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THE DESIGN OF HETEROLOGOUS ENZYMES SUITABLE FOR THERAPEUTIC APPLICATION

By

Kaelyn Boner Hadley

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ABSTRACT

The Design of Heterologous Enzymes Suitable for Therapeutic Application

by

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Scurvy in guinea pigs provides a convenient model of an inborn metabolic disease for the investigation of enzyme therapy protocols. The purpose of these studies was to examine chemical modifications of gulonolactone oxidase, the missing enzyme in ascorbic acid biosynthesis. Administration of the modified forms of this enzyme was tested in guinea pigs to examine such factors as stability of the enzyme, immune response, and the therapeutic response of the animal.

One modification of gulonolactone oxidase involves immunoprecipitation of the enzyme with specific antisera, followed by crosslinking the precipitate with glutaraldehyde. Following this modification, gulonolactone oxidase activity was protected against rapid inactivation at 37°C and also against trypsin digestion. Intraperitoneal injections of this modified enzyme complex to scorbutic guinea pigs, along with substrate supplementation, resulted in a three-fold increase in plasma ascorbic acid concentrations. Furthermore, repeated injections of the complex were tolerated. With this enzyme replacement therapy, the survival time of ascorbic acid-deficient animals could be prolonged to at least 100 days. Although the animals developed circulating antibodies against both the enzyme and antibody component, anaphylaxis was elicited only upon challenge with the antibody component. These results suggest that immobilization of the foreign

enzyme within a large stable complex was critical to reducing the toxicity of these foreign proteins. The complex may be oriented in such a way that the enzyme surface is covered with antibodies, leading to greater sensitization to this surface component.

Conjugation of gulonolactone oxidase with polyethylene glycol (PEG) was the second modification investigated. This modified form of the enzyme was suitable for intravenous administration and its infusion elicited ascorbic acid synthesis in a dose-dependent manner. However, the circulating half-life of enzyme activity was not prolonged by this modification. Proteolytic digestion may explain the disappearance of enzyme activity from the circulation, but tissue uptake could contribute initially. In contrast to results obtained with other enzymes, attachment of polyethylene glycol to gulonolactone oxidase did not abolish the enzyme's ability to react with pre-formed antibodies nor did it eliminate its immunogenicity. Apparently, all of the antigenic determinants on the enzyme were not covered by the polymer. Despite this, animals were able to tolerate a second injection of this complex.

To my husband, Robert

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LIST OF ABBREVIATIONS

AA	ascorbic acid
ANOVA	analysis of variance
GLO	gulonolactone oxidase
i.v.	intravenous
lsd	least significant difference
PEG	polyethylene glycol
PEG–GLO	PEG–gulonolactone oxidase
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
XL-IP	crosslinked immunoprecipitated

INTRODUCTION

The progression of events leading to our present understanding of inherited metabolic diseases has been described in great detail by other authors (Stanbury et al., 1983). However, in order to develop an awareness of the problems encountered in the treatment of these diseases, an abbreviated summary of the history of their identification is presented here. This is followed by a discussion of various approaches to the treatment of these diseases and the rationale for each. The focus of this dissertation involves testing enzyme replacement therapy protocols, using scurvy in guinea pigs as a model metabolic disease. The basis for the use of this model, as well as previous protocols which have been tested using it, will also be presented. The specific objectives of this investigation will then be outlined.

I. Inherited Metabolic Disease

A. History

The existence of inborn errors of metabolism was first described by Sir Archibald Garrod at the beginning of the twentieth century (Garrod, 1909). This description was based on his extensive studies of the condition alkaptonuria. Garrod observed that patients with alkaptonuria excreted large quantities of homogentisic acid in the urine. Unaffected individuals did not excrete this compound. He also noted that the condition had a familial distribution. This pattern of occurrence could be explained by the laws of genetics, if the condition were inherited as a recessive trait. From these studies Garrod postulated that

certain diseases were the result of the absence or abnormality of a particular enzyme which governed a single metabolic step. In the case of alkaptonuria, he hypothesized that the accumulation of homogentisic acid, and thus its excess excretion, was the result of the inability of the patient to oxidize this component. Fifty years later, this was indeed shown to be the case (LaDu et al., 1958). Investigators demonstrated the absence of homogentisic acid oxidase activity in the liver of a patient with alkaptonuria. Garrod also studied patients with cystinuria, pentosuria, and albinism. He suspected that these conditions were the result of a block in some metabolic pathway as well. Albinism was thought to be the result of a block in melanin formation. He proposed that pentosuria and cystinuria resulted from the excretion of excess substrates that accumulated proximal to a blocked metabolic step. In the case of pentosuria, this was later shown to be correct. L-Xylulose is excreted in the urine due to the block of its conversion to xylitol (Hiatt, 1978). Cystinuria was shown to result from a block in amino acid transport (Segal and Thier, 1983).

A basis for the explanation of these inborn errors of metabolism was provided when the relationship between genes and enzymes was postulated about 40 years later (Beadle, 1945). Beadle proposed that the information of one gene provided for the synthesis of one enzyme. This concept was developed further by Beadle (1959) and Tatum (1959). Evidence suggested that all biochemical processes are under genetic control. These biochemical processes take place through a series of chemical reactions, with each reaction in the series being controlled by a single enzyme. Mutation of a single gene, therefore, could result in the inability of an organism to carry out a single reaction. Thus, it appeared that inborn metabolic diseases, such as alkaptonuria, could be produced by mutations in genes encoding particular enzymes.

At about this time, the first enzyme defect in a human genetic disease was demonstrated by Gibson (1948). Gibson showed that methemoglobinemia was the result of a deficiency in an NADH-dependent enzyme responsible for the reduction of methemoglobin. Demonstrations of other conditions followed. In addition, evidence showing that mutated proteins do indeed show structural differences from the normal, native counterparts was presented (Pauling et al., 1949). Pauling showed that human mutations actually produce an alteration in the primary structure of the proteins. So it appeared that metabolic errors could result when mutant genes produced abnormal proteins whose functional activities were altered.

B. Incidence

Inborn errors of metabolism are genetic diseases and are categorized as monogenic disorders. That is, they are the result of a single mutant protein and show Mendelian patterns of inheritance. At this time, approximately 1400 such monogenic disorders have been identified. These diseases occur in approximately 1% of live births and account for significant morbidity in children.

The basic biochemical lesions responsible for these conditions involve a variety of proteins. Of the 250 diseases for which the specific defect has been identified, 170 of them involve abnormal enzymes. Although identifying and understanding the specific defect has provided for more accurate detection of carriers and earlier diagnosis of afflicted individuals, the lack of a specific therapy for many of these diseases remains. Therapeutic measures directed toward correcting the metabolic defect exist for only about 40 of these conditions. Since genetic diseases are a major cause of infant mortality in industrialized countries (Bart and Lane, 1985), research directed toward development of effective therapeutic measures must continue.

II. Treatment of Metabolic Disease

Thorough treatment of an inborn error of metabolism depends upon an accurate diagnosis and an understanding of the pathophysiology of the disease. Currently, most of these diseases are not fully understood. A complete understanding would mean knowing the exact aberration at each level; at the DNA level, the protein level, and also a knowledge of how the aberrant gene product affects cell function. Although the specific enzymatic defects and accumulating metabolites have been identified for approximately 170 of these metabolic diseases, in some cases it remains unclear how the particular alteration produces the effects of the disease. In addition, the exact disruption at the DNA level has not been deciphered. Despite the fact that some of the information is lacking, attempts are being made to treat these genetic diseases. Investigations are also being carried out to understand their causes more fully.

A. Gene replacement therapy

With the advances in recombinant DNA techniques and molecular biology, attempts are being made to correct inherited enzyme deficiency diseases by replacement of the defective or missing genetic information. The goal is toward achieving permanent restoration of the particular enzyme activity. Directly reversing the deficiency in this manner would appear to be potentially curative. However, problems with this method still exist. Appropriate genetic information may not be available. Presently, genes encoding for the needed enzymes have not been located and cloned in most cases (Anderson, 1984). Even if the DNA has been isolated, one must be able to correctly insert this information into the appropriate cells and assure that it remains there to be effective.

The techniques for gene replacement procedures must be perfected. At this time, techniques for inserting the genes into a particular chromosome,

with the assurance that they will be expressed in an orderly and controlled manner, are not available. The expression of the newly inserted genetic information must be appropriately regulated. Cells into which the genetic information has been transferred may have to carry out post-translational modifications to provide a properly functioning enzyme and produce an appropriate amount of activity. Also, there is not yet a reliable way to introduce the corrected cells so that they will survive while the defective cells are eliminated. This may be necessary if corrected cells do not have a growth advantage over the endogenous, defective cells. The defective cells might have to be removed so that the growth and expansion of the corrected cell population would be favored.

Finally, localization of the enzyme to specific sites may pose a problem. Currently the only human tissue used for gene transfer are bone marrow cells. These cells can be extracted easily and then reinserted after genetic manipulation (Stanbury et al., 1983; Anderson, 1984). In some disorders though, the functioning gene must be located in other organs to be effective. With current techniques this is not possible. Therefore, in view of the problems that must be resolved with genetic restitution and considering the vast number of diseases for which the genetic information has not yet been found, investigation of other therapeutic regimens is necessary.

B. Other therapeutic approaches

A variety of approaches have been investigated in an effort to treat inborn metabolic diseases (Chang, 1977; Watts, 1982; Stanbury et al., 1983). Presently, however, a single method is not applicable to the treatment of many such disorders. A brief discussion of several treatment methods is presented here. The rationale for each of these and an example of its use is included.

One method of therapy involves the dietary restriction of the substrate that accumulates prior to the metabolic block. For some genetic disorders,

reduction of the accumulating substrate can prevent the clinical manifestations associated with the disease. An example of this is shown by treatment of the condition phenylketonuria (PKU). Patients with PKU have a deficiency of the enzyme phenylalanine hydroxylase. This enzyme catalyzes the formation of tyrosine from phenylalanine. As a result of this enzyme deficiency, the substrate, phenylalanine, accumulates and an excess of its metabolites appear to contribute to the pathology of the disease. There is evidence that phenylalanine derivatives disrupt myelin formation and this can lead to the mental and growth retardation associated with PKU. Restriction of dietary phenylalanine in these patients, so that normal blood levels of this amino acid are maintained, has been shown to prevent these effects. To achieve optimal efficacy, strict adherence to the dietary regimen is required, beginning within 8 weeks after birth and continuing until at least 10 years of age. After this, the diet may be discontinued gradually with no deterioration in the patient's condition (Bickel and Schmidt, 1982). However, problems may arise when the female patient reaches reproductive maturity. High blood phenylalanine levels in untreated PKU mothers can adversely affect the fetus. Even though the infant may not have PKU, it can suffer mental retardation, congenital malformations, and even early death, as a result of exposure to high maternal phenylalanine levels. Therefore, it is imperative to impose the strict dietary restrictions again. Treatment should begin no later than the third week of pregnancy, and, preferably, before conception. Delivery of a healthy baby is still not guaranteed. An increased frequency of microcephaly and mental retardation has been noted in babies whose mothers comply with the diet. So, problems remain with dietary management, especially regarding maternal PKU and preventing damage to the unborn child. A more reliable means of controlling phenylalanine accumulation is necessary to ensure tight control of phenylalanine levels and prevention of these effects.

Replacement of the deficient end-product is another approach to therapy. A metabolic block causes a decrease in the initial product of the particular reaction and, possibly, in subsequent products of the metabolic sequence. If the absence of these products contributes to the pathologic effects, then their replacement should alleviate the disease state. Orotic aciduria is an example of a case in which such therapy is effective. In this condition, orotic acid can not be converted to uridine due to defects in two sequential enzyme steps. The product, uridine, is necessary for the synthesis of pyrimidines, which are important for nucleic acid synthesis. Administration of uridine overcomes this block and corrects the symptoms of the disease. It also results in a diminished synthesis of orotic acid, so the excretion of this acid in the urine is decreased.

In some diseases, the clinical symptoms are the result of accumulation of stored metabolites in the tissues of the body. Removal of these excess stored materials may alleviate the problems associated with the disease. In a case such as this, the goal of therapy is to deplete the accumulated metabolites. A classical example of a condition where depletion of a stored substance can be effective is Wilson's disease. Copper is stored in excess in afflicted patients and eventually its accumulation leads to severe neurologic problems and liver damage. Removal of excess copper is effected by the agent penicillamine. This agent chelates the copper, increasing its subsequent excretion and alleviating symptoms of the disease.

Inhibition of metabolic pathways is another approach that is useful in the treatment of certain disorders. If the substrate which accumulates as a result of the metabolic error is toxic, control of its production may ameliorate the symptoms of the disease. To this end, inhibition of an enzyme critical for the synthesis of the particular accumulating substance may provide therapy. The use

of hematin in the treatment of acute hepatic porphyrias is an example of this (Watts, 1982). The enzymatic defect in acute hepatic porphyrias is a deficiency of porphobilinogen (PBG) deaminase activity. Symptoms of the disease occur when δ-aminolevulinic acid (ALA) and porphobilinogen (PBG) accumulate as a result of the PBG deaminase deficiency. Certain hormones and drugs can precipitate porphyric attacks. They do this by stimulating the synthesis of ALA and PBG through the induction of ALA synthetase. Hematin inhibits ALA synthetase activity and, therefore, reduces levels of ALA and PBG. Inhibiting the metabolic pathway in this manner and preventing the formation of the accumulating substances can lessen the symptoms of the acute porphyric attack.

Organ transplantation has also been considered as a therapeutic measure for certain metabolic diseases. Transplanting an organ which can normally synthesize the deficient enzyme has been done. Kidney transplants have been attempted in cases of Fabry's disease and these patients show measurable levels of α-galactosidase activity after the procedure (Groth, 1982 and Stanbury et al., 1983). Other examples include splenic transplantation and renal transplantation as treatment for Gaucher's disease (Desnick, 1980; Groth, 1982). However, the results do not warrant the use of organ transplantation on a routine basis. Rejection of the transplanted organ by the patient's body presents a major difficulty in the use of this approach. Demonstration of clear therapeutic benefit is lacking as well. Although an increase in enzyme activity following transplantation is noted in some cases, actual improvement in the clinical symptoms of the disease has not been shown. Transplanting allogenic bone marrow to patients who are afflicted with certain immunodeficiency states provides another example. In some cases efficacy has been demonstrated, but problems in finding compatible donors remain.

C. Enzyme replacement therapy

Enzyme deficiency diseases should be treatable by administration of the missing enzyme. Studies of methods by which this can be accomplished are the subject of this dissertation research. In addition to its usefulness as treatment for inborn metabolic disorders, enzyme administration may be used as therapy for other human diseases, such as certain cancers. The treatment of acute lymphocytic leukemia with asparaginase is one such example. The rationale for using asparaginase in cancer chemotherapy is that certain tumor cells are dependent upon exogenous sources of the amino acid asparagine, whereas normal cells are not. Asparagine is depleted by the administered asparaginase, depriving the tumor cells of this necessary nutrient and ultimately causing their death (Uren, 1981). Another experimental application for administered enzymes is the detoxification of foreign chemicals in vivo (Goedde and Altland, 1971). The use of organophosphate hydrolyzing enzymes to treat organophosphate poisoning is an example of this. Early studies suggest that these enzymes may be effective in the prevention of organophosphate toxicity.

While administering enzymes appears to be a relatively simple concept, a number of major difficulties are encountered. First, adequate quantities of pure enzyme suitable for administration are difficult to obtain. Enzyme purification procedures are time-consuming and frequently result in low yields. Considering that affected individuals may require treatment over an entire lifetime, large quantities of enzyme may be necessary. The stability of injected enzymes may also present difficulties. Administered enzymes are susceptible to denaturation and protease digestion and thus may have brief half-lives. Furthermore, if repeated injections are required immunologic complications may occur. If the injected enzymes are recognized as foreign by the body, an immune response can be elicited and an allergic reaction initiated upon subsequent

injections of the particular enzyme. Degradation of the injected enzyme activity may also be accelerated as a result of the immune response. Directing the administered enzyme to the appropriate site for its action presents problems in the therapy of certain inborn metabolic diseases. Modification of the particular enzymes may be required in order to target them to specific sites. In summary, there are various problems which need to be overcome to render enzymes suitable for routine therapeutic use.

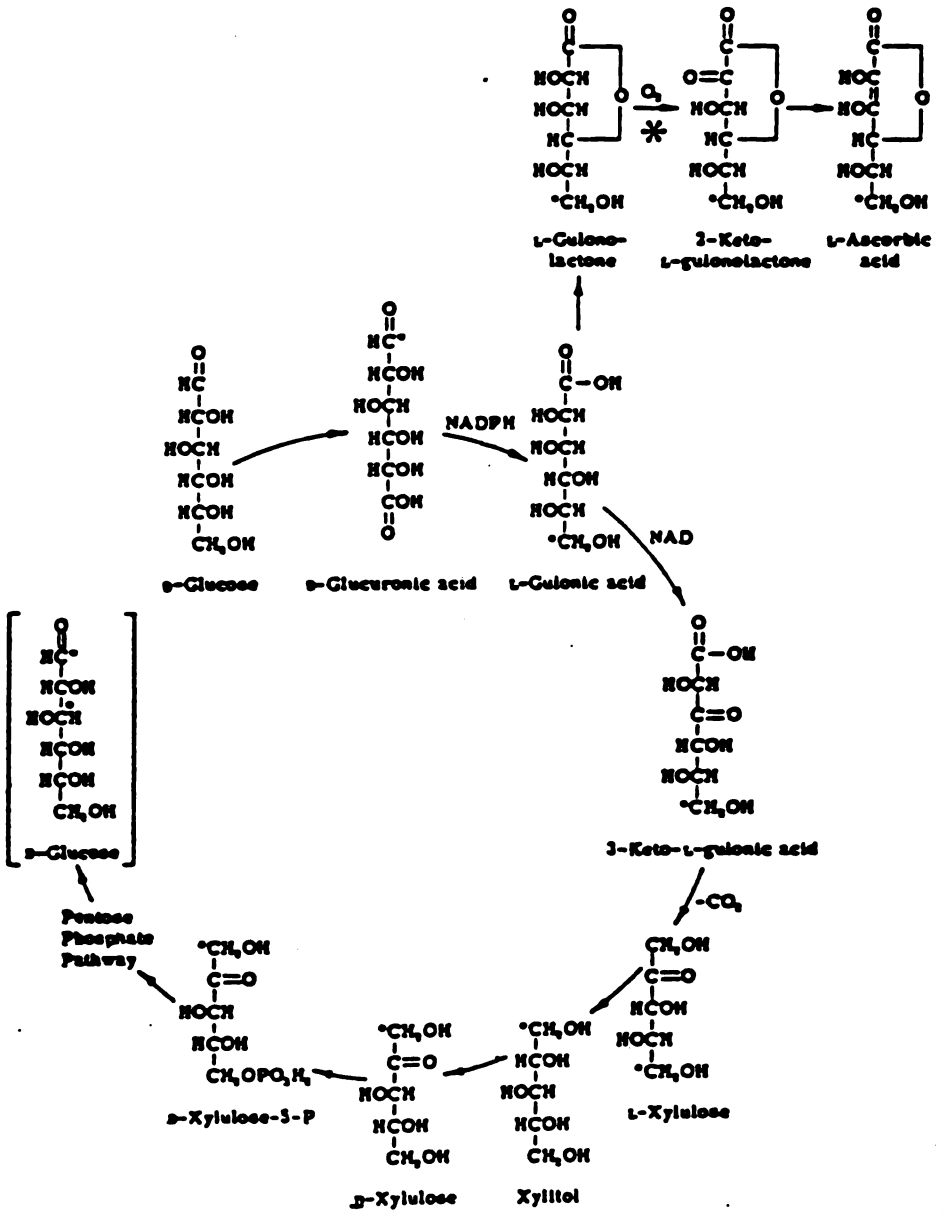
Attempts at using enzyme replacement therapy to treat patients affected by enzyme deficiencies have been reported (Desnick, 1980; Brady, 1983). The outcome has been disappointing, however. These trials often represent the first time that the efficacy of such therapy has been tested. Patients affected by the diseases receive enzyme replacement therapy without evidence that it is safe and will indeed be therapeutically beneficial. The effectiveness of these treatments is ambiguous. While some evidence of increased enzyme activity and subsequent substrate depletion is shown, clinical improvement in the patients' conditions has not been demonstrated. Furthermore, toxicity to the enzyme usually develops. A more reasonable approach would be to test the safety and effectiveness of enzyme therapy in animals before initiating therapy in humans. Although some animal models exist (Migaki, 1982), difficulties are involved in obtaining suitable animals for studies. Many of the animals are heterozygotes and the disease traits are recessive. Careful breeding of animals is often necessary in order to obtain homozygotes that express the disease. The animals' genotype must be determined as well. In the studies presented in this dissertation, the disease scurvy in guinea pigs is used as a model inborn error of metabolism. The rationale for its use is discussed below.

III. Scurvy - A Model Inborn Metabolic Disease

Scurvy in guinea pigs provides a convenient model for the study of enzyme therapy protocols. This disease, which results from the lack of ascorbic acid, has been determined to be an inborn error of metabolism (Burns, 1956). Guinea pigs, like man and other primates, have lost the capacity to synthesize this vitamin. Therefore, they require a dietary intake of ascorbic acid. Their inability to synthesize this vitamin is the result of an enzyme deficiency. Gulonolactone oxidase, the missing enzyme, catalyzes the final step in ascorbic acid biosynthesis (Burns, 1956; Sato et al., 1976). Ascorbic acid is synthesized from glucose by the glucuronic acid pathway as shown in Figure 1 (Burns, 1975). The liver microsomal enzyme gulonolactone oxidase catalyzes the conversion of gulonolactone to ascorbic acid. Scurvy-prone species do not express the gene coding for this enzyme (Sato and Udenfriend, 1978a). Administering this enzyme to scorbutic guinea pigs should give them the capacity to synthesize their own ascorbic acid and thus alleviate scurvy.

There are a number of advantages to using scurvy as a model in the study of enzyme therapy regimens. Guinea pigs are a common, readily available laboratory animal. Scorbutic animals are easily obtained simply by maintaining them on an ascorbic acid-deficient diet. Importantly, guinea pigs are homozygous for the enzyme deficiency trait. This eliminates the necessity of determining their genotype and carrying out the selective breeding procedures that are necessary with other animal models. Scurvy is a also a lethal disease, so there is a clear endpoint to evaluate the effectiveness of the enzyme replacement therapy. In addition, gulonolactone oxidase is the final enzyme in the biosynthesis pathway and it does not require other cofactors for activity. For this reason, it is not necessary to target the enzyme to a particular site for optimal activity.

Figure 1. Glucuronic acid pathway of glucose metabolism. Ascorbic acid is synthesized from glucose via this pathway. The point of conversion of gulonolactone to ascorbic acid is indicated by an asterisk. The microsomal enzyme, gulonolactone oxidase, catalyzes this reaction. Taken from Burns (1975) with permission.



Glucuronic Acid Pathway of Glucose Metabolism

Figure 1

In the studies presented here, various therapy regimens are tested using this scurvy model. The efficacy and toxicity of the protocols can be compared and, if necessary, adjustments or variations in a protocol can be instituted. After testing the treatment regimens in this animal model, perhaps they can be applied to the treatment of other inherited metabolic diseases.

IV. Treatment of Scurvy

A number of different methods of enzyme replacement therapy have already been investigated using the scurvy model. These methods will be discussed here briefly.

Implantation of the enzyme contained within a dialysis bag is one approach that was examined (Sato, 1980). Administration of the enzyme in this way was designed to protect it from proteolytic attack and degradation. Furthermore, the animal would not be exposed to the foreign protein and this should prevent the initiation of an immune response. Gulonolactone oxidase, purified from rat liver, was placed in a dialysis bag which had been surgically implanted within the peritoneal cavity of the animal. The substrate, gulonolactone, was then injected into the peritoneum. The dialysis bag should be permeable to the small molecules of substrate and product, but impermeable to the enzyme macromolecule, antibodies, and proteolytic enzymes. Control animals were treated similarly, except that bovine serum albumin was contained within their dialysis bags. In vivo enzymic activity was demonstrated by increases in plasma and tissue concentrations of ascorbic acid following gulonolactone oxidase treatment. In the enzyme-treated animals, a four-fold increase in plasma ascorbic acid concentrations occurred, while the vitamin concentrations in the plasma of control animals remained relatively constant. Tissue concentrations of ascorbic acid in the enzyme-treated animals were also significantly higher than those in the control

group. Multiple injections prolonged the survival of scorbutic animals to twice the normal time of survival on the deficient diet, demonstrating a therapeutic effect. Scorbutic animals usually do not survive beyond 23-28 days (Barnes et al., 1973; Jones et al., 1973). Gulonolactone oxidase contained within the dialysis bag is not antigenic nor does sera from the animals contain detectable precipitating antibody against the enzyme. Therefore, using the scurvy model, it was shown that the enzyme administered in this manner catalyzes the synthesis of ascorbic acid and has potential clinical value.

The feasibility of using an intestinal segment as an artificial organ for enzyme replacement was also tested (Shelt et al., 1982). One problem with the use of the implanted dialysis bag was that the animals mounted a response against the foreign dialysis bag in the long-term studies. This impaired its permeability and effectiveness. It was postulated that by using a membrane from the animal, the problem of a tissue response would be avoided. In addition, this membrane, like the dialysis membrane, should allow passage of substrate and product, while preventing direct exposure of the animal to the foreign enzyme. For these studies, a surgical procedure was developed to prepare a pouch from an intestinal segment of the animal, leaving the blood supply to it intact. A silastic tube attached to this pouch permitted access for injection of the enzyme. Attempts to demonstrate in vivo synthesis of ascorbic acid by increases in plasma ascorbic acid concentrations following this treatment were unsuccessful. However, one animal did survive for 57 days while being fed the vitamin C-deficient diet and receiving enzyme replacement therapy. So, ascorbic acid synthesis may have occurred. Perhaps the vitamin did not enter the plasma at a rate fast enough to detect an increase in the plasma during the time of the experiment. The vitamin may also have been converted to a different form, for instance dehydroascorbic acid, which would not be detectable in the assay used. Some of the treated

animals did not appear to produce antibody against the foreign enzyme. However, results from immunodiffusion tests show that antibodies were raised against the enzyme in other animals. This may have resulted from the diminished integrity of the segments, allowing enzyme to leak into the peritoneal cavity. Despite the presence of antibody, animals did not exhibit an allergic reaction upon intravenous challenge with the enzyme. To summarize, this method does not appear to be feasible for administration of gulonolactone oxidase in amounts adequate to allow detectable in vivo ascorbic acid synthesis. It does appear to decrease the antigenicity of the foreign protein and it could be useful in the treatment of other metabolic disorders.

Additional work has focused on the development and investigation of chemical modifications of enzymes that make them suitable for therapeutic application. Efforts are directed toward improving the stability of the injected enzyme activity in this way. Decreasing the immunogenicity and allergenicity of the foreign enzyme is a goal also. One modification that has been studied involves the immunoprecipitation of the enzyme with specific antisera followed by crosslinking the precipitate with the bifunctional reagent glutaraldehyde. Crosslinking is intended to reinforce the interactions between antigen (enzyme) and antibody.

