Ann. Rev. Pharmacol. Toxicol. 1977. 17:97–116

ENZYMES AS DRUGS

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"Enzymes far exceed man-made catalysts in their reaction specificity, their catalytic efficiency, and their capacity to operate under mild conditions of temperature and hydrogen-ion concentration" (1). These properties have prompted investigators to use specific enzymes as therapeutic agents since the beginning of this century. Undoubtedly for centuries, proteolytic enzymes were part of mixtures of animal and plant materials used topically.

In pioneering experiments in the 1930s, Avery & Dubos isolated and characterized a bacterial enzyme which degraded the capsular polysaccharide of type III pneumococcus. Parenteral administration of this enzyme protected experimental animals from infection by this type of pneumococcus (2). Subsequently, enzymes have been used to promote fibrinolysis, proteolysis, and food digestion. In recent years, enzymic degradation of amino acids and folic acid derivatives have been used in cancer chemotherapy. An exciting new direction is enzyme replacement therapy in certain genetic diseases.

Enzymes have some unique disadvantages as drugs. For parenteral administration, they must be exhaustively purified to eliminate contaminating toxic materials. Enzymes are generally quite costly to prepare and are degraded in the body. They are large molecules with limited distribution within the host. In addition, the enzymes that are foreign proteins to the host are antigenic. The development of antibodies may prevent the use of the drug for prolonged periods of time as a result of decreased enzyme activity or severe hypersensitivity reactions.

In this review, we concentrate on the practical aspects of enzyme therapy by discussing (*a*) development of new enzymes, (*b*) *in vivo* pharmacology, pharmacokinetics, and therapeutic effects of these agents, and (*c*) experimental attempts to

overcome the problems of instability, short half-life, limited distribition, and immunogenicity. These aspects are illustrated mainly from experiments with amino acid–degrading enzymes. We briefly discuss the advances and special problems in the use of enzymes as fibrinolytic agents and as replacement in genetic diseases. A detailed discussion of enzyme therapy can be found in a review by Cooney & Rosenbluth (3). Enzyme therapy of cancer recently has been reviewed by Uren & Handschumacher (4). Asparaginase and glutaminase enzymes as antineoplastic agents have been reviewed in this series in 1970 (5) and elsewhere (6, 7).

DEVELOPMENT OF NEW ENZYMES

Sources and Purification of Therapeutic Enzymes

Microorganisms generally constitute the most practical source for producing large amounts of an enzyme for therapeutic use. Large quantities of a microorganism can be cultured at relatively low cost and in little time. Often the yield of enzyme produced may be greatly increased (10 to 100 fold) by manipulation of culture conditions or by selection of a desirable mutant strain. When bacteria are used to produce enzymes that are suitable for parenteral administration, it is essential that the final product be exhaustively purified to remove endotoxins. Enrichment culture techniques (utilizing the substrate compound as the sole carbon and/or nitrogen source) are utilized for selection of useful organisms and for enzyme induction. Microorganisms are ruptured by sonication or homogenization, and the enzymes are purified through the use of procedures that give high recoveries and can be readily scaled-up. These include precipitation by salts, organic solvents, heat treatment, or pH adjustment, and chromatographic separation with ion exchange, gel filtration, and affinity binding.

Replacement of defective lysosomal enzymes in genetic diseases presents special problems, because these enzymes are glycoproteins with determinants that are responsible for efficient incorporation into the lysosomes (8). Bacterial enzymes may lack these determinants. So far the human placenta has been the major source of these enzymes (9).

Characteristics of Therapeutically Useful Enzymes

Enzymes that degrade selected nutrients or metabolites in circulation should have the following characteristics:

1. High activity and stability at physiological pH.
2. Retention of activity and stability in animal serum and whole blood.
3. High affinity for the substrate (low K_m).
4. Slow clearance from the circulation when injected into animals.
5. No inhibition by its products or other constituents normally found in body fluids.
6. No requirement for exogenous cofactors.
7. Effective irreversibility of the enzymatic reaction under physiologic conditions.
8. Availability from a nonpathogenic organism that contains little endotoxin.

A number of amino acid–degrading enzymes that do not exhibit antitumor activity also fail to meet at least one of these criteria. For instance, *Escherichia coli* glutaminase has a pH optimum of 5 and essentially no activity at physiologic pH. An ineffective form of *E. coli* asparaginase has a K_m over 1 mM. Asparaginase enzymes from a yeast, *Bacillus coagulans,* and *Fusarium tricinctum* all have excessively rapid clearance rates in mice (7).

Clearance rates in mice bearing transplantable tumors may be misleading, since most of these tumors contain the lactate dehydrogenase–elevating virus. Infection by this virus greatly slows the clearance of some enzymes (10). Slow clearance in infected mice does not necessarily correlate with a long half-life in other animals.

The activity of some enzymes is tightly controlled by product inhibition, cooperative substrate kinetics, or other effectors. During treatment of mice with glutaminase-asparaginase, the glutamate level rises over 100-fold and greatly exceeds the glutamine concentrations. A glutaminase inhibited by its products would rapidly lose its effect under these conditions (6). A cysteine-degrading enzyme from *Salmonella typhimurium* shows a sharp fall in activity at cysteine concentrations below 0.1 mM (11). The sigmoid kinetics would greatly decrease its ability to deplete cysteine in circulation. The activity of glutaminase B from *E. coli* is allosterically regulated by carboxylic acids, divalent cations and adenine nucleotides (12). Such an enzyme might have very little activity in vivo.

