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Medical Applications of Microbial Enzymes

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I. Introduction

The modification of foods by microbial enzymes prior to ingestion was practiced in ancient times and occurred long before primitive man had any knowledge of what an enzyme was or how it operated. Early examples of enzyme application were in cheese manufacture, bread-making, bating of hides, and a host of fermentation processes involved in the brewing of beer, wine, spirits, and vinegar. Microbial enzymes as digestive aids were slow to be scientifically applied but despite this fact were sometimes effective as incidental contaminants of food. Yeast has been used medically not only as a source of vitamins but also to combat constipation and to stimulate normal digestion by the action of yeast proteases and amylases.

Since animal and plant enzymes as well as microbial enzymes are widely used in medicine, one must ask how microbial enzymes differ, if at all, from the rest. In general, there is a remarkable similarity between microbial enzymes and those of higher organisms. Enzyme parameters, such as pH optimum, activation energy, temperature sensitivity, inhibition, substrate specificity and affinity, are likely to be quite comparable but by no means identical properties of enzymes in all living organisms (Gutfriend, 1965). At the molecular level the same is also true. The amino acid sequence of a particular enzyme bears considerable resemblance in different organisms although the further apart two species are phylogenetically the greater the number of amino acid substitutions which have occurred in the primordial peptide chain (Byers *et al.*, 1970). In the case of enzymes which exist in a polymeric form, the active form may not always be identical in the

microbial and animal species. For example, alcohol dehydrogenase of yeast has a molecular weight of 153,000 and contains four molecules of NAD (nicotinamide-adenine dinucleotide) and four atoms of zinc, while that of horse liver has a molecular weight of 73,000 and contains two residues each of NAD and zinc (Drum and Vallee, 1970). Although the differences between microbial enzymes and those of higher forms may seem small, the variations may well be significant enough to make it critically important in medical applications to use one enzyme in preference to another. Since most enzymes which are used medically are not crystalline, the impurities in the preparation cannot be ignored since they may interfere with the catalyzed reaction. Bacterial enzymes in particular may contain pyrogens or toxins which may have serious adverse effects on the patient. Hence, microbial enzymes often must be extensively purified before they can be used; e.g., collagenase from *Clostridium welchii* is too dangerous for human applications until all the toxin has been removed by successive purification steps (Mandl, 1961). Since all enzymes are proteins, they behave as antigens when injected and eventually stimulate the production of antibodies which, in addition to causing the usual allergic reactions, may combine with the enzyme and inactivate it. As might be predicted, a patient who has in this way become allergic or refractory to an enzyme from one organism may still react beneficially to the comparable enzyme from another species (Sizer, 1964).

A convenient way to review the medical applications of microbial enzymes is to consider them with reference to systems of the body or, in some cases where this is impractical, to deal with individual diseases. In the case of certain enzymes which exert beneficial activity on several body systems, mention will be made in several places in this review.

II. Enzymes Active in the Digestive System

Enzymes taken orally may be useful because of their activity in the digestive tract or because of their action on some other part of the body after their absorption from the small intestine. In either case they may need to be protected from hydrolysis by pepsin or pancreatic proteases through the use of enteric coatings or by binding the enzymes to insoluble particles such as plastics or porous glass (Weetall, 1969).

Microbial enzymes which supplement the action of amylase in saliva or pepsin in gastric juice are not very effective digestive aids because of the short time available for action in the mouth and be-

cause most are inert in the highly acidic stomach (pH about 2.0). Of some interest is the use of enzyme preparations from *Aspergillus oryzae* and *A. niger* in the form of a toothpaste by scientists in several different pharmaceutical companies. These fungal enzymes produced a significantly greater retardation of calculus (tartar) formation and the accumulation of soft accretions on teeth as compared with controls. In addition, tobacco staining was also lessened by the enzyme preparation. Along similar lines, Fitzgerald *et al.* (1968) used a culture filtrate of *Penicillium funiculosum* containing the enzyme dextranase. When caries (induced by *Streptococcus* infections) of hamster teeth were treated with dextranase (dispersed in food or drinking water), coronal plaque deposits on the molar teeth decreased and the progression of the disease was retarded. It is suggested that the enzyme brings about this beneficial effect by degrading the microbially produced extracellular polysaccharides of the dextran type which are present in the plaque matrix.

