NOP

Animal-Derived Enzymes TAP Review

Compiled by OMRI
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Enzymes, Animal-derived

Processing

Seven animal-derived enzymes were chosen for this TAP review: rennet, bovine liver catalase, animal lipase, pancreatin, pepsin, trypsin, and lysozyme. This document has rennet as the "model" enzyme.

Identification

Chemical Name(s): Rennet (animal-derived)

CAS Number: 9001-98-3

Other Names: Bovine rennet, rennin, chymosin, prorennin, rennase

Other Codes:
Enzyme Commission: 3.4.23.4

Summary Recommendation

Synthetic / Non-Synthetic:	Allowed or Prohibited:	Suggested Annotation:
Non-synthetic *(consensus)*	Allowed *(consensus)*	*Enzymes derived from animals that are not genetically engineered may be used in processed foods labeled as "Organic." Incidental additives, including processing aids used in the production of enzyme preparations, must be non-synthetic or be substances that appear on the National List of ingredients allowed for use in foods labeled as "Organic." (consensus)* *Only animal-derived enzymes that are affirmed Generally Recognized As Safe (GRAS) by the Federal Food and Drug Administration may be used in organic food (1 in favor, 1 against, and 1 did not vote).*

Characterization

Composition:
Rennet and bovine rennet are commercial extracts containing the active enzyme rennin, also known as chymosin. Rennet is the aqueous extract prepared from cleaned, frozen, salted, or dried fourth stomachs of calves, kids, or lambs. Bovine rennet is the product from adults of the animals listed above. Both products are called rennet.

Rennin is the milk-coagulating enzyme of the mucosa of the fourth "true" stomach (abomasum) of young calves. The structure of rennin consists of a single polypeptide with an internal disulfide bridge (Budavari, 1996).

Properties:
Rennet and bovine rennet are clear amber to dark brown liquid preparations or white to tan powders.

How Made:
The mucosa of the abomasum of young calves is minced. The rather thin 'brei' is adjusted to pH 2-3 with hydrochloric acid and incubated at 42°C (110°F) to convert the zymogen ["enzyme maker"] prorennin to rennin. It is next adjusted to pH 5.5 with sodium phosphate. In the presence of phosphate, the mixture becomes fluid and is dried in a vacuum and powdered. The product contains some fat, which is removed from the dried powder by solvent extraction. The solvent is usually acetone or alcohol, residues of which are easy to remove from the preparation (Balls, 1947).

Alternative method of preparation: The calf stomach is dried and ground. The powder is then stirrred for several days with a solution of sodium chloride containing a preservative. The extract is separated from the undissolved tissue, and acidified with sufficient hydrocloric acid to precipitate the mucin. The enzyme is next precipitated by the addition of enough sodium chloride to saturate the solution. The precipitate is filtered off and dried at room temperature. The product contains considerable salt but less protein impurity that that obtained by the first method (Balls, 1947).

Note: Animals used for the production of rennet and bovine rennet must be free of bovine spongiform encephalopathy (cattle) or scrapie (sheep, goats).

Specific Uses: cf. Title 21, Code of Federal Regulations, Parts 131 (Milk and Cream) and 133 (Cheeses)

§131.160	Sour cream		§133.141	Gorgonzola		§133.182	Soft ripened cheese
§131.162	Acidified sour cream		§133.144	Curd cheese		§133.183	Romano cheese
§133.102	Asiago cheese		§133.147	American cheese		§133.184	Roquefort cheese
§133.106	Blue cheese		§133.149	Gruyere cheese		§133.185	Samsoe cheese
§133.108	Brick cheese		§133.150	Hard cheeses		§133.187	Semisoft cheese
§133.111	Caciocavallo cheese		§133.152	Limburger cheese		§133.188	Semisoft partskim
§133.113	Cheddar cheese		§133.153	Monterey cheese, monterey jack		§133.190	Spiced cheeses
§133.118	Colby cheese		§133.155	Mozzarella cheese		§133.195	Swiss, emmentaler
§133.127	Cook cheese		§133.156	Mozzarella cheese			
§133.129	Dry curd cottage cheese		§133.162	Neufchatel cheese			
§133.133	Cream cheese		§133.164	Nuworld cheese			
§133.136	Curd cheese		§133.165	Parmesan cheese			
§133.138	Edam cheese		§133.181	Provolone cheese			

Action:
Rennet is a coagulant used to curdle milk to be made into cheese or sour cream. The milk-clotting effect of rennin is due to a specific and limited hydrolysis of the K-casein surrounding the protein micelles in milk. As a result the micelles lose their electrostatic charge and are able to aggregate with the help of calcium and phosphorus ions to form a network that traps the fat micelles. A gel structure is thus formed (Nielsen, 1992).

Combinations:
Enzyme preparations usually contain diluents, preservatives (to prevent microbial growth in liquid preparations), antioxidants, and other food grade substances consistent with current good manufacturing practice (Pariza and Foster, 1983; FDA, 1995). Among the substances used in commercial rennet preparations include salt (sodium chloride), propylene glycol, sodium benzoate, and sodium propionate (White and White, 1997).

Status

OFPA
The substance is used in handling and is non-synthetic but is not organically produced: 7 USC 6517(b)(1)(C)(iii).

Regulatory
Animal-derived rennet and bovine rennet are direct food substances affirmed as Generally Recognized As Safe (GRAS) (FDA, 1995); see Title 21, Code of Federal Regulations, Section 184.1685(a)(1).

