are stirred gently at 4°C for 3–4 hr after which 300 mg of glycine is added to stop the reaction. Following an overnight dialysis at 4°C against 1% NaCl and 1% glycine, unreacted protein and small polymers are separated by ultrafiltration using Amicon XM300 filters. The resultant polymers may then be sized using Biorad Biogel or Pharmacia Sepharose molecular seive chromatography.

The above recipe is the optimum conditions which we found for cross-linking human albumin and human placental alpha-glucosidase. Including large amounts of substrate (natural or artificial) was always required to optimize enzyme recovery. Conditions for other enzymes required adjusting conditions and concentrations to regulate size and enzyme activity. While glutaraldehyde always worked as a cross-linking agent, it was sometimes more efficient to use either a water soluble carbodiimide or sodium periodate.

Table 3 demonstrates the enzyme recovery of a number of enzyme preparations following cross-linking. In each case it may have been necessary to adjust the cross-linking conditions to maximize the yield. In all cases we have been able to achieve yields at least as high as 50%. In order to achieve high yields it was necessary to carry out the cross-linking reaction in the cold and in the presence of large concentrations of substrate to protect the active sites of the enzyme during the conjugation procedure. Figure 2 demonstrates the changes in the pH profiles that can be achieved by use of appropriate cross-linking conditions. Similar reports have been described using other enzymes in conjugation with polyethylene glycol (Abuchowski and Davis, 1981) and in the conjugation of papain to solid supports (Goldman *et al.*, 1968). This property of the cross-linking procedure may have important physiological consequences. In the case of hog liver uricase, the pH optimum is 10.5 with only 5–10% residual enzyme activity at pH 7.4. By carrying out the cross-linking reaction at pH 9.1 we were able to broaden the optimal

TABLE 3. *Enzyme Recovery Rates Following Cross-Linking with Albumin*

Enzyme	Cross-Linking 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*	65
α-Glucosidase (yeast)	pH 6.8 + PnPG	Glycogen†	2
α-Glucosidase (human)	pH 6.8 + Maltose	PNPG*	0
α-Glucosidase (human)	pH 6.8 + Maltose	Maltose†	84
α-Glucosidase (human)	pH 6.8 + Maltose	Glycogen†	63

*Enzyme assay run at pH 6.8.

†Enzyme assay run at pH 4.8.

The uricase enzyme requires oxygen as a cofactor and running the cross-linking 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 alpha-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.

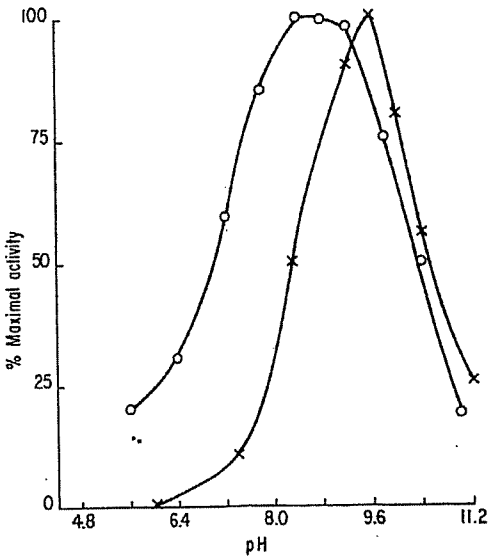

FIG. 2. pH dependence of uricase (hog liver) activity in its free or naked form (×) and in its immobilized form as a soluble conjugate with a ten fold excess of albumin (○). Cross-linking was performed using glutaraldehyde at pH 9.1. At its maximum activity the polymeric form of the enzyme retains 66% of the maximum activity of the enzyme free in solution (Table 3). Both curves are reversible and the enzyme activities measured at room temperature.

pH range for maximum activity such that the enzyme in the conjugated form retains as much as 60% of its optimal activity at pH 7.4. The exact reason for this shift is not well understood. Either the pH of the microenvironment of the cross-linked enzyme has been changed such that at physiological pH the enzyme still sees a basic microenvironment or else the enzyme may have been immobilized (albeit in a soluble form) in a more active state at the basic pH of the cross-linking procedure and that state is retained even when the pH of the medium is reduced to pH 7.4.