Enzymatic digestive aids effective in the small intestine have been extensively used for a long period of time. They are most often fungal in origin and preparations from *Aspergillus oryzae* and *A. niger* are the most commonly used, because of their high content of amylase and protease. Certain foods which contain indigestible cellulose fibers such as cucumbers, cabbage, and radishes may produce dyspepsia, flatulence, and other digestive disturbances. This condition may be relieved by cellulase of *Aspergillus oryzae* or *Tricoderma viride* but the latter has been approved for human use only in Japan (Cayle, 1962). Lipase from *Aspergillus oryzae* or *Candida lipolytica* taken orally may be useful in patients who have fatty stools, but careful limitation of fat in the diet is also indicated (Underkofler *et al.*, 1958). Of special interest is the possibility of treatment of genetic disease in which a single enzyme is lacking in the body. Administration of the missing enzyme by an oral or intravenous route may prove useful, and considerable progress in this area can be expected in the near future. An example is seen in the treatment of children who are unable to digest lactose in milk because of a deficiency in lactase (β-D-galactosidase). Addition of a crude lactase preparation from *Aspergillus niger* to the diet results in the digestion of lactose and the absorption of the glucose and galactose from the intestine (Cayle, 1962).

As in the case of enzymes which can be added to foods, the FDA (Food and Drug Administration) severely limits the enzymes on the GRAS (Generally Recognized As Safe) list which can be taken orally to those from *Saccharomyces cerevisiae* and S. *fragilis*, *Aspergillus niger* and *A. oryzae*, and *Bacillus subtilis*.

III. Enzymes Active on the Blood Coagulation System

The major problem in the cardiovascular system is the formation of blood clots in the arteries and veins and smaller blood vessels of the body. Thromoembolism can be serious wherever it occurs in the body, but can be quickly fatal if it occurs in the coronary artery of the heart; or branches of the carotid artery of the brain. Although the blood coagulation system is most complicated and involves many enzyme-catalyzed steps, major attention has focused upon the last step: the conversion of soluble fibrinogen to insoluble fibrin, the major component of the clot. Enmeshed in the fibrin network of the clot are blood cells, especially erythrocytes, white blood cells, platelets, and plasma proteins. Microbial enzymes have been employed both for blocking the coagulation system and for dissolving the clot after it has been produced in an insoluble form. Many of the enzymes injected into the blood to bring about the dissolution of clots exert their action by acting as proteolytic enzymes to split a single arginyl-valine bond of plasminogen (profibrinolysin) thereby converting it to plasmin (fibrinolysin). The plasmin is responsible for digesting the fibrin in the clot (Sherry, 1968) thereby effecting its dissolution. Once digestion of a thrombus (a clot on the wall of a blood vessel) begins it may become dislodged and carried to another resting place to become a dangerous embolus if it clogs a critical blood vessel. Hence, it is crucial that a circulating blood clot be quickly and completely digested.

Although fibrinolysin can be used directly for the lysis of blood clots (Chazov, 1966), typically enzymes have been used which convert the normally occurring blood zymogen (profibrinolysin) to fibrinolysin. Most extensively employed for this purpose is streptokinase from a β-hemolytic *Streptococcus*. Until recently this preparation of streptokinase has been very crude (Varidase) and is contaminated by streptodornase (deoxyribonuclease) in large amounts. A relatively pure streptokinase has been prepared by Heimberger *et al.*, of Behringwerke, A. G. (Editorial, *Chem. & Eng. News*, 1971) who found that the enzyme molecule is fairly small (mol. wt. 47,000) consisting of a single polypeptide chain stabilized by disulfide bonds. The rate of conversion of plasminogen to plasmin by streptokinase is accelerated by *o*-thymoctic acid (Editorial, *Chem. & Eng. News*, 1971). Partially purified streptokinase may be administered buccally, intradermally, intramuscularly, parenterally, or intravenously—the latter is the method of choice when rapid action is required.

Despite the fact that millions of dollars have been spent on research and thousands of patients have been treated with streptokinase, clinical experience with this enzyme varies tremendously (Hume,

1970). On the negative side impurities in the poorly standardized preparations can cause trouble; action of streptokinase is not confined to fibrin (fibrinogen and other plasma proteins may also be hydrolyzed); some patients are sensitive to this bacterial preparation while others become immunized against streptokinase so that it is no longer effective. Highly purified (pyrogen-free) and carefully standardized streptokinase can, however, produce a satisfactory plasma thrombolytic state that is usually well tolerated except for impaired hemostasis. In a typical patient a high level of streptokinase in plasma can incite lysis of experimental and naturally occurring thromboemboli. The enzyme has found use in the treatment of a great many types of thromboembolic problems in several different organs of the body. For example, in a recent extensive study using streptokinase in acute myocardial infarction the mortality from this condition was halved.