Using crosslinked immunoprecipitated enzymes, several of the basic problems encountered in enzyme administration can be addressed. Immunoprecipitation facilitates the isolation of large quantities of enzyme that may be needed for therapy. Furthermore, enzyme activity is significantly stabilized to heat denaturation and trypsin digestion by a number of different modifications (Holcenberg, 1982; Poznansky, 1983), including immunoprecipitation (Snyder et al., 1974) and crosslinking (Snyder et al., 1974; Poznansky, 1979; Klibanov, 1979; Wold,

1973). The characteristics of crosslinked immunoprecipitated gulonolactone oxidase were examined as well as its potential for use in therapy.

Work using this modification for gulonolactone oxidase demonstrates that crosslinked immunoprecipitates are effective in reversing scurvy in guinea pigs (Sato and Walton, 1983). Their weight gain is restored following a single dose, as compared to the continuing decline of body weight observed in control animals. A three-fold increase in plasma ascorbic acid concentrations also occurs following the enzyme therapy. Furthermore, repeated injections of this modified enzyme are tolerated and are therapeutically beneficial in prolonging the survival of guinea pigs fed without ascorbic acid (Sato et al., 1986). These gulonolactone oxidase-treated animals survived for at least 100 days compared to the usual time of survival of guinea pigs on the ascorbic acid-deficient diet which is usually 23-28 days (Jones et al., 1973; Barnes et al., 1973). These studies show that the enzyme has been detoxified by this modification, yet substantial enzyme activity remains. Specific guinea pig antisera was used to immunoprecipitate gulonolactone oxidase in these experiments. It is possible that use of homologous sera in the procedure is critical for the animals to tolerate repeated administration of the modified enzyme complex. Further investigation into the basis for the "detoxification" of gulonolactone oxidase by this modification are part of this dissertation research.

V. Objectives

The research presented in this dissertation is directed toward the investigation of chemical modifications of gulonolactone oxidase that render it suitable for therapeutic use. Work has continued with the crosslinked immunoprecipitated gulonolactone oxidase with efforts focused on determining the possible reasons for the "detoxification" of this modified enzyme complex. Efforts were directed

toward the development of a modified enzyme that would be suitable for intravenous administration as well. To this end, gulonolactone oxidase was conjugated to the polymer polyethylene glycol.

A. Heterologous immunoprecipitates

After completion of the studies using specific guinea pig antiserum for the modification of gulonolactone oxidase, it was postulated that the multiple injections were tolerated because the homologous antibody masked the antigenic sites of the foreign enzyme. The guinea pigs, therefore, do not recognize it as allergenic. Alternatively, the modified enzyme may be tolerated not because it is bound to homologous antisera, but because of some other characteristic of the immunocomplex, such as immobilization within a large stable complex. These studies were designed to investigate whether detoxification of the enzyme was dependent on the use of homologous antiserum. Using heterologous antiserum to modify the enzyme in these experiments should distinguish between these possibilities. Furthermore, when considering application to human therapy, the use of heterologous antiserum would be advantageous. Otherwise, large amounts of human serum would be necessary to prepare the enzyme-antibody complex. Using heterologous antiserum would be far more practical since large amounts of antibody specific for the enzyme can be raised in animals and then used for preparations to treat humans.

For these studies, specific antiserum against gulonolactone oxidase was raised in rabbits and horses and used for immunoprecipitation of the enzyme from solubilized chicken kidney microsomes. Chicken kidneys possess gulonolactone oxidase which has a high specific activity (Chaudhuri and Chatterjee, 1969; Chatterjee et al., 1975). The modification procedure was optimized with these components in order to maintain as much activity as possible and the characteristics of the resulting complex were examined. Experiments were conducted to

examine the ability of this modified enzyme complex to catalyze the in vivo synthesis of ascorbic acid and to be therapeutically beneficial to scorbutic animals. The effects of repeated administration of the complex were also tested since the ability of animals to tolerate repeated injections of the foreign proteins is an important consideration in enzyme replacement therapy. To be therapeutically beneficial, repeated administration of the foreign proteins may be required and, as a result, there may be immunologic complications. It is important that this be avoided so that allergic reactions do not interfere with subsequent treatments and efficacy. Studies of the toxicity and immune response to the modified enzyme, as well as its metabolic fate, are presented.

If a procedure is to have practical applicability, it should be suitable for modifying other enzymes that have therapeutic value. This is also tested in the experiments discussed here. Three other enzymes having potential applications in therapy were modified by immunoprecipitation and crosslinking. Included were the enzymes asparaginase, histidase, and serum cholinesterase. Asparaginase, which catalyzes the hydrolysis of asparagine, has possible uses in cancer chemotherapy. Histidase, the missing enzyme in the disease histidinemia, catalyzes the deamination of histidine, while administration of serum cholinesterase has potential use in the treatment of organophosphate poisoning. The characteristics of these modified enzyme complexes were examined in a further effort to test the adaptability of this procedure. In addition, the toxicity of these complexes was studied.

B. Polyethylene glycol (PEG)-conjugated gulonolactone oxidase

Studies were also conducted to modify gulonolactone oxidase to render it suitable for intravenous administration. Attempts to administer crosslinked immunoprecipitated gulonolactone oxidase via this route suggested that a more rapid and efficient synthesis of ascorbic acid would occur (Sato and Lindemann,

1986). Since infusion of that complex was found to be highly toxic, examination of another modification procedure was necessary.

Development of a modified form of gulonolactone oxidase that could be infused intravenously would permit comparison between the two routes of administration. Because of a more efficient synthesis by the enzyme administered intravenously, the same therapeutic benefit may be attained using smaller doses of the enzyme. The amount of ascorbic acid synthesis might be more predictable as well, because enzyme and substrate could interact almost immediately, whereas with the previous protocol, enzyme access to the substrate may have been delayed by slow absorption. In addition, potential problems with modified enzyme accumulating in the peritoneum could be avoided. Directing the enzyme to a site other than the peritoneum would be an option as well with administration into the vasculature.

Polyethylene glycol-enzyme adducts have characteristics suitable for enzyme administration and can be infused intravenously (Abuchowski et al., 1977; Savoca et al., 1979; Abuchowski and Davis, 1981). A number of other enzymes have been modified by attachment of PEG and their characteristics examined. The PEG-enzymes are less susceptible to proteolytic attack and covalent attachment of this polymer to certain proteins prolongs their circulating half-life. By comparison, the unmodified enzymes are inactivated rapidly by proteolytic digestion and have very short half-lives in the circulation. Importantly, the immunogenicity of these foreign enzymes is also diminished by the reaction with PEG. One hypothesis for the ability of PEG to confer these properties is that the polymer forms a protective layer around the enzyme. In this way, attack by proteolytic enzymes is prevented and recognition of the foreign enzyme by the immune system is avoided.

All of the properties discussed above are desirable when considering administration of an enzyme in therapy. The ability of PEG-conjugation to confer these characteristics may be variable however, since the catalytic properties of the bound enzymes may be affected to various extents by the modification. The degree to which PEG is able to decrease immunogenicity and enhance stability may differ with different enzymes. Rather than yielding a product of uniform properties regardless of the protein modified, it may depend upon the characteristics of the particular enzyme.

The effects of modifying gulonolactone oxidase with the PEG polymer were therefore examined. The product of the conjugation was characterized with respect to its catalytic properties and stability in vitro and in vivo. The results of administering this modified enzyme to vitamin C-deficient guinea pigs, as well as the immunogenicity of this PEG-enzyme, were also studied.

MATERIALS AND METHODS

I. General

A. Animals

English short hair guinea pigs (strain, Mdh:(SR[A])) were obtained from the Michigan State Health Laboratories (Lansing,MI). Animals used for enzyme administration experiments were 3-7 day old males weighing 130-170 g. They were depleted of ascorbic acid by feeding them either short-term or long-term ascorbic acid-deficient diets (ICN Nutritional Biochemicals, Cleveland,OH). Guinea pigs used for immunization were mature males (400-500 g). Male ICR mice weighing 18-22 g were obtained from Harlan Sprague-Dawley (Indianapolis, IN). New Zealand White male rabbits weighing 2.5-3.0 kg were purchased from Bailey Rabbitry (Alto, MI) and a horse was utilized from the College of Veterinary Medicine (Michigan State University, East Lansing, MI) for the purpose of raising antisera.

B. Surgical procedures

For some enzyme administration studies, guinea pigs were catheterized via the carotid artery in order to obtain plasma samples and via the jugular vein for the infusion of enzyme, substrate, or both. These surgical procedures were carried out using ketamine-acepromazine anesthesia (Shugard et al., 1975). Lidocaine (0.1 ml) was injected subcutaneously at the incision site to produce local anesthesia. The animals were allowed to recover for at least one day prior to treatment.

C. Preparation of enzymes

Gulonolactone oxidase (EC 1.1.3.8) was purified from chicken kidneys obtained from Pel Freez Biologicals (Rogers, AR). The purification procedure is similar to the one previously described by Sato and Grahn (1981) with some minor modifications. All procedures were carried out at 0-4°C and are described below.

Chicken kidneys (100 g) were thawed in 1.15% potassium chloride and homogenized in 4 volumes of 0.25 M sucrose. This homogenate was centrifuged at 103 g for 10 min, the resulting supernatant saved at 0°C and the pellets washed with 1.15% potassium chloride, 10 mM EDTA (pH 7.5) and centrifuged again (103 g for 10 min). This supernatant was then combined with the one from the previous spin and centrifuged for 45 min at 100,000 g. The resulting pellets was washed by homogenizing in 1.15% potassium chloride, 10 mM EDTA (pH 7.5) and centrifuged again for 45 min at 100,000 g. This pellet was suspended at a protein concentration of 10 mg/ml in 20 mM Tris-acetate buffer (pH 8.0) with 1 mM EDTA. Trypsin (0.1 mg/ml) was added to this suspension and it was stirred overnight, at 4°C, under nitrogen.

The trypsin-digested pellet suspension was centrifuged at 100,000 g for 60 min. The resulting pellet was suspended at a protein concentration of 10 mg/ml in 20 mM Tris-acetate buffer (pH 8.0) with 1 mM EDTA that contained 3% Tween 20 for solubilization and stirred for 30 min at 4°C. Following centrifugation at 100,000 g for 75 min, the pellet was discarded and the supernatant portion was frozen at -20°C overnight.

Ammonium sulfate fractionation was carried out by adding finely ground solid ammonium sulfate to the solubilized preparation, slowly, with constant stirring. The first addition of ammonium sulfate was 130 mg/ml, after which the preparation was centrifuged at 30,000 g for 15 min, and the pellicle removed. A second addition of ammonium sulfate (145 mg/ml) was carried out,

followed by centrifugation at 30,000 g for 30 min and the resulting pellet was dissolved in 7 ml of Tris-acetate buffer (pH 8.0), which contained 10 mM potassium chloride, 1 mM EDTA, and 0.4% of the detergent Brij 35. This preparation was dialyzed extensively overnight against 4 liters of the same Tris-acetate buffer in which it was dissolved. The dialysis buffer was changed once.

This preparation was passed through a DEAE Sephadex A-50 column (2.0x15 cm) equilibrated with the same Tris acetate buffer. The unadsorbed fractions which possessed high specific activity were combined and frozen at -85°C under nitrogen. This resulted in the purified enzyme preparation that was used for the PEG modification procedure. When a more highly purified preparation was necessary, the fractions from the DEAE chromatography step were dialyzed overnight against 10 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 0.4% Brij 35. Adsorption chromatography was carried out using a hydroxylapatite column (2.2x4 cm) that was equilibrated with the same 10 mM potassium phosphate buffer. Elution with 100 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 0.4% Brij 35 was performed. Fractions from the first peak and the elution peak were assayed for enzyme activity. The fractions with high specific activity were concentrated to greater than 0.5 mg/ml protein using an Amicon ultrafiltration apparatus equipped with a YM 5 membrane. Enzyme was stored frozen at -85°C under nitrogen.

Human serum cholinesterase (EC 3.1.1.8) was purified from frozen plasma by affinity chromatography as described by Lockridge and LaDu (1978) and was a generous gift from them.

Partially purified histidase (EC 4.3.1.3) from P. fluorescens, α-galactosidase (EC 3.2.1.22) from E. coli, and chromatographically purified asparaginase Type II (grade VIII) (EC 3.5.1.1) from E. coli were purchased (Sigma Chemical Co., St. Louis, MO).

D. Analytical methods

Protein concentrations were determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard. Published methods were used to assay asparaginase (Ho et al., 1970), histidase (Tabor and Mehler, 1955), serum cholinesterase (Kalow and Lindsay, 1955), and α-galactosidase (Petek et al., 1969). Gulonolactone oxidase activity was determined by the method of Sato and Udenfriend (1978b). The method of Zannoni et al. (1974) was used to measure ascorbic acid.

E. Statistical analysis

Kinetic data and tissue ascorbic acid concentrations are expressed as means ± S.E.M. In experiments where comparisons are made between two groups, the Student's t-test was used to compare the two means. Comparison of ascorbic acid synthesis between gulonolactone oxidase-treated and control animals was analyzed using a mixed design ANOVA and individual comparisons were made with either the least significance difference (lsd) test or Dunnett's test for between group comparisons (Steel and Torrie, 1980). Significance was established at $p < 0.05$.

II. Heterologous Immunoprecipitate Studies

A. Preparation of antisera

Gulonolactone oxidase, asparaginase, histidase and serum cholinesterase were electrophoresed in 7% polyacrylamide gels (Dewald et al., 1974). Gulonolactone oxidase gels contained 1.5% Tween 20. The location of this enzyme in the gels was determined by staining for activity (Nishikimi et al., 1976). Serum cholinesterase and asparaginase activities migrated in the gels with the only major 280 nm light-absorbing band. So, these enzymes were located by scanning the gels in a spectrophotometer at this wavelength. Histidase was

located by slicing the gels and assaying the individual segments for activity. Gel segments containing enzyme activity were excised and homogenized in equal parts of saline and Freund's complete adjuvant (GIBCO Laboratories, Grand Island, NY).

Guinea pigs received injections of the enzyme (40 µg) intramuscularly and rabbits were injected with 40 µg in the footpads. Three weeks later, similar booster injections were given. Guinea pigs were bled by cardiac puncture under anesthesia (Shugard et al., 1975) and rabbits were bled via the central ear artery one week after the booster injection. A horse was immunized and boosted with gulonolactone oxidase, according to a similar schedule, with gel segments containing 0.5 mg of the enzyme. It was bled from the jugular vein one week after the booster injection. One week later, the horse was boosted again and bled the following week. All sera were stored frozen at -20°C.

B. Purification of immunoglobulin G

IgG was purified from the horse antiserum by chromatography on QAE Sephadex (Joustra and Lundgren, 1969). Serum (50 ml) was thawed and centrifuged for 20 min. The clear supernatant was diluted with 100 ml of ethylene diamine-acetic acid buffer (pH 7.0). This solution was loaded onto a QAE Sephadex A-50 column (4.5x20 cm) equilibrated with the same buffer. The breakthrough fraction was immediately concentrated to approximately 10% of its original volume, using an Amicon ultrafitration cell equipped with an XM 50 membrane, and the fraction lyophilized. Using this procedure, the average yield of IgG from 50 ml of sera was 1.61±0.12 g dry weight.

C. Preparation of immunoprecipitates

For the studies using whole antiserum to prepare immunoprecipitates, chicken kidney microsomes (5 mg protein/ml) were solubilized using 0.7% sodium deoxycholate as described (Nishikimi and Udenfriend, 1976) and concentrated to 10 mg protein/ml. Immunoprecipitates were made by mixing antiserum (13 ml)

with solubilized microsomes (17 ml). The mixtures were allowed to stand overnight at 4°C. They were then centrifuged for 20 min at 30,000 g at 4°C, suspended to their original volume in 0.1 M potassium phosphate buffer (pH 7.4) containing 2 mM EDTA, and centrifuged again.

When immunoprecipitates were prepared with purified horse IgG, chicken kidney microsomes (10 mg protein/ml) were solubilized using 0.7% sodium deoxycholate. Purified horse IgG was dissolved in 0.1 M potassium phosphate buffer (pH 8.0) to a concentration of 20 mg/ml and mixed with the solubilized microsomes. For each ml of microsomes, 2.5 mg of IgG was used. The mixtures were allowed to react overnight at 4°C, centrifuged for 40 min at 100,000 g at 4°C, suspended to 60 ml in 0.1 M potassium phosphate buffer (pH 7.4) containing 2 mM EDTA, and centrifuged again. Inactive immunoprecipitates for the treatment of certain control groups were made using microsomes which had been obtained from kidney homogenates prepared in 1.15% potassium chloride with 10 mM EDTA at pH 4.5.

Immunoprecipitates of E. coli asparaginase were obtained by mixing 1.5 mg of asparaginase in 1 ml of 0.2 M Tris buffer (pH 8.6) with 13 ml of anti-asparaginase serum. Histidase immunoprecipitates were prepared using 30 mg P. fluorescens cells. These cells were suspended in 3 ml of 0.1 M Tris buffer (pH 9.0), sonicated for 2.5 min at 2°C, and centrifuged for 60 min at 100,000 g at 4°C. The supernatant was then mixed with 10 ml of anti-histidase serum. Serum cholinesterase was precipitated from 27 ml of human serum with 3 ml of antiserum. Reaction of enzymes with the respective antisera was carried out overnight at 4°C. The immunoprecipitates were isolated by centrifugation at 40,000 g for 30 min, washed by resuspension in their appropriate buffer, and recentrifuged.

D. Glutaraldehyde reaction

The procedure used was adapted from Habeeb and Hiramoto (1968). Immunoprecipitates made from whole antisera were suspended to 30 ml in 0.13 M potassium phosphate buffer (pH 6.5). Glutaraldehyde (25mM; Sigma Chemical Co. St. Louis, MO) was added slowly, with mixing, to a final concentration of 8.3 mM at 0°C. This was centrifuged for 25 min at 15,000 g at 4°C. Precipitates were then resuspended in 0.1 M potassium phosphate buffer (pH 7.4) containing 2 mM EDTA and centrifuged again. When pure IgG was used to obtain the immunoprecipitate, conditions were the same except that crosslinking was carried out at pH 7.0 to obtain a preparation that was considered to be crosslinked as extensively as possible, while maximally preserving enzyme activity. The crosslinked precipitate was centrifuged for 25 min at 50,000 g at 4°C. Catalytically inactive crosslinked immunoprecipitated gulonolactone oxidase was prepared by using 166 mM glutaraldehyde and carrying out the reaction for 1 hr at 25°C.

E. Enzyme administration

Glutaraldehyde-reacted immunoprecipitates of gulonolactone oxidase were suspended in 6% dextran in 0.9% sodium chloride (Abbott Laboratories, North Chicago, IL) at a concentration of 5.3 mg dry weight of precipitate per ml, when prepared from whole antisera. A total volume of 5 ml of the suspension was injected intraperitoneally into the guinea pigs. This amount of the modified gulonolactone oxidase is able to produce 0.07 mg ascorbic acid/ min at 37°C in <u>in vitro</u> assays.

Crosslinked immunoprecipitates, made using purified horse IgG, were also suspended in 6% dextran in 0.9% sodium chloride (Abbott Laboratories, North Chicago, IL) and injected intraperitoneally. The preparation for a single dose had a dry weight of about 20 mg per ml of suspension.

Crosslinked immunoprecipitates of serum cholinesterase, asparaginase, and histidase were suspended at concentrations of approximately 5.2, 2.2, and 0.1 mg dry weight/ml, respectively. Mice were given 1 ml and guinea pigs were given 5 ml of these suspensions by intraperitoneal injection.

F. Postmortem examination

Selected guinea pigs were sent to the Animal Health Diagnostic Laboratory (Michigan State University, East Lansing, MI) in order to determine their cause of death or to look for possible pathological changes caused by the enzyme replacement therapy. First, the occurrence of any gross lesions was noted. Histopathological and bacteriological examination of sections of various tissues was conducted. The tissues examined included the following: cerebrum, cerebellum, midbrain, duodenum, liver, spleen, kidney, lung, tongue, ileum, adrenal glands, skeletal muscle, bladder, bone marrow, lymph nodes, and stomach. Vitamin A and E levels were determined in the liver. The levels of selenium and other minerals were determined in the liver and kidney.

G. Radiochemical methods

^{14}C-Labelled enzyme complex was prepared by reductive alkylation of the immunoprecipitates following the glutaraldehyde crosslinking reaction. Immunoprecipitates were prepared and crosslinked with glutaraldehyde as described previously for the treatment of animals. Radiolabelling was carried out as described by Rice and Means (1971) using ^{14}C-formaldehyde (10 mCi/mmol, New England Nuclear, Boston, MA). ^{14}C-Formaldehyde was added to the suspended crosslinked immunoprecipitates and allowed to react for 10 min. Following this reaction, sodium borohydride was added to reduce the formaldehyde. The labelled precipitates were then washed extensively by suspension in 0.1 M potassium phosphate buffer (pH 7.0) and recentrifuged to remove the unreacted ^{14}C-label.

The washed, labelled precipitates were suspended and administered in the same way as the unlabelled immunoprecipitates.

H. Preparation of samples for liquid scintillation spectrometry

Guinea pigs that were treated with the ^{14}C-labelled crosslinked immunoprecipitated gulonolactone oxidase were housed in urine and feces collection cages. Plasma samples, urine, and feces from each animal were obtained each day. After the specified period of time, animals were sacrificed and tissues were obtained for counting.

Tissue samples of 200 mg were dissolved in 1.0 ml of TS-1 tissue solubilizer (Research Products International Corp., Mount Prospect, IL). Glacial acetic acid (0.2 ml) was added and samples allowed to stand at room temperature for 45 min to minimize chemiluminescence. Following this procedure, 10 ml of Safety Solve counting cocktail (Research Products International Corp.) was added to each vial and samples were counted as described below.

Plasma samples (0.5 ml each) were counted in the form of an emulsion (Kobayashi and Maudsley, 1974). Triton X-100 (Research Products International Corp.) (5 ml) was added to each sample , along with 5 ml of the Safety Solve counting cocktail. After thorough mixing, samples were heated for 20-30 min at 80°C, cooled for 2-4 hr at 4°C and then allowed to equilibrate to room temperature prior to counting. Urine samples (0.5 ml) were counted in 10 ml of counting cocktail.

Feces samples (20 mg) were prepared for counting by adapting the method of Mahin and Lofberg (1966). Water (0.180 ml), 0.2 ml of perchloric acid, and 0.4 ml of 30% hydrogen peroxide were added to the feces samples. They were heated at 80°C for 1-2 hr, and then allowed to cool to room temperature. Counting cocktail (10 ml) was added and after 45 min, the samples were counted.

I. Liquid scintillation counting

Carbon 14-labelled samples were counted using a Beckman LS7000 scintillation counter. Samples were counted in a full C-14 window (95% of all C-14 emissions). Accuracy of counting was ± 5%. Disintegrations per minute were calculated using a quench curve. The quench curve was generated by counting samples that contained varying amounts of tissue and a known amount of C^{14}. The H-number technique (Long, 1977) was then used to determine the degree of quench.

III. Polyethylene Glycol-Conjugated Enzymes

A. Modification of enzymes with polyethylene glycol

Gulonolactone oxidase was purified from chicken kidneys as described above. For this modification, adsorption chromatography through hydroxyapatite was not performed, however. After DEAE Sephadex chromatography, the fractions containing gulonolactone oxidase activity were concentrated, under nitrogen, using an Amicon ultrafiltration unit equipped with a PM 10 membrane. Prior to the modification procedure, the buffer of this enzyme preparation was changed to 0.1 M potassium phosphate (pH 8.0) using a spun column containing G-25 Sephadex. The primary amine groups of this enzyme preparation were measured using the fluorescamine method (Udenfriend et al., 1972; Bohlen et al., 1973). Polyethylene glycol (PEG) 5000, activated with succinimidyl succinate (Enzon, Inc., Piscataway, NJ), was reacted with the enzyme. An amount of this polymer representing a 5-fold molar excess of the primary amine groups in the enzyme preparation was used to modify the enzyme. The method of Abuchowski et al. (1977a) was adapted for this procedure. The activated PEG was reacted with the enzyme for 90 min in 0.1 M potassium phosphate, pH 8.0. Unreacted PEG and derivatives were removed by washing with 0.9% sodium chloride using an

ultrafiltration cell equipped with a PM 30 membrane and overnight dialysis against 0.9% sodium chloride. α-Galactosidase from E. coli (Sigma Chemical Co., St. Louis, MO) was modified by the same procedure.

B. Enzyme administration

PEG-reacted gulonolactone oxidase was infused at a rate of 0.2 ml/min into the jugular vein. Doses of modified gulonolactone oxidase that were able to produce 0.01 to 0.07 mg ascorbic acid per min at 37°C in in vitro assays were tested. The PEG-conjugated α-galactosidase was also given via the intravenous route.

RESULTS

I. Heterologous Immunoprecipitate Studies

Previous studies showed that guinea pigs could be maintained without dietary ascorbic acid by administration of crosslinked immunoprecipitated (XL-IP) gulonolactone oxidase (Sato et al., 1986). Specific guinea pig antisera were used to immunoprecipitate the enzyme in those experiments. It was concluded that the multiple injections were tolerated because homologous antibody masked the antigenic determinants of the foreign enzyme. Thus, the guinea pigs did not recognize the complex as being allergenic. An alternative explanation is that the modified enzyme was tolerated because it was immobilized within a large, stable complex. The studies described here were designed to test whether detoxification of the enzyme was dependent on the use of homologous antiserum or some other characteristic of the crosslinked enzyme immunocomplex.

A. Modification of gulonolactone oxidase

Gulonolactone oxidase was precipitated from chicken kidney microsomes using specific antisera raised in rabbits. This was followed by crosslinking the precipitate with glutaraldehyde. Reaction with this bifunctional reagent is intended to reinforce the interactions between the antigen (enzyme) and antibody. The scheme for this modification procedure is shown in Figure 2. The amounts of antisera and solubilized microsomes necessary to obtain maximum precipitation of enzyme activity were determined. Then, appropriate conditions in which to

Figure 2. Protocol for the preparation of crosslinked immunoprecipitates. The source of gulonolactone oxidase is solubilized chicken kidney microsomes. The enzyme is isolated by reaction of this crude tissue with antibody (IgG or serum) directed against the enzyme. The resulting immunoprecipitate is collected and washed by resuspension in potassium phosphate buffer followed by ultracentrifugation. It is then reacted with glutaraldehyde, a bifunctional reagent that reacts mainly with primary amines of proteins. Formation of these covalent crosslinkages is intended to reinforce the existing bonds between the enzyme and antibody. The crosslinked complex is suspended in dextran and injected intraperitoneally into the animal.