A dramatic increase in the resistance of the cross-linked enzyme to proteolytic degradation and heat denaturation was observed compared to equivalent amounts of free or non-conjugated enzyme. This holds true even when equivalent amounts of albumin are added to the enzyme in question without the benefit of cross-linking. No increase in the resistance of the enzyme superoxide dismutase (SOD) could be detected following cross-linking but this enzyme in itself is extremely resistant to degradation. Figure 3 demonstrates the increased resistance of α-1,4-glucosidase conjugates to trypsin at 37°C. Changes in heat resistance as well as enzyme kinetics have also been observed (see Poznansky and Bhardwaj, 1980; Poznansky *et al.*, 1982; Poznansky and Leighton, unpublished data). The heat resistance of the conjugated enzyme was always significantly higher (except for SOD) than free enzyme although the enzyme kinetics changed in rather unpredictable ways. For L-asparaginase, cross-linking with albumin caused no change in the V_{max} whereas the K_m increased from 4×10^5 to 6.5×10^5 not unlike the changes in enzyme kinetics which Abuchowski and Davis (1981) observed when they conjugated L-asparaginase with PEG. When hog liver uricase was conjugated with albumin the V_{max} decreased by a factor of 2 and the K_m increased from 3.5×10^5 to 8×10^5 again similar to the values reported for uricase–PEG conjugates by Abuchowski and colleagues. The small changes in enzyme activity following conjugation with carriers are more than offset by the increased stability of the enzyme in the conjugated form.

5.3. *IN VIVO* PROPERTIES

Earlier work in our laboratory dealt with the possibility of using uricase–albumin polymers to lower the uric acid levels in the plasma of dalmation coach hounds, a canine breed which suffers from chronic hyperuricemia (Poznansky, 1979). The results were

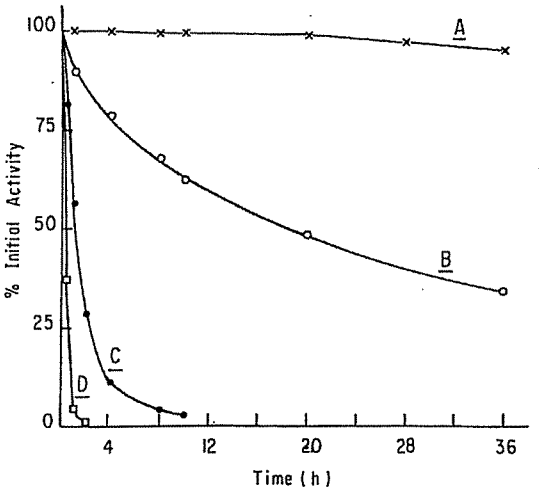

FIG. 3. The resistance of free α-1,4-glucosidase and α-1,4-glucosidase–albumin polymers to trypsin at 37°C. (A) 20 units of polymer in 3 ml PBS. (B) 20 units of polymer in 3 ml PBS with 5 units trypsin. (C) 20 units of enzyme in 3 ml PBS and (D) 20 units of free enzyme in 3 ml of PBS with 5 units of trypsin. Addition of free albumin to either C or D resulted in only negligible increases in enzyme stability.

encouraging though limited in number. The enzyme–albumin complex remained in the circulation for approximately five times longer than equivalent amounts of the free enzyme (half-life of 20 hr as opposed to 4 hr) and was more effective in lowering plasma uric acid levels. The protected enzyme lowered plasma substrate levels for as long as six days as opposed to two days for the free enzyme. Subsequent injections of enzyme–albumin polymer produced no decrease in effectiveness of the complex whereas subsequent injections of free enzyme saw a sharp drop in the circulation half-life of the enzyme, a drop in the enzymes' ability to lower plasma uric acid levels and finally after the third injection a severe anaphylactic-type reaction. We attributed the reaction and the progressive drop in the effectiveness of the free enzyme to be a result of antibodies being produced to the enzyme molecule, although no such measurement were made at the time.

