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Review 62

My Compliments

Determining the Safety of Enzymes Used in Food Processing

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ABSTRACT

Enzymes are proteins that catalyze chemical reactions. They are highly specific and needed in only minute quantities. Certain enzymes have long been used to produce specific foods (e.g., cheese). Today they have numerous applications and are increasing in commercial importance. There has never been a health problem traced to the use of an enzyme per se in food processing. However, it is important that scientific data be provided to show that enzyme preparations, particularly those lacking a long history of safe use, are in fact safe to consume. The purpose of this report is to propose guidelines for assessing enzyme safety. We conclude that the enzymes per se now used or likely to be used in the future in food processing are inherently nontoxic. Safety evaluation should focus on possible contaminants which could be present. Assuming that current Good Manufacturing Practices (CGMPs) are followed, toxic contaminants could only come from the enzyme source itself (animal, plant or microbial). Hence, the safety of the source organism should be the prime consideration. Enzymes from animals or plants commonly regarded as food need not be subjected to animal feeding studies. Some food plants produce toxins and chemical assays may be used in these cases to assess safety. For enzymes from bacteria, it should be shown that antibiotics and acute toxins active via the oral route (enterotoxins and certain neurotoxins) are absent. Small molecular weight toxins (< 500 daltons) may be produced by certain fungi and actinomycetes. It should be shown that enzymes from such organisms are free of these materials. If it is established that a microbial culture does not produce antibiotics or toxins active via the oral route, then enzymes manufactured from that culture using CGMPs may be regarded as safe for use in food processing.

BACKGROUND

To understand and apply the proposed guidelines for determining safety of enzymes used in food processing, it is necessary to consider what enzymes are, how they act, how they are prepared and how they are used. That is the purpose of this section.

General considerations

Enzymes are proteins which catalyze chemical reactions. Like all catalysts enzymes increase the rates at which reactions achieve equilibrium. For example, there are instances where certain enzymes increase the rates of specific reactions by 10 million times (*47*). Enzymes act by lowering activation energy. Since they cannot create energy, enzymes will only affect reactions which, because of a "downhill" net energy flow, could occur spontaneously. Like other catalysts, enzymes are not consumed by the reactions which they catalyze. Hence, one enzyme molecule can, through time, catalyze the transformation of many molecules of substrate (*47, 52*).

Most complex chemical reactions not controlled by catalysts produce a variety of products. However, in general, enzymes accelerate specific reactions which result in the generation of specific products. High degrees of specificity and strong catalytic activities are the most important functional properties of enzymes. Clearly, without enzymes DNA could not be replicated nor could RNA and proteins be synthesized and degraded. The controlled and orderly array of metabolic processes of living cells, which in fact define life, would not be possible. Life on earth is absolutely dependent upon enzymes. Every cell comprising every organism alive at this moment contains enzymes which are functioning in highly ordered and specific ways to transform one chemical into another as dictated by biological necessity.

Like all proteins, enzymes are synthesized inside cells by a complex process involving DNA, RNA, cellular structures called ribosomes, various small molecules such as amino acids, energy-rich phosphorus compounds and certain cations, and enzymes to catalyze specific reactions (*52*). The fact that enzymes are a necessary component in the biological mechanism which produces new enzymes underscores the fundamental importance of these remarkable biological catalysts.

After synthesis, enzymes may remain inside cells or they may be secreted into the extracellular milieu. Secreted enzymes are hydrolytic and their purpose is to decompose macromolecules into small units which then can be taken up by cells and used (under enzymic direction) as needed in metabolic processes. Enzymes which remain inside cells (intracellular) are of all classes and may be involved in synthesis or degradation of various substances. Economically important enzymes are found among both the intracellular and extracellular groups (*47*).

The name given to an enzyme is determined according to the reactions which is catalyzed. It is customary to attach the suffix "-ase" to the name of the principal sub-

strate upon which the enzyme acts; e.g., the sugar *lactose* is acted upon by *lactase*, *proteins* are degraded by *proteases*, intramolecular rearrangements *(isomerizations)*are catalyzed by *isomerases*. Additionally, many well-known and long-used enzymes have trivial (common, historical) names, e.g., papain from papaya. To minimize confusion, each enzyme activity is assigned a four-part number (called the IUB[1] number) and a systematic name based on the reaction. However, this system does not distinguish between different enzymes from different organisms which catalyze the same reaction (*47*).

All living organisms produce and contain many enzymes, but no one organism has enzymes for all or even most possible biotransformations. Organisms may produce one specific enzyme to act on a given substrate. Organisms may also produce two or more different enzymes which catalyze the same reaction; such enzymes are called isoenzymes. The reasons for this are not known, but it is believed related to the apparent necessity of organisms to maintain precise control over enzyme synthesis, degradation and activity (*52*). Although enzymes catalyzing the same reaction but produced by different species may be similar, it is also possible that they may be entirely different (*21, 52*). Similarities and differences between enzymes and other proteins is one way of estimating evolutionary divergence among species (*21, 52*).

Catalytic activity is ultimately derived from the sequence of specific amino acids which comprise an enzyme. Amino acid sequence, in turn, determines the shape of the enzyme molecule. The shape or configuration is all-important. Disrupting the shape destroys activity.

Enzyme activity is operationally defined by kinetic parameters such as maximum catalytic rate and the affinity of the enzyme for its substrate. Virtually any environmental factor (pH, ionic strength, temperature, etc.) affects enzyme activity. Enzymes are also subject to inhibition by various means (*47, 52*). These properties permit cells to regulate the activities of enzymes which they synthesize and contain. A thorough understanding of the properties of individual enzymes also permits their optimal use in industry.

Historical examples of enzyme use

Most of what we call "food" is really tissue derived from living organisms (animals or plants); in some cases (e.g., milk), food is a secretion from living cells. Many of the enzymes in the cells of tissues remain active after cell death. For example, meat is "aged" by hanging animal carcasses in refrigerated rooms for several days after slaughter. During this time cells in the tissues break down, freeing various degradative enzymes, which then partially digest the connective tissue to give a more tender product. The tenderizing process can be accelerated by adding proteolytic enzymes derived from other sources to the meat at various stages before consumption, such as injecting proteases into the vascular system of the animal before slaughter or sprinkling papain (protease from papaya) on the meat before cooking. The tenderizing process is simply the first step in digestion which continues in the gastrointestinal tract of the consumer.

Enzymes have always been present in human food even though they have only recently been recognized as such. In addition to tissue-derived enzymes, microorganisms (because they are ubiquitous) also pervade the food supply, and the enzymes in microorganisms can alter the character of food. It was discovered early in the development of human civilization that some microbial transformations are desirable.

One of the first to be recognized was the souring of milk, a necessary step in making cheese. According to legend, cheesemaking was discovered several thousand years ago when an Arabian merchant carried milk in a pouch made of sheep's stomach. Rennet in the lining of the pouch caused the milk to curdle. We must assume that microorganisms grew at the same time and produced other enzymic changes that came to be regarded as desirable.

During the intervening centuries, man has learned how to make hundreds of kinds of cheese by controlling the environment and by adding types of microorganisms that produce enzymes which can bring about desirable changes. Lipases and proteases from various animal and microbial sources can also be added to achieve certain desired qualities.

We now use the term "fermentation" to describe milk souring and similar processes invoiving mass growth of microorganisms to produce useful products (*52*). Originally, however, the term described the transformation of grape juice into wine. Production of wine from grapes through fermentation also has its origin in antiquity. Among the treasures placed in the tombs of Egyptian pharaohs were casks of wine. The ancient Greeks attributed to the god Bacchus the discovery of fermentation (*52*). We now know that it is not yeast per se, but rather a system comprised of several enzymes contained in yeast that is ultimately responsible for the production of ethanol and carbon dioxide from the sugar in grape juice. This enzyme system was one of the first to be extensively studied and characterized. In fact, the word "enzyme", introduced by Kuehne, means "in yeast," although it has been expanded and now applies to all proteinaceous catalysts from any biological source (*52*).

Other ancient processes of food alteration and/or preservation involving enzymic action include breadmaking (yeast) and the production of vinegar from wine (*Acetobacter*). Only within the past 100 years has it been recognized that enzymes exist as discrete entities, and can, in fact, function in isolated systems outside living cells (*52*). This realization has led to remarkable advances through technological application of enzymes to many areas of human need.

Modern uses of enzymes

Food processing. Fermentations involving living or-

[1]The enumeration system of the Enzyme Commission of the Third International Congress of the International Union of Biochemistry (*47*).