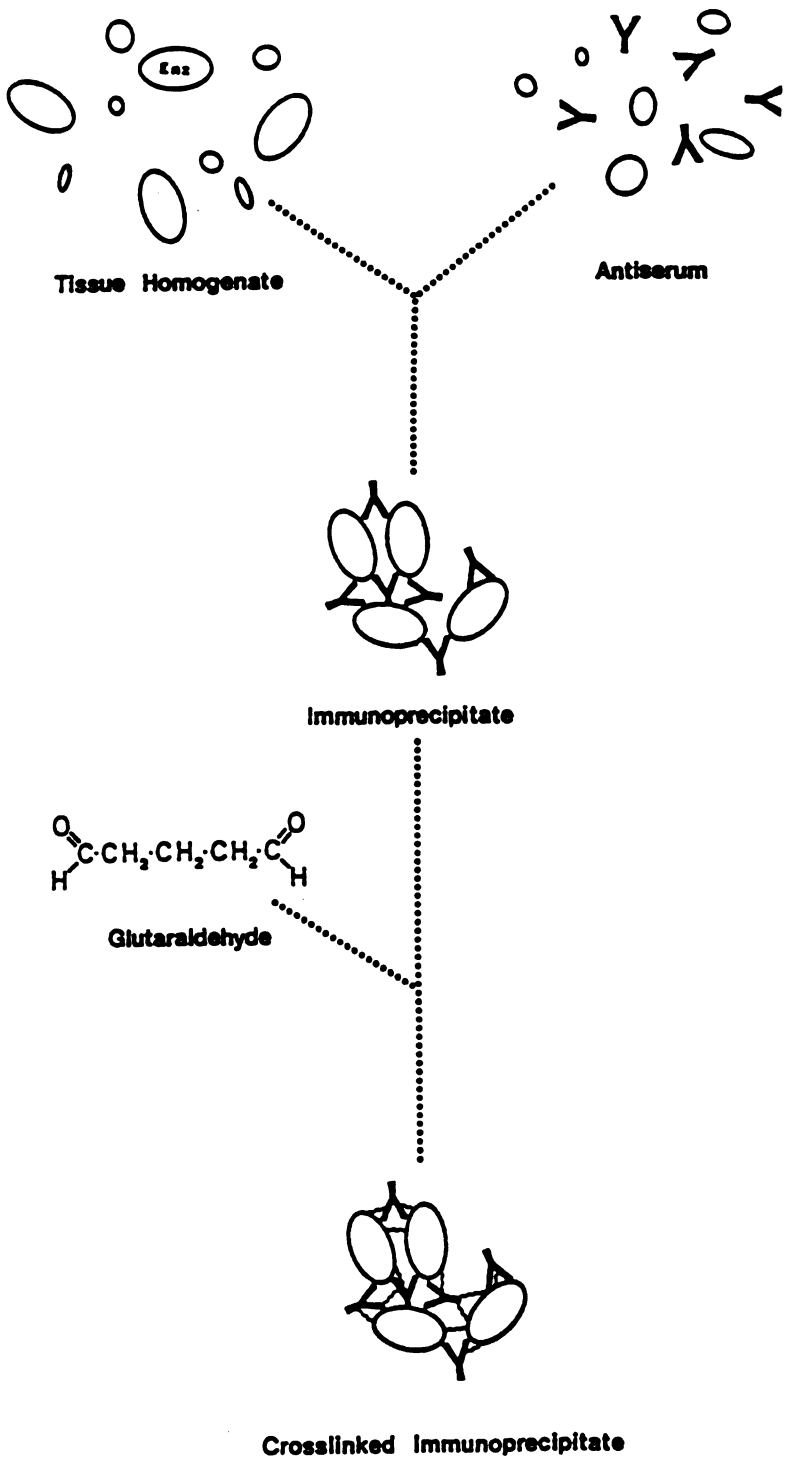

Enz
Tissue Homogenate
Antiserum
Immunoprecipitate
Glutaraldehyde
Crosslinked Immunoprecipitate

Figure 2

crosslink the enzyme-antibody complex with glutaraldehyde were studied. Conditions that provided extensive crosslinking with minimal inactivation of the enzyme were determined.

1. Crosslinking conditions

The effect of hydrogen ion concentration on the crosslinking reaction was examined. Glutaraldehyde (16.6 mM) was reacted with the immunoprecipitates at pH values ranging from 6.0 to 7.5 and enzyme activity tested. The results from one such experiment are shown in Table 1. The greatest enzyme activity was obtained when the crosslinking reaction was carried out at pH 6.5 or 7.0. Subsequently, this reaction was performed at pH 6.5.

Next, the concentration of glutaraldehyde was examined. Concentrations of this reagent ranging from 3.3 to 83 mM were reacted with the immunoprecipitates and enzyme activity was assayed. Also, the extent of the glutaraldehyde reaction was assessed by reacting the crosslinked immunoprecipitates with fluorescamine (Udenfriend et al., 1972; Bohlen et al.,1973). Glutaraldehyde reacts predominantly with the primary amines of proteins (Habeeb and Hiramoto, 1968). Fluorescamine also reacts with primary amines to yield a fluorescent product. Reaction of the complex with fluorescamine, therefore, should reflect the extent of the glutaraldehyde crosslinking reaction. The results from a representative experiment are shown in Figure 3. Both the enzyme activity and fluorescence decreased with increasing concentrations of glutaraldehyde. For subsequent crosslinking reactions, 8.3 mM glutaraldehyde was selected. At this concentration, 70% of the enzyme activity was retained, while there was a 70% decrease in fluorescamine-reactive groups.

2. Catalytic characteristics

The kinetic parameters of the enzyme were compared in order to examine how they had been affected at different steps of the modification.

TABLE 1

The effect of pH on the Glutaraldehyde Crosslinking Reaction

pH	Activity (nmol/min/mg)
6.0	0.080
6.5	0.125
7.0	0.120
7.5	0.090
Unreacted	0.330

Figure 3. Relationship between the extent of crosslinking and enzyme activity with increasing concentrations of glutaraldehyde. Immunoprecipitated gulonolactone oxidase was crosslinked using glutaraldehyde concentrations of 3.3 to 83 mM. Results from a representative experiment are shown here. Enzyme activity was determined following crosslinking and activity is expressed as nmol ascorbic acid formed/min/mg microsomal protein (●—●). Unreacted primary amines were determined using fluorescamine. The results are expressed as relative fluorescent units (○--○).

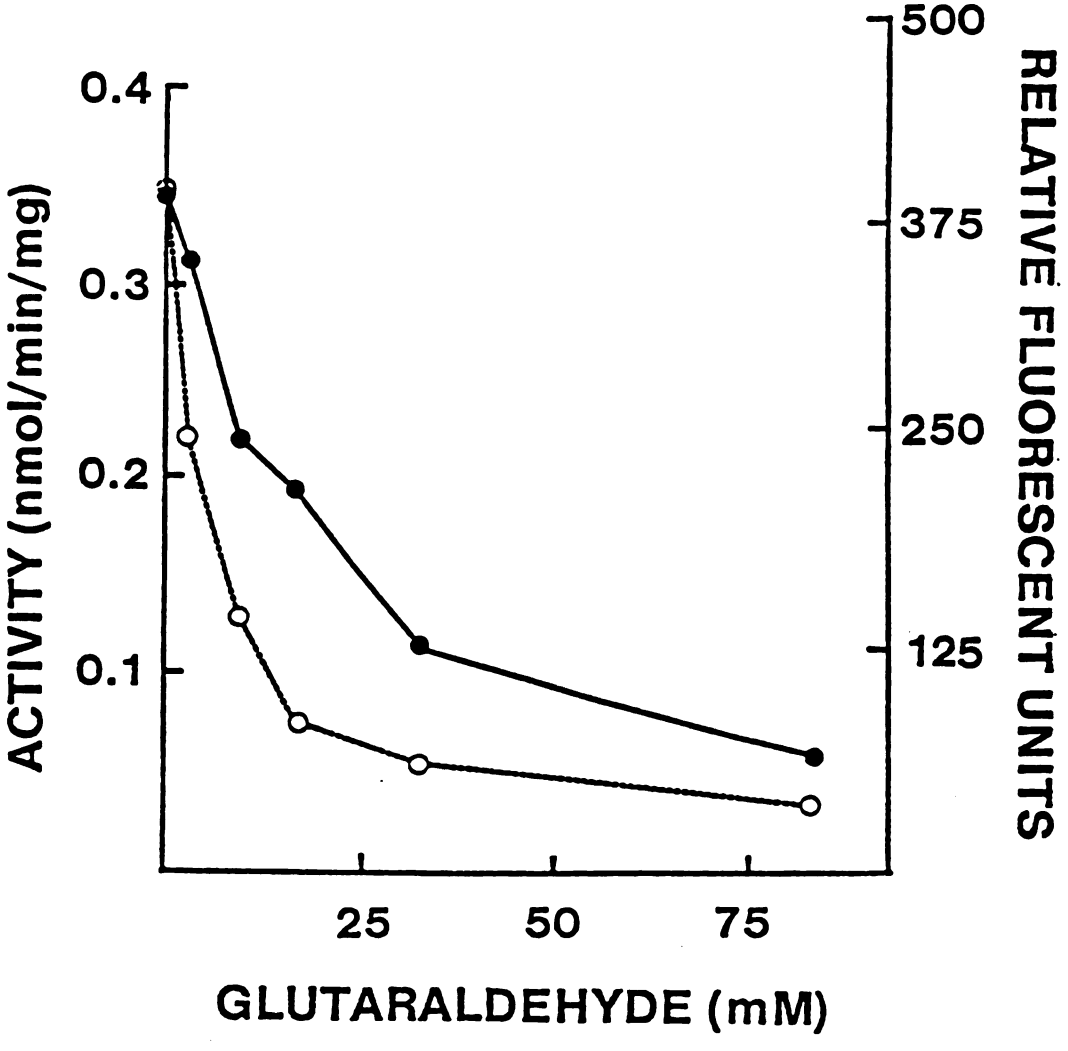

ACTIVITY (nmol/min/mg)
0.4
0.3
0.2
0.1
500
375
250
125
RELATIVE FLUORESCENT UNITS
25
50
75
GLUTARALDEHYDE (mM)

Figure 3

Activity of the modified and unmodified preparations was tested at varying substrate concentrations. The apparent Km and Vmax of the unmodified and modified enzymes were calculated from Lineweaver-Burk double reciprocal plots. Recovery and apparent kinetic constants were compared among the unmodified, immunoprecipitated, and crosslinked immunoprecipitated enzymes (Table 2). Recovery of activity was based on the amount of enzyme mixed with the antiserum, with the assumption that an antiserum concentration at which no detectable activity remained in the supernatants after centrifugation represented precipitation of all of the enzyme. To calculate recovery, activity of the modified enzyme was compared to unmodified enzyme at the same substrate concentration used in the usual assay. Upon immunoprecipitation, about 80% of enzyme activity was lost. However, no further decrease in enzyme activity occurred with crosslinking. Comparing the crosslinked immunocomplex to the microsomal preparations, Vmax decreased, but Km values were not significantly different.

B. Administration of modified gulonolactone oxidase

1. Single dose studies

Following development of this XL-IP gulonolactone oxidase, experiments were performed to determine whether a single dose was able to elicit ascorbic acid synthesis in guinea pigs. Vitamin C-deficient animals were injected intraperitoneally with the XL-IP enzyme complex suspended in dextran. The substrate, gulonolactone, was infused intravenously. Control animals were treated with histidase or asparaginase that had been modified similarly. Plasma was analyzed for ascorbic acid (Figure 4). During a 5-h period, plasma ascorbic acid concentrations of the gulonolactone oxidase-treated animals increased more than three-fold. At the end of the sampling period, plasma concentrations of ascorbic acid in these animals were 0.33 mg/100 ml, which approached concentra

TABLE 2

Comparison of Kinetic Parameters of L-Gulonolactone Oxidase and the Modified Enzyme

	Unmodified Enzyme	Immuno-precipitate	X-Linked Complex
Recovery	100	19.9±3.4	19.0±2.2
Km (mM)	0.06±0.01	0.08±0.02	0.07±0.01
Vmax (nmol/min/mg)	4.48±1.02	0.89±0.22	1.04±0.10

The apparent Km and Vmax of the modified and unmodified enzymes were calculated from Lineweaver-Burk double reciprocal plots. Recovery is expressed as a percentage of the unmodified enzyme activity at the substrate concentrations used in the usual assay. Km is expressed as a mM concentration. Vmax is expressed as nmol ascorbic acid formed/min/mg microsomal protein.

Figure 4. Synthesis of ascorbic acid in gulonolactone oxidase-treated animals. A group of four 14-day ascorbic acid-deficient guinea pigs was injected with the XL-IP chicken kidney gulonolactone oxidase (●—●). A control group of four 14-day ascorbic acid-deficient animals was given either histidase or asparaginase prepared similarly (O--O). Plasma ascorbic acid concentrations were measured. Gulonolactone (100 mg/2 ml) was administered intravenously immediately prior to injection of the enzyme and 800 mg/5 ml was infused over a 3-hr period afterward. At zero time, the enzyme suspension was injected intraperitoneally. Blood samples were taken every 30 min. Three samples were drawn before injection of the enzyme and sampling was continued for a 5-hr period thereafter. Statistics were done by mixed design analysis of variance and the vertical bar denotes $\pm$ SEM for between group comparisons. The 120-min and all subsequent time points of the gulonolactone oxidase-treated group are significantly higher than the control ($p < 0.05$). The standard error for within group comparisons is ± 0.02 for each group.

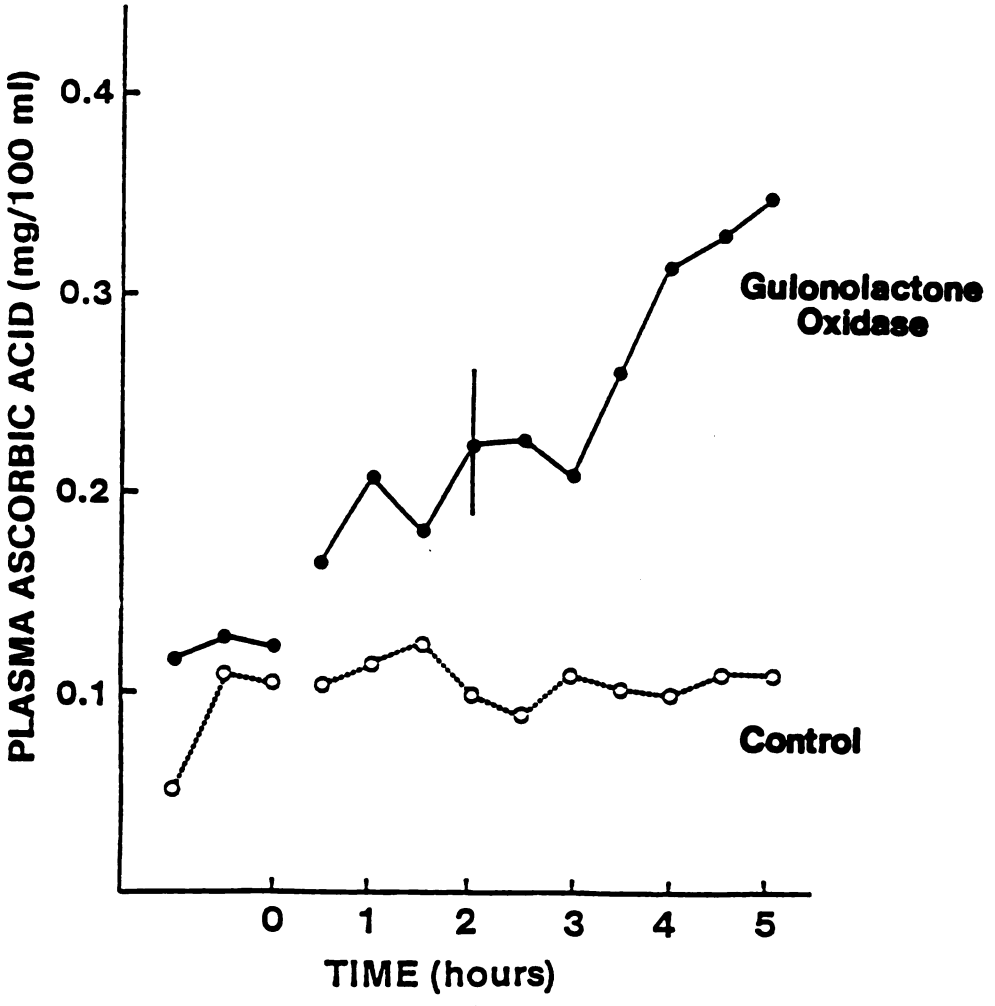

PLASMA ASCORBIC ACID (mg/100 ml)
0.4
0.3
0.2
0.1
Gulonolactone Oxidase
Control
0
1
2
3
4
5
TIME (hours)

Figure 4

tions found in guinea pigs fed a saturating amount of vitamin C, when analyzed using the same method (Zannoni et al., 1974). On the other hand, plasma ascorbic acid concentrations in the control animals remained relatively constant.

2. Effect of repeated doses of the modified enzyme

Since the XL-IP gulonolactone oxidase possessed in vivo activity, studies were planned to test whether repetitive treatments could be tolerated and therapeutically beneficial to the animals. The ability to tolerate repeated injections of foreign protein is a vital consideration in the treatment of enzyme deficiency diseases. Afflicted individuals may require therapy for their entire lifetime. To examine these aspects, guinea pigs were maintained on an ascorbic acid-deficient diet and given weekly injections of the XL-IP gulonolactone oxidase along with substrate supplementation. The growth curves of three animals treated by this protocol are shown in Figure 5 (A-C). These animals survived for at least 50 days on the ascorbic acid-deficient diet with enzyme replacement therapy. Ten animals received the control treatment that consisted of either histidase or asparaginase modified according to the same method. The average weight gain of these control animals is shown for comparison in part D of Figure 5. These animals survived an average of 23.6 ± 2.62 days on the deficient diet.

The weekly gulonolactone oxidase injections prolonged the survival time of the ascorbic acid-deficient animals. Guinea pigs ordinarily survive between 23 and 28 days on this vitamin C-deficient diet (Jones et al., 1973; Barnes et al., 1973). The fact that the gulonolactone oxidase treatment prolonged the survival of scorbutic animals demonstrates in vivo synthesis of vitamin C, as well as a therapeutic benefit to these animals.

3. Toxicity of modified gulonolactone oxidase

Fourteen animals were started on the therapy with this XL-IP gulonolactone oxidase; however, survival was prolonged significantly in only 7 of

Figure 5. Survival and prevention of scurvy in guinea pigs by enzyme administration therapy. Guinea pigs were placed on an ascorbic acid-deficient diet on Day 0 and weighed daily. On the days indicated by arrows, modified gulonolactone oxidase was administered intraperitoneally and gulonolactone (86 mg/0.6 ml) was injected at 20 min intervals subcutaneously over 2 hr. Parts A-C of this figure are the growth curves for 3 animals that survived for 50 days on this therapy. In D, the open circles represent the average body weight of 10 control guinea pigs. The standard errors of the mean body weights ranged from ± 6 to ± 8 grams. These animals received either histidase or asparaginase prepared by the same procedure and also supplementation of gulonolactone.

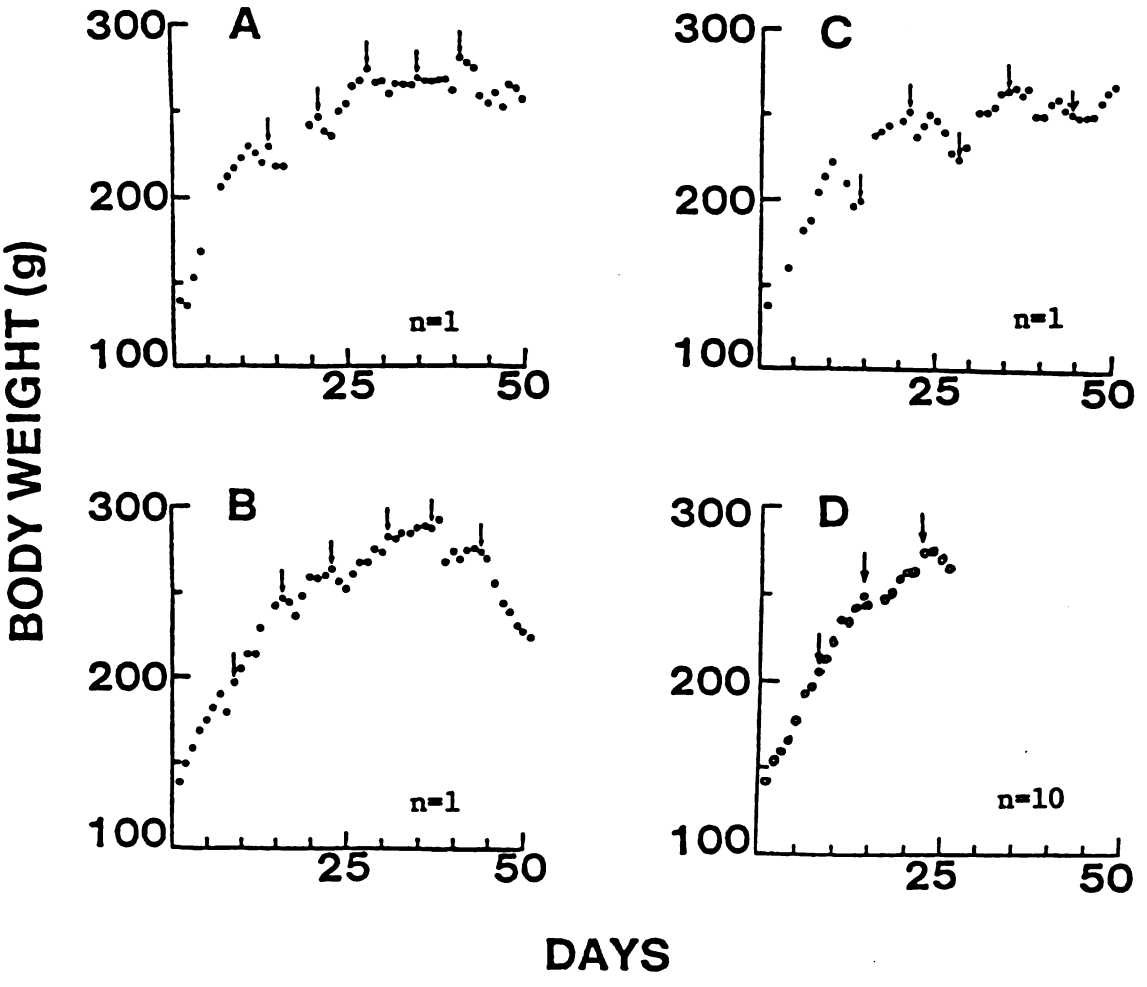


Figure 5

these animals, with only 3 surviving for the entire 50-day period. Since the therapy was beneficial for only some animals, the reasons for this limited success were investigated.

A possible explanation for the survival of only certain animals is that animals that died might have had an allergic reaction, whereas those that survived did not. To determine whether the surviving animals had been sensitized immunologically to the foreign proteins, their serum was tested for antibodies against the components of the modified enzyme. A serum sample was obtained from each animal after the 50-day treatment and Ouchterlony immunodouble diffusion tests were carried out. Serum was tested for antibodies against the enzyme source (chicken kidney microsomes) and rabbit antiserum directed against another enzyme, E. coli asparaginase. The results are shown in Figure 6. The precipitin line between the center well and the wells that contained microsomes in part C could not be seen unless stained with Coomassie Blue. Even then, the line was too faint to be seen in photographs of the plate, so the unstained plate is shown here. These guinea pigs formed antibodies against both microsomes and antiserum. Thus, the surviving animals did mount an immune response against these foreign proteins.

These animals were also tested to find out whether they had been allergically sensitized to the components of the XL-IP gulonolactone oxidase complex. Following the 50-day enzyme treatment, the animals were challenged first with an intravascular dose of a solubilized microsomal extract from chicken kidneys (1 mg protein). One day later, each animal received 0.1 ml of rabbit anti-asparaginase serum via the same route. Responses were recorded after each challenge and the data are presented in Table 3. No response was noted when the shocking doses of enzyme were administered. On the other hand, when serum from rabbits was injected, all three of the guinea pigs experienced anaphylactic

Figure 6. Ouchterlony immunodouble diffusion tests for antibody in serum of gulonolactone oxidase-treated animals. The wells contained the following: 1,4-saline; 2,3-solubilized chicken kidney microsomes; and 5,6-rabbit antiserum against asparaginase. Center wells contained serum from the treated guinea pigs. The plates are labelled A-C to correspond to the animals represented in A-C of the growth curves (Figure 5). The precipitin line between the serum and the microsome well of plate C is not visible in the photograph.

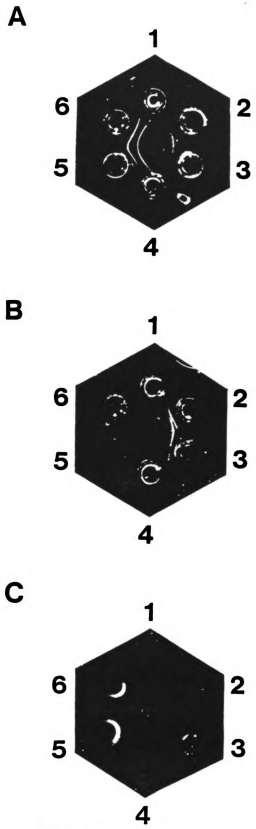


Figure 6

TABLE 3

Reaction of Guinea Pigs to Challenging Doses
of Components of Modified Gulonolactone Oxidase

Animal	Shocking Agent	Response
A	Enzyme	No reaction
	Rabbit Antiserum	Reaction -> recovery
B	Enzyme	No reaction
	Rabbit Antiserum	Reaction -> death
C	Enzyme	No reaction
	Rabbit Antiserum	Reaction -> death

One week after the last dose of XL-IP gulonolactone oxidase, the animals were challenged first with the solubilized microsomal extract (enzyme source) and, one day later, with rabbit anti-asparaginase serum. Challenging doses were administered intravenously.

reactions. The animal whose sera is tested in Fig. 5A recovered, while the other two animals had terminal reactions. These tests also show that the surviving guinea pigs have been sensitized by this XL-IP enzyme preparation.

The survival of the animals cannot be attributed to absence of an immune response against the foreign proteins. They tolerated multiple intraperitoneal injections of the complex, but not the antiserum component when it was injected separately into the vasculature. While serum antibodies were present against the microsomal extract, they did not experience allergic reactions when challenged with it.

C. Adaptability of the modification procedure to other enzymes

If this procedure is to have practical applicability, it must be adaptable to other enzymes and not uniquely suited to gulonolactone oxidase. Therefore, studies were conducted with other enzymes that have potential therapeutic application. Adaptability of this procedure was demonstrated by using asparaginase, histidase, and serum cholinesterase. These enzymes were modified with either guinea pig or rabbit antisera and crosslinked with glutaraldehyde. Kinetic parameters were determined for the resulting XL-IP enzyme complexes and compared to the native enzymes. Then, the toxicity of these XL-IP enzymes was tested in mice and guinea pigs.