Both *E. coli* asparaginase and *Acinetobacter* glutaminase-asparaginase are located near the surface of the bacterium, presumably in the periplasmic region between the plasma membrane and cell wall (13, 14). The enzymes have very low K_ms for these amino acids. An intracellular location of similar enzymes would require either their compartmentalization, repression of their synthesis, or some kinetic control of their activity to protect the pools of these amino acids from hydrolysis. Theoretically, enzymes secreted into the media or on the surface of the microorganism would not require these controls of their activity. One can select for these enzymes by searching for strains that demonstrate the activity in suspensions of whole cells or in the culture media.

Several amino acid–degrading enzymes require pyridoxal phosphate or other cofactors for activity. Meadows and his co-workers (15) have recently reported that tyrosine phenol-lyase from *Erwinia herbicola* inhibited growth of established B16 melanoma tumors. These workers, however, found that repeated injections of this enzyme caused only partial lowering of tyrosine in plasma of treated animals. The relatively weak depletion of tyrosine in vivo by this enzyme appears to be largely a result of its requirement for pyridoxal phosphate as a coenzyme. Administered pyridoxal phosphate has a half-life of only a few minutes in mice. Meadows and Elmer (16) have shown that the pyridoxal phosphate is rapidly stripped from the enzyme in vivo. The cofactor is also removed by incubation with albumin, alkaline phosphatase, or membrane preparations from liver. Limited antitumor activity has been noted with two other pyridoxal phosphate–requiring enzymes. Parodi et al (17) tested the therapeutic potential of *E. coli* tryptophanase. These workers reported that injections of very high doses [10,200 international units (IUs) per kilogram] of

purified tryptophanase into rodents produced only a 35% reduction in the plasma tryptophan level. Kreis & Hession (18) isolated a methionine-degrading enzyme from *Clostridium sporogenes* which partly inhibited the growth of the Walker 256 carcinosarcoma in rats. In addition to its pyridoxal phosphate requirement, this enzyme has a high K_m for methionine (90 mM). A depot form of this cofactor or covalent binding to the enzyme might greatly increase the activity of this type of enzyme.

Oki et al (19) reported that leucine dehydrogenase from *Bacillus sphaericus* was highly inhibitory to the Ehrlich ascites carcinoma. However, Roberts and his coworkers (20) found that a homogeneous preparation of this enzyme did not lower plasma leucine levels or inhibit tumor growth in mice. The lack of in vivo effectiveness of this enzyme may be explained by several factors. The concentration of its cofactor NAD is negligible in serum. The activity of this leucine dehydrogenase at pH 7.4 is less than 5% of that at its optimal pH of 11. Furthermore, the equilibrium constant of this reversible reaction indicates that very little leucine breakdown can occur at the pH and ammonia concentration of plasma.

Contamination by bacterial endotoxin represents a major problem with some enzymes. Loos et al (21) showed that many commercial preparations of *E. coli* asparaginase contain endotoxin. The Limulus gel test allows for much more rapid screening of preparations than did previously used pyrogen tests. Generally, enzymes from bacteria known to have high concentrations of endotoxin should be avoided.

IN VIVO PROPERTIES OF THERAPEUTIC ENZYMES

Pharmacology and Pharmacokinetics

All the asparaginase and glutaminase-asparaginase enzymes used for enzyme therapy are tetrameric molecules with a molecular weight of about 140,000 g/mole, similar to that of 7S gamma globulin (22). *E. coli* asparaginase has an absorption and distribution similar to that of other macromolecules of this size (23–25). In man and dogs, the volume of distribution of asparaginase is 20–30% greater than the plasma space. In man, intramuscular administration resulted in maximal plasma levels that were only half those observed at the same time after intravenous dosing. In contrast, plasma levels are comparable 24 hr after equal intravenous, intramuscular, and intraperitoneal doses in mice. Detailed studies in dogs showed that the enzyme slowly enters lymph and cerebrospinal fluid reaching a maximum 2–3 hr after an intravenous dose. The concentration in thoracic duct lymph rose to 25% of plasma, while neck and leg lymph did not exceed 5% and cerebrospinal fluid was less than 0.5% of plasma. The concentration in bile reached equilibrium rapidly but was never more than 5% that of plasma. Intra-arterial injections of either histamine or bradykinin caused increases of asparaginase activity in draining lymph.

Studies in patients showed slow appearance of the enzyme in thoracic duct lymph, very low levels in the cerebrospinal fluid, and no activity in the urine. When the enzyme was injected directly into the cerebrospinal fluid of the lateral ventricles, the

enzyme was rapidly transferred to the plasma so that by 24 hr no activity remained in the spinal fluid.

Schwartz et al (25) noted that pleural fluid in two patients had only 1–3% of the activity in plasma. Asparaginase, however, may persist within abnormal accumulations of body fluids. High levels of activity persisted for at least two days after injection of the enzyme directly into a pleural effusion. J. S. Holcenberg (unpublished observations) found a slow input and removal of several asparaginase and glutaminase enzymes in the peritoneal fluid of mice bearing the Ehrlich ascites tumor. Enzyme levels increased in this fluid for 24 hr and then decreased at a slower rate than plasma, so that the activity in the peritoneal fluid exceeded that in plasma by two to three days.

Extracts of normal and tumor tissues contain enzyme activity in excess of that in their residual blood content (26). We have measured the relative concentration of four asparaginase and glutaminase enzymes in extracts of subcutaneous EARAD tumors and plasma. The activity in tissue extracts was 10–15% of plasma 24 hr after a single dose of 300–2000 IU asparaginase per kilogram of each enzyme. It is not known whether this activity is only in the extracellular fluid or whether part of it is bound to the tumor cells.