TABLE 1. *Enzyme preparations used in food processing (3).*

Trivial name	Classification	Source	Systematic name (IUB)[a]	IUB No.[a]
α-Amylase	Carbohydrase	(1) *Aspergillus niger*, var. (2) *Aspergillus oryzae*, var. (3) *Rhizopus oryzae*, var. (4) *Bacillus subtilis*, var. (5) Barley malt (6) *Bacillus licheniformis*, var.	1,4-α-D-Glucan glucanohydrolase	3.2.1.1
β-Amylase	Carbohydrase	Barley malt	1,4-α-D-Glucan maltohydrolase	3.2.1.2
Bromelain	Protease	Pineapples: *Ananas comosus*, *Ananas bracteatus* (L)	None	3.4.22.4
Catalase	Oxidoreductase	(1) *Aspergillus niger*, var. (2) Bovine liver (3) *Micrococcus lysodeikticus*	Hydrogen peroxide: hydrogen peroxide oxidoreductase	1.11.1.6
Cellulase	Carbohydrase	(1) *Aspergillus niger*, var. (2) *Trichoderma reesei*	1,4-(1,3;1,4)-β-D-Glucan 3(4)-glucanohydrolase	3.2.1.4
Ficin	Protease	Figs: *Ficus* sp.	None	3.4.22.3
β-Glucanase	Carbohydrase	(1) *Aspergillus niger*, var. (2) *Bacillus subtilis*, var.	1,3-(1,3;1,4)-β-D-Glucan 3(4)-glucanohydrolase	3.2.1.6
Glucoamylase (Amyloglucosidase)	Carbohydrase	(1) *Aspergillus niger*, var. (2) *Aspergillus oryzae*, var. (3) *Rhizopus oryzae*, var.	1,4-α-D-Glucan glucohydrolase	3.2.1.3
Glucose isomerase	Isomerase	(1) *Actinoplanes missouriensis* (2) *Bacillus coagulans* (3) *Streptomyces olivaceus* (4) *Streptomyces olivochromogenes* (5) *Streptomyces rubiginosus*	D-Xylose ketolisomerase	5.3.1.5
Glucose oxidase	Oxidoreductase	*Aspergillus niger*, var.	β-D-Glucose: oxygen oxidoreductase	1.1.3.4
Hemicellulase	Carbohydrase	*Aspergillus niger*, var.	None	None
Invertase	Carbohydrase	*Saccharomyces* sp. (*Kluyveromyces*)	β-D-Fructofuranoside fructohydrolase	3.2.1.26
Lactase	Carbohydrase	(1) *Aspergillus niger*, var. (2) *Aspergillus oryzae*, var. (3) *Saccharomyces sp.*	β-D-Galactoside galactohydrolase	3.2.1.23
Lipase	Lipase	(1) Edible forestomach tissue of calves, kids, and lambs (2) Animal pancreatic tissues (3) *Aspergillus oryzae*, var. (4) *Aspergillus niger*, var.	Carboxylic-ester hydrolase Triacylglycerol acylhydrolase	3.1.1.1 3.1.1.3
Papain	Protease	Papaya: *Carica papaya* (L)	None	3.4.22.2
Pectinase[b]	Carbohydrase	(1) *Aspergillus niger*, var. (2) *Rhizopus oryzae*, var.	Poly (1,4-α-D-galacturonide) glycanohydrolase Pectin pectylhydrolase Poly (1,4-α-D-galacturonide) lyase	3.2.1.15 3.1.1.11 4.2.2.2

Pepsin	Protease	Porcine or other animal stomachs	None	3.4.23.1
Protease (general)	Protease	(1) *Aspergillus niger*, var. (2) *Aspergillus oryzae*, var. (3) *Bacillus subtilis*, var. (4) *Bacillus licheniformis*, var.	None	3.4.21.14 3.4.24.4
Rennet	Protease	(1) Fourth stomach of ruminant animals	None	3.4.23.4
		(2) *Endothia parasitica*		3.4.23.6
		(3) *Mucor miehei, M. pusillus*		3.4.23.6
Trypsin	Protese	Animal pancreas	None	3.4.21.4

[a]*Enzyme Nomenclature: Recommendations (1978) of the Nomenclature Committee of the International Union of Biochemistry*, Academic Press, New York. 1979.
[b]Usually a mixture of polygalacturonase, pectin methylesterase and pectate lyase.

ganisms are in wide use today, although it is now known that enzymes produced by these organisms are the actual agents responsible for the conversion of grapes to wine, milk to buttermilk or yogurt, etc. In addition to modern applications of ancient discoveries, enzymes extracted from living organisms also are widely employed in the food industry.

Enzymes used by food manufacturers are derived from edible and nontoxic plants, animals, and nonpathogenic, nontoxigenic microorganisms (*47*). Some of the enzymes used in food processing are given in Table 1 along with the sources of each. Because enzymes are catalysts, the amounts added to food (usually at an early or intermediate step in processing) represent only a minute fraction of the total food mass (*5*). Even this small amount may be reduced by further processing. For example, heating to produce desired organoleptic properties enhance shelf-life and ensure the absence of pathogenic microorganisms will denature or destroy the activity of most enzymes. The protein molecules which comprised the enzymes will still be present, but their physical shape will have been irreversibly altered by heating so that they no longer possess catalytic activity. There are also other methods of enzyme removal and/or inactivation such as raising or lowering the pH beyond limits which the enzyme can tolerate (*47*). Every enzyme exhibits a range of pH stability above or below which inactivation occurs. Many enzymes are inactivated by the acidity of the stomach.

The main organic constituents of foods are carbohydrates, proteins and lipids. It is often desirable to alter one or more of these constituents with enzymes during the conversion of raw to finished product. An important example of this involves the use of carbohydrases and isomerase to produce corn syrups from starch (*29, 32, 47*).

In one example of this conversion, alpha-amylase (IUB 3.2.1.1) first breaks long-chain starch molecules into shorter chains. Then glucoamylase (IUB 3.2.1.3) cleaves the individual glucose molecules from the chains. The resulting corn syrup has many commercial applications, but it is not as sweet as sucrose, the common table sugar obtained from sugar cane and sugar beets.

This deficiency of corn syrups has been overcome in recent years by the discovery of glucose isomerase (IUB 5.3.1.5), which converts glucose into fructose. The resulting high fructose corn syrup (HFCS) approaches the sweetness of sucrose and is less expensive. It is replacing the disaccharide in many applications.

There are many other novel and important applications of enzymes. For example, some foods and beverages do not store well in the presence of oxygen. By use of the enzyme glucose oxidase (IUB 1.1.3.4), which adds molecular oxygen to glucose to produce gluconic acid, it is possible to remove atmospheric oxygen safely and effectively from foods or beverages that are susceptible to oxygen.

Another interesting example is the production of juices from certain fruits and vegetables, where pectin content may become an important consideration (*47*). Pectin and pectic substances occur in plants. They are complex carbohydrates which are insoluble in water but nonetheless absorb water and, when dispersed, greatly increase viscosity. This is a desirable property for certain juices, such as those made from tomatoes, apricots and oranges, but the resulting lack of clarity is undesirable in apple and grape juices. Unfortunately, nature does not necessarily accommodate human taste. Raw apple and grape juice can contain considerable amounts of pectin even though most of us may not like them that way. For this reason, it is usually necessary to add pectic enzymes to raw apple and grape juices during processing to hydrolyze the pectin. Additionally, considerable amounts of juice can remain trapped in masses of pectic material. Through the use of pectic enzymes, such trapped juice can be freed. This makes juice extraction more efficient and economical, hence it lowers the price for consumers.

It is important to recognize that pectic enzymes (a mixture of three enzymes — see Table 1), as well as pectin, are naturally present in fruit juices, and where more enzyme activity is required, additional pectic enzymes may be added as indicated above. However, where high pectin content is preferred (e.g., apricot nectar, tomato and orange juices) the juice may be heated at an early stage in processing to denature native pectic enzymes and thereby

preserve natural pectin content. Another variation is used in jelly manufacture. Here, the native pectin is hydrolyzed by pectic enzymes, and then, after heating to denature the enzymes, commercial pectin possessing certain desirable properties is added to produce jelly of consistent quality.

Pharmaceutical/medical applications. Because of the great versatility of enzymes, their use is not restricted to food processing. Enzymes also have gained importance in the pharmaceutical/medical industry. For example, they are used in rapid and highly reliable clinical diagnostic tests. In one such test, the enzymes glucose oxidase and peroxidase (IUB 1.11.1.7) have been combined in a specific and sensitive assay for glucose in urine (a symptom of diabetes). The glucose oxidase/peroxidase test is superior to urine-glucose tests based on chemical reduction of glucose (*9, 25*). It has also recently been applied to the detection and quantitation of glucose in blood. Other enzymes which catalyze different reactions with glucose also are used in glucose determinations. Moreover, many physiologically important substances, such as blood urea nitrogen (BUN), triglycerides and glycerol, cholesterol, uric acid, and several physiologically important enzymes, can be rapidly and specifically assayed with commercially available enzyme-based tests.

Enzymes also are employed in antibiotic manufacture to alter the chemical structure of antibiotics and thereby increase the range of microorganisms which the antibiotics can control. A related and particularly interesting example is the therapeutic application of beta-lactamase (formerly penicillinase) (IUB 3.5.2.6), an enzyme which destroys penicillin. The gene which codes for penicillinase is found on certain plasmids (extrachromosomal DNA) and the acquisition of such plasmids by pathogenic bacteria confers penicillin resistance. However, the purified enzyme can also be used to treat people who are hypersensitive to penicillin but were inadvertently exposed to the drug (*47*). Thus, imaginative application has resulted in health benefit from an enzyme which functions in nature to the detriment of human health.

There are many other similar examples of the therapeutic uses of purified enzymes from pathogenic microorganisms, from the venom of poisonous snakes, from human urine and from a variety of other plant, animal and microbial sources (*19*). Enzymes may be used in the treatment of human maladies ranging from cancer and thrombosis to prevention of tooth decay (*19, 47*).

Enzyme detergents. The addition of enzymes to laundry products to aid in stain removal was developed by Rohm, who patented the idea in 1913. Various improvements were made on the original concept, and, by 1969, enzyme detergents claimed 50% of the market in Europe and almost 45% in the United States (*49*). Then, following widely circulated, unfavorable publicity concerning the possible development of allergies to enzymes inhaled as a result of dust formation, the use of enzymes in laundry products in the United States declined dramatically. However, an expert committee, with support from the United States Food and Drug Administration (FDA), has concluded that irritation from enzyme detergents does not exceed that of detergents which do not contain enzymes (*15*). In addition, methods have been developed to encapsulate enzymes in polymeric matrices which are too large to be dispersed in air as dust particles, yet retain enzyme catalytic activity in the laundry product. Hence, it is now possible to produce an essentially dust-free enzyme detergent (*49*).