Superoxide dismutase was tested as a possible anti-inflammatory agent in the conjugated form with albumin (Wong *et al.*, 1980). The free form of SOD was cleared from the circulation with a $t_{1/2}$ of less than 6 min whereas the SOD–albumin conjugates were cleared with $t_{1/2}$ ranging from 4 hr for the large complexes to 15 hr for the smaller complexes (MW = 300,000). The anti-inflammatory properties of the enzymes were tested by determining their ability to inhibit carrageenan-induced paw edema. The SOD–albumin complex could inhibit over 50% of the inflammation at a dose of 1000 U/kg while a dose of 1500 units produced an inhibition of 65%, as good an inhibition as any other anti-inflammatory drug can claim. The free SOD had a barely significant anti-inflammatory effect when given by the same route of administration (10–14% inhibition at a dose of 3300 U/Kg). The albumin itself was without effect. The enormous increase in the activity of SOD as an anti-inflammatory agent in conjugated form with albumin is attributed primarily to its ability to remain in the circulation when complexed with albumin.

In experiments with α-1,4-glucosidase (Poznansky and Bhardwaj, 1980, 1981) we have investigated the possibility of delivering enzymes to lysosomes to determine the feasibility of using the enzyme–albumin approach for the treatment of inborn errors of metabolism manifested as lysosomal storage diseases. The concepts of targeting are dealt with in a following section. Once targeted to liver tissue, we see that enzyme activity within a lysosomal fraction can be observed. Figure 4 demonstrates the access of the ^{125}I-labeled enzyme–albumin complex to a lysosomal fraction of either isolated rat hepatocytes or homogenized whole liver. If a shorter incubation period of polymer with liver or hepatocytes is used, or if the incubation is carried out below 17°C where internalization

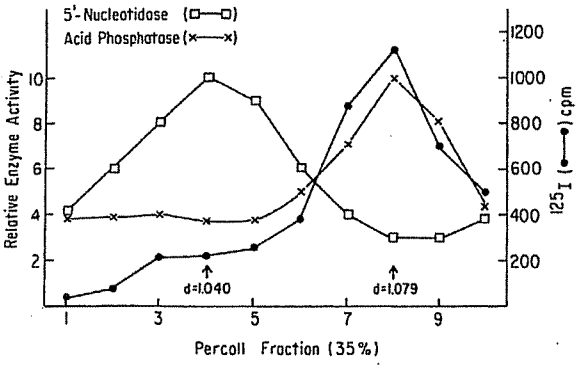

FIG. 4. Association of ^{125}I-labeled α-1,4-glucosidase–albumin-anti-hepatocyte antibody with various subcellular fractions following density gradient centrifugation using Percoll (Pharmacia). The plasma membrane fractionates with a density of 1.040, exhibiting the highest concentration of the marker 5′-nucleotidase whereas the lysosomal fraction, associated with the marker, acid phosphatase fractionates at a density of 1.079. The fractionation was performed 1 hr following injection of the polymer. When fractionation was performed 20 min following injection a greater proportion of the labeled polymer could be seen associated with the plasma membrane fraction.

is inhibited, then a greater fraction of the enzyme complex (in this case targeted by the use of antihepatocyte antibodies) is found associated with a plasma membrane fraction (denoted by a lower density and a high concentration of the plasma membrane marker 5′-nucleotidase) than with the lysosomal fraction, rich in acid phosphatase. Table 4 demonstrates that the increased uptake of complex by lysosomes as denoted by ^{125}I uptake in Fig. 4 can be demonstrated by looking at enzyme activity within liver lysosomes following administration of either a yeast α-1,4-glucosidase preparation with or without conjugated albumin, or an enzyme preparation from human placenta in conjugated form with albumin. These experiments were carried out without attempts at targeting. Both the free enzyme and the conjugated enzyme (from yeast) were cleared from the circulation and associated with a liver lysosomal fraction.