1. Catalytic characteristics

Apparent kinetic constants were affected to varying degrees by the modification (Table 4). The Vmax values varied from almost complete inhibition of activity to values that approached those of the unmodified enzyme. All of the enzymes tested had a lower Vmax after they were reacted with antibody. The glutaraldehyde reaction had little further effect on this parameter. Km values varied widely as well. Serum cholinesterase modified using guinea pig antisera showed only small decreases in affinity, while asparaginase modified with

TABLE 4

Kinetic Parameters of Modified Enzymes

Enzyme		Modified Using					
		Guinea Pig Antisera			Rabbit Antisera		
		Unmodified	Immuno-precipitate	X-Linked Immuno-precipitate	Unmodified	Immuno-precipitate	X-Linked Immuno-precipitate
Asparaginase	Recovery	100	1.1	31.2	100	10.2	9.4
	Km	2.0	30.1	3.1	3.3	27	67
	Vmax	348	91.4	71.3	526	168	386
Serum Cholinesterase	Recovery	100	57	48	100	60	48
	Km	0.008	0.011	0.013	0.011	0.040	0.067
	Vmax	0.73	0.38	0.33	0.83	0.50	0.40
Histidase	Recovery	100	2.1	1.8	100	4.2	3.4

Apparent kinetic constants were determined from Lineweaver-Burk double reciprocal plots. Recoveries are expressed as a percentage of the unmodified enzyme activity at the substrate concentrations used in the usual assays. Km is expressed as a mM concentration. Vmax is expressed as μmole product formed or substrate metabolized (serum cholinesterase)/min. Asparaginase is expressed per mg protein and unmodified enzyme is purified enzyme. Unmodified serum cholinesterase is serum and Vmax is expressed per ml of serum.

rabbit antisera had a much lower affinity. Modified histidase was not sufficiently active to allow determination of its kinetic constants.

2. Toxicity of these modified enzymes

Mice and guinea pigs were given repeated intraperitoneal injections of these modified enzymes to test the toxicity of such XL-IP complexes. The animals received either three or four injections of enzyme complex at two-week intervals. Mice received multiple injections of the enzymes modified by using either rabbit or guinea pig antisera, and guinea pigs were treated with rabbit antisera-enzyme preparations. All of the modified enzymes in this experiment were tolerated (Table 5). A total of 69 animals were treated without any observable allergic or other reaction to any of the treatments. These results suggest that such XL-IP enzyme complexes are safe for repeated administration. Furthermore, this modification procedure can be extended to enzymes other than gulonolactone oxidase.

Animals in this study were also tested for an allergic response to the components of the XL-IP complexes used in their treatments. Some of the animals were challenged with unmodified enzyme. Others were challenged with nonimmune serum from the same species that was used to obtain antiserum for modifying the enzyme that they received. Doses were approximately 1.5 mg protein/animal. The results are summarized in Table 6. The ratios are the number of animals that exhibited an allergic response immediately following the challenge over the total number of animals tested. Human sera was used as the enzyme source for serum cholinesterase. Most of the animals that did not exhibit any reaction to an initial challenge with enzyme were subsequently shocked with the appropriate nonimmune serum. This accounts for the higher total number of animals in this table. Characteristic signs of systemic anaphylactic shock were observed in the animals that reacted following the challenge.

TABLE 5

Reaction of Animals to Repetitive Injection of Enzymes Modified with Heterologous Antisera

ASPARAGINASE		
Species	Serum Source	Responding
Mice	Guinea Pig	0/7
Mice	Rabbit	0/6
Guinea Pig	Rabbit	0/3
HISTIDASE		
Species	Serum Source	Responding
Mice	Guinea Pig	0/13
Mice	Rabbit	0/6
Guinea Pig	Rabbit	0/3
SERUM CHOLINESTERASE		
Species	Serum Source	Responding
Mice	Guinea Pig	0/12
Mice	Rabbit	0/13
Guinea Pig	Rabbit	0/6

Animals were injected 3 or 4 times with the modified enzyme at 2-week intervals. Mice received enzyme modified with guinea pig or rabbit antisera. Guinea pigs were given enzyme modified with rabbit antisera. The ratios are the number of animals that experienced symptoms characteristic of anaphylactic shock over the total number of animals challenged.

Table 6

Reaction of Animals to Challenging Doses of
Components of the Modified Enzyme Complexes

Species	Shocking Agent	Responding
Guinea Pigs	Heterologous sera	7/10
	Histidase or Asparaginase	1/5
	Human sera	5/5
Mice	Heterologous sera	29/38
	Histidase or Asparaginase	0/6
	Human sera	11/13

Two weeks after the last dose of modified enzyme animals were challenged with either the enzyme or the nonimmune serum from the species from which the antisera was obtained to modify the enzyme. Human serum was used as the source for serum cholinesterase. Ratios are the number of animals showing symptoms characteristic of an anaphylactic reaction over the total number of animals challenged.

Like the gulonolactone oxidase-treated guinea pigs these animals also have been allergically sensitized to some component of serum, but not to the administered enzyme. When challenged with an intracardiac (guinea pigs) or intravenous (mice) injection of enzyme, only 1 of 11 animals (a guinea pig) showed a reaction. It is not clear whether this reaction was a true anaphylactic reaction. It may have been related to the cardiac puncture. In contrast, 52 of 66 animals shocked with either the antisera or the human serum clearly experienced symptoms characteristic of an anaphylactic reaction.

D. Modification of gulonolactone oxidase using purified IgG

Since these studies showed that a component of antisera was capable of initiating an allergic reaction, IgG was purified from antisera in an effort to eliminate potentially toxic components. It was postulated that using purified IgG to modify the enzyme might result in a more consistent product and improve the success of the XL-IP gulonolactone oxidase therapy. For these experiments, antiserum against chicken kidney gulonolactone oxidase was raised in a horse. The IgG was purified using QAE Sephadex chromatography, according to the method of Joustra and Lundgren (1969). Polyacrylamide gel electrophoresis showed that the product obtained was essentially homogeneous (Figure 7).

1. Characteristics of the XL-IP enzyme complex

Gulonolactone oxidase was precipitated from chicken kidney microsomes with the purified IgG. Optimum glutaraldehyde concentrations and crosslinking conditions were established. Electrophoresis of the resulting immunoprecipitate revealed fewer components in this preparation compared to those prepared using whole antiserum (Figure 8).

The stability of this XL-IP gulonolactone oxidase preparation to heat denaturation and trypsin digestion was examined and compared to that of the unmodified enzyme in <u>in vitro</u> studies. Purified gulonolactone oxidase and the

Figure 7. Polyacrylamide gel electrophoretic analysis of immunoglobulin G purified from horse serum. IgG, purified from horse antiserum against gulonolactone oxidase by the method of Joustra and Lundgren (1969) (Panel A), and a standard horse IgG preparation (Miles Scientific, Naperville, IL) (Panel B) were electrophoresed on nondenaturing gels and compared. The gels were stained with Coomassie blue and scanned at 600 nm in a spectrophotometer with gel scanning attachment. Migration is from right to left.

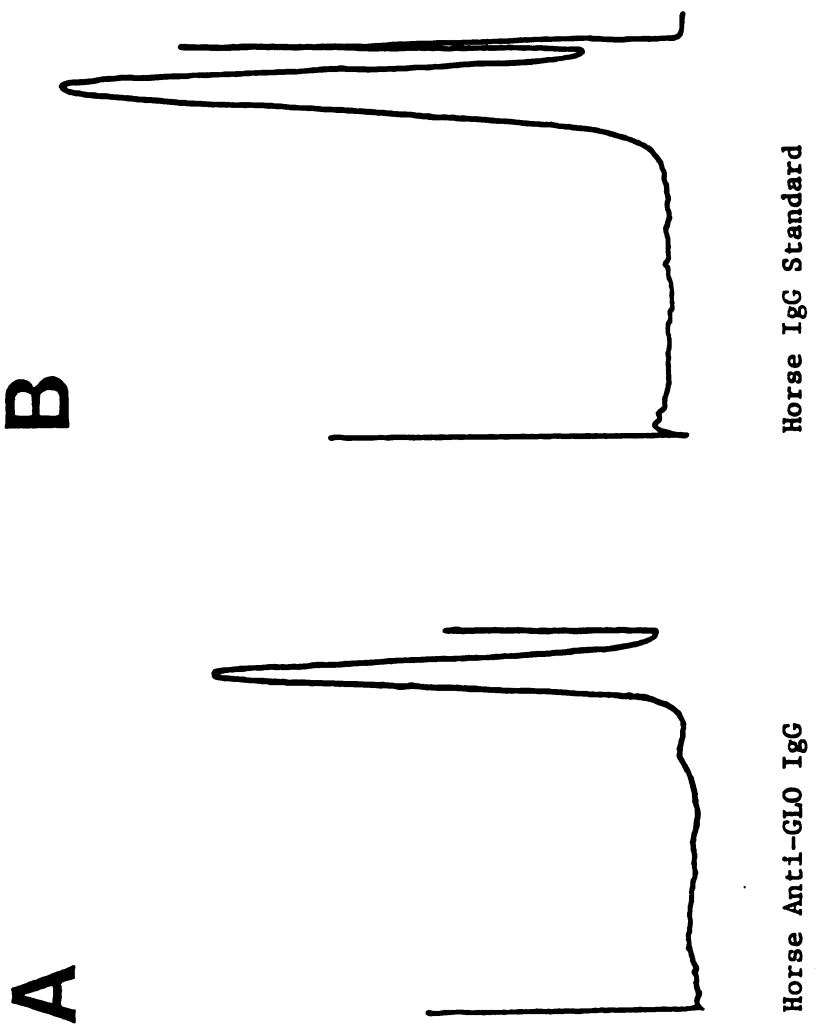


Figure 7

Figure 8. SDS-Polyacrylamide gel electrophoresis of gulonolactone oxidase immunoprecipitates. Samples were dissociated in the presence of mercaptoethanol and electrophoresed in 7.5% polyacrylamide gels in the presence of 0.1% SDS, according to the method of Nishikimi et al. (1977). Scan A is purified chicken kidney gulonolactone oxidase. An immunoprecipitate prepared from whole antiserum is shown in scan B and scan C is an immunoprecipitate made using purified IgG. Scan D is the purified IgG component alone. The gels were stained with Coomassie Blue and scanned at 600 nm in a spectrophotometer with a gel scanning attachment. Migration is from right to left.

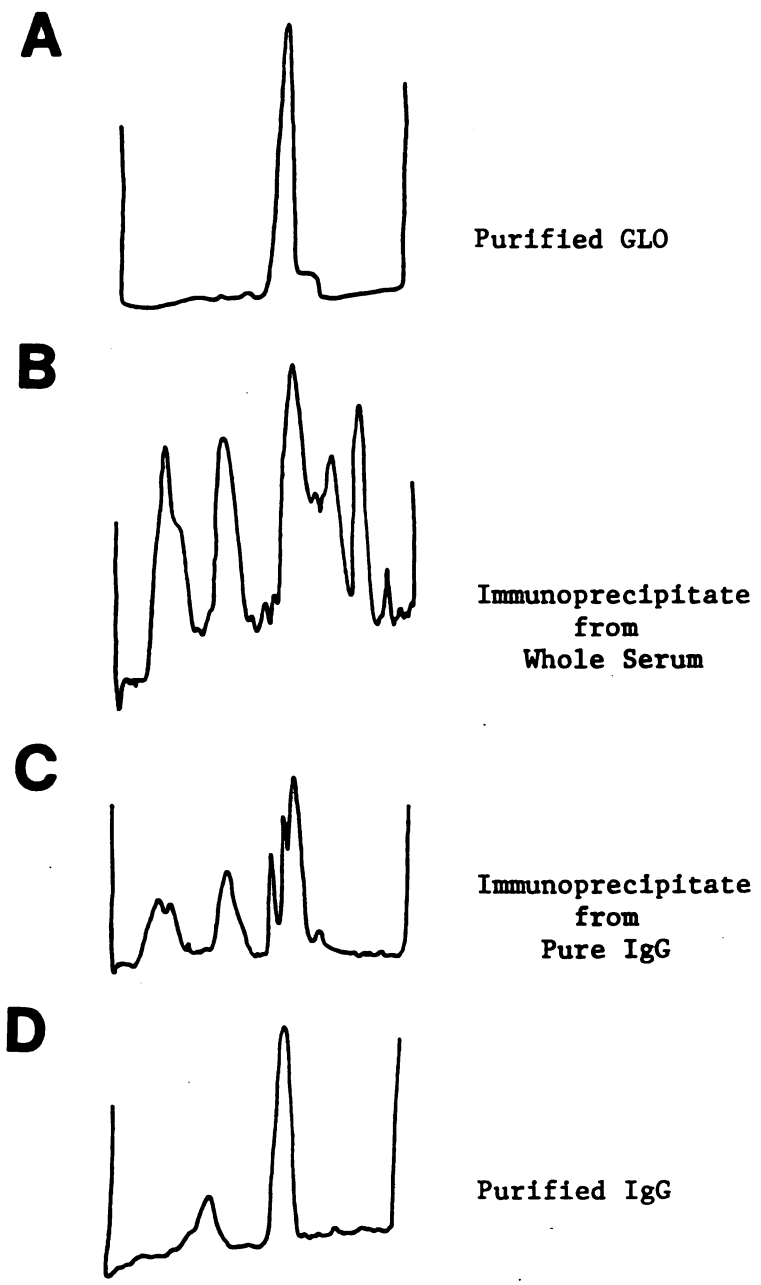

A
Purified GLO
B
Immunoprecipitate
from
Whole Serum
C
Immunoprecipitate
from
Pure IgG
D
Purified IgG

Figure 8

XL-IP enzyme were incubated alone and in the presence of the proteolytic enzyme trypsin at 37°C. Enzyme activity was assayed at selected times over a 48-hr period. The results from a representative experiment are shown in Figure 9. The activity of the unmodified enzyme decayed rapidly at 37°C (t 1/2 = 12 min) and in the presence of trypsin (t 1/2 = 9 min). In contrast, there was virtually no decay in the activity of the modified gulonolactone oxidase over a 3-hr period. The half-life of activity of this XL-IP enzyme was on the order of 24 hours. Even in the presence of trypsin, its half-life was approximately 3 hr. Thus, compared to the unmodified enzyme preparation, gulonolactone oxidase activity in the XL-IP enzyme complex was greatly stabilized and protected from proteolytic degradation to some extent

2. Administration of the less contaminated complex

This XL-IP gulonolactone oxidase was administered to guinea pigs to test its in vivo activity. The scorbutic guinea pigs also received subcutaneous injections of the substrate, gulonolactone. Elevations of plasma ascorbic acid in guinea pigs after a single intraperitoneal injection demonstrated that this modified enzyme had ascorbic acid synthetic capability (Figure 10). Over the 5-hr period, there was a two-fold increase in plasma concentrations of the vitamin.

In order to show that this complex could be given safely over an extended period, normal guinea pigs were treated with this XL-IP gulonolactone oxidase. A group of four animals received repeated injections of the modified enzyme for a period of 100 days, while being maintained on a normal vitamin C-containing diet. All four animals survived up to 14 weekly injections and they gained weight at a rate comparable to untreated normal guinea pigs (Figure 11).

Figure 9. Increased stability of XL-IP gulonolactone oxidase to incubation at 37°C and trypsin digestion. The results from a representative experiment are shown here. Purified gulonolactone oxidase (0.2 U activity/ml) was incubated alone (●—●) and in the presence of the proteolytic enzyme, trypsin (0.5 mg/ml) (○-○) in a water bath maintained at 37°C. The immunoprecipitated, crosslinked enzyme (0.07 U activity/ml) was also incubated alone (▲—▲) and with trypsin (0.5 mg/ml) (□-□) under the same conditions. One unit (U) of activity equals 1 umole of ascorbic acid formed per minute. Enzyme activity was assayed at various times over a 48-hr period and is expressed as a percent of the initial activity on a log scale.

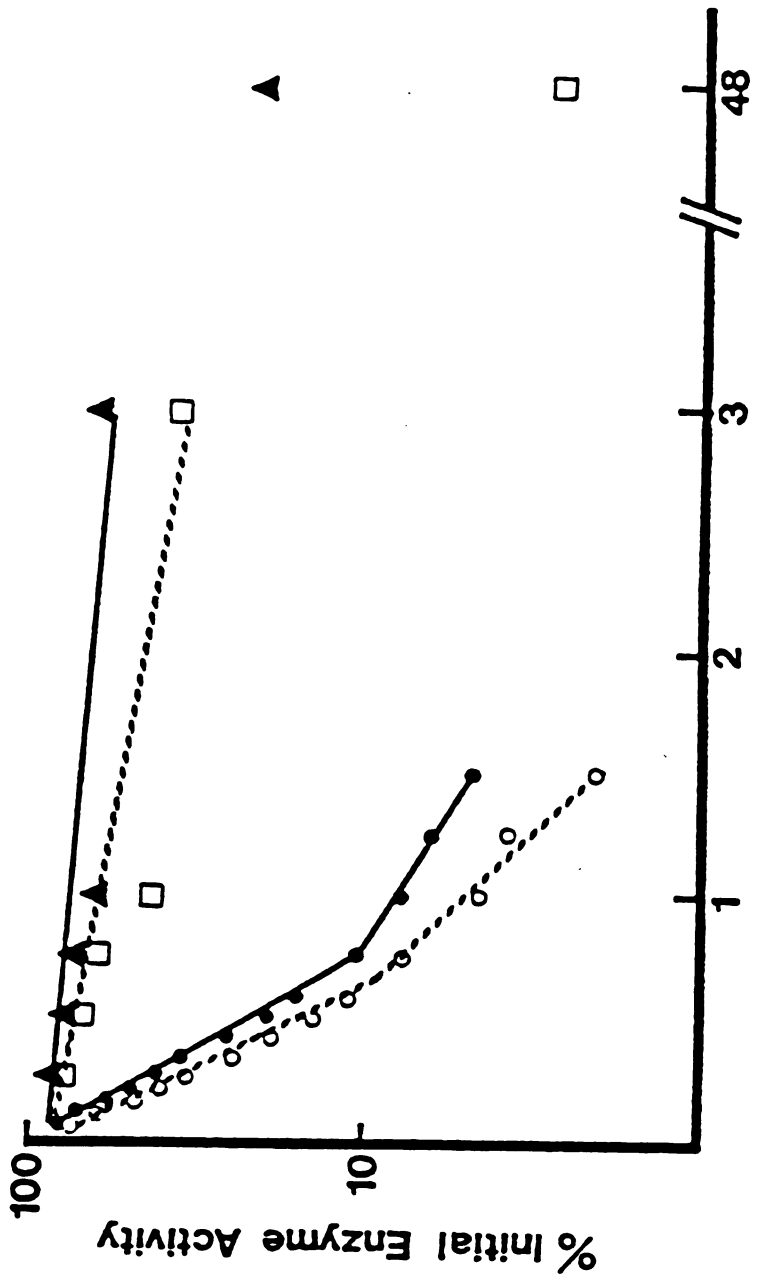


Figure 9

Figure 10. Synthesis of ascorbic acid by guinea pigs treated with gulonolactone oxidase modified using purified horse IgG. Two 11-day ascorbic acid-deficient guinea pigs received intraperitoneal injections of XL-IP gulonolactone oxidase (●—●). The substrate, gulonolactone (100 mg/1 ml), was injected subcutaneously prior to the enzyme injection. Subcutaneous injections (0.6 ml) of gulonolactone (800 mg/5 ml) were given at 20 min intervals for a 3-hr period afterward. Two plasma samples were tested to establish pretreatment ascorbic acid levels. Plasma was tested at 30 min intervals following enzyme injection and ascorbic acid was measured by the method of Zannoni et al. (1974). It has been demonstrated previously that control animals given modified proteins other than gulonolactone oxidase and substrate maintain relatively constant plasma ascorbic acid concentrations (——) (Hadley et al., 1987).

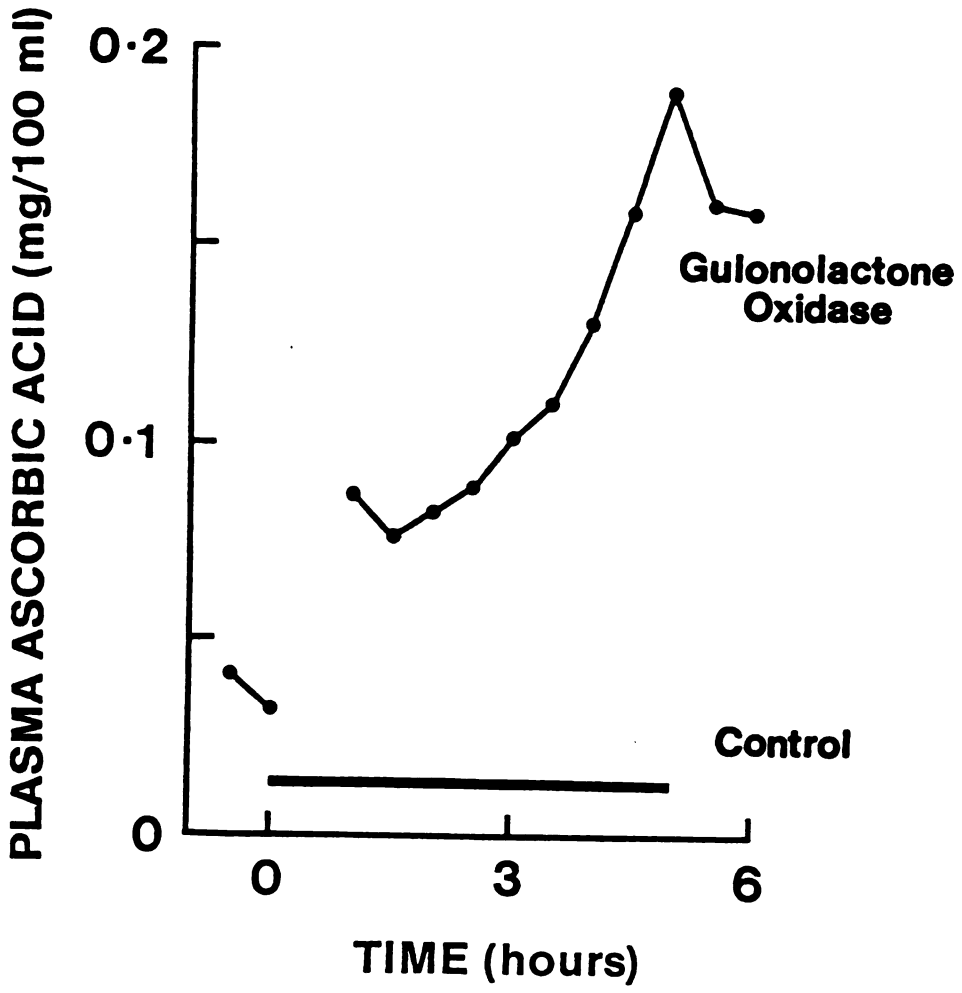

PLASMA ASCORBIC ACID (mg/100 ml)
0·2
0·1
0
0
3
6
TIME (hours)
Gulonolactone Oxidase
Control

Figure 10

Figure 11. Growth rate of enzyme-treated normal guinea pigs and enzyme-treated scorbutic guinea pigs compared to untreated normal control animals. The open squares represent the average body weights of 4 animals treated for 100 days with XL-IP gulonolactone oxidase, while being maintained on a normal diet. The solid line is a growth curve for untreated normal control animals provided by the animal supplier (Michigan State Department of Health). For comparison, the average body weights of 4 ascorbic acid-deficient animals that received gulonolactone oxidase replacement therapy are shown by closed circles (Sato et al., 1986).

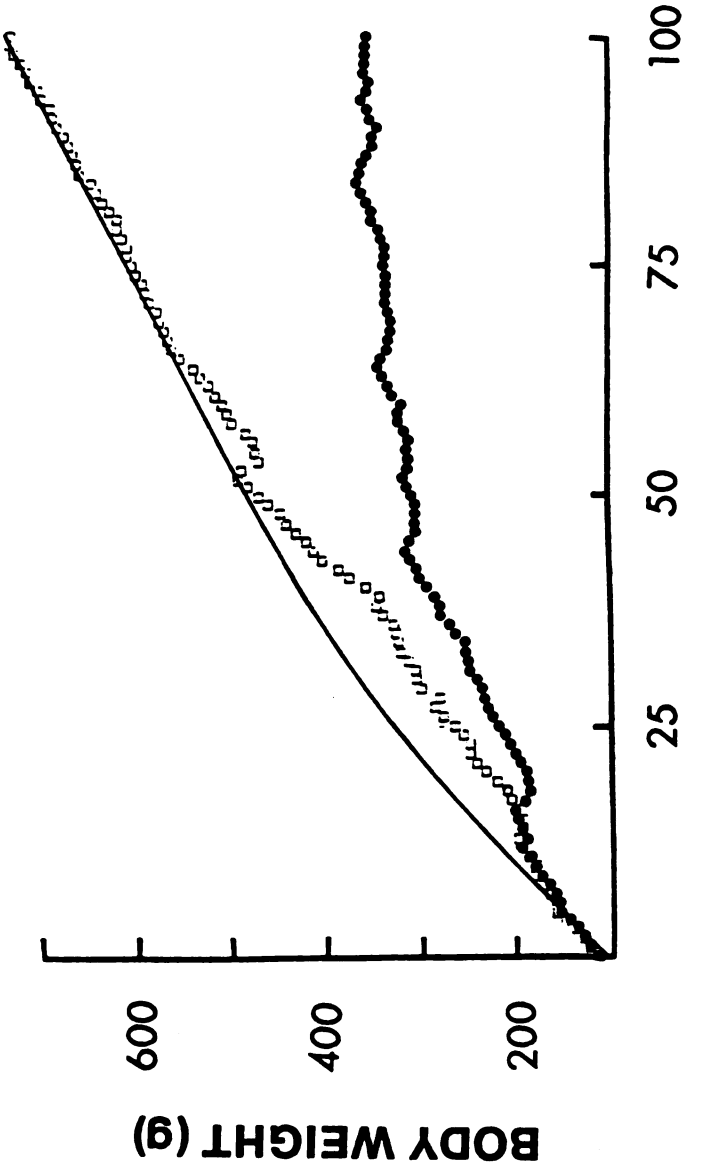


Figure 11

Pathologic examination of these enzyme-treated animals at the end of this 100-day period confirmed that the enzyme was not toxic. Thus, this preparation was safe for long-term administration.

A product that contained less contaminants had been prepared, its synthetic capability demonstrated and safety established, so the possibility of treating scurvy was tested. Attempts to show a long-term therapeutic benefit were unsuccessful, however. The success rate of this therapy was not improved compared to that of the protocol using enzyme modified with whole antiserum. None of four guinea pigs treated with this preparation survived for 50 days on the therapy, while being maintained on the ascorbic acid-deficient diet. From these studies, it must be concluded that the poor rate of successful therapy was not the result of a few preparations containing a toxic component.

3. Analysis of doses of enzyme activity administered

The possibility that inadequate ascorbic acid synthesis contributed to the early death of gulonolactone oxidase-treated ascorbic acid-deficient guinea pigs was also considered. Surviving animals may have been better able to tolerate marginal vitamin C deficiency. Based on the plasma and tissue ascorbic acid concentrations of the vitamin, the amount of vitamin C synthesis catalyzed in the enzyme-treated animals was believed to be adequate however. Comparisons were also made between the doses given to survivors of a 100-day enzyme therapy protocol and those given to the nonsurvivors (Sato et al., 1986). There was no correlation noted between successfully treated animals and those that received the highest amounts of activity. Furthermore, the average doses were not significantly different. The average dose given to surviving animals was 0.69 ± 0.06 U/treatment, while animals that did not survive received 0.66 ± 0.03 U/treatment. It was concluded, therefore, that the doses of enzyme activity administered were adequate.