The use of enzymes in laundry products offers prospects for decreasing energy (heating) costs as well as minimizing water pollution (diminishing the need for other chemical additives). Enzymes are being used widely and successfully in laundry products without evidence of adverse health effects in consumers (*49*).

Other uses. There are many other practical applications of enzymes. For example, enzymes are used widely in the textile and leather industries to remove undesirable substances from products during manufacture. Additionally, commercial enzyme preparations are available for use in septic tanks. Such preparations often contain many enzymes for decomposing complex carbohydrates, proteins and lipids, as well as viable microorganisms which use the enzyme-liberated products as nutrients and produce additional degradative enzymes to continue the cycle. Microorganisms producing appropriate enzymes are also used to detoxify pesticides, and other bacteria can remove nitrate and nitrite from water supplies (*47*). Certain microorganisms and their enzymes are gaining particular attention in the production of alcohol as fuel as well as in the production of food from inedible materials or by-products (*47*).

Future applications of enzymes

It is now apparent that additional useful and important applications of enzymes to societal improvement are limited only by the depth of our imagination and our resolve as a nation to encourage experimentation and innovation. Technological application of enzymology is a direct outgrowth of our scientific preeminence, and once reasonable safety has been established, new developments should be allowed to proceed unfettered. Many problems which disturb us and plague much of the rest of the world, such as unavailability of food, fuel, adequate medical and pharmaceutical supplies, clean water and pollution control, are amenable to enzyme technology. Enzymes are an immensely valuable renewable natural resource, and their imaginative use in improving human welfare should be nurtured.

By way of specific example, one area of great potential is enzymic nitrogen fixation. Nitrogen is an essential element for life [indeed, all enzymes contain about 16% nitrogen (*52*)], yet atmospheric nitrogen cannot be utilized by animals, plants and most microorganisms. Nitrogen can be "fixed" as ammonia (a biologically usable form of the element) by industrial processes which consume much energy (*31*). In contrast, blue-green algae and certain species of bacteria can produce ammonia from nitrogen and hydrogen in a much more efficient manner, although energy is still required (*52*). Hence, an important challenge is the

harnessing of the enzymic process of nitrogen fixation for industrial-scale production of ammonia. Such a development would go far towards alleviating global food shortages.

As the example given above illustrates, enzymes in the broadest sense are really inexpensive alternatives to energy-requiring physical processes, such as the application of heat and/or high pressure. This is because enzymes accelerate reactions which would proceed only very slowly, or not at all, under ordinary conditions. Moreover, because enzymes are so specific in the reactions which they catalyze, many important and highly useful chemical transformations could not be accomplished without them. For these reasons, the future of enzyme technology seems exceedingly important and bright.

MANUFACTURE, COMPOSITION AND CONSUMPTION OF ENZYME PREPARATIONS

Enzymes are manufactured because we need highly specific catalysts which are safe to use. Two considerations are of primary importance: (a) catalytic activity must be preserved during production and (b) the intended and proper use of enzyme preparations must pose no health risk for plant workers or consumers. These two central principles underlie enzyme manufacture and use.

Like all biological materials, enzymes are affected by the conditions under which they are produced and handled. Economically important enzymes are obtained from animals, plants and microorganisms. In the manufacture of enzymes there must be strict adherence to current Good Manufacturing Practices (CGMPs). (*8*).

Enzymes from animals

One of the first intentional developments by man of what could be called an "enzyme preparation" was rennet, a crude extract of the lining of the fourth stomach of ruminants. This extract contains various proteolytic enzymes which cause milk to curdle, a step essential for cheese production. Rennet is still obtained from this traditional source except that modern methods of enzyme manufacture and quality control are applied to ensure a product of consistent activity which is free of pathogenic bacteria and toxic substances (*3*, *4*, *8*).

Other crude enzyme mixtures are also obtained from animals at slaughter, such as pancreatin from the pancreas (contains several proteolytic, amylolytic and lipolytic enzymes), pepsin from hog stomachs, lipase from the throat glands of young ruminants and hyaluronidase from bovine seminal vesicles (used medically to facilitate the diffusion and adsorption of local anesthetics). An important perspective of enzyme production from animals is evident from the fact that in 1975, in the Federal Republic of Germany alone, pancreas glands from 13.3 million animals were required for the production of just 100 kg of pancreatin (*44*). As in the manufacture of calf rennet, high standards of quality are maintained throughout the production process to ensure the safety and efficacy of the final enzyme preparations.

Enzymes from plants

Enzymes of commercial importance are also obtained from edible nontoxic plants. The terms *edible* and *nontoxic* are both important, since some edible plants can contain toxic substances (e.g., potatoes and rhubarb) (*13*). However, the plants used for food enzyme manufacture are not known to produce or contain such toxins. Three plant proteases (bromelin, papain and ficin) are obtained, respectively, from the stalks of pineapple plants, the fruit of papaya and the sap of fig trees. Additionally, horseradish roots serve as the source of horseradish peroxidase (an important analytical and research enzyme), and barley seeds are the source of malt which contains amylase activity and is used in brewing (*47*).

Imported raw materials are surveyed for possible insect-derived contamination. If found, the product is processed to remove the contaminant. Another consideration common to all agricultural products is possible pesticide residues or mycotoxins in plant-derived enzyme preparations. Enzymes often are separated from other plant constituents by precipitation with organic solvents such as ethanol, acetone or isopropanol (*47*). Any organic toxins initially present are likely to be separated from the enzyme-containing protein fraction which precipitates.

Enzymes from microorganisms

Microorganisms are the most important source of commercial enzymes. Virtually any enzymic activity of industrial importance may be produced by one or more species of microorganism. This does not mean that microorganisms naturally synthesize animal or plant enzymes, but rather that microorganisms may produce their own enzymes to catalyze reactions that are also catalyzed by structurally different enzymes from animals or plants. Microorganisms are readily grown and manipulated on an industrial scale, and the synthesis of specific products, including enzymes by these organisms, can be regulated by using selected or genetically-engineered strains and/or varying growth conditions. Hence, the uniformity of composition of microbial enzyme preparations can be maintained.

Organism selection. Manufacturing a microbial enzyme begins with well-characterized pure cultures isolated from various sources. There are many cultures currently in use (Table 1). Microbial cultures used in food enzyme manufacture should have been tested to establish that they are nonpathogenic, nontoxigenic and do not produce antibiotics (*3*, *4*, *7*, *45*, *47*). Specific cultures often will have been subjected to many tests, and there should be little doubt that the microorganisms listed in Table 1, when handled under CGMPs, are safe for food enzyme manufacture. Cultures of the same or different species isolated anew from natural sources may also be of potential importance in food enzyme manufacture. The guidelines and procedures which we present below can be used to assess the safety of new isolates.

A culture (currently in use or isolated anew) will have been selected on the basis of its ability to synthesize a desired enzyme. However, the enzyme may be produced at

only relatively low rates. Moreover, the culture may also produce other undesired enzymes. For example, microbial rennet preparations often contain unwanted enzymes which can produce off-flavors in cheese on prolonged aging (*47*). Hence, it is common practice to attempt to improve the desirable qualities of the isolate by altering growth conditions, usually in conjunction with strain selection by mutation or other types of genetic manipulation. The result can be a special strain that will not survive in nature but is very useful from a commercial standpoint.

Laboratory-generated mutant strains characteristically lack certain functional or regulatory properties.[2] While the primary structures of proteins can be altered within limited ranges by mutagenesis, mutants possessing enzymes with improved catalytic activity for their normal substrates have not been reported (*30*). Moreover, no one has ever reported a mutation which transformed an otherwise nontoxic enzyme or protein into a toxin. It is now possible to introduce foreign genes into microorganisms by using DNA cloning techniques so that entirely new proteins are produced, but this should not be confused with mutagenesis where the intrinsic DNA of an organism is altered.

A useful mutant strain might be one which has lost a regulatory function that limits the synthesis of a desirable enzyme so that the mutant cannot stop synthesizing the enzyme and continues to produce it in great excess of biological need. The mutant may also have lost the ability to synthesize one or more unwanted enzymes. Additionally, it may have been manipulated genetically so that more than one copy of the gene coding for the desired enzyme is present, hence, there are more "blue-prints" available (*47*). Such organisms are really genetically impaired and are maintained in the laboratory or industrial setting by using specific, well-controlled growth conditions. These microorganisms have not been found in nature probably because they cannot compete successfully with the wild-type (nonmutant) parent or other microorganisms. It is also important to note that when the parental isolates are pathogenic, the derived mutant strains are characteristically less hazardous. Of course cultures used for food enzyme manufacture are not pathogenic, but by way of example, mutant strains of *Salmonella typhimurium* developed for routine mutagenesis testing are far less virulent than *S. typhirmurium* found in nature (*1*). Therefore, in choosing innocuous isolates for enzyme production, the process of enzyme manufacture from microorganisms becomes inherently safer.

The nonpathogenic, nontoxigenic microbial cultures traditionally used in enzyme manufacture are also ideal candidates for cloned DNA. For example, the gene for a useful enzyme that is not synthesized by *Bacillus subtilis* could be introduced into the organism. The new "strain" would then produce the new enzyme product and would not present a pathogenic or toxigenic risk greater than that of its "parents," the nonpathogenic *B. subtilis* and the gene for the useful enzyme.