NOTE: Chymosin produced by fermentation of microorganisms genetically engineered to produce the calf prorennin/prochymosin molecule, with subsequent acid hydrolysis of the prochymosin to chymosin, also is included in 21 CFR184.1685. Thus, it is critical to specify "animal-derived rennet," defined in section 184.1685(a)(1).

Catalase, bovine liver	21 CFR 184.1034
Animal lipase	21 CFR 184.1415
Pancreatin	21 CFR 184.1583
Pepsin	21 CFR 184.1595
Trypsin	21 CFR 184.1914
Lysozyme	GRAS application to FDA pending (63 *Fed. Reg.* 12421)

Status Among U.S. Certifiers
Most US certifiers have allowed the use of animal-derived enzymes documented to not be from genetically engineered sources. Specific conditions for extractions and incidental additives does not appear to be uniform among U.S. certifiers at this point.

Historic Use

A: Calf rennet has been used since ancient times for milk coagulation in cheese production.
B: Catalase has been used to treat food wrappers to prevent oxidative deterioration in food (Sarett, 1956) and to remove traces of hydrogen peroxide (Budavari, 1996).
C: Purification of lipase from castor beans predates the isolation from animal sources in 1959 (Budavari, 1996).
D: Method to produce pancreatin from cow or pig pancreas patented in 1965 (Budavari, 1996).
E: First recorded isolation of pepsin occured in 1930 (Budavari, 1996).
F: A stable form of trypsin was patented in 1960 (Budavari, 1996).
G: Lysozyme was first discovered by Fleming in 1922 (Budavari, 1996). First isolation from chicken egg white recorded in 1945 (Budavari, 1996). A lysozyme preparation to control undesirable organisms in cheeses and other dairy products was patented in 1979 in the UK (Ferrari and Dell'Acqua, 1979, quoted in Green, 1995). Commercial use in wine, meat, and other applications appear to be limited but are potentially significant (Green, 1995).

International

In general, enzyme standards for international trade are set by the Joint FAO/WHO Expert Committee on Food Additives (1990). The Codex Alimentarius Commission organic food guidelines allow "[a]ny preparations of microorganisms and enzymes normally used in food processing, with the exception of microorganisms genetically engineered/ modified or enzymes derived from genetic engineering" (Joint FAO/WHO Food Standards Programme, 1999). The most recent edition of the IFOAM *Basic Standards* considers enzymes acceptable for use in organic food processing provided they are based on the established Procedure to Evaluate Additives and Processing Aids for Organic Food Products (IFOAM, 2000). These standards are parallel to, but not exhaustively covered by the OFPA criteria. Enzymes combined with small amounts of preservatives, such as sodium benzoate, are considered to be acceptable under the standards set by the German umbrella group AGÖL (Beck, 2000).

Criteria from the February 10, 1999 NOSB Meeting

A PROCESSING AID OR ADJUVANT may be used if;

1. *It cannot be produced from a natural source and has no organic ingredients as substitutes.*
   Animal-derived enzymes are produced from a natural source. Animal-derived enzymes are non-synthetic materials that are treated in certain cases with solvents or stabilized with synthetic antioxidants or preservatives.

   Animal-derived enzymes could be produced from organic livestock but the number of organic livestock slaughtered at any one time and in any one place is probaby not adequate to satisfy the commercial demand for organic dairy products (particularly cheeses) that require animal-derived rennet for their production and existence.

2. *Its manufacture, use, and disposal do not have adverse effects on the environment and are done in a manner compatible with organic handling as described in section 6513 of the OFPA.*
   Enzyme production must be accomplished in sanitary conditions and under good manufacturing practice in order to be suitable for use in human food processing. Both enzymes and the waste from organ extraction are biodegradable. Normal food factory waste treatment in industrialized nations reduces biological oxygen demand and thus practically eliminates the risk of environmental contamination.

   Enzymes are catalysts and are use in catalytic (i.e., small) amounts to achieve the desired effect. For example, the maximum amount of rennet used to clot milk is 0.036% (Pariza and Foster, 1983).

   Enzymes are undenatured proteins. Heat, light, and air can cause irreversible degradation of enzyme activity. Thus, enzyme preparations should be protected from heat (normally they are kept under refrigeration) and stored in the dark. As proteins, enzymes are biodegradable.

3. *If the nutritional quality of the food is maintained and the material itself or its breakdown products do not have adverse effects on human health as defined by applicable Federal regulations.*
   The primary purpose of rennet is to coagulate milk to form cheese. Milk is perishable; many cheeses can be stored for long periods of time so cheese production is an ancient form of food preservation and thus of maintenance of the food value of the milk. Cheeses are wholesome foods regulated by FDA in Part 133, Title 21, Code of Federal Regulations.

Enzyme preparations can produce sensitivity reactions (including allergy) upon inhalation or skin contact. Reports of allergies and primary irritations from skin contact with enzymes or inhalation of dust from concentrated enzymes relate primarily to workers in production plants and are not relevant to an evaluation of safety of ingestion of such enzymes in food (FDA, 1995).

Animal-derived enzymes are naturally occurring proteins that are ubiquitous in living organisms. They are derived from animals that have been used as sources of food and have been safely consumed as part of the human diet throughout human history (FDA, 1995).

Animal-derived enzymes are used extensively as medical adjuvants. Pancreatin, a mixture of lipase, proteases, and amylase, is used as a supplement by patients with cystic fibrosis to improve the digestibility of food. One enzyme component of pancreatin, trypsin, is also used alone to improve protein digestibility.

4. *Its primary purpose is not as a preservative or used only to recreate/improve flavors, colors, textures, or nutritive value lost during processing except in the latter case as required by law.*
The primary purpose of rennet is to coagulate milk to form cheese. Milk is perishable; many cheeses can be stored for long periods of time so cheese production is an ancient form of food preservation.