4. Supplementation of ascorbic acid during gulonolactone oxidase therapy

To further examine the possibility that insufficient doses of enzyme activity and, subsequently, inadequate vitamin C synthesis contributed to the death of the gulonolactone oxidase-treated animals, an additional study was performed. A group of guinea pigs was maintained on the ascorbic acid-deficient diet and given injections of modified gulonolactone oxidase every 4 or 5 days. In addition to the enzyme therapy, these guinea pigs received 6 mg of vitamin C mixed with the dose of substrate and injected subcutaneously. This amount of vitamin C approximated both the minimum requirements of this vitamin for animals of this size (Chatterjee, 1967), as well as the estimated amount of the vitamin synthesized by a single enzyme injection. If the reason for the low rate of therapeutic success involved inadequate ascorbic acid synthesis in the enzyme-treated animals, then vitamin C supplementation should remedy the situation. After several weeks on this therapy, the condition of these animals suggested that simply providing adequate amounts of ascorbic acid was not equivalent to treating animals fed the normal vitamin C-containing diet.

5. Supplementation of other vitamins during enzyme therapy

Another difference between these two groups was their diets. Animals fed the ascorbic acid-deficient diet might not have been obtaining adequate nutrition if the ascorbic acid-deficient diet was deficient in nutrients other than vitamin C. Other evidence supported this hypothesis. For example, even the deficient animals that survived the previous 100-day protocol (Sato et al., 1986) did not grow at the same rates as untreated and enzyme-treated normal guinea pigs (Figure 11). In addition, postmortem examination of animals that did not survive the gulonolactone oxidase treatment revealed that these animals were deficient in vitamins A and E. Perhaps supplementation of other nutrients is

necessary for the treated animals that are maintained on the vitamin C-deficient diet.

Studies were designed to examine whether the success of the XL-IP gulonolactone oxidase therapy could be improved by supplementing the animals maintained on the ascorbic acid-deficient diet with additional nutrients. Improvement of the nutritional status of such animals might be a critical factor for increasing the rate of success of the enzyme replacement therapy protocol. Five guinea pigs were started on the ascorbic acid-deficient diet and treated weekly with active XL-IP gulonolactone oxidase. In addition to the enzyme therapy, these animals were given injections of vitamins A, B, D and E and selenium once every week. Appropriate doses of each of these vitamins were given to meet the weekly requirements (Fox et al., 1984). Control animals were given preparations of XL-IP gulonolactone oxidase which had been inactivated. These inactivated preparations contained 0.3% of the activity given to the other animals. Weekly vitamin supplements were also given to the control animals.

The growth curves for these animals are presented in Figure 12. Four of the animals started on the therapy with active XL-IP gulonolactone oxidase survived for at least 100 days (parts a-d) and the fifth animal (part e) survived for 68 days. Importantly, all of these animals appeared healthy and showed growth rates approaching those of animals fed a normal diet. In contrast, the five control animals survived an average of only 33.6±0.78 days (part f) . This is similar to the usual time of survival on an ascorbic acid-deficient diet (23-28 days) (Barnes et al., 1973; Jones et al., 1973), showing that the vitamin supplementation (A, B, D, E and selenium) itself does not protect from scurvy. The fact that the control animals survived slightly longer than usual could be the result of the small amount of gulonolactone oxidase activity that they received. It appeared that supplementation with other vitamins was necessary to ensure

Figure 12. Growth curves of nutrient-supplemented guinea pigs on enzyme replacement therapy. Five guinea pigs were placed on an ascorbic acid-deficient diet and treated with XL-IP gulonolactone oxidase on the days indicated by arrows. Animals shown in part A-E were given active preparations. Treatment was terminated after 100 days. The animal in part e died on day 68. Five control animals received inactive XL-IP gulonolactone oxidase and an average of their body weights is shown in part F of the figure. The standard errors of the mean body weights of the control animals ranged from $\pm$8 to $\pm$20 grams. Body weights were determined daily.

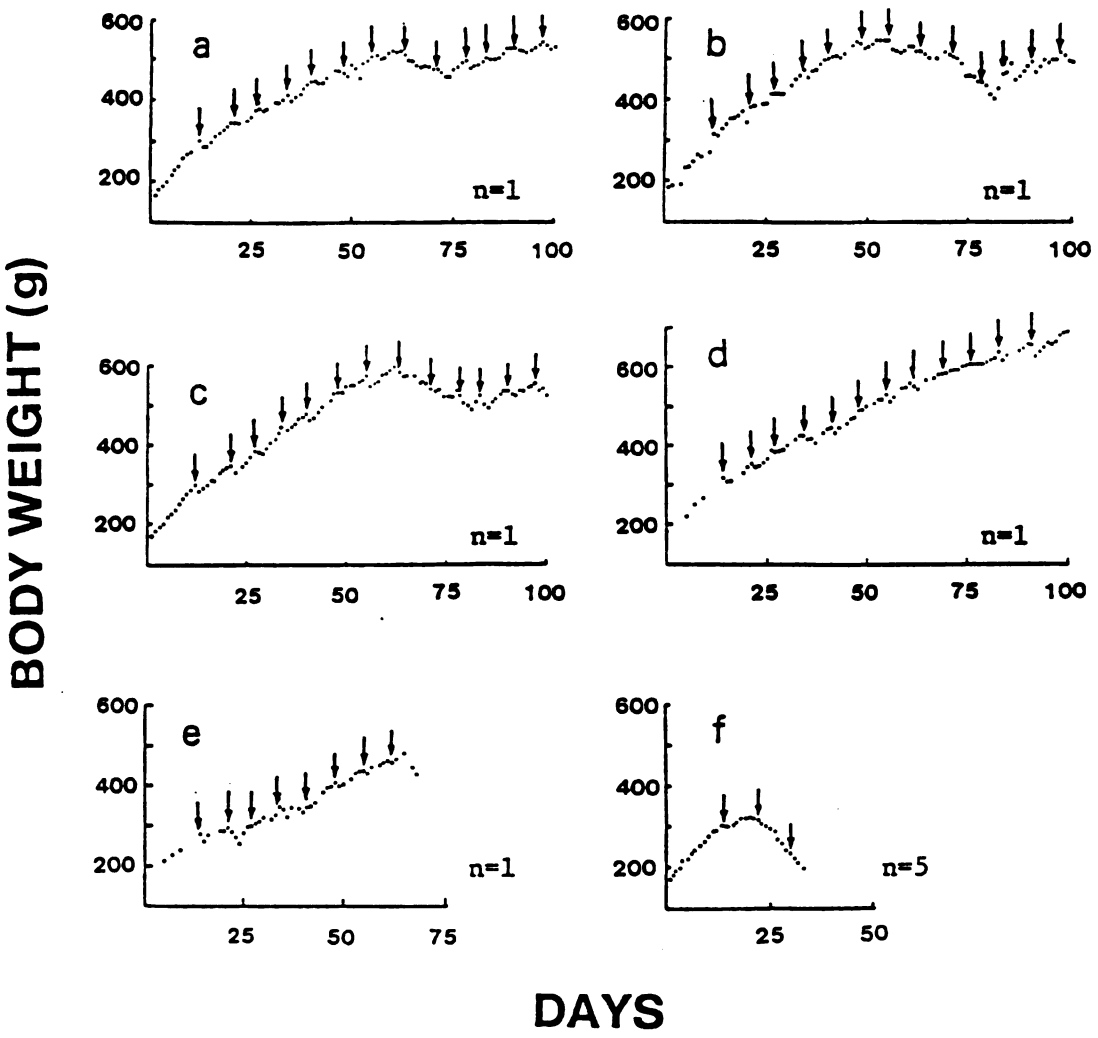

BODY WEIGHT (g)
600
400
200
a
n=1
25
50
75
100
b
n=1
c
n=1
d
n=1
e
n=1
f
n=5
DAYS

Figure 12

that animals fed the ascorbic acid-deficient receive adequate nutrition. The success rate of the gulonolactone oxidase therapy was greatly improved.

The cause of death of the guinea pig that did not survive the entire 100 days of the protocol (part e of Figure 12) was most likely a bacterial infection (Enterobacter cloacae) in the lungs and liver. Enterobacter cloacae is a common inhabitant of the guinea pig intestinal tract. Internal examination of this animal showed that the abdominal cavity contained blood, peritonitis and adhesions between the liver and gastrointestinal tract. These symptoms were not necessarily an effect of the modified enzyme, since one of the other animals in the study was examined and did not exhibit these signs. The presence of this infection was consistent with a puncturing of the gastrointestinal tract during intraperitoneal injection of the enzyme.

Partial analysis of the vitamin and mineral content of the ascorbic acid-deficient diet was performed also. There were some disparities between the contents of this diet and those of the normal guinea pig chow. For the most part, however, nutrient levels were similar. The diet was considered to be adequate. Nutrient analyses provided by the manufacturers confirmed this belief. However, after 14 days of vitamin C deficiency, guinea pigs become anorexic and enter a chronic fasting state (Peterkofsky et al., 1986). As a result, additional nutritional deficiencies may be imposed. The frequent intraperitoneal enzyme injections may have complicated this situation by causing irritation and lowering the appetite further. It may be that supplementation of additional vitamins to the ascorbic acid-deficient animals prevented more generalized malnutrition.

E. Comparison of gulonolactone oxidase therapy regimens

A comparison of the XL-IP gulonolactone oxidase therapy protocols described is presented in Figure 13. The survival time of all of the animals in

Figure 13. Comparison of the survival time of ascorbic acid-deficient animals treated with the different gulonolactone oxidase therapy regimens and their respective control groups. Animals in part A were given weekly injections of XL-IP histidase or asparaginase and served as controls for animals in part B. Weekly injections of XL-IP gulonolactone oxidase were administered to animals in part B. Part C represents animals treated with inactivated preparations of the XL-IP gulonolactone oxidase and weekly vitamin (A, B, D, E and Se) injections. These animals served as controls for the animals in part D. The animals in part D received active XL-IP gulonolactone oxidase and the weekly vitamin (A, B, D, E, and Se) supplementation. The substrate, gulonolactone, was given to all animals with each enzyme treatment. The mean survival time is shown for each group of animals. Survival time which is significantly longer is indicated by an asterisk ($p < 0.05$). Animals in part B are compared to their respective controls in part A and animals in part D are compared to those in part C.

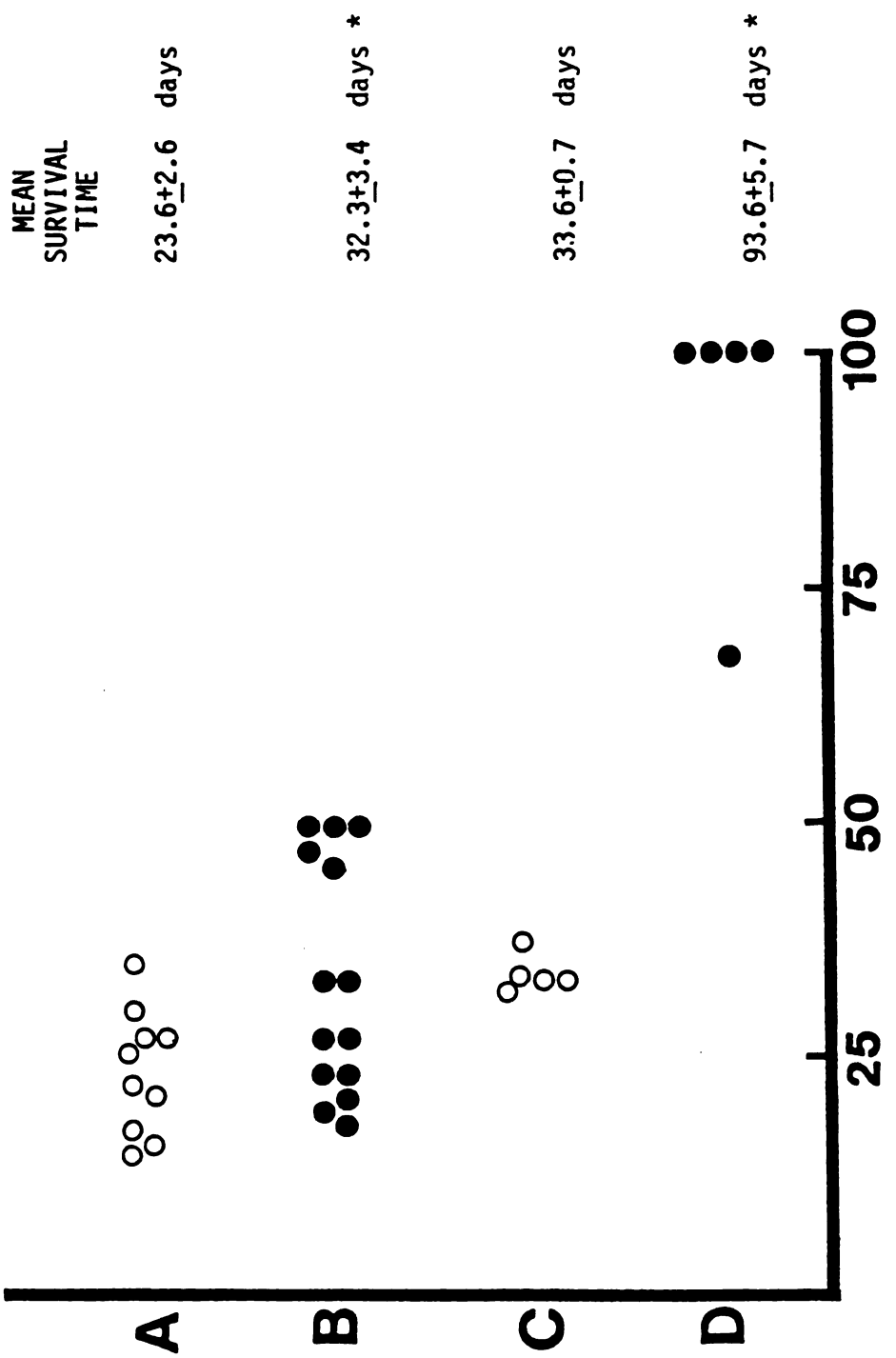

MEAN
SURVIVAL
TIME
23.6±2.6 days
32.3±3.4 days *
33.6±0.7 days
93.6±5.7 days *
A
B
C
D
25
50
75
100
Days

Figure 13

each gulonolactone oxidase-treated group and its respective control-treated group is shown. The mean survival time for each group is presented, as well. Gulonolactone oxidase replacement therapy significantly prolonged the survival time of ascorbic acid-deficient guinea pigs. Both treatment protocols indicate this. However, with the first gulonolactone oxidase therapy regimen (part B), many animals still died early in the study. Seven guinea pigs survived 33-50 days which was significantly longer than untreated scorbutic animals, but only 3 survived for the entire 50-day treatment period. The enzyme therapy was not effective in many of the animals. It is evident, however, that the vitamin supplemented-animals (part D) represented a great improvement in the success of the therapy. The animals in this study received supplementation of other vitamins (A, B, D, E, and selenium) to ensure their adequate nutrition. All of the animals that received this vitamin supplementation along with the enzyme replacement therapy survived significantly longer than their respective control animals and untreated scorbutic animals.

F. Disposition of crosslinked immunoprecipitates of gulonolactone oxidase

Having shown that XL-IP gulonolactone oxidase can be given safely over a long period of time, the distribution and elimination of this complex, following intraperitoneal injection, was examined. To do this, the crosslinked enzyme-antibody complex was radioactively labelled with ^{14}C-formaldehyde and then administered to guinea pigs. Distribution was monitored for a period of 10 days, with the sacrifice of one animal each day. The counts in plasma, urine, and feces were monitored daily.

1. Recovery of administered radioactive dose

The percent of the administered dose of radioactivity recovered in tissues and excretion products increased over the 10-day period, with the

greatest recovery on the last day (Table 7). This suggests that the immunoprecipitate was slowly removed from the peritoneal cavity. The percent of the dose of radioactivity recovered from each individual tissue is plotted for each day of the experiment (Figure 14). The greatest percentage of label appeared in the urine, kidney, and liver. Muscle seemed to contain a great amount of label, even though the concentration in this tissue is quite low, due to its larger mass compared to the other tissues. Fewer than 0.2 percent of the counts were detected in each of the following: heart, brain, adrenal glands, gall bladder, and feces, and these are omitted from Figure 14. The lung is not included because less than 0.3 percent of the counts was detected in this tissue.

2. Concentration of radioactivity in tissues

Figure 15 illustrates the concentrations of radioactivity recovered from individual tissues on a daily basis. This gives some indication of the uptake and metabolism of the modified enzyme complex. Within the first day following administration, the greatest amount of label was found in the spleen. Over the next few days, larger concentrations were noted in the liver, kidney, and urine. Gradually, concentrations of label were increased in the gall bladder and lungs, then the levels in these tissues declined. Concentrations also increased in the adrenal gland over the 10-day period. Label detected in both plasma and feces peaked at day 3 and declined thereafter. Very low concentrations of radioactivity were found in the heart, muscle, and brain.

II. Polyethylene Glycol(PEG)-Conjugated Gulonolactone Oxidase

Administration of crosslinked immunoprecipitated gulonolactone oxidase, along with substrate supplementation, enabled guinea pigs to synthesize their own ascorbic acid. The enzyme modified in this manner must be injected intraperitoneally, however. When infused intravenously, this modified enzyme complex was

Table 7

Recovery of Administered Radioactivity

	Day									
	1	2	3	4	5	6	7	8	9	10
% Dose Recovered	4.30	9.80	11.4	34.4	34.4	32.2	52.6	43.1	45.4	54.2

Recovery of ^{14}C-label is based on the values obtained from single animals on each respective day of the study.

Figure 14. Recovery of the dose of radioactivity from individual tissues and excretion products. The percent of the total administered dose of radioactivity that was recovered from individual tissues on each day of the 10-day study is shown. Each point represents the determination from a single animal. Estimation of total muscle mass of individual animals is based on their body weight (Breazile and Brown, 1976). Tissues shown in part A include: liver (□); muscle (●); and spleen (▲). In part B, plasma (○), kidney (▲), and urine (●) are shown.

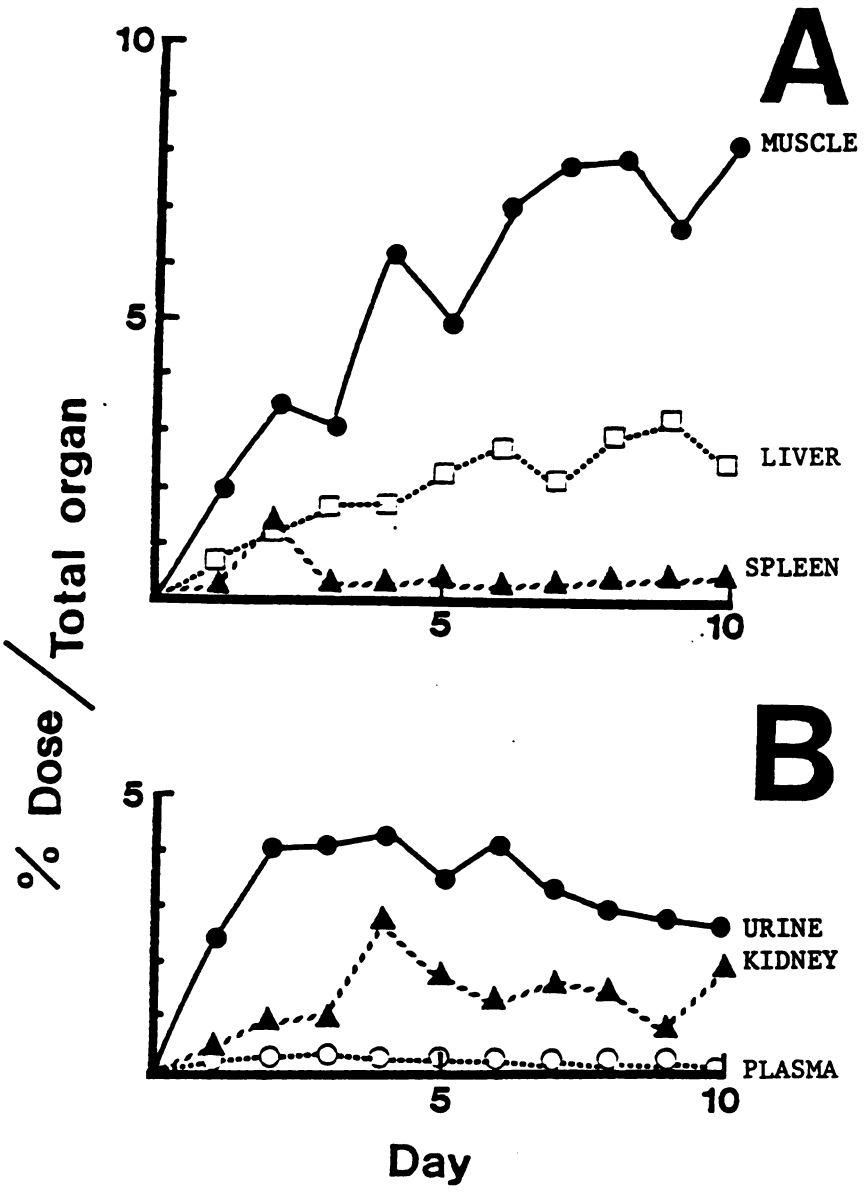

A
10
5
MUSCLE
LIVER
SPLEEN
5
10
% Dose / Total organ
B
5
URINE
KIDNEY
PLASMA
5
10
Day

Figure 14

Figure 15. Concentrations of radioactivity found in individual tissues and excretion products. The concentrations of radioactivity found in individual tissues is plotted for each day of the 10-day period. Each point represents the determination from a single animal. Only very low concentrations were detected in the heart, muscle, and brain. These are not included in the figure. Tissues represented in part A of the figure include: kidney (●); liver (▲); and spleen (○). In part B, gall bladder (□), plasma (○), and urine (●) are shown. Adrenal glands (○); lung (●); and feces (□) are presented in part C.

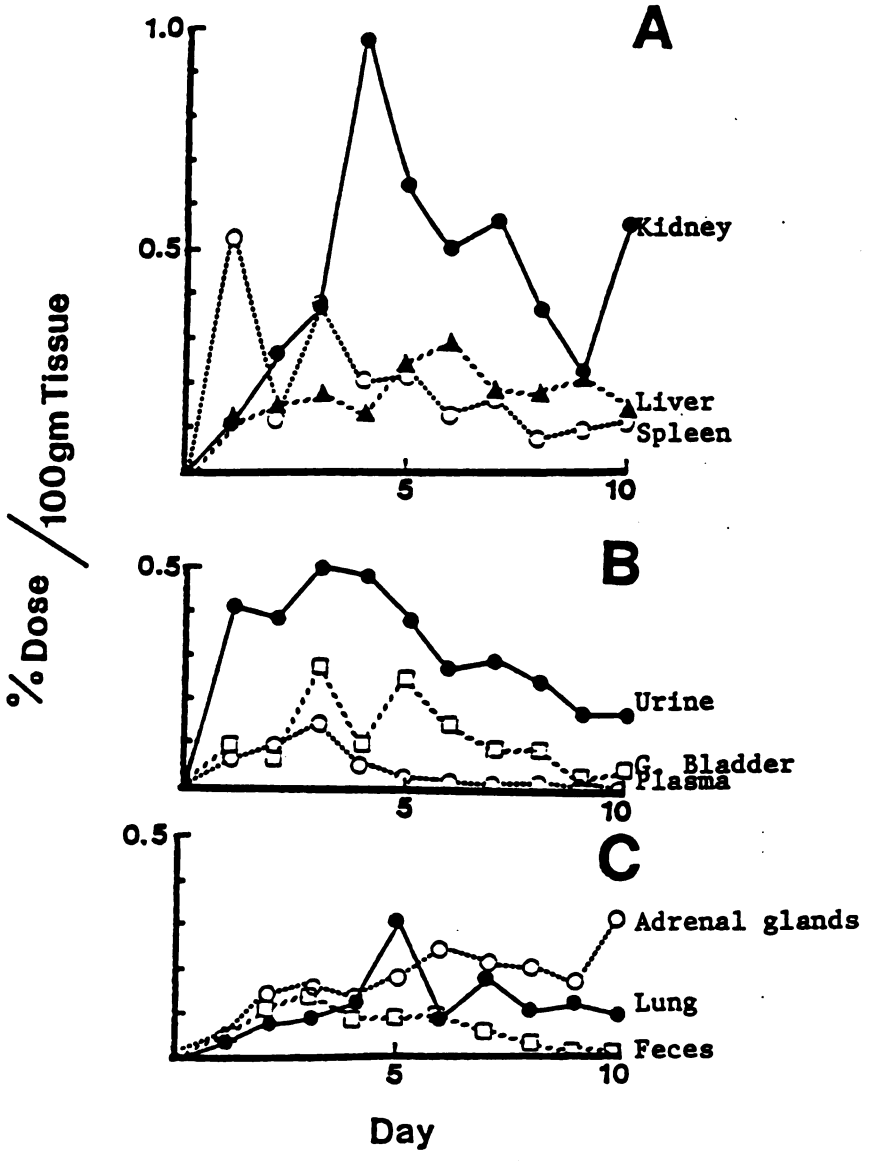

A
1.0
0.5
Kidney
Liver
Spleen
5
10
B
0.5
Urine
G. Bladder
Plasma
5
10
C
0.5
Adrenal glands
Lung
Feces
5
10
% Dose / 100gm Tissue
Day

Figure 15

found to be toxic (Sato and Lindemann, 1986). Prior to their death, the animals in these experiments showed a rapid increase in plasma ascorbic acid, suggesting a more rapid synthesis of the vitamin than following intraperitoneal injections. Therefore, experiments were designed to modify gulonolactone oxidase so that it would be suitable for intravenous administration.

PEG-enzyme conjugates have characteristics that are suitable for infusion via this route. Studies with other proteins show that covalent attachment of PEG increases their circulating lifetime, lessens their reactivity with preformed antibodies against the protein and their susceptibility to proteolytic attack, and decreases their immunogenicity (Abuchowski et al., 1977; Abuchowski and Davis, 1981). In the studies discussed below, the characteristics of a PEG-gulonolactone oxidase conjugate were investigated. The effects of its administration are described as well.

A. Attachment of PEG to gulonolactone oxidase

1. Characteristics of the PEG-conjugate

Highly purified gulonolactone oxidase was coupled to the PEG polymer in an effort to obtain a modified enzyme possessing characteristics suitable for intravenous administration. PEG activated with succinimidyl succinate reacts covalently with primary amine groups of proteins. The scheme for this reaction is presented in Figure 16. The extent of the reaction was determined. The number of primary amines of the enzyme that are blocked by reaction with PEG was estimated by reacting the modified enzyme with fluorescamine and measuring the resulting fluorescence. Fluorescamine should react only with the remaining unreacted primary amines. Based on the fluorescence of unconjugated enzyme, there were 39 primary amines per 50,000 daltons of protein. Following conjugation, there were 21 primary amines, a decrease of 47$\pm$4.8%. It is unclear whether this represents the addition of 18 polymers or if a

Figure 16. Scheme showing attachment of the polymer polyethylene glycol (PEG) to a protein.