Large-scale growth. There are two ways to grow microorganisms on an industrial scale. One way is to use liquid medium which is agitated and aerated, and the other way is to use solid or semi-solid medium held in large trays or drums (*16, 47*). In both cases, it is necessary to control environmental factors such as temperature, pH and degree of aeration. Equipment must be designed for easy cleaning and sterilization. Conditions must be employed which minimize the growth of contaminating microorganisms that will ruin the fermentation. During growth, cultures are routinely sampled and tested for possible contamination (*16, 47*).

All ingredients used to formulate the growth medium should be free of toxic contaminants (*7, 8, 16, 45, 47*). It is important that any "carry-over" of growth medium into the final enzyme preparation not bring with it possible toxic substances, especially when the enzyme being manufactured is intended for food processing.

Enzyme extraction, concentration and standardization. The desired enzyme may be present in the medium or inside the cells. Enzymes secreted into solid or semi-solid medium, and most intracellular enzymes, are extracted before further processing. In this context, extraction means to "wash out" and solubilize the enzyme in an aqueous solution (*16, 47*). Where the enzyme is secreted into a liquid growth medium, an extraction step is not necessary.

Enzymes secreted into solid or semi-solid media may be extracted directly into water solutions using a counter current system which filters as well as extracts (*16, 47*). Alternatively, solid or semi-solid media containing the microorganisms may be dried, ground and treated with water solutions to solubilize the desired enzyme. This method can be used to recover both intra- and extracellular enzymes. In the case of intracellular enzymes from microorganisms grown in liquid media, the cells are first collected by centrifugation or filtration and then ruptured by any of a number of physical and/or chemical procedures (*16, 47*). The enzymes are then extracted from ruptured cells with aqueous solutions.

After extraction, enzyzme solutions are usually concentrated to reduce volume. It is common to use ultrafiltration to reduce the amount of water and substances below specified molecular weights (e.g., salts, small organic molecules and peptides). Sometimes enzymes are concentrated by precipitation with salts or organic solvents, but because of organic solvent cost this method is not as common today as it was 10 years ago (*47*). In other cases, con-

[2]Under certain conditions an inducible enzyme can be made constitutive by mutation in the regulator, operator or (more rarely) the promoter region of the genetic operon. The enzyme will then be expressed in the absence of the inducer. Thus, under fermentation conditions used to produce an enzyme, production of "new" enzymes or proteins can be made to occur. These proteins or enzymes were originally present in the genetic material of the parent and would be normally synthesized under the right fermentation conditions without mutation. In addition, mutation induces minor changes in base sequence of DNA encoding for proteins and enzymes (base change, deletion, etc.). Thus, minor changes in protein structure are possible as a result of mutations affecting the structural gene. These changes can lead to increased enzymic activity or they may decrease or destroy enzymic activity (*18*).

centration is accomplished simply by removing water through evaporation. Preservatives are almost always added during processing, and optionally in the final preparation, to prevent microbial growth and to stabilize and maintain the desired enzymic activity. Proper and appropriate use of preservatives and stabilizers serve to protect the consumer from unsafe or ineffective enzyme products (*7, 8, 16, 47*). When the enzyme is intended for addition to food, all such additives and diluents must be acceptable to the FDA for use in food. They must be of food grade quality and the levels used must not exceed specified limits.

Most industrial enzymes are not purified to any significant extent because purification is not necessary to achieve safe and useful products (*3, 4, 16, 47*). However, it is sometimes desirable to remove or destroy unwanted enzyme activities which would otherwise interfere with effective use of the desired enzyme preparation. For example, rennet produced by some microorganisms contains lipase activity which will make the finished cheese rancid. By carefully exposing the crude rennet to heat or low pH, the lipase can be inactivated without affecting the protease activity. In this example, the unwanted lipase is not physically removed (as in purification); the protein remains but is no longer catalytically active (*47*). Because of expense, physical separation normally is accomplished only when there is a market for the individual separated enzymes, although some manufacturers do highly purify certain enzymes of particular economic importance. For example, one company produces a very pure, crystalline glucose isomerase preparation for its own use (*47*).

Following extraction, concentration and stabilization, enzyme preparations are standardized (*3,4,47*). Because enzymes are catalysts, they are marketed in terms of units of catalytic activity rather than by weight or volume. A unit of catalytic activity for an enzyme preparation is defined in terms of the transformation of a given amount of substrate during a specified period of time under stated reaction conditions. Biochemists often use a unit defined by international convention, which is the amount of enzyme required to transform one micromole of substrate per minute under specific reaction conditions. However, this definition is not applicable to many commercial uses where the substrate is part of food (e.g., Swift's hamburger test for papain; *47*). Hence, many assays for industrial enzymes are based on specific application rather than uniform convention.

The standarization procedure consists of using a specific quantitative assay to determine the level of enzyme activity per milliliter or gram of the final enzyme preparation and then adjusting the activity (usually by dilution of the enzyme preparation) to conform with a desired level of activity which is convenient to use. Unstandardized enzyme preparations may also be sold, and, in this case, total activity is stated and will vary between lots.

Given that enzymes are marketed on the basis of activity rather than weight or volume per se, it follows that the activities and amounts of other enzymes, as well as the levels of nonenzymic catalytically inert materials, may vary from lot to lot and almost certainly from source to source (*47*). Moreover, since enzyme preparations are almost always relatively crude mixtures, it is apparent that anything produced by the source organims, and anything purposely or inadvertently introduced into the system during enzyme manufacture, may end up in the final enzyme preparation. For this reason, it is important that the source organism not produce or contain toxins. To avoid inadvertent contamination with unsafe substances, it is necessary that CGMPs be followed during enzyme manufacture. There are strict limits on the levels of heavy metals which will be tolerated, and there are requirements for demonstrating microbiological safety (absence of salmonellae, etc.) (*3, 4, 16, 45, 47*).

Immobilized enzymes

Some enzymes are sold in an immobilized form, i.e., products containing enzymes that have been immobilized by adsorbtion, entrapment, reaction with cross-linking agents or covalent attachment to insoluble supports (*29*). The safety evaluation of products such as these may require consideration of factors other than the safety of the enzyme, its source and the by-products of the production methods. For this reason, safety evaluation of immobilized enzymes will not be included in this paper.

Consumption levels

Total Organic Solids (TOS). Enzymes are marketed by units of activity rather than by weight or volume, and enzyme preparations always contain other substances (salts, preservatives, stabilizers, carriers, nonenzymic organic material, etc.) (*16, 45, 47*). Further, some enzymes are added to food and remain there, although they may be inactivated by heat or other treatment in the finished food product. On the other hand, some enzymes only come in contact with the food (immobilized enzymes) but do not stay there. For these reasons, it is not an easy matter to estimate total enzyme use and consumption.

The most logical means currently available for arriving at a reliable estimate of enzyme use and consumption was developed by the Ad Hoc Enzyme Technical Committee (AHETC), a trade group representing companies that produce or distribute enzymes for food use. AHETC set forth the concept of Total Organic Solids (TOS; *5*) as a means of determining the toxicological significance of material derived from the enzyme source. TOS is defined as the sum of the organic compounds, excluding diluents, contained in the final enzyme preparation. It is derived experimentally as follows:

$$\text{TOS}\,(\%) = 100 - A - W - D$$

where A = % ash contained in the extract or isolated enzyme, W = % water in the extract or isolated enzyme, D = % diluents (if any, or carrier if enzyme is immobilized).

The 1978 Enzyme Survey. The Food and Nutrition Board (FNB) of the National Research Council's Assembly of

TABLE 2. *Selected enzymes and their maximum use in various foods based on TOS (Total Organic Solids) (5).*

Enzyme	Food category	Maximum use[a]
Papain	Baked goods	0.0078%
	Meats/meat products	0.0044%
	Beer/ale/malt beverages	0.0045%
Rennet (and other milk clotting enzymes)	Cheese	0.036%
	Gelatins/puddings/custards	0.0040%
Bromelain	Candy	0.000016%
	Fats and oils	0.000084%
	Snack foods	0.00056%
Pectinase	Baked goods	0.00000026%
	Fruits/juices	0.0035%
	Non-creamed soups	0.060%
Invertase	Candy	0.0078%
α-Amylase	Breakfast cereals	0.0030%
	Sugars/frostings	0.052%
	Gelatins/puddings/custards	0.0000020%
	Corn syrup	0.052%

[a]Percent of food based on TOS.

Life Sciences has undertaken several surveys of industrial use of food additives. In 1977, the FNB's Committee on GRAS List Survey — Phase III was asked by the FDA to organize an extensive survey of enzyme use in food processing. The Committee worked closely with AHETC and the FDA in developing questionnaires; then the AHETC distributed the survey forms to users and manufacturers of enzymes on a confidential basis. The FNB Committee received the completed forms directly for the respondents, reviewed and analyzed the data, and submitted a report to the FDA. The document is entitled *The 1978 Enzyme Survey (5)*.

The survey report contains extensive information on 23 enzymes and an analysis of their use in a detailed list of specific food items. Average and maximum use levels are estimated by TOS. Removal and inactivation of the enzymes by further processing is also tabulated. Table 2 contains some examples from this survey demonstrating the low levels at which enzymes are added to foods.