Enzymes with high catalytic activity toward both asparagine and glutamine have less antitumor activity than *E. coli* asparaginase toward asparaginase sensitive subcutaneous tumors (27, 28). J. Roberts has shown that asparagine levels in subcutaneous EARAD tumors are lowered more by *E. coli* asparaginase than the glutaminase-asparaginase enzymes (unpublished results). In tissue culture, the dose of *E. coli* asparaginase needed to kill P5178Y cells is one tenth the dose needed for enzymes with high glutaminase activity. Amino acid levels in the tissue culture media show that asparagine is depleted at a slower rate by the glutaminase-asparaginase enzymes (29). Kinetic models predict this difference since the higher level of glutamine in culture media and body fluids effectively competes with asparagine for hydrolysis by the mixed substrate enzymes, but not with *E. coli* asparaginase.

The disappearance of *E. coli* asparaginase activity in mice infected with LDH-elevating virus and in dogs can be described by a single exponential function with a half-life of 24 and 14 hr, respectively (10, 23). After large doses in man the decay of plasma activity follows a biexponential function with half-lives of about 0.5 and 2.5 days (24, 30). Holcenberg (31) has described a model for asparagine concentration during asparaginase treatment. The plasma asparagine concentration at any time is dependent upon the rates of net input of the amino acid into the plasma and its hydrolysis by the enzyme. Woods & Handschumacher (32) have shown that the liver closely regulates the plasma asparagine concentration in the rat. This type of control can be described by the following equation:

$$\text{net plasma input} = I_{max} \cdot (1 - A/\bar{A}), \qquad 1.$$

where I_{max} is the maximal rate of asparagine input into the plasma, A is the asparagine concentration, and $\bar{A}$ is the normal controlled concentration of this amino acid. This equation fits the observations that the maximal input of this amino acid occurs when plasma asparagine concentration is low. When plasma asparagine

exceeds the controlling concentration $\bar{A}$, plasma input becomes negative, corresponding to the observed uptake of the amino acid by liver (32). The model assumes a constant maximal input rate for asparagine. Actually, synthesis of asparagine increases during periods of asparagine depletion.

L-Asparaginase activity follows Michaelis-Menton kinetics with no product inhibition. Therefore, the decay of enzyme activity in plasma with time can be described by the following equation:

$$\text{enzyme activity} = -A/(K_m + A) \cdot V_{max} \cdot \exp(-0.693\ t/T_{½}) \qquad 2.$$

where A is the concentration of asparagine, t is time, K_m is the Michaelis-Menton constant for the enzyme, $T_{½}$ is the half-life for decay of enzyme activity in the plasma, and V_{max} is the maximal velocity of the enzyme at an initial time. In man, two exponential terms are needed to describe the decay of enzyme activity with time.

The change in asparagine concentration dA/dt, is equal to the sum of the input term (Equation 1) and the enzyme activity term (Equation 2). Asparagine concentration at any time can be calculated by integration of this equation with an iterative process. Since the decay rate of the enzyme in plasma is considerably slower than its catalytic activity or the rate of input of asparagine, the calculation can be simplified by first determining the value for the exponential term at a particular time (Equation 2). Equilibrium is rapidly approached between enzyme activity and asparagine input. Therefore, the asparagine concentration can be calculated without integration by assuming that dA/dt equals zero.

The input rate can be determined experimentally by simultaneous measurement of the plasma asparagine and asparaginase activity. The maximal input rate is equal to the least enzyme activity needed to deplete the asparagine level to zero. Measurements indicate that the maximal asparagine input in mice is 1–5 nmole/min/ml plasma. This value is in the same range as the maximal input of asparagine from rat liver during perfusion studies (32). Using this input rate and the observed half-life of the enzyme, this model predicts closely the asparagine levels observed during treatment of mice and patients. The model may be useful in calculating dosing intervals needed to maintain a specific asparagine concentration and in the comparison of different enzymes. Similar calculations with a glutaminase enzyme indicate that the input rate of glutamine into plasma is approximately five times that of asparagine.

These input rates may also explain the inability of hemodialysis to deplete circulating levels of asparagine despite removal of large amounts of asparagine. An input of 2 nmole/min/ml plasma is equivalent to a total input of about 6 μmole/min in man. The clearance rate of asparagine by currently available dialysis techniques is far below this level.

Very little is known about the site or mechanism of clearance of natural or exogenous proteins (33). Modification of the reticuloendothelial system by zymosan did not change the clearance rate of L-asparaginase in dogs or guinea pigs (23). In mice without LDH-elevating virus infections, the half-life of *Acinetobacter* glutami-

nase-asparaginase is about 60 min. When the enzyme was labeled with I^{125} and injected into mice, the label did not concentrate in any organ but rapidly appeared in the urine as iodinated tyrosine (A. Montanaro, J. DiGiovanni, and J. Holcenberg, unpublished observations). The clearance of this enzyme was not affected by aggregated albumin which blocks Kupffer's cells pinocytosis or by desialylated α_1 acid glycoprotein which blocks the specific glycopeptide receptors on liver parenchymal cells. Thus, these proteins do not appear to be degraded by a selective process in the liver. A fruitful area of study would be the mechanism by which the LDH-elevating virus slows the clearance of this and other enzymes.

Lysosomal glycoprotein enzymes have a unique distribution and clearance after parenteral administration (8, 34). An intravenous dose of hexoseamidase A, ceramide trihexosidase, or glucocerebrosidase was very rapidly cleared from the blood of patients with genetic defects involving these enzymes. These enzymes appear to be incorporated into lysosomes primarily in the liver, but some activity is retained in the liver for prolonged times after plasma activity falls to control levels (34). Study of fibroblasts from patients with lysosomal enzyme defects indicates that the uptake of replacement enzymes is a highly specific process (35, 36). Further study is needed to define the receptors for this uptake and the characteristics of the enzymes that allow their incorporation into and persistence in lysosomes.