Because of the difficulties obtained with a pyrogen-containing crude bacterial enzyme and because human blood contains some antistreptokinase, many workers have recently turned to the use of urokinase, a human enzyme obtained from urine (Sherry, 1968) with some initial success in converting plasminogen to plasmin. In view of the fact that thrombosis is the primary health hazard of white adults it is essential that research continue on thrombolytic enzymes with emphasis on both streptokinase and urokinase.

Because of the significance of the problem a variety of other microbial enzymes are being explored for possible use in thromboembolic diseases. Pisano *et al.* (1963) have reported on the interesting fibrinolytic properties of three different fungi of the genus *Cephalosporium*. Similarly other workers have obtained lysis of blood clots in cancer patients using a filtrate of a culture of *Aspergillus oryzae* B-1273. This enzyme preparation was only slightly toxic and was nonantigenic. Doubtless other useful microbial fibrinases will be found in the near future (Sakakihara *et al.*, 1970).

IV. Use of Microbial Enzymes in the Treatment of Infection, Inflammation, and Burns

In most types of infection and trauma there is an accumulation of pus, microorganisms, blood cells, serum, clots, and other kinds of debris. In view of this situation, it is not surprising to learn that enzymes, especially proteases, have been widely used clinically to treat such conditions. Although enzymes are most successful when applied locally, they have also been administered by other routes. In the case of enzymes which have been used as antiinflammatory agents they are often taken orally. Despite the fact that typical enzymes are

inactivated in the digestive tract and would not be expected to be absorbed into the blood stream, many clinicians nonetheless have reported good results in treating inflammation by administering enzymes buccally. Although plant and animal enzymes seem to be preferred for the oral route, some use has also been made of microbial enzymes, especially streptokinase-streptodornase (Varidase) and *Aspergillus* enzymes (Bioprase, Nargase) (Underkofler *et al.*, 1958; Sameshima, 1966). Bruises, edema, sprains, and infectious areas of the body are often improved after such treatment. These same enzymes have also been applied locally for cleaning up dirty wounds, ulcers and necrotic tissue, infections, and for the debridement of third degree burns. When injected systemically the streptokinase-streptodornase preparation can relieve pain in traumatized areas (e.g., "black and blue" areas of the skin) by dissolving the blood clot. This enzyme preparation has also been used in the treatment of corneal herpes, facial edema, urinary and genital infections, acute otitis, and a variety of infectious conditions. While proteases are most commonly used for these conditions, other types of enzymes have also been employed in special conditions. For example, hyaluronidase, which hydrolyzes mucopolysaccharides, has been used as a "spreader" to assist local anesthetics in penetrating tissues. Similarly lysozyme from *Micrococcus lysodeikticus*, which attacks glycoproteins of bacterial walls, has proven valuable in the treatment of eye infections and of gastrointestinal disease. It is suggested that lysozyme may have some antiviral as well as antibacterial activity (Oldham, 1967).

Microbial enzymes used to treat infection are commonly administered in conjunction with other agents such as antibiotics. A very interesting medical application of an enzyme is its administration to those patients who are highly sensitive to penicillin or who inadvertently have received an overdose of it. Administration of penicillinase brings quick relief to such individuals (Jarvin and Berridge, 1969).

V. Use of Microbial Enzymes in the Treatment of Cancer

Differences between malignant and normal cells are quite subtle and are usually associated with biochemical factors related to the very rapid rate of growth of tumor cells. With this in mind streptodornase (Varidase) has been applied to Ehrlich tumor cells to hydrolyze their DNA (Nuzhina *et al.*, 1970). Lysozyme has also been used in the treatment of some types of cancer (Oldham, 1967). In situations where necrosis is involved in association with the tumor the proteolytic enzymes discussed in the previous section may find limited applica-

tion in "cleaning up" the area. On the whole, however, these digestive enzymes have not been highly successful in causing the tumor to disappear.