ENZYME SAFETY

Current status

Exhaustive literature reviews commissioned by the FDA for food enzymes from microbial (*43*) and nonmicrobial (*11, 44*) sources support the proposition that enzyme preparations from nontoxigenic, nonpathogenic organisms are safe to consume. This conclusion is strengthened by the report of the Joint FAO/WHO Expert Committee on Food Additives, which evaluated both published and unpublished data (*12*). There are numerous GRAS affirmation petitions currently before the FDA which also contain safety data on enzyme preparations (*46*).

It is not surprising that the enzymes used in food processing have proven to be nontoxic when tested in animals. In fact, very few toxic agents have enzymatic properties and those that do, e.g., diphtheria toxin and certain enzymes in the venoms of poisonous snakes catalyze unusual reactions which are completely unrelated to the kinds of catalytic transformations that are desirable in foods. Hence, the only relevant issue is whether enzyme preparations contain toxic contaminants. It follows that, if the source organisms do not produce toxins and if CGMPs are followed during manufacture, then the resulting enzyme preparations will not contain hazardous materials.

In practice, industrial enzymes have a strong record of safe use in food processing. However, as with all food components, it is important that scientific data be provided to show that enzyme preparations, particularly those lacking a long history of safe use, are safe to consume. To develop a logical approach to this issue, we shall first consider the factors which bear on the safety of enzymes and then present guidelines for assessing enzyme safety.

Safety considerations

Safety of source organism. The safety of the source organism should be the prime consideration in assessing the probable degree of safety of an enzyme preparation intended for use in food. For example, if the source organism is a food animal, an edible and nontoxic plant, or a nontoxigenic and nonpathogenic microorganism which does not produce antibiotics, then it follows that enzyme preparations obtained from that source organism using CGMPs (*8*) will be safe to consume at the low levels encountered in processed foods. Moreover, in other instances

where toxic contaminants are present, they may be removed during manufacture.

With regard to microorganisms used in enzyme manufacture, we have discussed previously our contention that mutagenesis in the laboratory does not result in the acquisition of new genes, so it is not possible for an isolate to acquire a new toxin gene by mutation. It may be theoretically possible for a mutation to alter the structure of an otherwise nontoxic enzyme in such a way that the enzyme becomes toxic (*10*), but there is no experimental basis for this notion and we consider it to be remote. Advances in DNA sequencing may ultimately be useful in providing definitive proof of nontoxicity.

Proving that a new microbial isolate does not produce a toxin elaborated by other strains in the same species is complicated by the fact that toxin production may be affected by growth conditions. Under some conditions, toxin synthesis may be high, whereas under other conditions, it may be low or undetectable. Hence, to establish that an isolate is nontoxigenic in an absolute sense may not be possible strictly from data on toxin expression. By assaying toxin production under a variety of growth conditions, the probability of demonstrating toxigenic potential is increased. Moreover, if an isolate is grown under conditions where other closely related organisms elaborate a toxin, the reliability of a negative result is strengthened even further.

In practice, enzyme preparations will not contain all of the substances that a source organisms is able to produce. For example, enzymes which are concentrated by ultrafiltration or precipitation will contain far fewer low molecular weight components than are present in crude enzyme extracts. For this reason, even if an organism produces low levels of a potentially hazardous substance, the amount of a finished enzyme preparation needed to produce a deleterious effect in animals likely will be far above the low concentrations at which enzyme preparations are employed in food processing. Published animal feeding studies and summaries of unpublished experiments reviewed by expert committees (*12, 43, 44*) fully support this conclusion.

Pathogenicity. If an isolate is known to be or suspected of being a human pathogen, it will almost certainly not be further considered for commercial enzyme production unless it is the singular source of a unique and useful enzyme. The problems inherent in maintaining and handling cultures of pathogenic organisms on an industrial scale make it unlikely that they will ever be used in the manufacture of enzymes for food processing, and there are federal regulations concerning this issue (*7*). However, high purified enzymes from pathogenic bacteria are produced commercially and used with medical supervision in the treatment of disease (*19*).

Carcinogens and mutagens. No one has ever reported an enzyme which when fed was mutagenic or initiated carcinogenesis.[3,4] Given our current understanding of the processes of carcinogenesis and mutagenesis (*34, 51*), it is implausible to expect that the protein component of an enzyme or protein with such activity will ever be discovered[4]. Rather, attention should be directed towards the relatively small organic molecules (in general, MW <500 daltons) that possess carcinogenic or mutagenic activity and which might reasonably be expected to contaminate a given enzyme preparation.

Enzymes from mammals commonly used as food in the United States will not contain mutagens or substances which can initiate [3] carcinogenesis as long as CGMPs are followed. Some plants are known to produce carcinogens (*13, 34*), but the pineapple, fig, barley and papaya are not among them. The fungal and bacterial enzyme sources listed in Table 1 also are not known to produce carcinogens or mutagens. However, fermentative yeasts, such as *Saccharomyces cerevisiae*, may produce low levels of urethan (*37*), a carcinogen which is not mutagenic in the Ames test (*1*), as a natural by-product of fermentation. For this reason bread, wine and beer often contain low levels of urethan (*37*). There are no reports of urethan in yeast enzyme preparations. Moreover, where yeast enzyme preparations are concentrated by ultrafiltration or precipitation, small molecular weight compounds, such as urethan, will be removed or greatly decreased in concentration. For this reason it is unlikely that urethan levels in yeast enzyme preparations would exceed the levels found naturally in bread, wine and beer.

Several long-term animal studies (>90 days) have been conducted with enzyme preparations from microorganisms, and none showed evidence of carcinogenicity or chronic toxicity (*12, 43*). It is necessary to conduct such long-term tests for each new microbial culture, or for each new enzyme? We think not. For example, we have been unable to locate a single confirmed report of a carcinogen or mutagen produced by bacteria, other than certain *Actinomycetales*, particularly *Streptomyces*, when grown in ordinary culture media. When nitrite and secondary amines are added to culture media, a few bacterial species appear capable of generating nitrosamines through unknown mechanisms (*35*). However, there is no reason for nitrite and secondary amines to be added to culture media intended for use in food enzyme manufacture. Nitrosamines,

[3]It is important to recognize that the process of carcinogenesis as now understood consists of two stages. The first stage is called *initiation*, the second *promotion (39)*. Some animal products, e.g., certain fats and hormones, may at high doses and in certain well-defined experimental systems promote specific types of cancers. However, it has not been shown that these substances can initiate cancer, and it is commonly accepted among experts in this field that they are not complete carcinogens. Animals exposed to carcinogens may metabolize them to other forms which retain carcinogenic activity, e.g., aflatoxin M_1 in the milk from cows exposed to aflatoxin B_1 in their diets; (*42*). Animals may also generate nitrosamines from nitrite and secondary amines in their gastrointestinal tracts (*35*). However, mammals are not known to produce substances as normal body constituents which experts would classify as carcinogens.

[4]It is possible for certain enzymes that act on nucleic acids, such as DNA-dependent DNA ploymerase, to be altered by mutation in such a way as to become error-prone, thus resulting in further mutation in the organism containing the error-prone polymerase (*48*). However, such enzymes would not be produced for use in food processing. Moreover, should such enzymes be present in food enzyme preparations, they would almost certainly not enter human cells and produce an adverse effect. They are also produced by some *Streptomyces* sp. antibiotic proteins with mutagenic and DNA-damaging activities due to the presence of nonprotein prosthetic chromophores, i.e., the apoproteins themselves are without such activity (*25a, 39a*).

or any other classes of carcinogenic or mutagenic chemicals, should not be considered either a real or potential problem area in enzyme manufacture from bacteria (other than certain *Actinomycetales*).

In contrast, some antitumor agents and antibiotics produced by *Actinomycetales*, particularly certain *Streptomyces*, are weakly carcinogenic, e.g., azaserine (*34*). Moreover, some mycotoxins have carcinogenic and mutagenic activities (*33, 34, 42*). If there is reason to believe that such substances might be produced by a new culture under test, then specific chemical, biochemical or biological tests for the substances should be conducted.

Teratogens and reproductive effects. Various dietary deficiencies and excesses, hormones, drugs, agricultural and industrial chemicals, naturally-occurring toxins, and physical and biological agents produce, under some circumstances, teratogenic effects or reproductive deficiencies in experimental animals (*20, 27*). Some of these agents or conditions, such as German measles, alcohol abuse, and certain drugs and antibiotics, produce similar effects in humans. However, enzymes are not among the substances which have been shown to cause teratogenesis or reproductive deficiency. In fact, in a four-generation study in rats, a rennet preparation from *Mucor pusillus* produced no evidence of teratogenicity or toxicity towards the reproductive system (*12*), and similar negative data have been obtained for various enzymes from other microbial (*43*) and nonmicrobial (*11*) sources. Those microbial metabolites which could pose such a risk should be detected either as certain specific antibiotics (*20, 27*) or as acute/subchronic toxins (*42*).

Antibiotics. Antibiotics are chemicals produced by various species of microorganisms which kill or inhibit the growth of other microorganisms. They are really a special class of toxic agents which are useful to man in the control of disease. It is well-documented that a sensitive microorganism can acquire plasmids which confer antibiotic resistance on the host (*40*). For this and other reasons enzyme preparations intended for use in food processing should not contain antibiotics. There are methods for assessing enzyme preparations for antibiotic activity (*4*).

Allergies and primary irritations. Industrial enzymes are foreign (nonhuman) proteins, and as such, may be allergenic for humans under certain conditions. The group most likely to be affected are plant workers (*11, 15, 47, 49*). There are methods and procedures for protecting workers from this potential hazard and it is considered to be a manageable problem (*15, 47, 49*).