Liquid preparations of enzymes may be prone to spoilage by microbial contaminant. For this reason, preservatives are almost always added during processing and after final preparation (Pariza and Foster, 1983; FDA, 1995).

5. *Is Generally Recognized as Safe (GRAS) by FDA when used in accordance with Good Manufacturing Practices (GMP), and contains no residues of heavy metals or other contaminants in excess of FDA tolerances.*
Animal-derived rennet has been affirmed as Generally Recognized As Safe (GRAS) by the U.S.A. Food and Drug Administration; see 21 CFR 184.1685. The other animal-derived enzymes listed below also have been affirmed as GRAS (FDA, 1995).

Catalase, bovine liver	21 CFR 184.1034
Animal lipase	21 CFR 184.1415
Pancreatin	21 CFR 184.1583
Pepsin	21 CFR 184.1595
Trypsin	21 CFR 184.1914

Lysozyme is not affirmed as GRAS. The FDA published a notice to affirm it as such on March 13, 1998 (63 *Fed. Reg.* 12421). The FDA tentatively placed lysozyme on the GRAS list at 21 CFR 184.1550 in that notice. This had not been made final as of the date of this review.

Enzymes are unchanged by their action on their substrates; they remain as they are, and active, until denatured by heat or other factors, or until the substrate is exhausted. Depending on the process, enzymes may be removed from the final product, or denatured and left in, or may even be potentially active. How they are labeled in final product formulations should be dependent on the specific outcome for the product in question. As was mentioned above, carriers, preservatives, or other commercial enzyme formulation components are also potential residues in finished foods.

The Food Chemicals Codex (Food and Nutrition Board, National Academy of Sciences, 1996) places the following limits on residues:

Coliforms: not more than 30 per g.
Heavy metals as lead: not more than 30 mg/kg.
Lead (Pb): not more than 5 mg.kg.
Salmonella spp: Negative by test in 25 g.

The Food Chemicals Codex also states that "[a]lthough tolerances have not been established for mycotoxins, appropriate measures should be taken to ensure that the products do not contain such contaminants" (Food and Nutrition Board, National Academy of Sciences, 1996).

An important consideration with animal-derived enzymes is the need for the animals to be from areas and from herds free of diseases communicable to humans, particularly BSE, or bovine spongiform encephalopathy, in cattle and scrapie in sheep.

6. *Its use is compatible with the principles of organic handling.*
Animal-derived enzymes have been used for thousands of years and constitute a valuable use of an animal organ (the abomasum in the case of rennet) not normally used as human food at the current time.

Enzymes have been used in organic processing for as long as organic processed food has been marketed, and are currently being used by certified organic processors. An industry survey of organic food processors regarding the compatibility of various processes found that enzymes were rated between 2.5 and 2.7 on a scale of 1 to 5, or approximately mid-range, as compatible with organic processing (Raj, 1991).

7. *There is no other way to produce a similar product without its use and it is used in the minimum quantity required to achieve the process.*
Cheese is the food created by the clotting action of rennet on the milk of cows, sheep, and goats. The only alternative to animal-derived rennet for producing cheeses with appropriate characteristics is genetically engineered chymosin, which the NOSB previously reviewed and determined is not compatible with organic food handling (voted synthetic, prohibited at Indianapolis, 1996).

Enzymes are catalysts, and the economics force the use of the minimum amount possible. Since enzymes at high levels may act on other components in the food, use of excessive levels of enzymes can create off-flavors that detract from the commercial value of the cheese. Thus, the least amount required is used.

TAP Reviewer Discussion

TAP Reviewer Comments

OMRI's information is enclosed in square brackets in italics. Where a reviewer corrected a technical point (e.g., the word should be "intravenous" rather than "subcutaneous"), these corrections were made in this document and are not listed here in the Reviewer Comments. The rest of the TAP Reviewer's comments are listed here minus any identifying comments and with corrections of typos.

Reviewer #1
[Ph.D. Nutritionist with food industry experience.]
The information provided indicates that the five animal-derived enzymes other than Rennet [Lipase, Catalase, Pepsin, Pancreatin, Trypsin] are not used extensively in food processing at the present time. My personal communication with one industrial enzyme supplier led to the comment that organic food processors avoid animal-derived products. They prefer microbial enzymes. They use animal-derived enzymes only if there is not a fermentation product available that will perform the specific function. This feedback is consistent with the EAFUS [Everything Added to Food in the United States] data. I suggest that organic food processors and the food enzyme industry be polled to determine if the organic food processing industry in fact needs or uses these five enzymes.

Rennet is critical for making cheese and non-animal sources are genetically engineered, so animal-derived rennet merits its own review document.

The other five animal-derived enzymes are less critical and can be consolidated into one document. The "Identification" and "Characterization" elements for each of these five (5) animal-derived enzymes with affirmed GRAS status (having specific sections in 21 CFR, Part 184) need to be kept separate but the "Status" and the "Criteria" sections could be consolidated as was done very nicely in the "Historic Use" subsection.

Lysozyme
I searched the Federal Register for any mention of lysozyme in any FDA notice over the past six years and found nothing. There is no 21 CFR Part 184 monograph on lysozyme that I could locate (and I tried). The 4th Edition of the Food Chemicals Codex (1996) does not include lysozyme in its p. 129 listing of "animal-derived preparations." In no case should lysozyme be included in the same document with animal-based enzymes whose FDA-affirmed GRAS status is so unambiguous, transparent, and independently verifiable.