Examples of Therapeutic Uses of Enzymes

THERAPY OF NEOPLASIA BY ENZYMIC DEPRIVATION OF NONESSENTIAL AMINO ACIDS Depletion of specific nonessential amino acids in body fluids by enzymes offers a potential for cancer therapy with relatively high specificity for the neoplasm. Certain neoplasms are critically dependent on extracellular asparagine for their survival. Because asparagine is synthesized by normal cells and enters the bloodstream, simple dietary deprivation is not effective. For therapeutic effectiveness the amino acid must be continuously degraded in the body fluids by parenteral administration of the enzyme. Asparaginase exploits this specified asparagine requirement of certain neoplastic cells and, thereby, offers a much higher degree of selectivity for the susceptible tumor than conventional chemotherapeutic agents. L-Asparaginase was the first enzyme with antitumor activity to be intensively studied in man. About two thirds of acute lymphocytic leukemia patients when treated with asparaginase achieve complete remission (37, 38), whereas other neoplasms in man are relatively insensitive to asparaginase (39). Recent studies have indicated that asparaginase can rescue normal cells from the toxicity of high-dose methotrexate. Combination therapy with high-dose methotrexate and asparaginase has produced encouraging antitumor effects in human neoplasms (40, 41).

Glutaminase enzymes should have a greater antitumor potential than asparaginase enzymes (42). Glutamine, like asparagine, is a nonessential amino acid in the diet of humans. However, glutamine participates in a wide variety of metabolic reactions in mammalian cells (43). Cells grown in tissue culture require over ten times more glutamine than any other amino acid (44, 45). One of the important functions of glutamine in the metabolism of certain tumors may be as a direct precursor of glutamic acid, which can then furnish the carbon for the partial

operation of the citric acid cycle (an important energy source) (46). When compared with other tissues, certain tumor cells appear to operate at a marginal level of glutamine availability as a result of a slow rate of synthesis and a rapid utilization (46, 47). Therefore, glutamine deprivation may have selective toxicity toward certain tumor cells.

In 1964 Greenberg and co-workers reported that a glutaminase-asparaginase preparation with a relatively high K_m (7 mM) for glutamine decreased the initial rate of growth of a number of tumors, including an Ehrlich ascites carcinoma, but caused no significant increase in the survival time of tumor-bearing animals (48). Roberts and co-workers (14, 49) isolated and crystallized glutaminase-asparaginase from an *Acinetobacter* soil organism with the desired kinetic and molecular characteristics for in vivo activity and showed that asparaginase-resistant Ehrlich ascites carcinomas regressed completely following treatment with this enzyme. In tissue culture, *Acinetobacter* glutaminase-asparaginase selectively killed human leukemic leukocytes at about one hundredth the effective concentration of asparaginase (50). Broome & Schenkein reported that a glutaminase-asparaginase preparation derived from a *Pseudomonas* species caused temporary regression in a number of mouse lymphomas which were resistant to asparaginase (27). Bauer et al reported that glutaminase-asparaginase purified from *Pseudomonas aureofaciens* was inhibitory to 9 of 16 tumors in mice and rats (51). An asparaginase-resistant mouse adenocarcinoma was found to be especially sensitive to this enzyme. Hardy et al, however, noted that glutaminase-asparaginase from *Pseudomonas aureofaciens* had severe animal toxicity (52).

Schmid & Roberts (28) studied the toxicity of highly purified *Acinetobacter* glutaminase-asparaginase in C3H/HeJ, CD1, and BDF_1 mice. Daily injections of 250 IU/kg in mice infected with the lactate dehydrogenase-elevating virus maintained plasma glutamine and asparagine at undetectable levels while glutamate levels rose to 1700–2800 nmole/ml. To explore the possible toxicity of such high glutamate levels, C3H/HeJ mice were injected i.p. daily for 14 days with various doses of sodium glutamate. None of the injections, including doses of 100 mg per mouse, caused any overt toxicity. During a 90-min i.v. infusion of 100 mg sodium glutamate per mouse, the plasma glutamate level rose from 73 to 1775 nmole/ml and then dropped to normal by 5.5 hr after infusion. Daily treatment with 250 IU/kg glutaminase-asparaginase caused a maximal weight loss of 6 g in C3H/HeJ mice but little weight loss in CD1 and BDF_1 mice. Neutrophil counts reached a nadir of 800–1000/mm^3 on day 5 in each strain. Spleen weights and lymphocyte counts also decreased significantly. The hematocrit values in all treated groups decreased less than 20% from the normal values. BDF_1 mice tolerated i.p. administration of 500 IU/kg of *Acinetobacter* glutaminase-asparaginase daily for seven days, and three of five mice survived after treatment with 1000 IU/kg/day for seven days. Single doses of up to 6250 IU/kg were tolerated by these mice with only slight transient weight loss.

Holcenberg et al (53) showed that treatment with this enzyme only partially depleted free glutamine concentrations in muscle, spleen, small intestine, and liver. Brain and kidney glutamine concentrations actually rose with treatment. Only

kidney showed a substantial increase in free glutamate and glutamyl transferase activity. Ammonia levels rose less than fourfold.

Daily injections of 250–500 IU/kg of *Acinetobacter* glutaminase-asparaginase for 7–14 days resulted in 90–100% reduction of total packed cell volume of the following asparaginase-resistant ascites tumors: Ehrlich carcinoma, Taper liver tumor, and Meth A sarcoma. Median survival time was increased up to twofold in glutaminase-treated mice. Combination with 6-diazo-5-oxo-L-norleucine, azaserine, or L-methionine-DL-sulfoximine enhanced the antitumor effects of glutaminase. The glutamine antagonists did not appear to interfere with in vivo glutamine breakdown by glutaminase since plasma amide levels were unaffected by their presence. The *Acinetobacter* glutaminase-asparaginase was found to have little or no effectiveness against solid tumors (28).