An exciting breakthrough in the enzymatic treatment of cancer has resulted from the discovery of a metabolic difference between certain tumor and host cells. The tumor cell requires an extracellular source of L-asparagine and in its absence protein synthesis fails to occur in the cancer. L-Asparaginase hydrolyzes L-asparagine to aspartic acid and ammonia and is an enzyme found in many bacteria, but is often lacking in mammals (Wade and Rutter, 1970). When asparaginase from *Escherichia coli* is injected over a period of days into mice suffering from certain types of leukemia, marked improvement and often apparent cures occurred. The regression of lymphoma tumors in mice was not always permanent, however, and indeed the malignant cells often became resistant to the asparaginase. Riley virus tumors in mice, however, were completely cured by this enzyme (Wade and Rutter, 1970).

Patients suffering from acute lymphoblastic leukemia and treated with *E. coli* asparaginase showed up to 60% remission which lasted for 1 to 8 months. Relapse usually occurred despite continued treatment (Page and Alvarez, 1970). Undesirable side effects in some patients included nausea and vomiting, anorexia, and a change in indices of coagulation. Since asparaginase is a foreign protein it acts as an antigen with the result that repeated administration may lead to allergic reactions and production of antibodies which may inactivate the asparaginase. Despite these many disadvantages asparaginase has proven useful in the treatment of several kinds of leukemia, especially when used in conjunction with other anticancer drugs, in particular predisone and vincristine (Scott, 1968; Beard, 1970).

Because of the problems with *E. coli* asparaginase, other sources have been examined in the hopes of finding an enzyme with a low molecular weight (150,000), high affinity for substrate, and low toxicity. A pathogenic bacterium, *Erwinia caratovorum*, which produces soft rot in many vegetables, contains asparaginase many times more active than the *E. coli* enzyme (Wade and Rutter, 1970). Large-scale production of the *Erwinia* enzyme has made possible successful trials in curing lymphosarcoma in dogs: The purified enzyme is now undergoing extensive clinical trial against leukemia in humans.

An important modification in the method of application of asparaginase is to microencapsulate it before intravenous injection (Chang, 1971). Spherical ultrathin polymer membranes were prepared by polymerizing 1.6 hexamethylenediamine in the presence of a stabi-

lized enzyme suspension. The semipermeable microcapsules containing the enzyme did not leak but permitted rapid diffusion of asparagine and end products into and out of the capsule. By using these microcapsules it is possible to avoid the hypersensitivity and immunological reactions normally produced by *E. coli* asparaginase. In addition, the encapsulated enzyme remained active in the blood for a much longer time. In comparison with the nonencapsulated enzyme the encapsulated form was much more effective in suppressing the growth of implanted mouse lymphosarcoma (Chang, 1971).

The possibilities of using microencapsulated enzymes for other medical applications are most intriguing, since such preparations will have the advantage of low toxicity, high stability, prolonged and controlled enzyme activity, and no foreign body reaction. Various techniques using a variety of plastics (especially nylon) are now undergoing development. Closely related are techniques for attaching enzyme molecules by chemical bonds to polymer molecules. Especially useful for this purpose is a porous type of glass granule being developed by Weetall and his associates (1970). As soon as enzymes which are either encapsulated or bonded to polymers become generally available they will be tried clinically for the treatment of a variety of medical problems.

Other biochemical differences between tumor and normal cells in addition to the ability to synthesize asparagine exist and are being actively explored. It appears that some myeloid leukemic cells cannot synthesize sufficient serine. Hence, the enzyme serine dehydratase might be of clinical value in treatment of this leukemia (Wade and Rutter, 1970). Similarly carboxypeptidase may inhibit the growth of leukemia cells by causing a depletion of cellular folic acid (Bretino *et. al.*, 1971).