Other Specific Comments

NOSB Criterion Number 2 states: "Its manufacture, use . . . are done in a manner compatible with organic handling . . . . " The addition of preservatives during the preparation of animal-derived enzymes is problematic. Preservatives must be added to avoid putrefaction of the minced animal tissue if a prolonged extraction process is required, as it is for pepsin and pancreatin. However, the specific animal tissues are edible and thus could be described as "meat." Adding synthetic preservatives to organic meat is incompatible with organic food handling and destroys the organic integrity of the food.

The five animal-derived enzymes other than Rennet [Lipase, Catalase, Pepsin, Pancreatin, Trypsin] are not used extensively at the present time. They can be replaced by microbial enzymes in most applications. Microbial enzymes are produced by fermentation of a pure culture of a single organism so the production process is more modern and sanitary and intrinsically less in need of "preservation." These five animal-derived enzymes should be allowed only in applications where enzymes from non-genetically engineered microorganisms are not suitable.

The preservatives and/or stabilizers in liquid forms of enzymes can be problematic as well. Benzoic acid is found in nature but the food additive is chemically synthesized. The propionates can be produced by fermentation, but also by chemical synthesis. Additives are required but use of non-synthetic additives should be encouraged.

TAP Reviewer "votes" on Animal-Derived Enzyme Preparations

PREPARATION	SYNTHETIC OR NON-SYNTHETIC	ALLOWED OR PROHIBITED
Rennet	synthetic (if synthetic additives are used); otherwise non-synthetic	Allowed.
Lipase	synthetic (if synthetic additives are used); otherwise non-synthetic	Allowed if microbial* enzyme is not suitable for the application.
Catalase	synthetic (if synthetic additives are used); otherwise non-synthetic	Allowed if microbial* enzyme is not suitable for the application.
Pepsin	synthetic (if synthetic additives are used); otherwise non-synthetic	Allowed if microbial* enzyme is not suitable for the application.
Pancreatin	synthetic (if synthetic additives are used); otherwise non-synthetic	Allowed if microbial* enzyme is not suitable for the application.
Trypsin	synthetic (if synthetic additives are used); otherwise non-synthetic	Allowed if microbial* enzyme is not suitable for the application.
Lysozyme	synthetic (if synthetic additives are used); otherwise non-synthetic	Prohibited; GRAS status by FDA has not been documented [affirmed]

* microbial enzyme must be from non-genetically engineered microorganism.

Reviewer #2

[Professor of Food Science]

Introduction and Overview

An enzyme is a protein in nature that by acting as a catalyst accelerates a biochemical reaction without changing the nature or quantity of products formed and without itself being consumed in the process. Therefore the fundamental property of enzymes is to increase the rate or velocity of reactions that occur in nature or in food systems by lowering the activation energy.

Overall, the generalized mechanism of enzyme action can be demonstrated by using Michaelis-Menton Kinetics as follows:

$$E + S \quad ES \quad P + E$$

Where E = enzyme; S = Substrate; ES = enzyme substrate complex; P = Products and E = Enzyme. Thus enzymes recognize their specific substrate to form an enzyme substrate complex that then forms product with the enzyme now in its free form. (Aurand et al, 1987)

Commercial Application of Enzymes

Enzymes were used in a variety of ways long before they were recognized as definite biochemical components in living cells. Processes for making bread, wine, cheese, vinegar, alcohol, sauerkraut, and pickles have been known

from antiquity. Enzymes have several characteristics that make them significant for use in industrial processes: (Aurand et al 1987)

1. They accomplish and accelerate a reaction efficiently.
2. The rates of the reaction can be readily controlled by adjusting temperature, pH, and reaction time.
3. Enzyme activity may be destroyed by heating to denaturation temperatures.
4. They are natural in origin and non-toxic and therefore may remain in the product without any harmful consequences.
5. They exhibit great specificity and can be used generally at levels of less than 1% of the commercial product batch.

Additional Functional Role of Enzymes in Food (J. M. de Man, 2)

1. Lipase: hydrolysis of triglycerides to create free fatty acids which oxidize by lipid oxidation to produce flavors in products such as cheese and cheese seasonings (i.e. enzymes modified cheese seasonings)
2. Pepsin, Papain: Example of proteolytic enzymes that are specific for the peptide bonds in proteins which links each amino acid together. Useful for production of soy sauce, miso, ketchup, soft cheese, chill proofing of beer, tenderization of meat and fish protein hydrolyzates.
3. Catalase: no additional function in food processing applications.
4. Pancreatin-trypsin: no additional role or function in food processing applications.
5. Lysozyme: no additional role or function in food processing applications.

Comments on TAP Review Documents

A. Overall I agree with the written analysis and have provided additional supporting documentation and explanation of role, function, and mechanism of enzyme action.
B. I have provided two additional references that support the need and use of the animal derived enzymes under review.
C. Overall, I support the use of animal derived enzymes in food processing applications for the following reasons:
    1) Animal-derived enzymes are produced from natural sources and during their isolation and purification are freed from residual contaminants during purification especially if purified by affinity chromatography.
    2) Their usage in food process systems is generally less than 1% and in most cases much less due to their specificity and cost considerations.
    3) All animal derived enzymes used in organic food systems must meet title 21 CFR requirements.
    4) Historically, enzymes have been used in organic processing for as long as organic processed food has been marketed and are currently being used by certified organic processors.
    5) All reactions catalyzed by animal derived enzymes ate similar to the type of reactions catalyzed *in vivo* in mammalian cell systems.
D. *[A]* major area of concern to organic integrity is the use of synthetic anti-microbial and/or antioxidants used to maintain storage stability of the enzyme prior to food use. In most cases synthetic preservatives are used at levels less than 1% on a weight/weight basis of the enzyme preparation, therefore the amount of preservative/antioxidant for all practical purposes, may be less than 0.01-0.001% in the organic food product. Representing very low levels, almost incidental in levels, in organic foods.