Recently Roberts isolated and crystallized a new glutaminase-asparaginase from a soil isolate organism *Pseudomonas* 7A (54). Although there are similarities between the glutaminase-asparaginase obtained from *Pseudomonas* 7A and other organisms, a comparison of physical, kinetic, and biological properties shows marked differences. The *Pseudomonas* 7A glutaminase-asparaginase possesses properties that make it ideally suited for therapeutic usefulness in larger animals and humans. This enzyme has an unusually long biologic half-life (13 hr in normal mice and 43 hr in mice infected with LDH-elevating virus). Physical studies showed that at high protein concentration, the enzyme polymerizes in the presence of asparagine or glutamine. Perhaps this property contributes to its unusually long half-life (55). In preliminary studies, *Pseudomonas* 7A glutaminase-asparaginase demonstrated substantial antineoplastic effectiveness against a variety of leukemias and solid tumors including L1210, EARAD-1, C1498 myeloid leukemia, B16 melanoma, and Walker 256 carcinosarcoma. The *Acinetobacter* glutaminase-asparaginase showed less or no effectiveness against these tumors (56). The *Pseudomonas* 7A glutaminase-asparaginase lowered free glutamine and asparagine in solid tumor tissue to a much lower level than *Acinetobacter* glutaminase-asparaginase (J. Roberts and J. Holcenberg, unpublished results). *Pseudomonas* 7A glutaminase-asparaginase, thus, appears to be quantitatively and qualitatively a much more potent antitumor agent than *Acinetobacter* glutaminase-asparaginase.

An important characteristic of glutaminase-asparaginase therapy is that tumors do not seem to develop resistance to this enzyme as they do with asparaginase or other antitumor drugs. *Pseudomonas* 7A glutaminase-asparaginase treatment of mice bearing 13 consecutive generations of EARAD-1 leukemia did not select or induce a resistant line. Drug resistance to *E. coli* asparaginase occurred during two treatment generations [(57) and J. Roberts and J. Holcenberg, unpublished results]. Combination therapy with glutaminase-asparaginase substantially delayed development of resistance of L1210 tumors to methotrexate and also lowered host toxicity to the antimetabolite (56).

Spiers & Wade recently reported that a glutaminase-asparaginase with a short half-life has an antileukemic effect against asparaginase-resistant cells in man (58). Preclinical toxicity studies with a long half-life form of *Acinetobacter* glutaminase-asparaginase are being conducted at the Memorial Sloan Kettering Cancer Center.

J. Roberts and co-workers (unpublished results) have isolated a novel histidine-degrading enzyme from a soil organism. This enzyme has a broad pH-activity curve between pH 7.2 and 9, with 75% of maximum activity at pH 7.2. Injections of this enzyme were well tolerated by mice and lowered plasma histidine to undetectable levels. Although this enzyme is being developed primarily as a potential new antineoplastic agent, it may also be ideally suited for treatment of the inborn error of histidine metabolism known as histidinemia.

There is some discord in the literature regarding the usefulness of arginase in tumor growth retardation in vivo (59). Burton reported that in contrast to the reversible inhibition of growth of normal lymphocytes and fibroblasts, a number of mouse and rat tumor cell lines were killed by exposure in tissue culture to arginase for 6 hr (60). The arginase inhibition was reversed with arginine (61). The tumor cells died when the arginine concentration in the medium was less than 8 μM. These studies utilized arginase from mammalian cells. This enzyme possesses unfavorable characteristics for antitumor effectiveness (K_m is 24 mM, and the pH optimum is near 10). Rosenfeld & Roberts have recently purified arginine desimidase from *S. lactis* and *S. faecalis* (unpublished results) and arginine decarboxylase from a *Pseudomonas* soil isolate (62). Preliminary studies showed that the desimidase and decarboxylase enzymes can deplete plasma arginine in mice. These enzymes have much more favorable K_m amd pH optimum values than the mammalian arginase and, therefore, are much better suited for future studies of the antineoplastic potential of arginine deprivation therapy.

Uren & Lazarus (63) reported that cystine- and cysteine-degrading enzymes inhibited leukemic cell growth in vitro. However, their enzymes did not appear to have the desired properties for therapeutic usefulness in vivo. J. Roberts (unpublished results) has isolated a serine dehydratase from a bacterial organism which lowered plasma serine when injected into mice.

THERAPY OF NEOPLASIA BY ENZYMIC DEPRIVATION OF ESSENTIAL AMINO ACIDS There are conflicting reports in the literature concerning the antineoplastic effects of maintaining tumor-bearing hosts on synthetic diets deficient in selected essential amino acids. Amino acid–deficient diets were first used for tumor control in animals nearly sixty years ago (64). Extensive work along these lines with different essential amino acids followed in a number of laboratories (65). Recently Schmid et al (66) tested the effects on normal and tumor-bearing animals of three synthetic diets lacking the specific amino acids phenylalanine and tyrosine, isoleucine, or threonine. These studies revealed that even after two to three weeks on deficient diets only partial deficiencies of individual amino acids were observed in plasma of mice.

Studies with amino acid–deficient diets on tumor-bearing mice revealed that tumors were able to grow at the expense of carcass weight and there was very little or no increase in survival time. Relatively few studies of this nature have been conducted in humans suffering from malignancies because the synthetic diets were found to be cumbersome, complex, and unpalatable. Administration of specific amino acid–degrading enzymes may circumvent these problems and also effectively block availability to the tumor of an essential amino acid derived from catabolism

of normal tissue protein. An important advantage of enzymically depriving tumor cells of an essential amino acid is that the tumor should not develop resistance to deprivation of an essential amino acid.