L-asparaginase therapy as a means of treating acute lymphocytic leukemia (ALL) was at one point one of the most encouraging forms of anticancer therapy, one that exhibited little or no generalized cytotoxicity towards normal cells (Mashburn and Wriston, 1964). Unfortunately the high incidence of severe immunologic reactivity to the enzyme (most often from *E. coli*) and the induction of asparagine synthetase activity in normal cells and L-asparaginase-resistant tumor cells have relegated the enzyme to second or third tier in the stockpile of anticancer munitions (Haskell *et al.*, 1969; Burchenal and Karnovsky, 1970; Pratt and Ruddon, 1979). We have examined the properties of a polymeric conjugate of L-asparaginase and albumin. The complex appears to be non-immunogenic (Poznansky *et al.*, 1982; Yagura *et al.*, 1981) and in C3H/Hej mice bearing 6C3HED tumor cells which

TABLE 4. *Lysosomal Uptake of α-1,4-Glucosidase Preparation*

Preparation	Activity in liver lysosomes	(% Injected)
Saline (yeast control)	2 units*/g	
α-1,4-Glucosidase (yeast)	8 units*/g	22
α-1,4-Glucosidase-albumin (yeast)	10 units*/g	33
Saline (human placenta control)	9.9 units†/g	
α-1,4-Glucosidase (human placenta)	13.5 units†/g	35
α-1,4-Glucosidase-albumin (human placenta)	15.1 units†/g	55

*PNPG as substrate, pH 7.4.
†Maltose as substrate, pH 4.8.
α-1,4-Glucosidase was injected intravenously at $t = 0$, and at time $t = 2$ hr the liver was excised and a lysosomal fraction prepared. The high background in the human placenta control is due to endogenous lysosomal activity to maltose at pH 4.8 as compared to the low activity to PNPG at pH 7.4 for the yeast control.

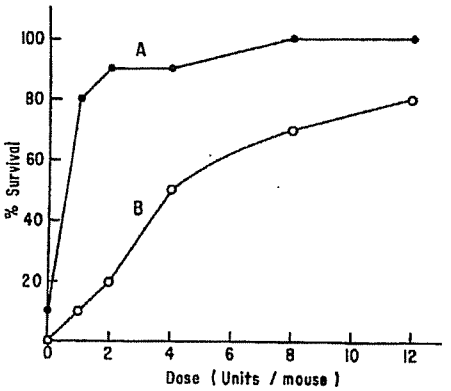

FIG. 5. Dose–response curve for L-asparaginase (B) and L-asparaginase–albumin polymer (A) in C3H/HeJ mice receiving 2×10^6 6C3HED tumor cells i.p. Varying doses of enzyme in 0.15 ml phosphate buffered saline were administered 2 hr after administration of the cells. The L-asparaginase used in these experiments was from *E. coli* and was purchased from Poulenc Ltd., Montreal. Percentage survival in each group of mice (10 mice/dose) was determined 40 days after treatment.

are L-asparaginase sensitive, the enzyme-albumin conjugate is between 20 and 40 times more effective than equivalent amounts of free enzyme (Fig. 5). We attribute this difference to the increased circulation times for the conjugated enzyme and to the increased stability of the enzyme to biodegradation. Therefore even in the absence of any targeting mechanism to deliver enzyme to a specific site, the method of conjugating the enzyme to an excess required to achieve a therapeutic effect. Not only does the enzyme appear to be nonimmunogenic in its conjugated form but less of the enzyme has to be given thereby further diminishing the possibilities of immunological complications. Abuchowski and co-workers (1979) demonstrate similar advantages for their L-asparaginase–PEG complexes.

There is some preliminary evidence, at least in tissue culture, that human pancreatic tumor cells may be L-asparaginase sensitive (Yunis *et al.*, 1977). We have verified this result using PANC-1 human pancreatic tumor cells (American Type Culture Collection) and demonstrated that L-asparaginase conjugated with human albumin is more effective as an antitumor agent (Poznansky *et al.*, 1982). Again in the tissue culture vessel we suggest that the conjugated enzyme is being protected from bioinactivation and thus is appearing to be a more effective antitumor agent.