Recommendations

I propose on the basis of all available scientific literature that animal lipase, pepsin, catalase, pancreatin-trypsin, and lysozyme as animal derived enzymes for food processing applications be considered non-synthetic and be allowed in organic food process applications. I would also suggest that all enzyme preparations, where practical, be frozen preparations and the use of synthetic preservatives and antioxidants be discouraged and/or removed where practical and cost effective for all enzymes used in organic food product systems.

Conclusions

Enzymes, animal derived: allowed, non-synthetic with annotation that commercial enzyme preparations not contain synthetic preservatives/antioxidants where practical and possible.

Reviewer #3
[Organic and natural food industry consultant.]
I agree with the summary recommendation. The product is non-synthetic, should be allowed and the suggested annotations are as follows: Enzymes derived from animals that are not genetically engineered as defined by the NOSB may be used in processed foods labeled as "Organic." Incidental ingredients used in the production of the enzyme preparations must b non-synthetic as defined by OFPA and the NOSB, or be substances that appear on the National List of ingredients allowed for use in foods labeled as "Organic." This includes water and other substances that are insoluble in the food but removed from the food after processing.

Comments
Animal-derived enzymes appear to have no incompatibility with organic processing. They are generally GRAS, used to produce traditional foods, such as cheeses, and their only substitutes in the marketplace are produced via genetic engineering techniques.

My recommendation would be to continue to use rennet as the "model" enzyme, but specifically spell out which enzymes are approved and what they are specifically used for. I think all of the enzymes can be incorporated into one TAP review and approval. Any new approvals should not be made until the individual enzyme and its use is specifically reviewed.

In conclusion, I agree with most of the analysis contained in the current TAP review document.

Conclusion

The TAP Reviewers recommend that enzymes derived from animals that are not genetically engineered as defined by the NOSB and are Generally Recognized As Safe by the FDA may be used in processed foods labeled as "Organic." Incidental ingredients used in the production of enzyme preparations must be non-synthetic as defined by OFPA and the NOSB, or be substances that appear on the National List of ingredients allowed for use in foods labeled as "Organic." This includes water and substances that are insoluble in food but removed from the foods after processing.

A number of incidental ingredients are found in commercial enzyme preparations. These include strong acids— such as hydrochloric acid— used to acidify the water used in hydrolysis. Preservatives, carriers and fillers are also commonly found in such preparations. These may be insoluble in food but removed from the foods after processing. Nonetheless, they have an effect on the food, and the reviewers shared a consensus that enzymes that contained such incidental ingredients should not be allowed for use in product labeled as "Organic." However, the reviewers could not agree on whether or not such ingredients should be allowed in foods labeled as "Made With Organic (specified ingredients)."

The Reviewers did not reach a consensus as to whether animal-derived enzymes that are not recognized by the FDA as GRAS should be prohibited or allowed in foods that are labeled as "Made With Organic Ingredients."

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Edited by RAYMOND E. KIRK
Head, Department of Chemistry, Polytechnic Institute of Brooklyn
and DONALD F. OTHMER
Head, Department of Chemical Engineering, Polytechnic Institute of Brooklyn

Assistant Editors
JANET D. SCOTT *and* ANTHONY STANDEN

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EXPLOSIONS

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Standard performance tests have been developed and approved by the industry for reflectance, gouge (hardness), and acid resistance; tentative standards have been developed for surface abrasion, warpage, impact resistance, torsion resistance; tests for adherence, resistance to thermal shock, and others are in the proving stage (4).

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R. M. KING

ENANTHALDEHYDE, $CH_3(CH_2)_5CHO$. See *Aldehydes*, Vol. 1, p. 336.

ENANTHIC ACID, $CH_3(CH_2)_5COOH$. See *Fatty acids.*

ENARGITE, Cu_3AsS_4. See *Arsenic*, Vol. 2, p. 114; *Copper*, Vol. 4, p. 395.

ENDELLITE, $Al_2O_3.2SiO_2.4H_2O$. See *Clays (survey)*, Vol. 4, p. 26; *Silica and silicates.*

ENSTATITE, $Mg_2Si_2O_6$. See *Silica and silicates.*

ENTHALPY; ENTROPY. See *Thermodynamics.*

ENZYMES AND ENZYMOLOGY

All living things make use of chemical reactions by which their life processes are carried out. When these reactions are duplicated in the laboratory it is generally discovered that they proceed at a reasonable speed only when they are performed at high temperatures or with very strong reagents. Neither of these conditions is compatible with cell life. In order to meet this contingency, living cells produce a series of catalytic agents whose purpose is to accelerate the reactions that occur under the conditions existing in living matter: at a temperature compatible with life, in the presence of

water, and usually at a nearly neutral pH. These biocatalysts are called enzymes or "ferments." They are made only by living cells, and all living cells contain them; but they can often be extracted from their original locations and made to catalyze chemical reactions in the test tube.

The various physiological processes of both plants and animals all seem to depend upon enzyme action. Thus interest in their growth and decay centers chiefly in an understanding of the appropriate enzyme systems involved. Of these general systems only that of carbohydrate metabolism has been worked out in considerable detail. The simple breakdown of fats and proteins appears to be clear enough, but little is known of the details of their fate in the organism or of their synthesis there. The individual metabolisms of specialized tissues, such as a nerve or tumor, are not known in detail, yet upon the former depends our defense against modern gas warfare and upon the latter hangs our chance of solving the cancer problem.