Prolonged deprivation of an essential amino acid will be toxic to the tumor-bearing host. However, deprivation of essential amino acids for short periods of time may be relatively well tolerated by the host. Selective inhibition of tumors could result if the tumor cells have a greater requirement for the amino acid than normal tissues. Abell et al have reported on in vivo inhibition of murine leukemia L5178Y by *Rhodotorula glutinis* phenylalanine ammonia-lyase (67); however, Roberts and co-workers (68) found that this enzyme only partially lowered free phenylalanine and tyrosine levels in tumor cells and normal tissues, and that treatment with this enzyme did not significantly increase the median survival time or long-term survival in mice bearing murine leukemias L5178Y, EARAD-1, or melanoma B16.

Poor results have been reported with enzymes requiring soluble or easily removed cofactors (see above). It is clear that new enzymes, with more favorable characteristics for in vivo activity, will have to be developed before the potential for treating neoplasms by deprivation of essential amino acids can be properly evaluated.

THERAPY OF NEOPLASIA BY ENZYMIC FOLATE DEPLETION Dietary deficiencies of folic acid (69), riboflavin (70), or pyridoxine (71) have been shown to inhibit tumor growth. Generally, it takes several weeks or months to deplete body stores of a vitamin when animals are fed vitamin-deficient diets. In some instances concomitant administration of a vitamin analogue can speed up this process. However, the most rapid and effective method of achieving a vitamin deficiency would be to administer a specific vitamin-degrading enzyme to tumor-bearing hosts. Bertino and his co-workers (72) have reported that carboxypeptidase G_1, a folate-hydrolyzing enzyme isolated from *P. stutzeri,* inhibited growth of murine leukemias L5178Y and L1210, the Walker 256 carcinosarcoma, and a human lymphoblastoid line propagated in vitro. Chabner et al (73) have shown that carboxypeptidase G_1 hydrolyzes methotrexate in vitro and in vivo. Lethal toxicity of large single doses of methotrexate in mice was prevented by carboxypeptidase G_1 given 24–48 hr following methotrexate, with no adverse effect on methotrexate antitumor activity.

Albrecht and co-workers (74) have recently isolated a carboxypeptidase from a water bacterium. This enzyme cleaves folic acid and methotrexate in vitro 20 times more rapidly than 5-methyltetrahydrofolate (the plasma folate). When injected into mice, this enzyme efficiently degraded methotrexate at concentrations as low as 1.5×10^{-8}M (A. Albrecht, personal communication). Further studies are in progress to ascertain the usefulness of this carboxypeptidase in ameliorating the toxicity of methotrexate, which is associated with the persistence of low plasma levels of the antifolate. Albrecht et al (75) have also isolated a folic acid deaminase from a *Pseudomonas* sp. This enzyme was reported to inhibit the growth of neoplastic cells in tissue culture.

ENZYMES AS FIBRINOLYTIC AND DEFIBRINATING AGENTS Fibrin clots are dissolved by the proteolytic enzyme plasmin which is formed in vivo from the inactive precurser plasminogen by endogenous activators. Fibrin lysis can be ac-

celerated by administration of plasminogen activators or other proteolytic enzymes capable of cleaving fibrin. The pharmacology, biochemistry, and clinical experience with these agents have been extensively reviewed (76, 77).

Streptokinase and urokinase are the major plasminogen activators. Both are proteins with a single polypeptide chain and molecular weight of about 50,000. They are rapidly cleared from circulation and, therefore, are administered by constant infusion. Urokinase is a natural product isolated from human urine. It is a trypsin-like enzyme which catalyses cleavage of plasminogen. As a normal constituent of urine, urokinase appears to be nonantigenic. Unfortunately its manufacture is quite costly and the drug is not available in commercial quantities. In contrast, streptokinase, a metabolic product of group C streptococci, is readily available in pure form. It does not have enzymatic activity alone, but forms an equimolar complex with plasminogen. Streptokinase probably induces a conformational change in the plasminogen, producing an active center which cleaves an internal peptide bond to produce plasmin (78). Most patients have circulating antibodies from previous streptococcal infections. These antibodies react with streptokinase and block its effect. Consequently, an initial loading dose must be given to overcome this neutralization.

The lysis of clots depends on activation of plasminogen within the fibrin network. Recently Kakkar and co-workers (79) showed improved lysis of deep-vein thromboses by sequential infusions of plasminogen and streptokinase. Clinically, the rate of lysis of pulmonary emboli and deep-vein thromboses is faster and more complete with the plasminogen activators followed by heparin than with heparin alone. Venous valves were better preserved with the combined therapy. The use of these thrombolytic agents is still controversial in myocardial infarction, retinal vessel thromboses, and arterial thrombi. In fact, a randomized, large cooperative study by the National Heart and Lung Institute showed that even though these agents increased the rate of clot lysis they did not improve survival of patients with pulmonary emboli. Their use in deep-vein thromboses depends on a balance of their therapeutic potential with their significant incidence of fever, allergic reactions, and bleeding (77).

The Malayan pit viper and a South American snake are sources of proteolytic enzymes Ancrod® or Arvin®, and Reptilase®, respectively. These enzymes cleave and clot fibrinogen and thereby produce marked hypofibrinogenemia (80). Many more fibrinolytic enzymes are under study from sources as diverse as molds and Brazilian blood-sucking leeches. The therapeutic role of these enzymes will depend on their antigenicity, ease of control, and toxicity.

REPLACEMENT THERAPY FOR INHERITED ENZYME DEFICIENCIES Lysosomal storage diseases caused by a single enzyme deficiency should be ideal candidates for enzyme replacement therapy. For successful therapy, it has been suggested that the following problems must be solved (81, 82):

1. Adequate amounts of human enzyme must be obtained. Currently, the major sources are placenta and urine. An enzyme must be chosen which is selectively taken up by cells and is stable within lysosomes.