5.4. IMMUNOLOGICAL PROPERTIES

The main purpose of producing enzyme–albumin polymers as a means of enzyme therapy was the possibility that the homologous albumin might serve to mask the antigenic sites of the foreign enzyme. It was not at all clear that this would occur since the act of cross-linking two homologous proteins might in fact yield new antigenic determinants. Such in fact is the case for the attachment of hapten molecules to carrier proteins to make the hapten molecules immunogenic (e.g. DNP added to bovine serum albumin). Our first test system was uricase, which was injected into rabbits (repeatedly over a 6 month period) either free, conjugated with rabbit albumin or conjugated with dog albumin as a control (Remy and Poznansky, 1978). Antigen–antibody reactions were determined by both double diffusion in agar gel and second antibody radioimmunoassay techniques. The enzyme itself was highly immunogenic (antiuricase antibody titres were high in these rabbits). Rabbits receiving uricase–rabbit albumin polymers produced no antibodies to either uricase or to uricase–rabbit albumin polymers, suggesting that the complex was indeed nonimmunogenic. Rabbits receiving injections of uricase conjugated to dog albumin produced strong antibody reactions to the uricase–dog albumin complex, to the dog albumin itself but not to the uricase molecule. This suggests that the enzyme molecule in the complex is nonimmunogenic even if the carrier molecules are immunogenic. We

TABLE 5. *Immunological Properties of Enzyme Albumin Polymers*

Enzyme preparation	Immune response
Uricase (hog liver)	+++
Uricase–albumin (1:10)	—
α-Glucosidase (yeast)	+++
α-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)	—

Antigen preparations were administered either i.p. (with Freund's adjuvant) or i.v. over a period up to 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. Yagura *et al.* (1982) have recently reported similar results with respect to the immonogenicity of L-asparaginase–albumin polymers using our cross-linking procedures.

concluded that the enzyme's antigenic sites were in fact masked by the excess of carrier albumin molecules since neither the uricase–rabbit albumin complex nor the uricase–dog albumin complex reacted in either test system to antibodies formed against free uricase. This suggests that in addition to being nonimmunogenic the complexed enzyme is also nonantigenic. At the time of this publication (Remy and Poznansky, 1978) we were careful to state that this finding related only to the system described; that is hog liver uricase linked to rabbit albumin and dog albumin as a control. Since then however, we have repeated these studies on a number of enzymes and in every case we have managed to produce enzyme–albumin complexes that are nonimmunogenic. The list of enzymes is included in Table 5. It is of interest to note that when SOD or α-1,4-glucosidase are conjugated with a minimum number of albumin molecules, the loss of antigenicity is not complete. Extensive study of the exact number of conjugated molecules required to reduce antigenicity has not yet been undertaken. Yagura *et al.* (1981) have duplicated our results with L-asparaginase in mice and produced L-asparaginase–mouse albumin polymers that are both nonimmunogenic and nonantigenic.

Although it seems quite clear at least in the case of uricase and superoxide dismutase (Wong *et al.*, 1980) that the antigenicity of the enzyme is lost upon complexing with homologous albumin, it may also be that we are producing tolerance to the conjugated protein by the use of 'self' carrier protein. It is well established that certain proteins (immunoglobulins) and a number of synthetic polymers including polyethylene glycol (PEG) and carboxymethyl cellulose (CMC) are capable of acting as toleragens to conjugated haptens (Borel, 1980; Abuchowski *et al.*, 1977; Diner *et al.*, 1979). Although we have yet to test the possibility that homologous albumin may also act as a toleragen for the enzyme that is being carried, we know that in at least two cases (from our laboratory) that 'self' albumin can in fact act as a toleragen. When the iron chelator desferrioxamine (DF) is covalently linked to mouse albumin (MSA), it does not act as a hapten and no anti-DF antibodies are formed following administration of the DF–MSA conjugates repeatedly into mice. When, however, bovine serum albumin (BSA) is conjugated to an excess of DF and injected into mice, large antibody titres are produced to both the DF molecule and to the carrier BSA. As a second injection of DF–BSA into the mouse that had received a previous injection of DF–MSA yielded an antibody production to the BSA but not to the DF; suggesting that the animal had been tolerized to the DF hapten and that in this case the MSA was acting as a toleragen. We have a second system where this mechanism appears to occur. In conjunction with Dr B. Singh and B. Cliffe of the Department of Immunology, University of Alberta, we have shown that mice can be tolerized to bovine insulin (BI) by conjugating it to MSA prior to