After an extensive study of enzymes it becomes apparent that not only are they capable of accelerating biochemical reactions, but they can also select one reaction for catalysis over other possible reactions that might go on between the same materials. In this way, enzymes direct the reactions that take place and this directive effect is known as specificity. Such specificity is by no means confined to enzyme catalysts. Many examples of specific catalysis are known in inorganic chemistry (see also *Catalysis*, Vol. **3,** page 245). Thus carbon monoxide and hydrogen with a platinum catalyst give methane and water; with zinc chromate, methanol; and with cobalt, benzene (as chief product). And just as with enzyme catalysts, the reaction involving the greatest loss of thermal energy is not always the reaction that takes place.

The number of enzymes in a cell is undoubtedly very great as there is a special enzyme for each chemical reaction, and biochemical changes are prone to occur in a stepwise fashion, even when more direct chemical routes are possible. For example, sugar may be burnt instantly to carbon dioxide and water in a furnace; but in biological oxidation it passes through a veritable multitude of intermediate substances before appearing as these end-products. (See also *Fermentation.*)

Enzymes as Catalysts. The applicability of thermodynamics to enzyme catalysis is unquestioned; yet it has not been possible to predict the specificity of such catalyses. Enzymically catalyzed reactions (like other catalyzed reactions) lead to equilibria, even though the equilibrium may be so far over on one side that it seems like a completed reaction. The reaction can go either way, depending on concentrations. If the cell is interested in the product of a reaction to the extent that it excretes it or stores it in a separate compartment and thus removes it from the system, the enzymic catalysis can then continue in one direction, either that of decomposition or that of synthesis. It happens that the living cell often has a means of disposing of either the synthetic or the hydrolytic end-products of a reaction; they may diffuse away, or be stored in a special place, or be changed at once by some other reaction; and in such cases an enzyme would appear to promote a reaction in only one direction.

Many substances, notably fats and glycosides, have been synthesized in the laboratory by the action of the same enzymes that under other circumstances of concentration would have hydrolyzed them. The same equilibrium point has been attained from both sides: this is the point at which there is no net change; apparently nothing is happening in the system—though in reality both hydrolysis and synthesis take place but exactly balance each other. The situation in living organisms is more complicated than this, however. To illustrate from our present meager knowledge, starch (more properly amylose) is synthesized in plants by one enzyme system (the phosphorylases) and hydrolyzed by another (the amylases). Each system has its own

equilibrium point. Either could break down starch under appropriate conditions, and probably does, but only the phosphorylase system has as yet been observed (in the laboratory) to synthesize starch.

Specificity. The specificity of enzymic catalysts early attracted attention. The best known description of enzymic specificity is the analogy of Emil Fischer, who likened substrate and enzyme to lock and key. The analogy is specially striking when the stereochemical specificity of enzymes is considered. Thus, L-leucylglycine is completely hydrolyzed by intestinal dipeptidase, exactly half of the racemic mixture DL-leucylglycine, and none whatever of the "unnatural" optical isomer D-leucylglycine. Many examples are known of the strict specificity of enzymes for the naturally occurring optical isomer. Clearly, an enzyme is able to distinguish between the architecture of two molecules when one is the mirror image of the other. This not only bespeaks a complex molecular architecture for the enzyme itself, but indicates that its activity is a consequence of some of these architectural complexities. The concepts of pure chemistry (by which is meant molecular structure and all its consequences) are today by far the most useful in attempting to picture enzyme action.

Many attempts have been made to visualize how an enzyme works; it is probably safe to say that none of them is an entire success. One reasonable concept is based on the theory that an enzyme combines with its substrate. One might imagine that the surface of the enzyme molecule is distorted in such a way that the substrate, after combining with it, is also distorted. Unfortunately for the substrate, this exposes to extra strain the particular bond which is destined to break. Since the reaction that results in breaking this bond will occur anyhow, although slowly (the enzyme is simply a catalyst), the added strain on the bond is like the extra straw that broke the camel's back. Such a conception quite naturally led to the further assumption that an enzyme should combine with its substrate in at least two places, and there is a good bit of evidence to indicate that, in some cases (the peptidases, for example), it does. In other cases, the available evidence is being disputed.

Definitions. Nomenclature. An *enzyme* (also correctly called a ferment, though the term is becoming obsolete in English) is a true catalytic agent manufactured by a living cell. It is not just a copper atom or a threonine molecule that the cell acquired by accident, although some enzymes do contain copper or threonine as parts of their structures. Since the cell manufactures the enzyme to accelerate a single reaction (or a group of very similar reactions), an enzyme is a *specific catalyst.* When several reactions can take place between two substances in a cell, the enzyme accelerates one of them so much that the others are practically left at the post, so that, by virtue of being a specific catalyst, an enzyme is also a *directive catalyst.* Enzymes whose action causes the breaking of a carbon-to-carbon linkage in the substrate, thereby causing disruption of its carbon skeleton, are frequently referred to as *desmolases.* As a whole, desmolysis involves considerable energy changes, and the characteristic processes of tissue oxidation and fermentation are sometimes treated under this head.

The substance, or substances, undergoing accelerated chemical change under the auspices of the enzyme are called its *substrates.* In a typical reaction, two substances are usually involved: fat hydrolysis, for example, is a reaction between fat and water. The substrate of the lipolytic enzyme in this case is the fat. Perhaps we should regard both the reactants (fat and water) as substrates, but as water is involved in all hydrolytic changes, and is generally present in great excess, it is not ordinarily considered as a substrate.