2. The enzyme should be nonimmunogenic. Even human enzymes may invoke an immunologic response. The mutation responsible for the enzyme deficiency may prevent entirely the production of enzyme protein, so that cross-reacting immunological material is absent.

3. The enzyme must reach the tissues in which storage is clinically important. The enzymes studied so far are rapidly taken up by liver. Unfortunately, the major storage problems in some of these diseases is in the heart, kidney, muscle, and central nervous system.

4. The enzyme in the lysosomal particle must fuse with the storage vacuole. In type II glycogenosis, enzyme replacement with purified human acid α-glucosidase resulted in high levels of enzyme in the liver but very little decrease in glycogen content of the liver or in the amount of intravacuolar glycogen (81).

For these reasons, injections of missing enzymes appears to be effective in very few lysosomal storage diseases. Enzyme therapy can cause temporary decreases in circulating levels of storage material in Fabry and adult Gaucher disease, conditions with slow progression and little central nervous system involvement. The prospects for improved therapy may be enchanced by new enzymes, enzyme modification, encapsulation, or entrapment in extracorporeal shunts and perhaps organ transplantation to supply the deficient enzyme (82).

METHODS FOR IMPROVEMENT OF ENZYME THERAPY

Soluble Chemical Modifications

The plasma half-life of *E. coli* and *Erwinia* asparaginases have been increased by deamination, acylation, and carbodiimide reactions with free amino groups (83, 84). Holcenberg et al (85) showed that *Acinetobacter* glutaminase-asparaginase could be modified by succinylation or by conjugation to low molecular weight glycopeptides, yielding enzyme derivatives with a 9- to 15-fold increased half-life in the circulation of mice, rats, and rabbits. Excessive reaction of the amine groups of each of these enzymes decreases the half-life or causes dissociation of the molecules (86). Cross-linking of lysine groups of *E. coli* asparaginase with dimethyl suberimidate decreased activity equally toward asparagine, glutamine, and diazooxonorvaline (87). In contrast, cross-linking by reaction of tyrosine residues with tetranitromethane altered the substrate specificity (88).

We have shown that succinylation of *Acinetobacter* glutaminase-asparaginase greatly decreases its susceptibility to digestion by trypsin (85). Cross-linking of α-galactosidase by hexamethylene diisocyanate stabilized the enzyme to trypsin digestion (89). Breakdown of these proteins in vivo is probably catalyzed by other proteolytic enzymes in the lysosomes. Gregoriadis (90) has shown that invertase cross-linked with glutaraldehyde is more stable in lysosomes. Such modifications which protect enzymes from digestion by lysosomal enzymes might greatly extend their duration of action.

Morell et al (91) have shown that removal of the terminal sialic acid residues of certain circulating glycoproteins causes their rapid uptake by specific galactose

receptors on liver parenchymal cells. Rogers & Kornfeld (92) showed that lysozyme and albumin were also selectively taken up by this liver system when coupled to the asialoglycopeptides of fetuin. G. Schmer and J. Holcenberg have bound asialoglycopeptides to *Pseudomonas* 7A and *Acinetobacter* glutaminase-asparaginase. These derivatives disappear from the blood with a half-life of less than 10 min and are concentrated in the liver. Homogenates of liver retain enzyme activity for about one hour (G. Schmer and J. Holcenberg, unpublished results). Succinylation and coupling of native gluteraldehyde-activated glycopeptides to the *Acinetobacter* enzyme did not change its distribution or effectiveness against solid tumors (85, 93).

Although *E. coli* asparaginase has immunosuppressive properties and retains half of its activity when complexed with antibody, immunologic effects have been shown to interfere with antitumor therapy in patients and experimental animals. Both anaphylactic hypersensitivity reactions and rapid enzyme clearance have been reported (94, 95). G. Schmer and D. Lagunoff (unpublished observations) have studied antibodies produced in rabbits to native *Acinetobacter* glutaminase-asparaginase and its succinylated and glycosylated derivatives. Antibodies to each enzyme preparation are equally effective in neutralizing and precipitating the natural and modified enzymes. Sela (96) has shown that covering the amino groups with polyalanine polymers can reduce the antigenicity of a protein or synthetic polypeptide antigen. Abuchawski et al (97) have reported that reaction of catalase with polyethylene glycol reduced its immunogenicity in mice. These techniques may have applications in the development of therapeutic enzymes.

Microcapsules, Liposomes, and Red Blood Cells

Enzymes have been encapsulated in inert and biodegradable capsules. Inert semipermeable ultrathin capsules of nylon or polyurea can separate the enzyme from proteolytic enzymes and prevent antibody response. The reaction rate of the encapsulated enzymes depends on diffusion of substrate and products across the confining polymer membrane. Thus, the K_m for L-asparaginase in capsules is 100 times higher than the native one (98). A further disadvantage of these capsules is the foreign body reactions they provoke after intraperitoneal administration. This reaction causes a clumping and sequestration of the capsules so that they are no longer capable of lowering asparagine in vivo (99).

Liposomes are small capsules composed of lipid bilayers alternating with aqueous compartments. The lipid layer is composed of egg lecithin, cholesterol, and a negatively charged lipid. Enzymes and water-soluble drugs can be entrapped in the aqueous phase. These liposomes remain intact in circulation without leakage of the protein. They are rapidly taken up by endocytosis into the liver and spleen. The liposomes then fuse with lysosomes and are eventually catabolized. Preliminary experiments have been carried out with biodegradable capsules loaded with asparaginase and purified lysosomal enzymes (90, 100). Manipulation of the composition of lipid membrane and incorporation of cell specific immunoglobulins or desiallylated glycoproteins offers promise of directing the liposomes to specific cells other than those of the liver and spleen. Gregonadis has shown that while protein entrapment in liposomes does not prevent an immune response, the stratagem may

decrease the severity of the hypersensitivity response upon repeated administration (90).