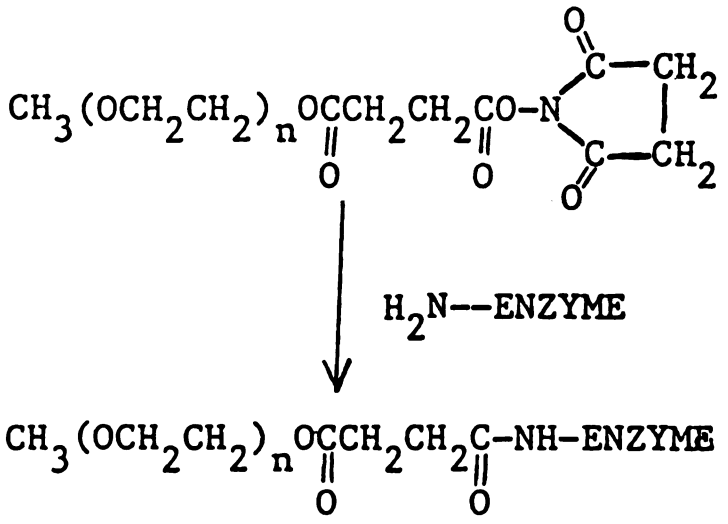

CH3(OCH2CH2)nOCCH2CH2CO-N
O
O
C—CH2
C—CH2
O
O
H2N--ENZYME
CH3(OCH2CH2)nOCCH2CH2C-NH-ENZYME
O
O

Figure 16

smaller number of attached PEG polymers prevents fluorescamine from reacting with a number of the unreacted primary amines.

In an effort to resolve this question, PEG-gulonolactone oxidase (PEG-GLO) was electrophoresed in SDS-polyacrylamide. Addition of each PEG should increase the molecular weight of the protein by 5,000 daltons. Assuming that enzyme with attached PEG migrates in SDS-polyacrylamide gels similarly to unmodified protein, comparison of the enzyme bands should provide an estimate of the number of PEG molecules attached to the protein. Eighteen PEG molecules per enzyme would add 90,000 daltons to the molecular weight for a total of 140,000 daltons. Results from such experiments have been inconclusive, however, perhaps because attachment of PEG changes the physical properties of the enzyme. Results from the electrophoresis of partially purified gulonolactone oxidase are shown (Figure 17). The protein appeared to be changed by the PEG modification. PEG was conjugated to a more highly purified form of gulonolactone oxidase and electrophoresed in further efforts to determine the molecular weight. This PEG-GLO preparation could not be detected in the silver-stained gels. Perhaps, it did not enter the gel or migrated as a very diffuse band making detection difficult. Determination of the molecular weight by using other methods may be necessary.

2. Catalytic properties

The kinetic parameters of PEG-GLO were also determined to examine how the enzyme had been affected by the modification. Analysis of the apparent kinetic constants showed little loss of catalytic properties. Apparent kinetic constants were calculated from Lineweaver-Burk double reciprocal plots. Results are summarized in Table 8. Upon modification with PEG, there was no significant change in the apparent Km. The enzyme activity decreased by approximately 30%.

Figure 17. Electrophoretic migration of PEG-gulonolactone oxidase compared to the unmodified enzyme in SDS-polyacrylamide gels. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out using a 7.5% gel. The lanes contained the following: A - PEG-gulonolactone oxidase; B - unmodified gulonolactone oxidase; C - a more highly purified gulonolactone oxidase; and D - calibration proteins bovine serum albumin, ovalbumin, and chymotrypsinogen A.

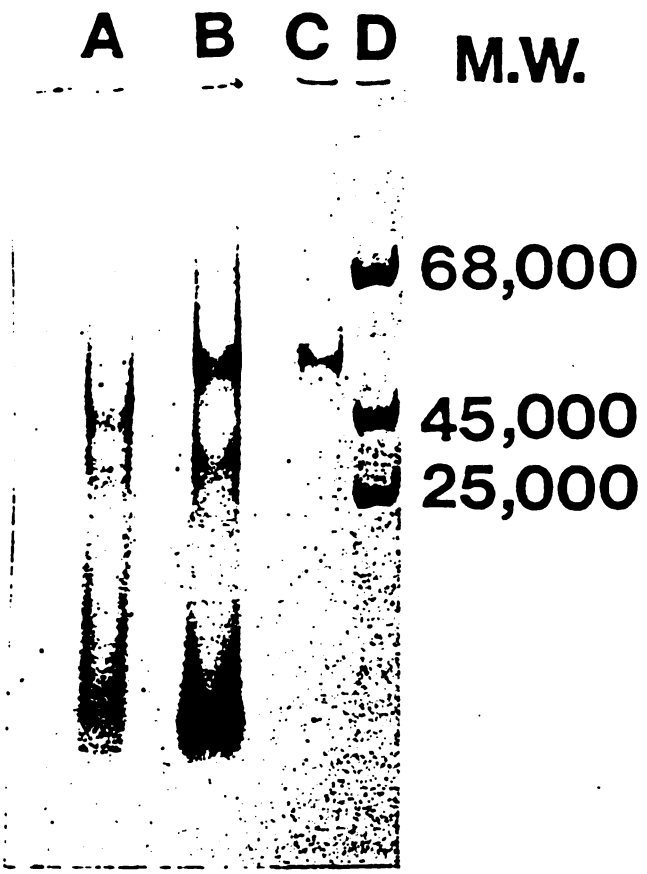


Figure 17

TABLE 8

Comparison of Kinetic Parameters of Gulonolactone Oxidase and PEG-Gulonolactone Oxidase

	Unmodified GLO	PEG-GLO
Recovery	100	73.9±3.9
Km (mM)	0.089±0.026	0.065±0.011

The apparent Km of the modified and unmodified enzyme was determined from Lineweaver-Burk double reciprocal plots. Recovery is expressed as a percentage of the unmodified enzyme activity at the substrate concentrations used in the usual assay. Km is expressed as a mM concentration.

B. Stability of PEG–gulonolactone oxidase

1. *In vitro* stability of PEG–GLO activity

The stability of PEG–GLO at 37°C and to trypsin digestion was compared to that of the unmodified enzyme in *in vitro* assays. The results are shown in Figure 18. Enzyme activity was greatly stabilized at 37°C by the modification. PEG–GLO retained 50% of its activity for more than 24 hr, whereas the unmodified enzyme lost 50% of its activity within 10 min. Little, if any, protection from trypsin digestion was afforded by conjugation with PEG. The activity of both preparations decayed to less than 50% within 6 min.

2. Circulating half-life of PEG–GLO activity

Experiments were continued to examine whether the circulating half-life of enzyme activity was extended by the modification. Conjugation of gulonolactone oxidase with PEG does not prolong the plasma half-life of activity in guinea pigs. The plasma half-life of PEG–GLO activity *in vivo* was not significantly different (54 min) from that of the unmodified enzyme (50 min) (Figure 19).

3. *In vitro* stability of PEG–GLO activity in plasma

To determine whether this loss of enzyme activity from the circulation was the result of the PEG–enzyme leaving the vasculature or being inactivated within the circulation, an *in vitro* experiment was conducted. PEG–GLO was incubated with fresh guinea pig plasma at 37°C. Enzyme activity was assayed at various times over a 4-hr period and compared to the results obtained from the *in vivo* half-life experiments (Figure 20). For the most part, the decline of enzyme activity in plasma *in vitro* paralleled that rapid decline in activity observed in the circulation. These results provide evidence that inactivation within the circulation may account for the rapid loss of PEG–GLO activity, as opposed to it leaving the vasculature. Since proteolytic degradation in the plasma

Figure 18. (A) Stability of unmodified gulonolactone oxidase to incubation at 37°C and trypsin digestion. Unmodified gulonolactone oxidase (0.2 U activity/ml) was incubated at 37°C alone (●) and in the presence of trypsin (0.5 mg/ml) (O). Enzyme activity was assayed at various times over a 90-minute period and is expressed as a percent of the initial activity. (B) Increased stability produced by modification with PEG. PEG-gulonolactone oxidase (0.02 U activity/ml) was incubated at 37°C alone (●) and in the presence of trypsin (0.5 mg/ml) (O) over a 24-hr period. Enzyme activity is expressed as a percent of the initial activity. One unit (U) of activity equals 1 μmole of ascorbic acid formed per minute.

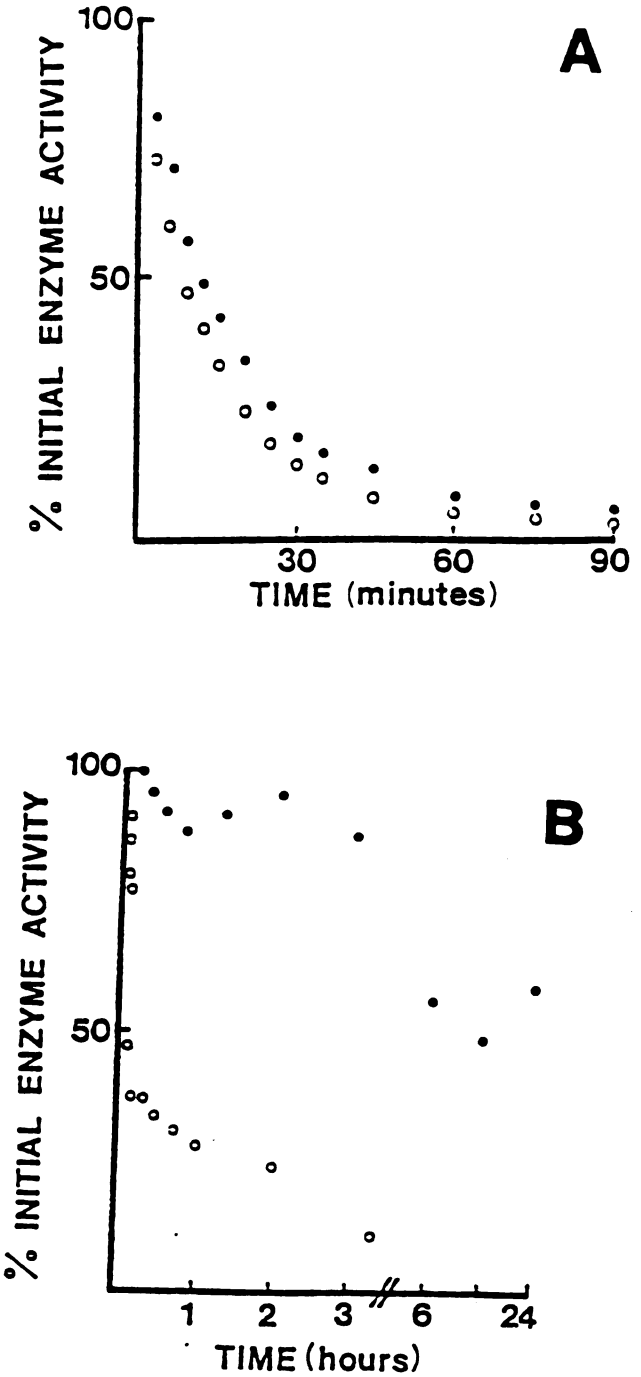

A
100
50
% INITIAL ENZYME ACTIVITY
30
60
90
TIME (minutes)
B
100
50
% INITIAL ENZYME ACTIVITY
1
2
3
6
24
TIME (hours)

Figure 18

Figure 19. Plasma half-life of PEG-gulonolactone oxidase compared to the unmodified enzyme. Doses of unmodified gulonolactone oxidase ranging from 0.16 to 0.36 U of activity were infused intravenously into 4 guinea pigs (●—●). Another group of 4 animals received i.v. infusions of 0.08 to 0.51 U of PEG-gulonolactone oxidase (○-○). One unit (U) of activity equals 1 μmole of ascorbic acid formed per minute. Blood was sampled at the time points indicated over a period of four hours and enzyme activity was determined in plasma. Activity is plotted as the percentage of the activity in the 10 min sample.

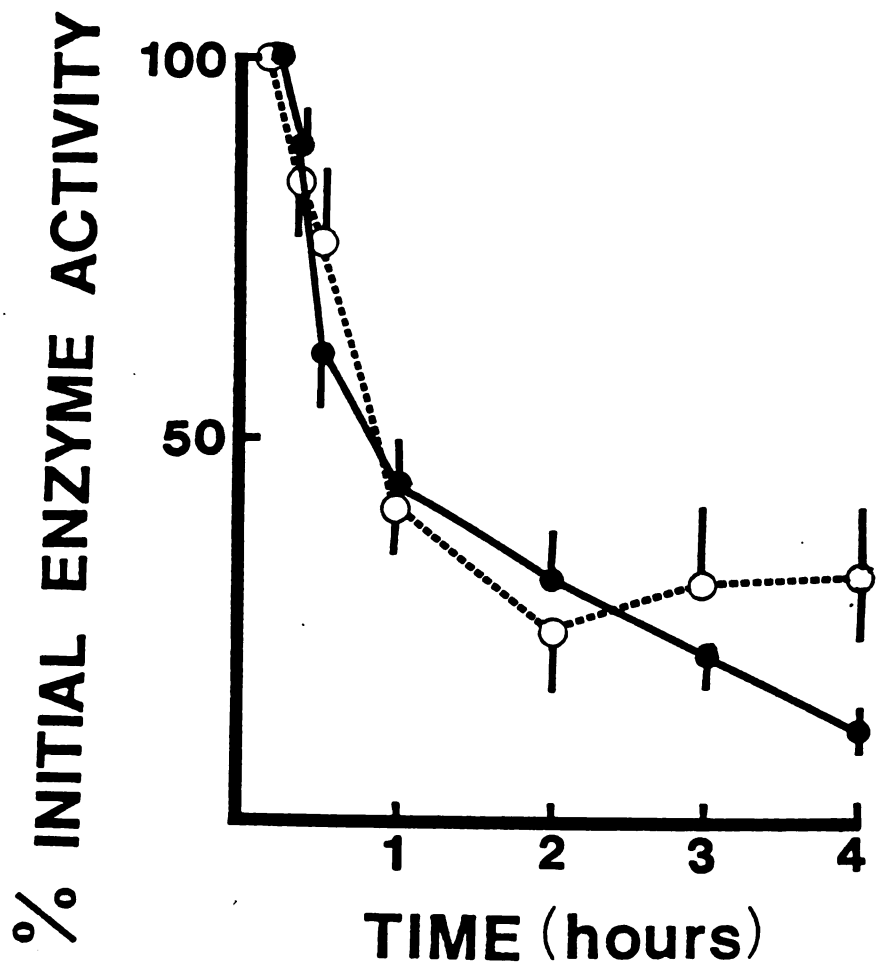


Figure 19

Figure 20. In vitro half-life of PEG-gulonolactone oxidase in plasma. PEG-gulonolactone oxidase was incubated with fresh guinea pig plasma at 37°C and enzyme activity was tested over a 4 hr period (●—●). Enzyme activity is expressed as a percent of the initial activity which was 0.007 U/ml. One unit (U) of activity equals 1 μmole of ascorbic acid formed per minute. The results from one experiment are compared with those obtained from in vivo studies previously shown in Figure 19 (O--O).

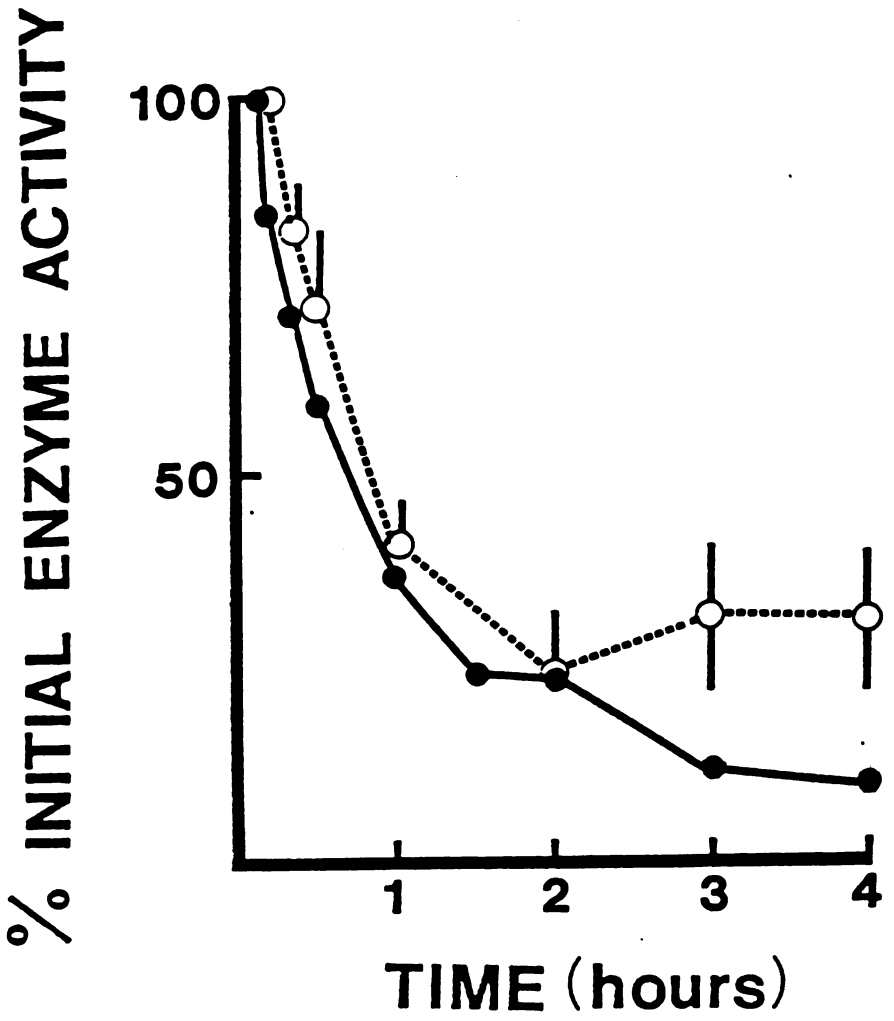


Figure 20

may be the basis for its short circulating half-life, conjugation of PEG to this protein did not appear to protect it from such degradation.

C. Administration of PEG–gulonolactone oxidase

1. Single dose studies

Intravenous administration of PEG–GLO along with infusion of substrate, to ascorbic acid–deficient guinea pigs elicited ascorbic acid biosynthesis. This was reflected by increases in plasma and tissues concentrations of the vitamin. Plasma ascorbic acid concentrations in PEG–GLO–treated guinea pigs increased rapidly to over four times their pretreatment values by the first sampling time point (45 min). Concentrations remained at that level through the second sample at 1.5 hr. After that, concentrations declined, but they remained significantly greater than the concentrations of control animals for a 3-hr period following infusion of the modified enzyme (Figure 21). The steady decline in plasma ascorbic acid concentrations was not surprising, given the rapid elimination of administered enzyme activity that was observed.

On the day following the plasma sampling experiments, tissue concentrations of ascorbic acid were determined in the PEG–GLO–treated and control animals. The control animals had been given modified α-galactosidase instead of the gulonolactone oxidase. All of the tissues tested, except for the brain, showed a significant increase in ascorbic acid concentrations as a result of the gulonolactone oxidase treatment (Table 9). Tissue concentrations following treatment were an indication of ascorbic acid biosynthesis elicited by the enzyme replacement therapy. These tissue ascorbic acid concentrations were 2-3 times those found in animals maintained on an ascorbic acid-free regimen (Veen-Baigent et al., 1975). This finding appeared to rule out the possibility that the observed increase in plasma ascorbic acid was a result of release of the vitamin from the tissues.

Figure 21. Intravenous administration of PEG–gulonolactone oxidase increases plasma ascorbic acid concentrations. A group of eight, 14-day ascorbic acid-deficient guinea pigs received an i.v. infusion of PEG–gulonolactone oxidase (●—●). Four control animals received α-galactosidase modified in the same manner (○-○). Plasma ascorbic acid concentrations were measured. Gulonolactone (100 mg/1 ml) was given immediately before administration of the enzyme and 800 mg/5 ml was infused intravenously over a 3-hr period after the enzyme injection. Three plasma samples were tested to establish pretreatment ascorbic acid concentrations and the enzyme was infused at zero time. Blood samples were taken every 45 minutes thereafter. Statistics were done using mixed design analysis of variance and individual comparisons were made using Dunnett's test for between group comparisons. The vertical bar denotes $\pm$ the SEM for between group comparisons. After the PEG–gulonolactone oxidase infusion, all the points up to and including the 3-hr time point are significantly higher than control ($p < 0.05$). These points are indicated by an asterisk. The standard error for within group comparisons is indicated for each group (SE_{WG}).

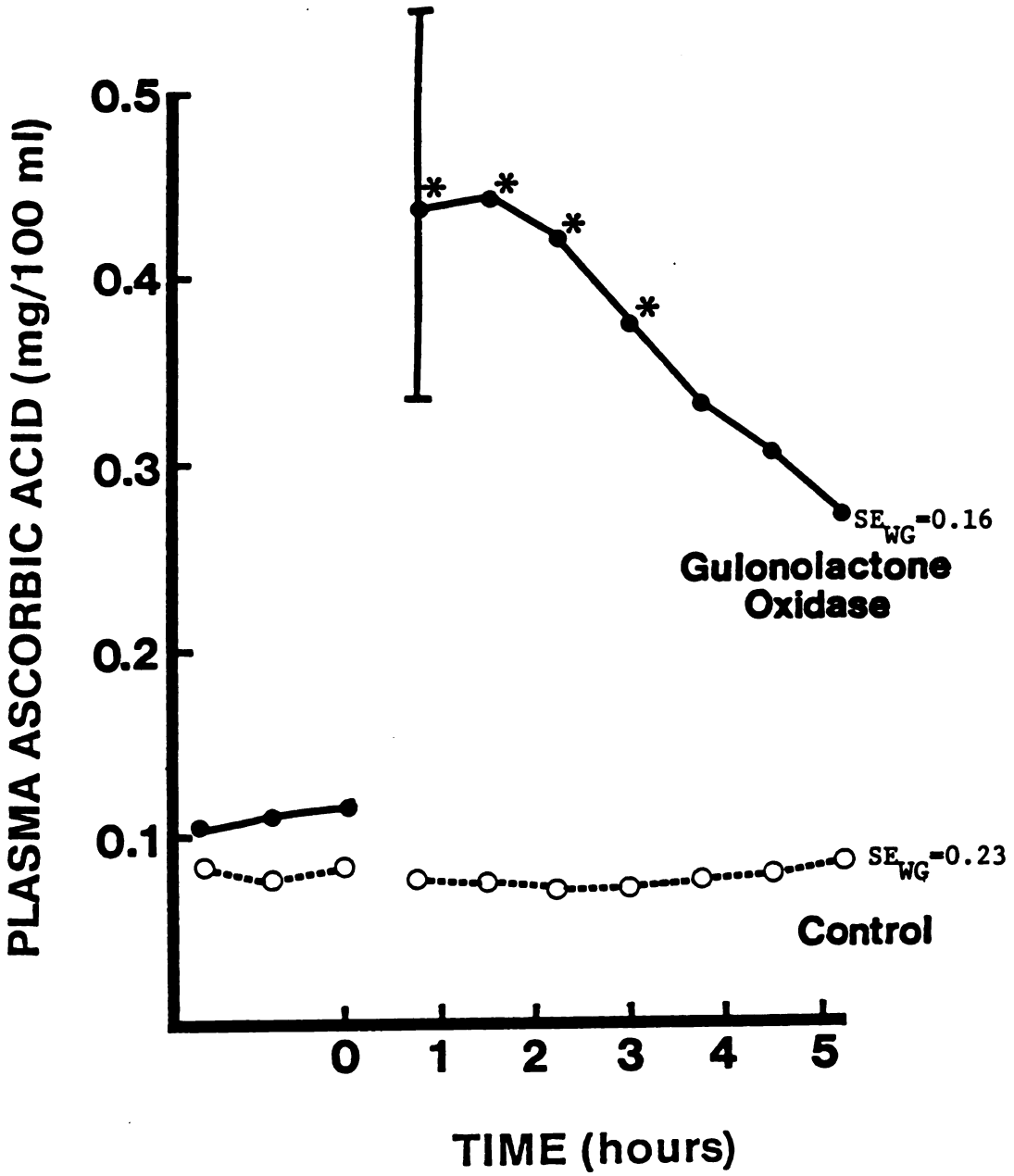

PLASMA ASCORBIC ACID (mg/100 ml)
0.5
0.4
0.3
0.2
0.1
0
1
2
3
4
5
TIME (hours)
SE$_{WG}$=0.16
Gulonolactone Oxidase
SE$_{WG}$=0.23
Control

Figure 21

TABLE 9

Tissue Ascorbic Acid Concentrations Increase Significantly as a Result of PEG-GLO Treatment

Tissue	PEG-GLO-Treated Animals	Control Animals
Liver	3.53±0.56*	1.84±0.21
Lung	2.39±0.55*	0.65±0.08
Adrenal	8.25±0.95*	3.77±0.44
Spleen	4.04±0.98*	1.05±0.09
Kidney	3.76±0.58*	1.34±0.15
Brain	10.25±0.97	9.48±0.88
Heart	1.16±0.21*	0.46±0.07

At the conclusion of the plasma sampling experiments, both PEG-GLO-treated and control animals were sacrificed and tissue concentrations of ascorbic acid measured. Concentrations of the vitamin are expressed as mg/100 g tissue ± SEM. Statistics were done using the one-tailed t-test for independent groups. Asterisks denote significance at $p < 0.05$.

2. Dose-response relationship

The relationship between the dose of enzyme activity and the amount of ascorbic acid synthesis was examined. Comparisons of the amount of synthesis that occurred in response to the various enzyme doses administered were based on the area under the plasma ascorbic acid concentration versus time curves of the individual animals. The results are shown in Figure 22. With the administration of increasing amounts of gulonolactone oxidase activity, ascorbic acid biosynthesis by the animal increased linearly. The magnitude of the increase in plasma ascorbic acid concentrations was dependent on the dose of activity administered. This relationship had a correlation coefficient of 0.98.

D. Immunogenicity of PEG-gulonolactone oxidase

Another characteristic which prohibits the routine use of enzymes in therapy is the possibility that such foreign proteins will elicit immunologic reactions. Immunologic reactions can lead to allergic reactions as well as accelerated degradation of injected enzyme activity.

1. Immunoreactivity of the PEG-enzyme

The immunoreactivity of the PEG-GLO was tested first. The ability of the PEG-enzyme to react with antibodies specific for chicken kidney gulonolactone oxidase from three sources was tested (Figure 23). Antisera against unmodified enzyme from rabbits and guinea pigs and IgG purified from horse antisera all reacted with the modified enzyme. Conjugation with PEG did not prevent the enzyme from reacting with antibodies directed against the unmodified enzyme. Following reaction of the PEG-GLO with antibody, activity was not lost.

2. Immunogenicity of the PEG-enzyme

The immunogenicity of PEG-GLO was then examined. Guinea pigs were immunized with the PEG-GLO that had been homogenized in Freund's complete adjuvant. Three weeks later, they received booster injections of the

Figure 22. Elevations in plasma ascorbic acid concentrations are dependent on the dose of activity. Eight, 14-day ascorbic acid-deficient guinea pigs received i.v. infusions of various doses of PEG-gulonolactone oxidase and supplementation with the substrate, gulonolactone. Plasma sampling experiments like those described in Figure 21 were conducted with each animal. The area under the plasma concentration vs. time curve was calculated for individual animals, subtracting pretreatment concentrations of the vitamin (see example in upper right portion of the figure). The dose of PEG-gulonolactone oxidase administered is expressed as units of activity/kg body weight.