Enzymes were originally named whatever pleased their discoverers. Pepsin, trypsin, and diastase (amylase) are old names that have persisted. Somehow "diastase" came to signify "enzyme" in general in French. English and German names ending in *in* were popular. Later it was agreed to name enzymes after their substrates by adding the suffix *ase*, a system of nomenclature that has worked reasonably well and is the one now in use.

The system led to difficulties when two enzymes were found to attack the same substrate, as with pectase and pectinase. When their manner of decomposing pectin became better understood, it was possible to call the former pectinesterase and the latter polygalacturonase. These names adequately describe the two enzymes, and also point out the relationship of the latter to the glycosidases. See Table I.

For a time when the synthetic action of an enzyme was under consideration, the suffix *ese* was used instead of *ase*.

The existence of substances known to increase or decrease the action of an enzyme has been recognized by the terms *coenzyme*, *activator*, and *inhibitor*. A coenzyme (see p. 740) is an organic molecule that takes part in the reaction by attaching itself to the enzyme, and thereby making the enzyme active. Any other substance that increases enzyme activity is called an activator. Both terms, however, are sometimes used loosely. When an enzyme has a coenzyme, the complex or complete enzyme is sometimes called the *holoenzyme*; and an (inactive) enzyme without the coenzyme is called the *apoenzyme*. A *proenzyme* or *zymogen* is a mother protein or precursor from which an enzyme may be formed. Proenzymes are usually designated by the suffix *ogen*. Thus, trypsinogen is the precursor of trypsin. Zymogens as such have no enzymic activity. See p. 741.

Inactivation, Inhibition, and Antienzymes. The action of an enzyme may be depressed or abolished in various ways, some of which are explained below. The terms *inhibition* and *inactivation* are often used, unfortunately, as though they were interchangeable. Strictly, inhibition is the partial depression of activity by a process that does not contemplate the actual destruction of the enzyme. Thus end-products of the reaction may inhibit. Inactivation is more inclusive, for it may be partial or total, reversible or irreversible.

Since enzymes are proteins, protein reagents are likely to reduce or destroy enzymic activity, and anything that leads to denaturation invariably does so. This explains the effect of heat, acid, alkali, supersonic vibration, and extremely high hydrostatic pressures. It is noteworthy, however, that all proteins do not react with equal readiness toward a particular form of treatment. Differences between enzyme proteins in this respect can be very marked and frequently form the basis for separating an enzyme from other proteins. Such methods of inactivation apply in principle to all enzymes, but special methods may apply to enzymes of a particular type, because of some peculiarity of the protein structure. Thus the papainases (papain, bromelin, etc.) are inactivated by oxidizing agents toward which other proteinases are stable. Enzymes carrying a heavy metal in their structures are for the most part inactivated by cyanide ion, because of the formation of metal cyanides. In other cases the enzyme may be inactivated by the addition of a heavy metal which combines with it. Thus invertase is inactivated by silver, but becomes active again if the silver is removed with hydrogen sulfide.

Antienzymes are certain inactivating substances found only in nature, whose

effects appear to be specific or nearly so. Two types of antienzymes may be distinguished: Those substances which occur in normal tissues, and those produced by the organism under the influence of an artificial stimulation, such as the injection of the enzyme. The trypsin inhibitor found in pancreas belongs to the first type of antienzymes. It has been isolated and found to be a proteinlike substance (mol. wt. 6000) that combines in equimolar proportions with trypsin to form an inactive compound, dissociable in acid solution (68). (The discovery of this reaction gives support to the theory that an enzyme combines with its substrate.) Besides antitrypsins from other sources than pancreas, other antienzymes have been recognized, notably antipepsin, anticatalase, and antihyaluronidase.

An inhibitor to hyaluronidase (quite distinct from the specific antibodies produced by injection) has been found in the blood of many species of animals. Hyaluronidase depolymerizes the complex polysaccharide hyaluronic acid, a component of connective tissue. This decomposition removes a normal barrier to the spreading of material that has been injected, for example, into the skin. Many pathogenic bacteria contain hyaluronidase, and it has been reasoned that their invasion of tissue is facilitated by this enzyme. Consequently hyaluronidase has also been referred to as the "spreading factor," and as "invasin." The antienzyme has been called "antinvasin" and has been regarded as an enzyme itself (21), though this conclusion has been disputed (15a).

When an antigenic substance is injected into an animal, one of the results is the appearance in the blood serum of specific antibodies. Such a serum neutralizes at least some of the physiological properties of the antigen, and can usually be recognized by the fact that it gives a precipitate when mixed with its antigen, whereas normal serum does not. The injection of enzyme proteins usually leads to the formation of substances in the serum which inactivate the enzyme and also precipitate it from solution. In the case of antiurease (72), the antiserum not only inactivates urease as an enzyme, but also protects the animal against otherwise lethal doses of this rather toxic protein. The precipitate formed by antiurease with urease solutions contains both enzyme and antienzyme. The latter appears to be a glycoprotein.

Enzymes as Proteins (*q.v.*). Some thirty-odd enzymes representing most of the important classes have now been crystallized. Because the crystals have invariably been identified as proteins, it is now regarded as highly probable that all enzymes are proteins. This cannot, of course, become an absolutely proved fact until many more enzymes are so identified, but it is nevertheless true that the general properties of enzymes, such as their stability and their behavior toward acid, alkali, and heat, all indicate that enzymes are protein in nature.