Proteins can be entrapped in erythrocytes by the technique of rapid reversible osmotic shock (101). Ideally, this technique should produce natural enzyme reactors with a prolonged circulation time. Unfortunately, the loading process appears to alter the red cells so that they often have a very short circulation time. Circulation time may be prolonged by modification of the method by including red cell enzymes, organelles, and substrates in the loading mixture, and size fractionation of the resealed ghosts. Thorpe et al (102) have utilized erythrocyte-entrapped β-glucuronidase to improve the treatment of β-glucuronidase-deficient mice. The erythrocyte-entrapped enzyme was retained fourfold longer in circulation, fivefold longer in hepatic tissue and appeared to deliver more enzyme to the lysosomes than the native enzyme. Suggestive evidence was presented that the enzyme-loaded erythrocytes are taken up intact by the liver cells and then slowly digested by the lysosomes. Thus, enzyme-loaded autologous erythrocytes may provide an intracellular depot form of the enzyme for treatment of lysosomal enzyme deficiencies. Nevertheless, the Kupffer's cells are likely to be largely responsible for the uptake of the red cell ghosts in the liver, and the therapeutic usefulness of loading these cells with enzyme is questionable.

Binding to Surfaces

Enzymes can be chemically attached to surfaces, incorporated within the matrix of the gel during polymerization, or bound to the surfaces in microspheres. Immobilized enzymes may be more stable and less likely to produce immune responses if the enzyme does not leach off the surface. Even if antibodies are formed, the complex of antibodies with the immobilized enzyme might still retain activity. Active anaphylaxis should not occur since the enzyme cannot reach sensitized mast cells. The problems associated with immobilized enzymes include changes in enzyme kinetics, leaching of the enzyme from the surface, bioincompatibility of the surface, and coating of the surface with circulating proteins with consequent inactivation of the enzyme reactor.

Allison et al (103) attached asparaginase to nylon tubing with glutaraldehyde. The immobilized enzyme was more stable but its K_m for asparagine was much higher than native enzyme and varied with flow rate. Horvath et al (104) embedded asparaginase within a polycarboxylic gel attached to nylon tubing. Incorporation of the enzyme within the matrix would be expected to prevent leaching from the surface and exposure of the enzyme to proteolytic enzymes and antibodies. In this case enzyme catalysis was also controlled by the rate of diffusion of the substrate into the matrix. Cooney et al (105) showed that asparaginase coupled to a dacron prosthesis by silanization, reduction, and diazotization gradually leached from the surface and brought about an immune response. In addition, accumulation of plasma protein and fibrin deposits on the surface of the graft impaired the catalytic capacity of the graft. This enzyme bound to glass plates by silanization and glutaraldehyde coupling also came off the surface (106).

D. Salley and J. Roberts (unpublished results) using emulsion polymerization prepared enzymically active, spherical (poly)acrylamide particles of controllable

size range, and studied the effects of the degree of cross-linking and density of the polymer gel on enzyme retention and catalytic activity in the gels. Therapeutic enzyme was also entrapped in (poly)N-vinyl pyrrolidone, (poly)hydroxyethyl methacrylate, and (poly)dimethyl siloxane (Silastic ®). These were chosen because of their previous use in biological applications. The polymers were prepared in a particle size suitable for i.p. injection. Injection of the (poly)acrylamide, (poly)vinyl pyrrolidone, and (poly)hydroxyethyl methacrylate enzyme polymers in mice caused a reduction in the plasma asparagine but for no longer than was brought about by injecting free enzyme. This finding is in qualitative agreement with that of O'Driscoll et al (107) who studied the antineoplastic effectiveness of *E. coli* asparaginase trapped in (poly)hydroxyethyl methacrylate. Those workers also reported some leakage of enzyme from the gel. Recently D. Salley and J. Roberts (unpublished results) observed that when a single cylinder of (poly)hydroxyethyl methacrylate gel (6 cm X 0.6 cm) of high asparaginase activity was inserted in the peritoneal cavity of a rat, the reduction in plasma asparagine persisted approximately five times longer than with native enzyme. When plasma asparagine had returned to the normal concentration, the gel cylinder still possessed 60–70% of its original activity. This decrease in activity in vivo could result from impaired diffusion caused by protein coating of the surface of the gel or increased synthesis of asparagine.

Further research is needed to develop better methods for coupling enzymes to matrices. This chemistry must produce biologically stable bonds, no leaching of the enzyme from the matrix, retention of effective enzyme activity, and retention of biocompatibility of the graft.

CONCLUDING REMARKS

Considerable progress has been made in the development and testing of enzymes for parenteral chemotherapy. The biologic obstacles of susceptibility to proteolytic degradation, restricted distribution, and immunogenicity have limited the uses of these highly efficient and specific catalysts. Further advances require the development and selection of enzymes with properties specifically suited for a particular therapeutic use. These enzymes may require chemical modification, encapsulation, or immobilization on biocompatible matrices to improve their stability and decrease the immunogenicity. Directing the enzymes to extrahepatic sites is a much more difficult problem. Tumor-specific antibodies have a limited distribution in the host and therefore may not improve the effect of antitumor enzymes. Certain drugs and hormones with specific high affinity receptors on the target cells may be ideal agents to bind to soluble or immobilized enzymes.

Acknowledgments

We wish to thank Drs. D. Lagunoff, G. Schmer, and D. C. Teller for their collaborative efforts and many helpful suggestions. This work was supported by NIH grants CA 11881, CA 15860, CA 08748, CA 25976, and CA 15674.

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