VI. Microbial Enzymes Active on Connective Tissue

Since collagen is the major component of connective tissue, one could have predicted that there might be medical applications of an enzyme which cleaves the peptide bonds of collagen. Native collagen is highly resistant, however, to enzymatic attack and hence it required intensive research before an effective collagenase became available for medical use. By far the best source is *Clostridium histolyticum* of which several strains are now available which produce collagenase in high yield with minimum toxicity. Because clostridial toxin is so lethal, however, it is necessary to carefully purify the enzyme before it can be used on humans. The medical applications of collagenase, which will be published as a monograph in 1972, were discussed in a

recent symposium. The various reports of the symposium have been summarized in a preliminary manner by Mandl (1970). Collagenase has proven to be a useful enzyme for growing cells in tissue culture since the enzyme does not damage cell membranes and hence can be used to disperse cells. Human skin fragments dispersed by bacterial collagenase could then be used as a multiple graft system to speed up the healing of third degree burns. The separated skin cells applied to the debrided burn site become foci for skin regeneration. In tissue culture collagenase can also be used to prevent the accumulation of connective tissue fibers in a human carcinoma as it is transferred through successive generations of rats, thereby rendering the tumor indefinitely transplantable.

In tooth transplantation Shulman *et al.* as reported by Mandl (1970) have treated the tooth allographs with bacterial collagenase prior to transplantation. As a result of the dissolution of collagen fibers in the periodontal ligament early rejection of the tooth was prevented in rhesus monkeys. In addition, the enzyme treatment reduced inflammation after transplantation, increased ankylosis, and resulted in prolonged survival of the tooth graft.

Collagenase may turn out to be a useful enzyme in the treatment of patients suffering from low back pain due to a "slipped disk." In this condition cartilage of the intervertebral disk protrudes and exerts pressure on the spinal nerve roots. Sterile collagenase injected into the nucleus pulposa between the vertebrae of dogs resulted in the dissolving of the cartilage without damage to surrounding tissue (Sussman and Mann, 1969). Similarly action by collagenase on the nucleus pulposa of cadavers was brought about with dissolution of fibrocartilage without action on hyaline cartilage or bone collagen. It should be noted that injection of chymopapain into the nucleus pulposa of patients suffering from "slipped disk" resulted in an attack on the chondromucoprotein and rapid relief of the prolapsed disk syndrome (Stern, 1969).

Cryoprostatectomy has been developed recently as a procedure for surgically removing the prostate after freezing it with the cryoprobe. In dogs the residual slough was subsequently dissolved by injection of collagenase into the prostate prior to inserting the cryoprobe. The collagenase prevented the residual slough from clogging the urethra yet did not damage vital tissues. Collagenase proved much more effective than other enzymes and produced total dissolution of the slough in 18 hours (Mandl, 1970).

Second and third degree burns have been treated with bacterial collagenase in a large number of patients prior to skin grafting. The enzyme appears to be equally useful in the treatment of human dermal

ulcers. Beneficial effects on burns and ulcers can occur in as short a time as 3 days. Overall satisfactory results were obtained in 80% of the patients in 14 days. Healing of the exposed underlying tissue progresses well with no keloid formation or contracture (Mandl, 1970). Action of other proteases on burns and ulcers has been discussed in a previous section of this paper.

VII. Medical Applications of Bacterial Enzymes *in Vitro*

Because of their high specificity and rapid action enzymes are especially useful for quantitative chemical assay in the clinical laboratory. Measurement of common and exotic components of blood and urine using microbial enzymes in the assay procedure is yielding to automation in the hospital laboratory. In addition, the measurement of normal and abnormal enzymes in blood or amniotic fluid has become a useful diagnostic tool. Details of the use of microbial enzymes in the clinical laboratory have been presented in a number of monographs on the subject (Abderhalden, 1961; Dioguardi, 1961; Cruickshank, 1965; Netter, 1966).

A very interesting application of microbial enzymes is their use in the biosynthesis of medically important compounds. Microbial enzymes have proven useful in the production of corticosteroids from inert precursors. In this regard enzymes can bring about steroid hydroxylation, hydrogenation and dehydrogenation, epoxidation and cleavage of side chains. The steroid raw material diosgenin (from yams) is converted chemically to progesterone which is then oxidized by a fungal enzyme to cortisone (Oldham, 1967). It is also possible to convert by the use of microbial enzymes certain antibiotics to derivatives which possess interesting antibiotic and medical properties. For example, penicillin can be hydrolyzed to 6-amino-penicillanic acid and phenylacetic acid by penicillin acylase of *E. coli* or *Streptococcus lavendulae*. The penicillanic acid can in turn be used for the synthesis of potent nonallergenic penicillin derivatives (Jarvin and Berridge, 1969). It seems highly likely that there will develop in the future an increase in the use of enzymes for bringing about specific steps in the synthesis of complex chemical compounds which exhibit high pharmacological activity.

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