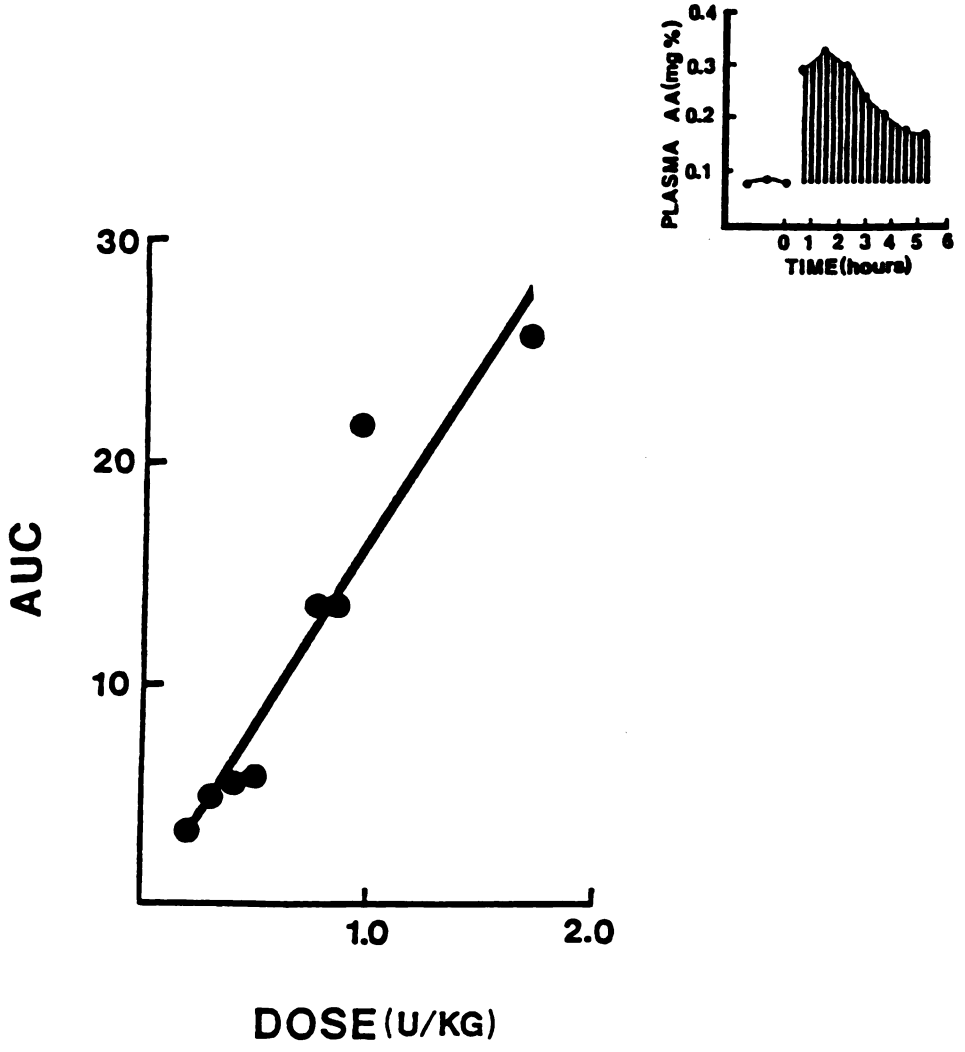

PLASMA AA(mg %)
0.4
0.3
0.2
0.1
0 1 2 3 4 5 6
TIME(hours)
30
20
10
AUC
1.0
2.0
DOSE(U/KG)

Figure 22

Figure 23. PEG-gulonolactone oxidase reacts with antiserum against the unmodified enzyme. Ouchterlony immunodouble diffusion tests were carried out to test the ability of PEG-gulonolactone oxidase to react with antiserum raised against the unmodified enzyme. The wells contained the following: 1 -horse anti-gulonolactone oxidase IgG; 3 - rabbit anti-gulonolactone oxidase serum; 5 - guinea pig anti-gulonolactone oxidase serum; and 2, 4, and 6 - saline. Center wells contained PEG-gulonolactone oxidase. Plate A was stained for enzyme activity and Plate B was stained with Coomassie Blue.

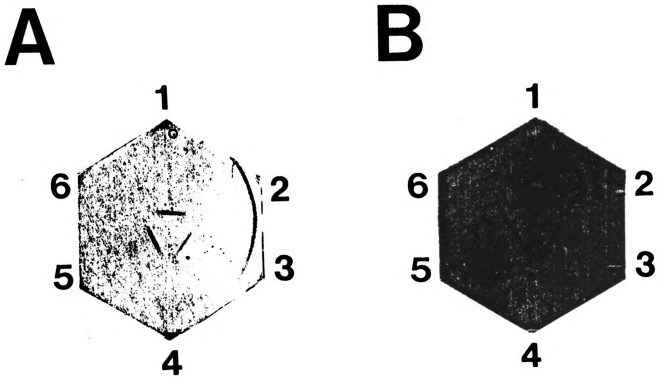


Figure 23

preparation. Serum samples were obtained one week after the booster injection. These immunized guinea pigs formed precipitating antibodies against the unmodified enzyme, but antibodies that reacted with PEG-GLO were not detected in Ouchterlony immunodouble diffusion tests (Figure 24). Despite the immunogenicity of this modified enzyme, animals were able to tolerate a second intravenous infusion of PEG-GLO.

Figure 24. Antibodies against the unmodified enzyme are detected in sera from guinea pigs immunized with PEG–gulonolactone oxidase. Ouchterlony immuno-double diffusion tests were carried out on sera from guinea pigs immunized with PEG–gulonolactone oxidase. The wells contained the following: 1, 4 -unmodified gulonolactone oxidase; 2 rabbit anti-gulonolactone oxidase serum; 3, 5 -PEG-gulonolactone oxidase; and 6 - horse anti-gulonolactone oxidase serum. A serum sample from a guinea pig immunized with PEG–gulonolactone oxidase was placed in the center well. The plate was silver stained. Faint precipitin lines are present between wells 2 and 3 and between 5 and 6, but can not be seen in this photograph.

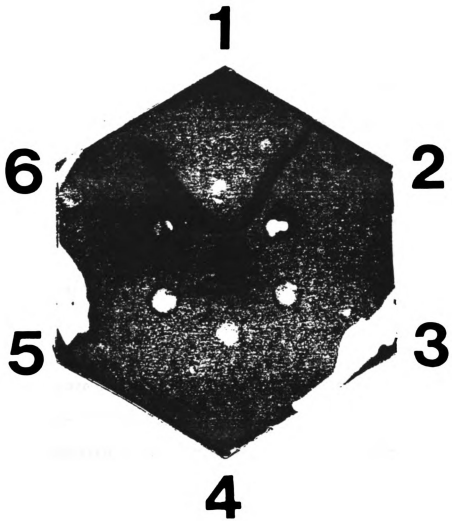


Figure 24

DISCUSSION

Scurvy in guinea pigs was used as a model inborn metabolic disease in studies to examine the suitability of various enzyme replacement therapy protocols. Chemically modified forms of gulonolactone oxidase, the missing enzyme in ascorbic acid biosynthesis, were administered to guinea pigs to test factors such as stability of the enzyme, and the immune response and therapeutic response of the animals. Modification has the potential to prolong the stability of enzymes upon injection as well as to decrease their toxicity, thereby improving their usefulness in therapy. Two enzyme modifications were examined in these studies and their safety and effectiveness for enzyme replacement therapy characterized.

I. Crosslinked Immunoprecipitated Gulonolactone Oxidase

A. Properties afforded by this modification

The crosslinked immunoprecipitated form of gulonolactone oxidase was studied extensively in these experiments. Several of the basic problems associated with enzyme administration can be overcome by using immunoprecipitation and crosslinking. For example, large quantities of pure enzyme for therapy are easily obtained. Only microgram amounts of enzyme are required for immunization and volumes of antisera sufficient to immunoprecipitate gram quantities of enzyme from crude tissue homogenates are obtained. The antibody reacts specifically with the enzyme protein, removing it from the tissue homogenate. Comparatively large amounts of enzyme can be obtained rapidly in this

way, as opposed to the low yields obtained using classical enzyme purification procedures. Rapid loss of enzyme activity presents another problem when considering the use of enzymes as therapeutic agents. Both thermal inactivation and degradation by circulating proteases contribute to the brief half-life of injected enzymes. Results from other investigators show that stability of enzyme activity is greatly enhanced under these conditions by immunoprecipitation. Covalent modifications, including crosslinking, also promote increased stabilization under conditions of heat degradation and protease digestion (Snyder et al., 1974; Poznansky, 1979; Wold et al., 1973; Klibanov, 1979). Although the procedures themselves result in an initial loss of enzyme activity, the remaining activity is greatly stabilized. It is believed that the covalent interactions and crosslinkages hold the enzyme structure together, making it less susceptible to breakdown.

The fact that foreign proteins are immunogenic also presents a challenge in their administration. Repeated injections of the foreign enzymes are often necessary to obtain the desired therapeutic effect. If an immune response is initiated, the patient may experience severe, and possibly fatal, anaphylaxis upon subsequent injections of the enzyme. As a result, therapy may have to be discontinued. Attachment of plasma proteins to the foreign enzyme may confer characteristics that make it appropriate for repeated administration (Borel, 1980; Poznansky, 1983). Conjugation of certain proteins, such as immunoglobulins or albumin, to foreign proteins is capable of rendering them nonimmunogenic. With this in mind, therefore, the suitability of crosslinked immunoprecipitated gulonolactone oxidase for enzyme replacement therapy was examined.

B. Modifying gulonolactone oxidase with homologous antiserum

Previous work from this laboratory confirmed that gulonolactone oxidase could be modified by immunoprecipitation and crosslinking. The modified

enzyme complex retains catalytic activity and can be therapeutically beneficial to scorbutic guinea pigs (Sato and Walton, 1983; Sato et al., 1986). In those experiments, the enzyme was immunoprecipitated with antisera raised in guinea pigs. Administration of this modified enzyme increases plasma ascorbic acid concentrations and reverses scurvy in guinea pigs. Survival of scorbutic animals can be extended to at least 100 days with this enzyme replacement therapy. It was concluded from these studies that animals tolerated the repeated injections of enzyme complex because homologous serum was used to modify the foreign enzyme. The homologous antiserum was proposed to mask the antigenic determinants of the enzyme and, as a result, prevent the guinea pigs from recognizing the enzyme as being foreign. It has been reported that when a foreign enzyme, such as uricase, is conjugated to homologous serum albumin (Remy and Poznansky, 1978) the antigenicity of the foreign uricase is greatly diminished.

The utility of the modification procedure could be limited if the use of homologous serum is an absolute necessity. To use this modified enzyme in human therapy, it would be necessary to obtain large amounts of human sera to perform the modification. This would not be practical. An explanation for tolerance of the modified enzyme complex by the animals is proposed by the results of this study. The enzyme preparation may be tolerated because it is immobilized within a large stable complex. Enzymes immobilized by a variety of methods have characteristics which make them useful for enzyme therapy (Chang, 1976). Administration of enzymes immobilized by adsorption or covalent linkage to certain polymers, entrapment within a synthetic matrix, and microencapsulation has been tested. If immobilization of the foreign enzyme is the reason that repeated injections are tolerated, then use of heterologous sera to modify the enzyme should also make the enzyme safe for therapy. Therefore, studies were performed to investigate whether heterologous serum could be used. Results from

these experiments should provide further insight into the reason for detoxification of the foreign enzyme by this procedure.

C. Modifying gulonolactone oxidase with heterologous antiserum or IgG

For the studies reported here, antiserum against gulonolactone oxidase was raised in rabbits and a horse. Under optimized conditions, the enzyme was modified using the specific antiserum or IgG purified from it. The resulting immunoprecipitate was crosslinked with glutaraldehyde. Covalent crosslinkages, introduced into the complex by this bifunctional reagent, should reinforce the interactions between the enzyme and antibody and slow the breakdown of the complex. In previous studies, immunoprecipitates that were not crosslinked were shown to be toxic when injected into guinea pigs (Sato and Walton, 1983). The toxicity of these immunoprecipitates was attributed to their subsequent breakdown.

Following modification, gulonolactone oxidase retained catalytic activity. The apparent affinity for substrate was not significantly changed by this procedure; there was a loss in activity, however. This decrease in activity occurred upon immunoprecipitation, with little additional decrease effected by the glutaraldehyde reaction. This loss in activity was probably brought about by changes in the enzyme structure as a result of antibody binding. The maximum velocity of the reaction could not be attained after immunoprecipitation. The affinity of the enzyme for the small molecular weight substrate, gulonolactone, was apparently unchanged however. While it is unfortunate that this activity was diminished after the procedure, it was not unexpected. Loss in activity frequently occurs following covalent modification of the enzyme protein. Other chemical modification and immobilization procedures cause similar or greater losses in activity (Uren and Ragin, 1977; Kamisaki et al., 1981; Nickle et al., 1982). If the modification improves the stability of enzyme activity, however, this initial

loss in activity becomes less important. Prolonging the half-life of enzyme activity upon injection into the animal may compensate for the initial loss during modification.

Results from experiments conducted in vitro showed that the stability of this XL-IP gulonolactone oxidase was improved. The stability of the enzyme modified using purified IgG was examined, alone and in the presence of the proteolytic enzyme trypsin, and compared to that of the unmodified enzyme under the same conditions. Immunoprecipitation and crosslinking enhanced the stability of gulonolactone oxidase. The half-life of the modified enzyme activity was nearly 150 times longer than that of the unmodified enzyme. In the presence of proteolytic digestion by trypsin, its half-life of activity was approximately 20 times greater than that of the unmodified enzyme. Although experiments to examine in vivo half-life were not done, these results give some indication that stability may be prolonged in the animal also. The observation that stabilization occurs even in the presence of proteolytic digestion supports this.

Results from single dose studies demonstrate that gulonolactone oxidase modified with whole antiserum retained activity in vivo. Intraperitoneal injections of XL-IP gulonolactone oxidase, along with substrate supplementation elicited a three-fold increase in plasma ascorbic acid concentrations. This increase, which occurred over a 5-hour period, suggests that the enzyme remained active at least over a period of several hours after injection. Ascorbic acid is rapidly absorbed after intraperitoneal injection and rapidly removed from the circulation (Hornig, 1975); the steady increase in plasma concentrations of this vitamin suggest continued synthesis. The potential for use of this modified enzyme complex to treat scurvy is also suggested by these results. A therapeutic effect was shown by the initial administration and no apparent adverse reactions were observed.

A critical consideration in the treatment of enzyme deficiency diseases is that the patient is able to tolerate repeated injections of the foreign enzyme. It may be necessary to treat these patients for their entire lifetime. With continued therapy, patients often become immunologically sensitized against the foreign protein and, upon subsequent injections, may experience severe allergic reactions. Long-term enzyme replacement therapy was initiated in order to test whether repeated injections of this XL-IP gulonolactone oxidase could be safe, as well as therapeutically beneficial. To this end, ascorbic acid-deficient guinea pigs received weekly injections of the XL-IP enzyme along with substrate supplementation. The ability of this therapy to prolong the survival of these animals for at least 50 days was examined. Without treatment, guinea pigs usually survive only 23-28 days on the ascorbic acid-deficient diet (Jones et al., 1973; Barnes et al,. 1973). The mean survival time of scorbutic pigs is 27.3±0.29 days. Of the fourteen gulonolactone oxidase-treated animals, only 3 survived for the 50-day period. Four more of the treated guinea pigs survived significantly longer than animals fed the deficient diet alone. The mean survival of these additional animals was longer than 31.4 days, which is outside the range of survival time of scorbutic animals. It was concluded, therefore, that this XL-IP enzyme could be therapeutically beneficial to some animals. There was concern that all or at least a greater number of the animals did not survive for the entire treatment period however. A higher percentage of successfully-treated animals would be required if the procedure were to be considered for use in the treatment of other metabolic diseases. Understanding the reasons for the early death of some animals was necessary.

It was postulated that some animals survived because they did not initiate an immune reaction against the foreign enzyme complex. Perhaps they had become immunologically tolerant; their immune processes did not recognize

the modified enzyme complex. The animals that did not survive, on other hand, may have experienced an immune response and died as a result of some delayed hypersensitivity reaction. These animals died 3-6 days after their last treatment, a period consistent with this hypothesis (Eisen, 1980). Testing the immune response of the three fifty-day survivors revealed that these animals did respond immunologically to the components of the modified enzyme complex. Serum samples obtained from these animals after their 50-day treatment period contained antibodies against both components of the XL-IP gulonolactone oxidase complex. These results confirm that the survivors also responded immunologically to repeated injections of the foreign proteins. Their survival, therefore, could not be attributed to the absence of an immune response. Furthermore, their response showed that the complex was not tolerogenic; the individual components of the complex retained antigenicity.

Additional tests of the surviving animals' response to these foreign proteins revealed that they experienced symptoms characteristic of anaphylactic shock when challenged with intravascular injection of the antiserum component of the complex. In contrast, the animals did not react when challenged with intravascular injections of the enzyme. These studies showed that the guinea pigs were sensitized to the serum component of the enzyme complex. Their survival, despite this sensitization, supported the conclusion that the other animals' deaths were probably not the result of an allergic reaction. Even the survivors were sensitized to the complex.

The reason for the early death of most of the XL-IP gulonolactone oxidase-treated animals in this study remained unclear. The possibility that absence of an immune response allowed a few animals to survive had been ruled out because it was demonstrated that the survivors were sensitized to the XL-IP enzyme complex. Another factor that may have contributed to the limited

success of this therapy was the possibility that this XL-IP enzyme complex was toxic. This consideration could be eliminated by the studies performed with other enzymes. These studies are discussed below. Inadequate ascorbic synthesis by the administered enzyme was another possibility and this was examined in continuing studies.

D. Application of the modification procedure to other enzymes

Experiments were carried out to determine whether the modification procedure could be used to modify other enzymes possessing therapeutic potential. Modification of three enzymes was performed and the toxicity of the resulting XL-IP complexes examined in mice and guinea pigs. Serum cholinesterase, histidase, and asparaginase were the enzymes studied.

The catalytic properties of these enzymes were affected to varying extents by this procedures. Histidase activity was, in fact, almost completely inactivated by the procedure. Kinetic parameters could not be determined for this modified enzyme complex. Neither changing the antiserum concentrations nor adding substrate, histidine, to the reaction mixture protected histidase from inactivation during the modification. The source of histidase for the experiments reported here was P. fluorescens cells. It is conceivable that, using another source of histidase, an active complex may be obtained from the modification. Covalent modification of histidase from Corynebacteriaceae with polyethylene glycol has been done with retention of 98 % of the original activity (Dwivedi and Davis, 1985), whereas histidase from P. fluorescens is inactivated by the PEG-conjugation.

Both serum cholinesterase and asparaginase retained sufficient activity to determine their kinetic parameters. These enzymes were affected to different extents by the procedure. Both enzymes lost activity upon modification. Fifty percent of the serum cholinesterase activity is retained even after cross-

linking. Asparaginase retained 20 to 70 percent as much activity as the unmodified enzyme. It was apparent from these studies that this procedure could be useful for modifying other enzymes. The effects of the modification varied according to the particular enzyme, but active enzyme complexes were obtained in several cases.

Studies were also conducted to examine the toxicity of these XL-IP enzyme complexes. The results suggested that the complexes would be safe for repeated administration. In these experiments, 69 mice and guinea pigs each received a series of intraperitoneal injections of a modified enzyme complex. Injections were given at 2-week intervals, a schedule consistent with eliciting allergic reactions (Kabat and Mayer, 1961). Mice were treated with enzymes modified using guinea pig sera and guinea pigs received injections of enzymes modified with rabbit antisera. In this way, all of the animals were treated with enzyme immunoprecipitates prepared from heterologous sera. None of these animals experienced an allergic reaction to any of the injections of XL-IP enzyme complex. In contrast, results from another study show that six of nine animals given similar amounts of unmodified asparaginase experience allergic reactions after the second or third injection (Sato and Lindemann, 1985). The results reported here provided additional proof that enzymes modified by this method were safe for repeated use.

The animals in these experiments were also tested for sensitization to the components of the XL-IP enzymes used in their treatments. Like the gulonolactone oxidase-treated guinea pigs, these animals were sensitized to some component of serum, but not to the administered enzyme. Although one out of 11 animals showed some reaction following injection of the unmodified enzyme, this may have been a result of the cardiac puncture. In contrast, 52 of the 66 animals challenged with the foreign serum component clearly experienced severe reac-

tions. Following the intravascular injection of serum, the animals showed acute, immediate shock-like reactions. They experienced convulsions, respiratory distress, and violent itching. While these symptoms are characteristic of the anaphylaxis mediated by reaginic antibodies and eventual mast cell release of mediators, similar severe and fatal reactions have been observed with serum sickness (Cecil and Loeb, 1959). With the high degree of sensitization that might occur upon repeated exposure to the foreign serum component of the XL-IP enzyme, immediate shock-like reactions could occur with subsequent intravascular administration of serum alone. In the sensitized animals, reaction of the circulating antibodies with the antigen (foreign serum proteins) could result in the formation of immune complexes that may trigger complement activation and formation of the anaphylatoxins associated with it. The immediate reactions observed in these animals could be this type of serum sickness reaction. The underlying mechanism for the shock-like reaction (reaginic antibodies or serum sickness) can not be determined from these studies. However, it is clear that the animals were sensitized to the heterologous sera used to modify the enzymes.

The fact that the animals experienced allergic reactions only to the serum component may have to do with the arrangement of the XL-IP enzyme complex. The complex may be formed in such a way that the enzyme surface was covered by antibodies. This could allow for greater sensitization to the surface antibodies than to the enclosed enzyme. This hypothesis is supported by evidence of increased sensitization to the surface components of a complex by the immune system (Novotny et al., 1986).

E. Administration of gulonolactone oxidase modified with purified IgG

The observation that most animals treated with the heterologous immunoprecipitates became allergically sensitized to the serum component of the complex led to attempts to remove shocking agent. The IgG fraction was purified

from the antiserum. It was postulated that removal of serum proteins other than IgG would result in a more uniform modified enzyme complex. Perhaps this would improve the safety of the treatment and the success of the gulonolactone oxidase replacement therapy. Following modification of the enzyme, the formation of a less contaminated preparation was demonstrated. This modified gulonolactone oxidase possessed catalytic activity as evidenced by plasma sampling experiments. Furthermore, it could be administered for at least a 100-day period without apparent adverse effects in normal guinea pigs. These results indicated its potential for use in the treatment of scurvy.

Despite the fact that a preparation containing fewer serum contaminants was obtained and shown to be safe in normal guinea pigs, the success of the gulonolactone oxidase replacement therapy was not improved at all. None of the ascorbic acid-deficient animals treated with this preparation survived for 50 days. It was concluded that early death in these animals was not a direct result of toxic components in the preparations used in their therapy. Other factors may have limited the survival of these animals.

Another factor that was considered to play a role in the limited success of the gulonolactone oxidase therapy was the dose of enzyme activity administered. Perhaps most animals were not able to synthesize sufficient quantities of ascorbic acid for survival with the doses of enzyme activity that they received. The few survivors may have been able to tolerate a marginal supply of the vitamin. It was believed that sufficient doses were being given, however, based on plasma and tissue levels of ascorbic acid following enzyme administration. A closer analysis was in order.

A detailed analysis of results from a previous study (Sato et al., 1986) permitted a comparison of the doses of enzyme activity administered to survivors and nonsurvivors of the enzyme therapy protocol. The doses received by animals

that survived for 100 days in this study were compared to those received by animals that did not survive. If the doses given to most of the animals were only marginal, one would expect that the survivors had received higher amounts of activity. This was not the case. There was no correlation observed between successfully treated animals and those that received the highest amounts of activity. There was no significant difference between these administered doses. The mean dose received by the survivors was 0.69 ± 0.03 units/treatment, while the non-survivors received 0.66 ± 0.04 units/treatment of enzyme activity.

Comparisons of the cumulative doses of activity received by each of these animals, throughout their treatment period, were made as well. This type of comparison permitted consideration of the possibility that the nonsurvivors received consistently lower doses of enzyme activity over extended periods, with only occasional high doses. Prolonged periods of treatment with insufficient enzyme activity may have been the reason for death in these animals. By simply comparing the average doses this could not be observed. The cumulative doses of enzyme activity administered to all of these animals were similar. In fact, some of the survivors had received lower cumulative doses of activity than did the nonsurvivors. Furthermore, many of the successfully treated animals received higher amounts of enzyme activity than did the survivors of the 100-day protocol. From this analysis it did not appear that inadequate enzyme doses were the major factor responsible for the death of these animals. The conclusion that insufficient ascorbic acid synthesis was the reason for the limited survival of gulonolactone oxidase-treated guinea pigs was not supported. There must be other factors that are important in determining their survival.

The condition of ascorbic acid-deficient animals that received the gulonolactone oxidase replacement therapy regimen and ascorbic acid supplements gave support to the hypothesis that factors other than dose were important.

Doses of ascorbic acid necessary to meet the minimum requirements of these animals were administered with each enzyme treatment (Chatterjee, 1967). This supplementation of vitamin C was intended to make up for the possibly inadequate vitamin synthesis catalyzed by the administered enzyme. It did not seem to accomplish this however. Despite ensuring adequate vitamin C intake, these animals did not appear to thrive as well as guinea pigs fed the normal diet. Taken together with the results from the activity dose analysis, it was concluded that another factor(s) must be critical for survival of these scorbutic guinea pigs.

Because insufficient ascorbic acid synthesis did not appear to be the cause of death of scorbutic XL-IP gulonolactone oxidase-treated guinea pigs, the possibility that inadequate supplies of other nutrients contributed to their early death was investigated. Several observations led to the consideration of this possibility. From the experiments with XL-IP asparaginase, histidase, gulonolactone oxidase, and serum cholinesterase, it was known that all of the animals fed the normal chow diet survived repeated injections of the modified enzyme complexes. In most cases, these animals looked like normal untreated animals and did not appear to suffer any adverse effects. Perhaps differences in the ascorbic acid-deficient diet contributed to nutritional deficiencies suffered by nonsurvivors.

Other factors may have played a role as well. For example, it is known that guinea pigs maintained on an ascorbic acid-deficient diet become anorexic after a period of approximately 14 days (Peterkofsky et al., 1986). Perhaps the guinea pigs on the vitamin C-deficient diet began to experience this anorexia about the time that treatment was initiated and, therefore, did not eat sufficient amounts of food to maintain adequate nutrition. Furthermore, these animals received the doses of enzyme via intraperitoneal injection. Such injections may have been irritating and further aggravated the anorexia. Patholo-

gic examinations of several gulonolactone oxidase-treated animals that had died early in the studies were also done. Death of these animals was attributed to deficiencies of other vitamins. Specifically, these guinea pigs died from complications resulting from vitamin A and E deficiencies. It was, therefore, postulated that supplementation of these vitamins and possibly others may be necessary for the animals maintained on the ascorbic acid-deficient diet.

Comparisons of the vitamin and mineral contents of the two diets revealed few differences. Except for vitamin C content, the nutrient levels were similar. Overall, the ascorbic acid-deficient diet was concluded to be adequate. Data from the suppliers supported this conclusion. Thus, specific differences in the diet were not thought to cause the deficiencies detected in the postmortem examinations of these animals.