There are no confirmed cases of allergies or primary irritations in consumers caused by enzymes used in food processing. This is probably due, in part, to the low levels of enzymes added to foods. Foods naturally contain a wide variety of foreign (nonhuman) proteins, many of which are present at levels far higher than the industrial enzymes added as processing aids. Allergies and primary irritations from enzymes used in food processing should be considered a low priority item of concern except in very unusual circumstances. There is no justification for requiring routine testing of enzyme preparations for allergic responses or primary irritations relative to consumer safety.

Toxins involved in food posioning. A few bacterial species produce toxic proteins or peptides which can cause food poisoning. These include both enterotoxins and neurotoxins (*41*). There are immunological assays or animal systems for detecting such toxins. Within a bacterial species known to cause food poisoning via a toxin, usually only some, but not all, strains produce the toxin. Hence, nontoxigenic strains can be isolated (*41*). Some bacterial toxins are actually coded for in bacteriophage DNA which has become integrated into the bacterial genome as a prophage. "Curing" the organims of the prophage results in loss of toxicity (*41*).

Bacterial toxins which cause food poisoning are, by definition, substances which produce acute toxic responses following introduction into the gastrointestinal tracts of sensitive animals. The nature and severity of the toxic response may vary among animal species under test, as well as the amount of toxin required to produce a measurable effect.

Products of enzymic reactions. Enzymes are used in food processing because they produce desirable changes in the natural food constituents. They are usually inactivated or removed before the final food product is marketed. As such, enzymes should be classified as *processing aids* or *secondary direct additives*. Declaring their presence on the label of a food product, in most cases, would be incorrect, since only rarely is the active enzyme present in the final product. This unique status of enzymes can lead to a new question, however. Are the products of the enzymic reaction safe? Developing an answer to this question requires an understanding of what the enzyme is doing in producing an apparently favorable transformation in the food.

Most of the enzymes used in food processing are degradative enzymes which split macromolecules, i.e., proteins, complex carbohydrates and lipids, into smaller subunits. Another important example is glucose isomerase, which catalyzes the conversion of glucose into its isomer fructose. Both glucose and fructose are nutritive and nontoxic. Only one enzymic reaction used in food processing is known to yield a potentially toxic product. Pectic enzymes increase the methanol content of treated fruit products, but the amount produced is far below the hazard level (*47*). There are reliable and rapid assays for methanol in food.

The question of hypothetical, potentially hazardous enzyme reaction products is difficult to evaluate, but probably its importance is marginal. For example, proteases from all sources degrade proteins into peptide fragments and amino acids. However, different proteases attack proteins at different sites and may produce different sets of peptide fragments from the same protein substrate (*52*). There are many biologically active peptides in nature which serve in various metabolic regulatory capacities. One may wonder if the peptides produced by proteases have any biological properties of their own. Until recently, most biochemists would have considered as highly remote the possibility that toxic peptides might be generated from

otherwise nontoxic proteins, and, indeed, it should still be considered speculative. However, a recent report (*53*) indicates that peptides with neuropharmacological properties are generated by the action of the natural animal digestive enzyme, pepsin, on wheat gluten or casein, i.e., the major protein of milk. The peptides are called "exorphins" because they mimic in vitro the action of opioid-like peptides, the endorphins, which are produced naturally by animals. It is suggested that such peptides may form during digestion of some food proteins in the human gastrointestinal tract, and could have physiological significance (*53*). The possibility of such peptides forming in processed foods treated with proteases was not considered.

This example illustrates the difficulty that arises when one attempts to establish absolute safety. Such a goal would be extremely difficult for a static system, and is clearly impossible when dynamic forces, such as basic scientific inquiry, continually expand our understanding and knowledge. However, there is also no reason, on the basis of available information, to fear that processed foods treated with proteases might pose a hazard, especially one that is greater than that posed by our own digestive systems. This is clearly a research area which deserves further support, especially as it relates to human physiological significance and development of specific and relevant assays.

Interactions between enzymes and other food components. It is well-known that certain drugs are not compatible with one another and that combinations of such incompatible drugs can result in interactions which are toxic (*28*). It has been suggested that such interactions might also occur between enzymes and other components of beverages or food products (*6*). However, there is no scientific basis for such speculation. It is extremely unlikely that enzymes, which are used at very low concentrations and are almost always inactivated or removed before the finished food or beverage is marketed, could produce a toxic effect due to interaction with another substance. Given the high specificity of enzyme action, it is difficult to imagine such an occurrence. The highly improbable possibility of toxic interactions involving food enzymes should not be afforded serious consideration unless supporting data appear in respected and well-refereed scientific journals.

Direct effects of food enzymes on consumers. Under the usual conditions of use in foods, enzymes do not pose a hazard for consumers. For example, ingesting an active protease at relatively low levels could hardly affect the human gastrointestinal tract, where many potent proteases, such as trypsin and pepsin, already are present at levels sufficient to digest food. This view is supported by the report of an expert committee (*11*). Proteases may adversely affect the skin, mucous membranes of the nose and throat, and lungs, and such effects are sometimes seen workers who handle large quantities of proteases. However, such occurrences are extremely rare in consumers who use much lower levels of active enzyme (*11, 15*), and it is not possible for heated foods containing inactive proteolytic enzymes to pose such a threat. Active proteases are, of course, widely distributed in fresh fruits, vegetables, cheeses and other uncooked foods which may be consumed.

We know of no reported adverse effects on humans from lipase/esterases or carbohydrases in foods. Moreover, many enzymes are inactivated in the gastrointestinal tract and digested as protein.

Concept of relative safety

The terms *nontoxigenic* and *nonpathogenic* should not be considered in an absolute sense. In the real world they are relative concepts which convey certain probabilities. A nontoxigenic organism is one which does not produce injurious substances at levels that are detectable or demonstrably harmful under ordinary conditions of use or exposure. In the same vein, a nonpathogenic organism is one that is very unlikely to produce disease under ordinary circumstances. Thus, *Aspergillus oryzae* should be considered nontoxigenic because it does not produce detectable levels of aflatoxin (*23, 50*) and is not listed with molds known to produce other mycotoxins (*42*). Strains in commercial use did not produce detectable levels of beta-nitropropionic acid (*36*) and there are no reports of this organism producing adverse effects in animals. Likewise, *S. cerevisiae* should be considered nontoxigenic even though low levels of the carcinogen urethan are produced during fermentation (*37*) because, as far as we can tell, the amount of urethan is too low to be significant. Applying an absolute definition in this case would result in the banning of bread, wine and beer. There is no reason to believe that such an extreme measure would make our lives safer! As long as the levels of urethan in fermentative yeast enzyme preparations do not exceed those found in fermented foods and beverages, they should not be a cause of concern.

Aspergillus niger produces low levels of toxic substances (*22*), but it is only after such substances are extracted and concentrated that toxicity can be demonstrated. This example points up the important distinction between *toxin*, a chemical entity, and *toxic effect*, a biological phenomenon produced by toxins only at effective doses. Synthesizing low levels of toxins per se should not be sufficient to support redefining *A. niger* as a toxigenic organism, and it should remain classified as nontoxigenic. In the same way *B. subtilis* should be considered nonpathogenic even though one could imagine an individual with an extremely compromised immunological system succombing to a *B. subtilis* infection. Under more ordinary circumstances, *B. subtilis* does not cause disease.

These concepts are important in considering safety assessment. Absolute safety is not achievable and cannot be our goal. Rather, we should think in terms of probabilities tempered with common sense.

Animal testing for toxins

The purpose of animal testing is to assure that toxic effects are not produced by non-enzyme substances in enzyme preparations under realistic projections of use. There

is no basis for concern that the enzymes under consideration in this report are themselves toxic. Acute and subchronic oral toxicity studies (to be proposed) should be conducted with two animal species (*24*). This is necessary to compensate for possible species variation in toxic response. For example, rats are much more sensitive to aflatoxin B_1 than mice, whereas dogs are more sensitive than rats to ochratoxin A (*42*). There are also species variations in response to the protein/peptide enterotoxins and neurotoxins of bacteria (*41*). Additionally, some animal species are capable of emesis, e.g., dogs and pigs, whereas others are not, e.g., rodents. Selection of appropriate test animals should be based on two criteria: (a) which toxins could be produced by the source organism and (b) which toxins have already been eliminated from further consideration by the use of specific chemical/biochemical assays. In many instances, rats and dogs may be the most appropriate test animals (*24*).

Guidelines for determining enzyme safety

Basic premises. In developing guidelines to assure the safety of enzymes used in foods, we have adopted the following basic premises to guide our thinking. The rationale for each of these premises can be found in preceding sections.

1. Enzymes are naturally occurring proteins. Only a very few, highly unusual enzymes are toxic and they would not be used in foods.
2. There is no basis for concern that enzymes acting on otherwise wholesome food constituents will generate harmful products. Hence, there is no reason to test enzyme-treated foods for toxicity.
3. New enzymes could be derived from animals, plants or microorganisms. However, for technical reasons it is likely that most new enzyme preparations will be derived from microbial sources, in many instances new microbial species or strains.
4. Enzymes are added to food at very low levels. Failure to demonstrate harmful materials in, or toxic effects from, concentrated enzyme fractions, which when diluted yield finished enzyme preparations for marketing, gives reasonable assurance of their safety. Alternatively, failure to demonstrate harmful materials in, or toxic effects from, cultures or crude extracts of a proposed source microorganism, gives reasonable assurance of safety for any enzyme preparation which may be produced from that source organism using CGMPs.
5. If a microbial culture does not produce known toxins and if its metabolites are nontoxigenic in the sense that they do not produce food poisoning, intoxication or illness when ingested, then enzymes derived from that culture using CGMPs will be safe for use in food processing.
6. If there are toxigenic strains of the species to which the new culture belongs, then growth conditions under which those strains produce toxins should be tested. The condition(s) to be used for enzyme manufacture would, of course, be included. It is also prudent to test mutants for toxins produced by other *strains* of the same *species* even if the parent culture is negative for such substances.
7. Certain microbial species produce antibiotics, which are detectable in appropriate bioassays.
8. Some of the filamentous fungi and *Actinomycetales* produce toxins. A few of these substances are carcinogenic, e.g., aflatoxin, and some also possess antitumor and antimicrobial activity, e.g., azaserine. Such metabolites may be detected with specific chemical, biochemical or biological assays.
9. Bacteria other than *Actinomycetales* may also produce acute toxins. Of specific concern are the peptide/protein toxins that act via the oral route, e.g., enterotoxins and certain neurotoxins. Toxins associated with foodborne illness can be detected with serological or animal assays.
10. Bacteria as a group (other than *Actinomycetales*)are not known to produce carcinogens or mutagens when grown in ordinary culture medium which does not contain nitrite and secondary amines.
11. Yeasts as a group are not known to produce toxins, although some yeasts are pathogenic. The carcinogen urethan may form at very low levels in yeast fermentations. Urethan can be detected by chemical assay.

Microbial enzymes. Guidelines for determining safety of microbial enzymes are shown in Table 3. These guidelines may be applied to concentrated enzyme fractions which are diluted to produce finished enzyme preparations. Alternatively, the guidelines may be applied to crude culture extracts or whole cultures from which enzymes are manufactured. If the crude culture extracts or whole cultures are judged to be safe, then enzymes can be manufactured from these sources without further testing.

It is important to note the following features concerning the guidelines in Table 3.

1. All test materials must be evaluated for antibiotic activity.
2. No test material can pass through the Decision Tree without being tested for toxic constituents.
3. Two animal bioassay systems are proposed. The first is a single oral challenge. The purpose of this assay is to evaluate the test material for food poisoning toxins, specifically enterotoxins and certain neurotoxins, which are protein or peptide toxins produced by a few bacterial species. The second proposed bioassay is a subchronic feeding study in two appropriate animal species. The purpose of this procedure is to detect mycotoxins and other toxic substances which might not produce acute toxicity. All

TABLE 3. *Guidelines for determining the safety of microbial enzymes*[a].

A. *Decision Tree*	If yes —proceed to—	If no
1. Is the test material free of antibiotics?[b]	A,2	D
2. a. *For bacteria and yeast*, is the test material:		
i. Free of toxins[c] known to be produced by other strains of the same species?	A,3	D
ii. If there are no known toxins[c,d] produced by other strains of the same species, is the no-adverse effect level in a single oral challenge at least 100 times greater than the estimated mean human consumption level?[e,f]	B	D
b. *For molds*, is the test material free of detectable levels of aflatoxin B_1, ochratoxin A, sterigmatocystin, T-2 toxin, zearalenone and any other toxins known to be produced by strains of the same species?[g]	C	D
3. Is the no-adverse effect level in subchronic (90-d) feeding sutdies at least 100 times greater than the estimated mean human consumption level?[f,h]	ACCEPT	D
B. *Special considerations for certain yeasts and bacteria*		
1. If the source culture is well-known, widely distributed, nonpathogenic yeast, e.g., certain species of the genus *Saccharomyces*, or if it belongs to a bacterial species that is well-characterized, commonly present in foods, has a history of safe use in food enzyme manufacture, and has never been implicated in foodborne disease, e.g., *Bacillus coagulans, Bacillis licheniformis, Micrococus lysodeikticus*, and *Bacillus subtilis (17)*, the test material can be ACCEPTED at this point.		
2. Test material from other bacteria and yeasts must be considered under part A,3.		
C. *Special considerations for certain molds*		
1. If the source culture is well characterized, commonly present in food, has a history of safe use in food enzyme manufacture, and has never been implicated in foodborne intoxication or disease, e.g., *Aspergillus oryzae, Apergillus niger* and *Rhizopus oryzae (16,23,36,41,42,43,45,47,50)*, the test material can be ACCEPTED at this point.		
2. Test material from all other species of molds must be considered under A,3.		
D. *Disposition of materials that fail any Decision Tree requirement*		
A negative answer to questions 1, 2 or 3 signifies the presence of an undesirable substance and the material is not acceptable for use in food. If the undesirable substance can be removed, the purified material must be passed through the system again beginning at the point of the original negative answer.		

[a]These guidelines are intended for crude culture extracts, for whole cultures, and for concentrated enzyme fractions which, when diluted, become enzyme preparations suitable for marketing.
[b]As determined by (*4*) or comparable methods.
[c]For the purposes of these guidelines, the term "toxin" refers to a substance which is regarded by experts as a cause of food poisoning, intoxication or illness when ingested. Examples are staphylococcal enterotoxins, botulinal neurotoxins and mycotoxins.
[d]Certain cultures in this category are acceptable on the basis of a single acute oral toxicity test, as explained in part B,1. Cultures that fall under part B,2 can go directly to part A,3 without an acute oral toxicity test. This is permissible because the subchronic feeding specified in part A,3 is more rigorous and more meaningful than the acute oral toxicity test embodied in part *A,2,aii*.
[e]Expressed as mg/kg body weight and determined using two appropriate animal species.
[f]Estimated mean consumption level is calculated from the sum of the intakes for each food category in which the material is expected to be used. An example of such determination is: (USDA mean portion size) × (Maket Research Corporation of America eating frequency for the entire population) × (the usual level of use expressed as TOS for the enzyme in question)(*2,14*).
[g]As determined by (*38*) or comparable methods.
[h]Expressed as mg/kg body weight/day, and determined using two appropriate animal species.

known microbial toxins active via the oral route and present at effective levels will be detectable by these procedures. It should be pointed out that preparations will be tested in these proposed feeding studies only after first being assayed for toxins which might reasonably be expected, using chemical, biochemical or biological methods. For example, all test material from fungal sources should be assayed for certain known mycotoxins (*4, 38*).

4. In establishing an Acceptable Daily Intake for microbial enzymes based on the animal feeding studies which we have proposed, there should be no adverse effect at a dose which is 100 times the estimated mean human exposure (based on TOS). This criterion applies to the single oral challenge and to the subchronic feeding study, and is based on the traditional 100-to-1 safety factor for food chemicals (*26*).
5. The only test materials which can pass through the Decision Tree without a subchronic feeding study are those which satisfy the criteria of B.1 or C.1, i.e., certain bacteria, yeast and molds, which are well-known and have never been associated with foodborne illness or disease. However, as stated above, bacteria and yeast that meet these criteria still must pass the single oral challenge test, and molds must give negative test results for a battery of known mycotoxins.

Nonmicrobial enzymes. As indicated previously, meat animals, e.g., cattle, swine and sheep, and edible and nontoxic plants, e.g., papaya, pineapple, barley and fig, have long histories as sources of enzymes used in food processing (*3, 4, 16, 45, 47*). These traditional sources need not be subjected to toxicity testing.

For the purposes of this paper, it is assumed that only animals commonly regarded as food will be employed in enzyme manufacture. As long as CGMPs are followed during manufacture, enzymes derived from food animals may be assumed to be safe for use in food processing. Animal testing for possible toxicity is not warranted.

With regard to new plant enzyme sources, it is assumed that only edible plants will be considered. If the edible plant has been well-studied, is widely consumed without apparent harm, and does not produce toxic substances, then no animal testing should be required. However, if the plant is known to produce toxins, then care should be taken not to concentrate the toxic substances during enzyme manufacture. The final enzyme preparation should not contain toxic substances in quantities that might represent a hazard to health.