The efficacy of gulonolactone oxidase therapy in prolonging the survival of scorbutic guinea pigs was greatly improved by supplementing the animals with other vitamins. Guinea pigs that were maintained on the ascorbic acid-deficient diet and treated with weekly injections of XL-IP gulonolactone oxidase also received injections of vitamins A, B-complex, D, and E and selenium. Quantities approximating the weekly requirement of these vitamins and selenium were injected with each enzyme treatment (Fox et al., 1984). All of the animals survived significantly longer than animals maintained on an ascorbic acid-deficient diet alone. Four of them survived for at least 100 days and the fifth survived for 68 days. The fifth animal probably died as a result of an infection (Enterobacter cloacea) and complications of peritonitis. This was thought to result from puncturing the intestine during injection of the enzyme suspension. Otherwise, in most cases, the appearance of these animals was similar to that of untreated normal guinea pigs.

F. Metabolic fate of the administered enzyme complex

The metabolic fate of the crosslinked immunoprecipitated gulonolactone oxidase was examined. After development of a complex that was safe for administration, it was important to study its metabolic fate. Such studies might indicate a potential of this XL-IP enzyme to cause toxicity over a long period of time. There did not appear to be any toxicity or lasting effects noted in the necropsy reports of animals from the 100-day experiments. However, if the therapy were used in the treatment of humans, it may be continued for a much longer period of time. An individual could require treatment for his or her entire lifetime, amounting to years of therapy.

To examine distribution, guinea pigs were treated with a radioactively labelled XL-IP gulonolactone oxidase. The XL-IP enzyme was labelled with 14-C prior to injection. Distribution was monitored for a period of 10 days; one animal was sacrificed each day. Excretion of counts in the urine and feces was also monitored. Over the 10-day period, the percent of the radioactive dose recovered from the tissues and excretion products increased. The greatest recovery was on the tenth day. From individual tissues, the greatest percentage of the dose appears in the urine, kidney and liver. Large concentrations of the radioactive dose appeared in the spleen, liver, kidney, and urine.

These results give some idea of the uptake and metabolism of this XL-IP enzyme complex. The observation that the total dose is recovered slowly over a 10-day period, with only 50% of the dose being recovered at that time, suggests that the immunoprecipitate is only slowly removed from the peritoneum. This is similar to observations from the implantation of other immobilized enzymes into the peritoneal cavity (Chang, 1976). If the particulates are small, they may pass directly into the lymphatic duct and into the systemic circulation. However, particulates of greater than 20 um, especially if rigid, do not pass into the

lymphatic system, but stay in the peritoneal cavity. With time, they may be surrounded by phagocytes, and depending on the compound, may persist there for many months. Crosslinked immunoprecipitates may behave similarly upon injection into the peritoneal cavity. This modified form of the enzyme is solid and prior to injection, it was suspended in dextran. The particles may have been of varying sizes and thus remained in the peritoneal cavity for more or less time, depending on their size. The fact that it takes at least 10 days to recover up to 50 % of the administered dose supports this.

The radiolabelled XL-IP enzyme complex appeared in the highest concentrations in the spleen, initially, followed by high concentrations in the liver, kidney, and urine. This distribution is similar to that of other administered heterologous proteins (Fletcher et al., 1958; Halpern, 1974). Tissues that are rich in mononuclear phagocytes, for instance the liver and spleen, have the highest concentrations of radioactivity at the earliest times following administration. Thus, the complexes might ultimately be transported to the liver and spleen for further degradation and metabolism and then to the kidney and excreted in the urine.

In retrospect, a method of washing the peritoneal cavity should have been attempted in an effort to remove any residual counts from this area. In this way, the suggestion that the complex remained in the peritoneal cavity could be confirmed. Perhaps the total administered dose could be accounted for also. There is also the possibility that some of the dose could have been accounted for by analysis of the expired carbon dioxide. A larger number of animals might also be examined to obtain a sample population on which statistics could be performed. However, these results do give some indication of the fate of this XL-IP enzyme complex after its administration. Since its half-time of residence in the

peritoneum appeared to be on the order of 7-10 days, it may present a problem when considering repeated injections in humans over an entire lifetime.

G. Reasons for detoxification of the modified enzyme complex

Studies were conducted in an effort to determine the mechanisms by which crosslinked immunoprecipitated enzymes were made safe for therapeutic use. Two possible mechanisms of "detoxification" were proposed. The use of homologous sera for the modification procedure in previous studies (Sato and Walton, 1983; Sato et al., 1986) was concluded to be a critical factor in detoxification of the complex. The homologous antibodies were thought to cover antigenic determinants of the foreign enzyme. In this way, the enzyme could not be recognized as foreign by the guinea pig. The second possibility was that immobilization of the enzyme within a large stable complex was critical for "detoxifying" of the foreign enzyme. To differentiate between these possibilities, heterologous sera was used for the modification procedure in these studies. If homologous sera is necessary, then a complex formed using heterologous sera would be toxic. It is reported here that, even with the use of sera from a heterologous source, the XL-IP enzyme complex was rendered safe for repeated administration. This was demonstrated using four different enzymes to treat over 80 mice and guinea pigs. Animals can be injected repeatedly with the complex and do not suffer any observable allergic or other reactions to these injections. Homologous antisera does not appear to be the crucial factor in rendering the complex safe. It can be concluded that immobilization of the enzyme within a large complex must be involved in its "detoxification".

One reason for the ability of animals to accept repeated injections of the modified enzyme complex could be that the complex is tolerogenic. Under certain conditions, foreign antigens act as tolerogens (Eisen, 1980) in which case the animal fails to form antibodies against the particular foreign antigen.

Induction of tolerance may occur when a hapten is bound to either homologous or heterologous immunoglobulin carriers (Venkataraman et al., 1977). This did not occur in these studies however. The enzyme attached to heterologous immunoglobulin was not tolerogenic. The animals treated with the heterologous crosslinked immunoprecipitates formed antibodies to both the enzyme and antibody components of the complex. This complex retained its immunogenicity and did not act as a tolerogen.

Despite the immunogenicity of this XL-IP enzyme, repeated injections of the complex as a whole did not elicit any allergic reactions. Nor was there any reaction upon challenge with the enzyme component of the complex. Intravascular injection of the antiserum component alone did, however, precipitate an immediate shock-like reaction similar to anaphylaxis. Since the complex in its entirety did not elicit such a reaction, its stability is such that either it does not release fragments recognized by reaginic or other antibodies or perhaps the fragments are released in such a way that they are unable to precipitate an immediate reaction. Reaction of antibodies with the XL-IP enzyme complex may have occurred so slowly that anaphylaxis was not precipitated. Anaphylaxis depends on the rapid formation of antigen-antibody complexes. This causes the release of chemical mediators involved in the allergic reaction. Alternatively, perhaps the complex is nonimmunogenic as long as it remains together. The immune reaction may not take place until the complex begins to break down. At that time, antibodies may form against both components of the modified enzyme. So the complex itself will not precipitate an anaphylactic reaction.

Another interesting observation is that the antiserum, but not the enzyme, component of the modified enzyme complex can precipitate an immediate hypersensitivity reaction. Whether it was anaphylaxis or an acute serum sickness reaction can not be determined. However, the high degree of sensitiza-

tion to the serum component might be explained by the final formation of the crosslinked immunoprecipitate. If the antibodies are on the surface, enclosing the enzyme, this may provide for their greater interaction with the immune processes and subsequently greater sensitization to this component. However, it was demonstrated that the animals were also immunologically sensitized to the enzyme. They formed serum antibodies against this component. An explanation for this is that the animals actually became desensitized to the enzyme. It is possible that the amount of enzyme in the complex is small compared to the amount of antibody. The animals still responded to it as an immunogen, forming antibodies against it, but the repeated injections of these relatively small amounts effected desensitization to this component. One could compare it to allergy desensitization schedules, where small quantities of an allergen are injected at predetermined intervals in an effort to make the individual less sensitive to the particular antigen. Upon subsequent exposure to the particular antigen an allergic response is prevented.

The possibility that the immune response against the antibody component of the complex is greater than that against the enzyme component was not examined. The type and amount of particular classes of antibodies against each component is not known. Perhaps, this could help in determining why only the antibody component elicited an immediate hypersensitivity reaction and what type of reaction it was. The antibody component may have elicited the formation of more reaginic or other antibodies than did the enzyme. Delineation of the particular classes would be necessary to draw this conclusion, however. Quantitative analysis of antibody classes cannot be obtained with Ouchterlony immunodouble diffusion tests.

II. PEG-Conjugated Gulonolactone Oxidase

The purpose of these studies was to develop a modified form of gulonolactone oxidase that would be soluble and appropiate for intravenous administration. Various polymers have been attached to enzymes in an effort to improve their utility in therapy (Holcenberg et al., 1975; O'Driscoll et al., 1975). While some of these modifications have been shown to extend the half-life of enzyme activity in plasma and prevent proteolytic degradation, the immunogenicity of these complexes was not tested. Polyethylene glycol has been used to modify a number of enzymes of potential therapeutic value (Abuchowski et al., 1981; Chen et al., 1981; Davis et al., 1981; Naoi et al., 1984). This polymer is uncharged and has a terminal hydroxyl group which can be activated for attachment to the amino groups of proteins. Also, this conjugation reaction can be carried out under relatively mild conditions and perhaps not affect enzyme activity adversely.

For the most part, the PEG-enzymes have been shown to possess characteristics which greatly improve their utility in therapy. Notably, following conjugation to PEG, the circulating half-life of enzyme activity is prolonged and the enzyme is less susceptible to thermal degradation and proteolytic digestion. Immunogenicity, which limits the use of these foreign proteins in therapy, is also diminished by this modification procedure.

A soluble modified form of gulonolactone oxidase was obtained by covalent attachment of PEG to partially purified chicken kidney gulonolactone oxidase. PEG that was activated with succinimidyl succinate was reacted with the enzyme in a 5-fold molar excess of the primary amines of the enzyme preparation. This particular activated PEG preparation, rather than cyanuric chloride-activated PEG, was used because a greater percentage of enzyme activity is preserved (Abuchowski et al., 1984).

A. Characteristics of the PEG-gulonolactone oxidase conjugate

Following attachment of the PEG polymer to gulonolactone oxidase, various properties were examined to find out how it had been affected by the modification. The catalytic properties were tested and efforts to determine the molecular weight were attempted.

The catalytic properties of gulonolactone oxidase were not affected drastically by the modification. Seventy percent of the activity was recovered following the reaction and there was no significant change in the apparent affinity for substrate. This occurs even though nearly 50% of the primary amines of the enzyme are reacted with the polymer. Thus, a modified enzyme which retained activity in vitro had been attained. With this outcome, tests could continue to examine the activity of this modified enzyme in vivo.

Knowing the number of reactive amino groups on the molecule and the extent to which PEG reacted with these groups, the number of PEG molecules attached to the enzyme molecule could be estimated. It was estimated that 18 polymer molecules were attached to the enzyme molecule, with each one being 5000 daltons. This would be an addition of 90,000 daltons to the protein. The molecular weight of the enzyme is on the order of 50,000. Therefore, the conjugated enzyme would have a molecular weight of 140,000 daltons. Efforts to confirm this by SDS-polyacrylamide gel electrophoresis were not successful. Protein bands of the PEG-modified partially purified preparation did migrate differently through the gel, but molecular weight could not be calculated from this preparation. The highly purified enzyme, modified with PEG, did not show up on the gel.

Attachment of the PEG polymer likely effects changes in the protein, making it difficult to use this method to determine the molecular weight of the conjugate. SDS is a negatively charged moiety and covalently attaches to all of

the positive charges on the protein molecule. In this way, the proteins being analyzed take on a uniform negative charge and migrate based on their molecular weight. It is possible that once PEG is attached to the protein, SDS can not interact as well with it. PEG is an uncharged moiety covalently attached to the protein. It is not a linear polymer, either (Maxfield and Shepherd, 1975) and this could affect the migration of the modified enzyme through the gel. Once PEG is covalently bound, it may also prevent the interaction of SDS with protein. So the PEG-protein may retain its charge properties, which could interfere with its migration. Many PEG enzymes migrate much slower than expected based on their estimated molecular weight. Therefore, with SDS polyacrylamide gel electrophoresis a falsely high value is obtained for the molecular weight (Dr. Robert Yue, Enzon Inc., personal communication). Results using gel filtration have been variable as well, again probably due to the changes induced by the PEG polymer. Thus, currently the best estimate of molecular weight that we have is based on the number of reactive primary amines in the preparations before and after the reaction. From this, the number of PEG molecules bound to the protein can be calculated.

B. Stability of PEG-gulonolactone oxidase

Enzyme therapy is often limited because of the brief half-life of the enzyme activity in the circulation. The enzyme can be rapidly inactivated by proteolytic digestion as well as thermal degradation. One goal of this modification was to improve the stability of gulonolactone oxidase activity in the circulation. The stability of other enzymes is greatly improved under these conditions following modification with PEG (Abuchowski et al., 1977b; Davis et al., 1981; Ho et al., 1986).

The stability of the PEG-gulonolactone oxidase (PEG-GLO) activity was first tested under in vitro conditions at 37°C alone and in the presence of the

proteolytic enzyme trypsin. Attachment of PEG stabilized enzyme activity to incubation at 37°C, but enhanced stability of the modified enzyme to trypsin digestion was not observed. The concentration of trypsin used in these experiments was 10-fold less than that used in experiments with other enzymes (Abuchowski et al., 1977b) and these enzymes still showed increased stability. Furthermore, the action of trypsin may be somewhat limited compared to other proteases because of the specificity of its action. Other proteolytic enzymes could be even more effective in degrading PEG–gulonolactone oxidase. Although PEG confers stability to other proteins, the activity of this enzyme is not greatly stabilized under these in vitro conditions by this modification.

Experiments were continued to see if improvement in the circulating half-life of gulonolactone oxidase was observed following the modification. In previous studies with catalase, despite some destabilization in vitro, circulating half-life of the PEG–catalase is prolonged (Abuchowski et al., 1977b). Guinea pigs were given infusions of either the unmodified or the PEG–GLO and activity in plasma was tested for a 4-hour period afterward. No significant prolongation of the half-life of enzyme activity resulted from the modification. The half-life of enzyme activity was virtually the same for both the unmodified and modified preparations. These results were also different compared to the results obtained from the modification of other enzymes with PEG. The circulating half-life of PEG–arginase and PEG–asparaginase is extended up to hours (Savoca et al., 1979) and days (Ho et al., 1986), respectively.

Although it was not surprising that PEG–GLO had a brief circulating half-life, given the results from in vitro studies, preliminary experiments were performed in order to determine whether proteolytic digestion was responsible for this phenomenon or whether tissue uptake played a role. PEG–GLO was incubated in fresh guinea pig plasma at 37°C and enzyme activity was tested at various

times over a 4-hour time period. The decline in enzyme activity which occurred under these conditions paralleled that which occurred in the circulation. These results suggested that degradation in the plasma, perhaps by proteases, may have been entirely responsible for the rapid disappearance of enzyme activity from the circulation. It is, however, possible for PEG-enzymes to cross biological barriers (Abuchowski, 1981); PEG-asparaginase enters the circulation following intraperitoneal injection. There may be little doubt that degradation in the plasma contributes greatly to the steadily declining activity in the circulation, but tissue uptake can not be ruled out entirely. Observations following the administration of a single dose of PEG-GLO to guinea pigs support further examination and are discussed below.

C. Administration of PEG-gulonolactone oxidase

Scorbutic guinea pigs treated with intravenous infusions of PEG-GLO along with substrate supplementation showed significant increases of plasma ascorbic acid. Plasma concentrations of ascorbic acid increased immediately as a result of administration of this modified enzyme. The four-fold increase in vitamin C levels is similar to the saturating concentrations found in guinea pigs fed a normal vitamin C-containing diet (Zannoni et al., 1974). Plasma ascorbic acid concentrations in these animals remained significantly greater than the concentrations found in control animals for three hours following the PEG-GLO infusion. The observed steady decline in plasma ascorbic acid is not unexpected given that PEG-GLO appears to be rapidly degraded. However, it is interesting that the vitamin C concentrations continued to rise through the second sample at 1.5 hours. From the circulating half-life studies, one notes a steep decline in circulating enzyme activity during this period. So it is curious that the plasma ascorbic acid concentration continued to increase. An explanation for this involves possible tissue uptake of the infused enzyme. Perhaps the PEG-enzyme

was taken up immediately upon intravenous infusion by the reticuloendothelial system. At that location it retained activity and thus continued to catalyze ascorbic acid synthesis, resulting in the maintained level of the vitamin throughout this time point. After 1.5 hours, concentrations of the vitamin began a steady decrease. This decline may represent actual degradation of the enzyme. Future experiments directed toward investigating the distribution of this modified enzyme following infusion may confirm this explanation.

The doses used for these experiments were less than or equal to those used in the studies with the XL-IP gulonolactone oxidase. Based on plasma ascorbic acid concentrations following a single dose, a similar therapeutic effect might be achieved with a smaller single dose of the PEG-GLO than with the same dose of the crosslinked immunoprecipated form of the enzyme. Using smaller doses would be advantageous. Intravenous administration of the enzyme obviously has the potential for greater efficacy, however, stability of the enzyme needs to be improved.

D. Immunogenicity of PEG-gulonolactone oxidase

The ability of patients to tolerate repeated doses of the modified enzyme is of paramount importance when considering administration of a foreign protein over an entire lifetime. If an immune response occurs in response to the initial injection of the foreign protein, there is the possibility of subsequent hypersensitivity reactions. These reactions can range from mild bronchspasm to severe, even fatal, anaphylactic shock. Thus, another major goal of modifying foreign proteins is to confer properties that make them less able to elicit immune and allergic responses. Covalent attachment of PEG to proteins has been shown to accomplish this for some enzymes. The reason for this is not clear. It is hypothesized that the polymer forms a protective shell around the enzyme preventing the immune processes from interacting with it (Abuchowski et al.,

1977a). PEG-conjugates may also exert their effects by inducing the formation of T suppressor cells (Sehon, 1982). These T-cells are instrumental in suppressing an immune response. Reaction of a protein with PEG may not only prevent immune responses, it is also proposed to prevent the protein from reacting with pre-formed antibodies against the native enzyme. If the patient has been previously sensitized to the unmodified form of the enzyme, then the PEG-conjugated form can be used to continue the therapy without the body reacting against it.

Reaction of gulonolactone oxidase with PEG did not abolish its ability to react with pre-formed antibodies against the unmodified enzyme. PEG-GLO reacted with antisera on IgG against the unmodified enzyme from three species. The PEG-enzyme retained immunogenicity as well. Antibodies against the unmodified enzyme were detected in serum from guinea pigs immunized with this conjugate. Attachment of the polymer to approximately 50% of the available amino groups did not prevent immunoreactivity or abolish immunogenicity. Contamination of the PEG-GLO preparation with unreacted enzyme could lead to these observations. However, all of the enzyme protein is believed to be reacted with the PEG polymer. It is believed that all of the antigenic determinants of gulonolactone oxidase were not covered by the PEG molecules.

The 50% reaction of amino groups may not be sufficient for this enzyme. Uricase, for example, must have up to 60% of the available amino groups reacted before it is incapable of either eliciting antibody formation or reacting with pre-formed antibodies (Chen et al., 1981). A similar situation exists with catalase (Abuchowski et al., 1977b). Even a 43% modification of the amino groups on this enzyme is insufficient to inhibit an antigen-antibody reaction or prevent an immune response. Perhaps providing for a more extensive reaction of gulonolactone oxidase with PEG would render it nonimmunogenic and abolish its ability to react with pre-formed antibody.

E. Comparison of gulonolactone oxidase with other PEG-enzymes

In these studies, it has been demonstrated that gulonolactone oxidase does not behave the same as many other enzymes that have been modified with PEG. Attaching PEG to this enzyme does not improve its stability or abolish its immunogenic properties. Comparing gulonolactone oxidase to the other enzymes may provide insight into the basis for these differences.

Further examination of the other enzymes in their native state shows that they are more stable prior to the modification than gulonolactone oxidase. Gulonolactone oxidase has a half-life of approximately 12 minutes at 37°C and must be frozen at -85°C to maintain activity. Asparaginase, on the other hand, is much more stable prior to PEG-modification. This enzyme, in its native form, resists denaturation by heat and acid (Cooney and Davis, 1970). When boiled in water for 20 minutes, 25% of the original activity is retained. Gulonolactone oxidase and other proteins are denatured under such conditions. Another example is bovine liver catalase. In its native form it remains relatively stable at 55°C (Abuchowski et al., 1977b). Unmodified β-galactosidase is also very stable compared to gulonolactone oxidase (Wallenfels and Malhotra, 1970). This enzyme loses no activity when incubated at 40°C for 30 minutes. Other enzymes that have been conjugated to PEG also exhibit enhanced stability prior to the modification (Abuchowski and Davis, 1979).

Another difference in gulonolactone oxidase may be the source from which it is isolated. Gulonolactone oxidase is a microsomal enzyme. It is isolated from the membrane of the smooth endoplasmic reticulum. While residing in this membrane, it would be surrounded by membrane lipids. These membrane lipids may aid in maintaining the enzyme in a conformation necessary for optimum activity. Removal of the enzyme from this environment results in destabilization. The enzyme may not maintain the appropriate conformation to retain activity. In

addition, the unfolded enzyme is more likely to be broken down (Tombs, 1986). Perhaps the PEG destabilizes the gulonolactone oxidase structure, contributing to its greater instability following modification. PEG is a hydrophilic molecule and confers characteristics of hydrophilicity to the enzyme when it is covalently attached (Maxfield and Shepherd, 1975). By making this enzyme more hydrophilic, its structure may be compromised even further.

SUMMARY AND CONCLUSIONS

The research presented in this dissertation involved investigation of enzyme therapy protocols, using scurvy in guinea pigs as a model metabolic disease. Gulonolactone oxidase, the missing enzyme in ascorbic acid biosynthesis, was chemically modified by two different methods in an effort to make it suitable for enzyme administration.

The first modification examined involved immunoprecipitation of gulonolactone oxidase with heterologous antisera followed by crosslinking the precipitate with glutaraldehyde. Enzyme activity was greatly stabilized by this modification. At 37°C, the half-life of activity was approximately 24 hours and in the presence of trypsin, the half-life was 3 hours. Unmodified enzyme, on the other hand, loses 50% of its activity within 12 minutes and 9 minutes, respectively, under the same conditions. Following intraperitoneal administration of the XL-IP gulonolactone oxidase along with substrate supplementation, scorbutic guinea pigs showed elevations in plasma ascorbic acid concentrations that were three times those of control animals. The XL-IP gulonolactone oxidase was also suitable for long-term therapy and prevention of scurvy in guinea pigs. The survival of ascorbic acid-deficient animals was prolonged to at least 100 days by the enzyme replacement therapy, compared to the usual time of survival of untreated scorbutic guinea pigs which is 23-28 days. Weekly supplementation of vitamins A, B-complex, D, and E and selenium was necessary for the successful treatment of these animals however. Besides the fact that guinea pigs maintained on this diet may become anorexic, the intraperitoneal injections may have resulted in additional irritation

and appetite suppression. With XL-IP gulonolactone oxidase replacement therapy and vitamin supplementation, the animals maintained on the ascorbic acid deficient diet grew at a rate comparable to normal, untreated guinea pigs.

Asparaginase, histidase, and serum cholinesterase, other enzymes having potential therapeutic value, were modified by immunoprecipitation and crosslinking. The catalytic characteristics of these enzymes were affected to varying extents by the modification. Histidase was completely inactivated by the procedure. However, asparaginase and serum cholinesterase retained 20-50% of their original catalytic activity following modification. A total of 69 mice and guinea pigs received repeated injections of these XL-IP enzyme complexes and none experienced allergic reactions during the series of injections. These results indicate that the modification procedure can successfully be adapted for use with other enzymes and render them safe for repeated administration.

Immobilization of the foreign enzyme within a large stable complex appeared to be the critical factor in "detoxification" of these foreign enzymes. The enzyme complexes as a whole were not toxic, even though they were prepared using the heterologous sera. The components of these complexes retained immunogenicity however, as the animals formed antibodies to both the enzyme and antibody components of the XL-IP enzyme complex. Upon challenge with each component individually, 52 of 66 animals responded with a shock-like immediate hypersensitivity reaction to the antibody component. Only 1 of 11 animals responded to the challenge with the foreign enzyme however. It was concluded that the complex may be formed so that the antibodies cover the surface of the enzyme. In this way, greater sensitization to this antibody component may occur. Furthermore, the enzyme may be present in relatively small amounts in the complex compared to the amount of antibody. Repeated injections of the complex might result in the animals becoming desensitized to the

enzyme component. This would explain why the animals do not repond to challenges with the enzyme alone. The complex in its entirety remains safe for repeated use.

Gulonolactone oxidase was also reacted with the polymer polyethylene glycol to obtain a modified enzyme appropriate for intravenous administration. Approximately 50% of the available amino groups of the enzyme molecule were reacted with this polymer. The stability of enzyme activity to protease digestion was not improved; 50% of the enzyme activity was gone within 10 minutes. Its stability to incubation at 37°C was increased as the PEG–GLO had a half-life on the order of 24 hours. Despite protection to incubation at 37°C, the circulating half-life of enzyme activity was not prolonged by the modification. PEG–GLO and the unmodified enzyme had similar circulating half-lives. Conjugation of gulonolactone oxidase with PEG could destabilize it. PEG is a hydrophilic polymer and by conferring such properties to the enzyme may contribute to its rapid degradation.

Proteolytic degradation may explain the rapid disappearance of enzyme activity from the circulation, however, tissue uptake may contribute somewhat. Intravenous administration of PEG–GLO to ascorbic acid-deficient guinea pigs along with substrate supplementation elicited at least a 4-fold increase in plasma ascorbic acid concentrations. These concentrations were maintained at this level for 1.5 hours and were significantly greater than those of control animals for 3 hours following PEG–GLO infusion. The elevated concentrations of the vitamin through 1.5 hours suggested continued synthesis of the vitamin despite the rapid decline in enzyme activity in the circulation during this period. The enzyme may have been taken up by the tissues where it remained active for a period of time.

Following modification with PEG, the ability of the gulonolactone oxidase to react with antibodies and elicit an immune response was still present. PEG–GLO

reacted with antisera against the unmodified enzyme. Precipitating antibodies against the unmodified enzyme were detected in serum from guinea pigs immunized with PEG-GLO. Despite the fact that PEG was attached to 50% of the amino groups of the enzyme, its antigenic determinants must not have been covered entirely. Some antigenic determinants remained exposed and reactive following the modification.

Enzyme replacement therapy is one approach toward the treatment of inborn errors of metabolism. Like other methods of therapy, it may not be applicable to the treatment of every metabolic disorder. Enzyme therapy may, however, be beneficial in the treatment of disorders where the abnormal substrate or metabolite is in equilibrium with the circulation. Freely diffusible substrates/metabolites could be acted upon by enzymes administered intravenously or intraperitoneally. Examples of diseases that may be amenable to enzyme replacement therapy include: adenosine deaminase deficiency, which results in immune dysfunction; phenylketonuria; and histidinemia. Gaucher's disease and Fabry's disease, conditions where abnormal lipid accumulation is the basis of the pathology, may also be treated with the particular missing enzyme. Initiation of therapy early may decrease the rate of accumulation of the abnormal substrates in tissues and plasma. The stability of injected enzymes as well as decreasing their immunogenicity must be accomplished before they can be used routinely.

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