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REFERENCES

1. Ames, B. N., J. McCann, and E. Yamasaki. 1975. Methods for detecting carcinogens and mutagens with the salmonella/mammalian-microsome mutagenicity test. Mutat. Res. 31:347-364.
2. Anonymous. 1982. U.S.A. comment to the ad hoc working group on food additive intake. 15th Session Codex Committee of Food Additives, Joint FAO/WHO Food Standards Programme, Hague, Netherlands.
3. Anonymous. 1981. Food chemicals codex, 3rd ed. National academy of Science/National Research Council, Food and Nutrition Board, Committee on Codex Specifications, National Academy Press, Washington, DC. pp. 107-110, 480-481.
4. Anonymous. 1981. Specification for identity and purity of carrier solvents, emulsifiers and stabilizers, enzyme preparations, flavoring agents, food colours, sweetening agents and other food additives. Food and nutrition paper no. 19. 25th session of the Joint Expert Committee on Food Additives, FAO/WHO, Rome. pp. 215-218.
5. Anonymous. 1981. The 1978 enzyme survey, summarized data. National Academy of Sciences/National Research Council, Food and Nutrition Board, Committee on GRAS List Survey, Phase III. National Academy Press, Washington, DC.
6. Anonymous. 1981. CSPI asks hearing on proposal to rescind alcohol ingredient labeling. Food Chem. News 23(23):29-30.
7. Anonymous. 1982. Code of federal regulations. 21 CFR 173.110, 120, 130, 135, 145, 150, 160, 165. Office of the Federal Register, National Archives and Records Service, General Services Administration, Washington, DC.
8. Anonymous. 1981. Code of federal regulations. 21 CFR Sec. 110.1. Office of the Federal Register, National Archives and Records Service, General Services Administration, Washington, DC.
9. Anonymous. 1979. Modern urine chemistry. Ames Division, Miles Laboratory Inc., Elkart, IN.
10. Anonymous. 1978. Evaluation of certain food additives. pp. 8-9. *In* Evaluation of certain food additives, Technical report series no. 617. World Health Organization, Geneva.
11. Anonymous. 1977. Evaluation of the health aspects of papain as a food ingredient. Select committee on GRAS substances. National Technical Information Service Document PB-274-174. U.S. Dept. of Commerce, Springfield, VA.
12. Anonymous. 1975. Toxicological evaluation of some food colours, enzymes, flavor enhancers, thickening agents, and certain other food additives. pp. 11, 124-141. *In* World Health Organization Food Additive series, No. 6. World Health Organization, Geneva.
13. Anonymous. 1973. Toxicants occurring naturally in foods. National Academy of Science, National Research Council, Food and Nutrition Board, Committee on Food Protection, Washington, DC.
14. Anonymous. 1972. A comprehensive survey of industry on the use of food chemicals generally recognized as safe (GRAS). Comprehensive GRAS survey. National Academy of Sciences/National Research Council. National Technical Information Service PB-221 - 949. U.S. Dept. of Commerce, Springfield, VA.
15. Anonymous. 1971. Enzyme-containing laundering compounds and consumer health. National Academy of Science, National Research Council, Division of Medical Sciences, Ad Hoc Committee on Enzyme Council, Division of Medical Sciences, Ad Hoc Committee on Enzyme Detergents. National Technical Information Service, Document PB-204-118. U.S. Dept. of commerce, Springfield, VA.
16. Beckhorn, E. J., M. D. Labbe, and L. A. Underkofler. 1965. Production and use of microbial enzymes for food processing. J. Agr. Food Chem. 13:30-34.
17. Buchanan, R. E., and N. E. Gibbons (eds.). 1974. Bergey's manual of determinative bacteriology, 8th ed. Williams and Wilkins Company, Baltimore, MD.
18. Clarke, P. H. 1978. Experiments in microbial evolution. pp. 137-218. *In* I. C. Gunsalus, L. N. Ornston and J. R. Sokatch (eds.) The bacteria, vol. VI. Academic Press, New York.
19. Cooney, D. A., G. Stergis, and H. N. Jayaram. 1980. Enzymes, therapeutic. pp. 225-240. *In* Kirk-Othmer encyclopedia of chemical technology, 3rd ed., vol. 9. John Wiley & Sons, New York.
20. Dixon, R. L. 1980. Toxic responses of the reproductive system. pp. 332-354. *In* J. Doull, C. D. Klaassen and M. O. Amdur (eds.) Toxicology, 2nd ed. Macmillan Publishing Co., New York.
21. Doolittle, R. F. 1981. Similar amino acid sequences: chance or common ancestry? Science 214:149-159.
22. Ehrlich, K., A. J. DeLucca, and A. Ciegler. 1981. Isolation of toxic metobolites from *Aspergillus niger* recovered from stored cottonseed. Abstracts of the 95th Annual meeting of the Association of Official Analytical Chemists, Washington, DC. p. 67 (Abstract No. 195).
23. Fennel, D. I. *Aspergillus oryzae* (NRRL Strain 1988): a clarification. Science 194:1188.
24. Food Safety Council. 1978. Proposed system for food safety assessment. Food Cosmet. Toxicol. 16 (Suppl.):1-136.
25. Free, A. H., and H. M. Free. 1978. Urodynamics. Ames Division, Miles Laboratory, Inc., Elkhart, IN.
25a. Goldberg, I. H., T. Hatayama, L. S. Kappen, M. A. Napier, and L. F. Povrick. 1981. Protein antibiotics as DNA-damaging agents. pp. 163-191. *In* A. C. Sartorelli, J. S. Lazo, and J. R. Bertino (eds.) Molecular actions and targets for cancer chemotherapeutic agents. Academic Press, New York.
26. Hall, R. L. 1977. Safe at the plate. Nutrition Today 12 (6):6-9, 28-31.
27. Harbison, R. D. 1980. Teratogens. pp. 158-175. *In* J. Doull, D. C. Klaassen, and M. O. Amdur (eds.) Toxicology, 2nd ed. Macmillan Publishing Co., New York.
28. Hartshorn, E. A. 1973. Handbook of drug interactions, 2nd ed. Hamilton Press, Hamilton, IL.
29. Keyes, M. H. 1980. Enzymes, immobilized. pp. 148-172. *In* Kirk-Othmer encyclopedia of chemical technology, 3rd ed., vol. 9. John Wiley & Sons, New York.
30. Koch, A. L. 1981. Evolution of antibiotic resistance gene function. Microbiol. Rev. 45:355-378.
31. LeBlanc, J. R., Jr., S. Madharan, and R. E. Porter. 1978. Ammonia. pp. 470-516. *In* Kirk-Othmer encyclopedia of chemical technology, 3rd ed., vol. 2. John Wiley & Sons, New York.
32. MacAllister, R. V., E. K. Wardrip, and B. J. Schnyder. 1975. Mod-

ified starches, corn syrups containing glucose and maltose, corn syrups containing glucose and fructose, and crystalline dextrose. pp. 331-359. *In* Enzymes in food processing, 2nd ed. Academic Press, New York.

33. Miller, E. C., and J. A. Miller. 1979. Overview on the revelance of naturally occurring carcinogens, promoters, and modulators of carcinogenesis in human cancer. pp. 1-17. *In* E. C. Miller, J. A. Miller, I. Hirono, T. Subimura and S. Takayama (eds.) Naturally occurring carcinogens-mutagens and modulators of carcinogensis. University Park Press, Baltimore.
34. Miller, J. A. 1970. Carcinogenesis by chemicals: an overview. Cancer Res. 30:559-576.
35. Mills, A. L., and M. Alexander. 1976. N-nitrosamine formation by cultures of several microorganisms. Appl. Environ. Microbiol. 31:892-895.
36. Moskowitz, G. J., and T. Cayle. 1974. A method for detection of beta-nitropropionic acid in crude biological extracts. Cereal Chem. 51:96-105.
37. Ough, C. S. 1976. Ethyl carbamate in fermented beverages and foods. I. Naturally-occurring ethyl carbamate. Agr. Food Chem. 24:323-328.
38. Patterson, D. S., and B. A. Roberts. 1979. Mycotoxins in animal feedstuffs: sensitive thin layer chromatographic detection of aflatoxin, ochratoxin A, sterigmatocystin, zearalenone, and T-2 toxin. J. Assoc. Off. Anal. Chem. 62:1265-1267.
39. Pitot, H. C. 1978. Fundamentals of oncology. Marcel Dekker, New York.
39a. Povirk, L. F., and I. H. Goldberg. 1982. Covalent adducts of DNA and the nonprotein chromophore of neocarzinostatin contain a modified deoxyribose. Proc. Natl. Acad. Sci. (USA) 79:369-373.
40. Reanney, D. 1976. Extrachromosomal elements as possible agents of adaption and development. Bacteriol. Rev. 40:552-590.
41. Riemann, H., and F. L. Bryan (eds.). 1979. Food-borne infections and intoxications, 2nd ed. Academic Press, New York.
42. Rodricks, J. V., C. W. Hesseltine, and M. A. Mehlman (eds.). 1977. Mycotoxins in human and animal health. Pathotox Publishers, Park Forest South, IL.
43. Rogers, R. W. 1977. Monograph on microbial enzymes. National Technical Information Service Document PB-289-661. U.S. Dept. of Commerce, Springfield, VA.
44. Rogers, R. W. 1978. Monograph on nonmicrobial proteolytic enzymes exclusive of papain. National Technical Information Service, Document PB-289-397. U.S. Dept. of Commerce, Springfield, VA.
45. Roland, J. F. 1981. Regulation of food enzymes. Enzyme Microb. Technol. 3:105-110.
46. Rothchild, L. (ed.). 1982. FCN/PTCN annual yearbook. Food Chemical News and Pesticide & Toxic Chemical News, Washington, DC. pp.173-224.
47. Scott, D. 1980. Enzymes, industrial. pp. 173-224. In Kirk-Othmer encyclopedia of chemical technology, 3rd ed., vol. 9. John Wiley & Sons, New York.
48. Speyer, J. F. 1965. Mutagenic DNA polymerase. Biochem. Biophys. Res. Commun. 21:6-8.
49. Starace, D., and H. C. Barfoed. 1980. Enzyme detergents. pp. 138-148. *In* Kirk-Othmer encyclopedia of chemical technology, 3rd ed., vol. 9. John Wiley & Sons, New York.
50. Stoloff, L., P. Mislivec, and A. F. Schindler. 1977. *Aspergillus oryzae* (NRRL Strain 1988). Science 196:1354.
51. Thilly, W. G., and H. L. Liber. 1980. Genetic toxicology. pp. 139-157. *In* J. Doull, C. D. Klassen and M. O. Amdur (eds.) Genetic toxociology, 2nd ed. Macmillan Publishing Co., New York.
52. White, A., P. Handler, and E. L. Smith. 1973. Principles of biochemistry, 5th ed. McGraw-Hill Book Company, New York.
53. Zioudrou, D., R. A. Streaty, and W. A. Klee. 1979. Opioid peptides derived from food proteins, the exorphins. J. Biol. Chem. 254:2446-2449.