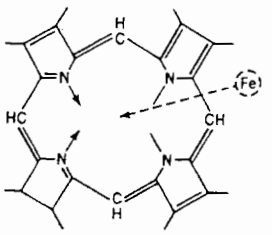
Enzymes do not all belong to any one type of proteins as these are ordinarily classified. For example, some enzymes are globulins (carboxypeptidase) and others behave like albumins (the pancreatic proteinases). The molecular weights of enzymes also vary enormously. A pancreatic enzyme that acts on ribonucleic acid has a molecular weight of only about 15,000. It is soluble in 50% alcohol and has been precipitated from such solutions in crystalline form by the addition of more alcohol. It is one of the few enzymes that is very resistant to heat, for its solutions in water may be boiled for some minutes without loss of activity. On the other hand, carboxypeptidase has a molecular weight of about 200,000. The pure protein is practically insoluble in water, and in fact dissolves readily only in solutions of lithium salts.

Some of the enzymes already isolated (like trypsin and pepsin; see Table I)

appear to be simple proteins (see p. 753). Despite the fact that their extraordinary catalytic properties mark them as different from ordinary proteins, nothing in their known constitution appears to be unusual. Others of the isolated enzymes, however, particularly those connected with oxidation and reduction, are complex proteins containing metals (like polyphenol oxidase, which contains copper; see Table I). Still other enzymes consist of a protein that is combined chemically with a relatively small nonprotein group which is not actually an integral part of the protein but is merely attached to it; some of these enzymes may also contain a metal (for example catalase, the attached group of which is an iron-porphyrin; see Table I). The nonprotein groups are often referred to as *prosthetic groups* (see below), and it is interesting that these groups in enzyme proteins are very often related to one of the known vitamins; the prosthetic group of acetaldehyde dehydrogenase, for instance, is related to nicotinic acid. It is frequently assumed, and seems logical, that one of the reasons why vitamins are a dietary necessity is that they enable the organism to complete the synthesis of enzymes it needs and would not otherwise be able to make. See *Vitamins*.

Enzymes, like other proteins, can undergo denaturation. When this occurs their activity is lost. If activity reappears, as sometimes happens, it means that the protein has reverted to its native form. Like other proteins also, enzymes have isoelectric points, and it is not surprising that the electrical charge of the protein influences its catalytic activity. There is, therefore, under any given set of conditions, a pH level at which the enzyme is most active—the so-called pH optimum, which also depends on media and substrates and is not a means of identifying an enzyme, although it can serve to differentiate some enzymes from others. Some enzymes (urease, for example) have very sharp pH optima and others (β-amylase) show their maximum activity over quite a wide pH range. The pH of maximum activity is not necessarily that at which the protein is most stable, as in the case of trypsin, which is most stable at pH 3.4 and most active at a pH of about 8.

Coenzymes and Prosthetic Groups. Broadly speaking, a prosthetic group could be any nonprotein group attached to an enzyme protein. However, it usually signifies a group wherein a chemical change takes place during catalysis, so that without the group the protein would be inactive. Some of the groups seem to be attached rather



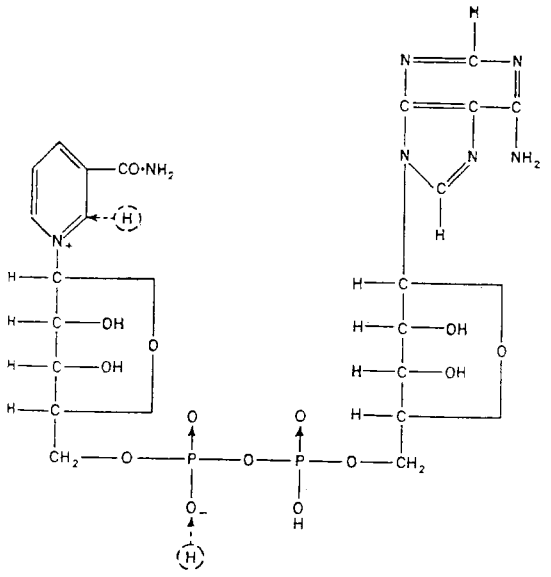
(1) Porphyrin skeleton, indicating position of iron in a hemin.

firmly, whereas with others the combination is easily reversible and a measurable equilibrium exists in solution between apoenzyme, holoenzyme, and free group. It is then usually easy to inactivate the enzyme by dialysis and to reactivate it by putting the dialyzate back again. (Similarly certain metal-containing enzymes on dialysis lose the metal, particularly if it is magnesium.) While there can be no definite line of demarcation, it is customary to refer to the more firmly attached groups as *prosthetic*

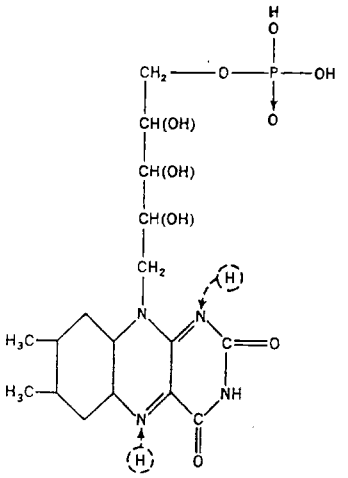
groups and to the less firmly held groups as *coenzymes*. Prominent among the prosthetic groups are the iron-porphyrins or hemins, and among the coenzymes are: (*1*) diphosphopyridine nucleotide (DPN), also called coenzyme I; (*2*) triphosphopyridine nucleotide (TPN), called coenzyme II; and (*3*) several isoalloxazine nucleotides, the coenzymes of the "yellow ferments." (See also *Fermentation*.)

The porphyrin skeleton is the basis of the structure of hemins, as shown in formula (1). When iron is introduced to form a hemin, it is held between the 4 nitrogen atoms of 4 pyrrole nuclei. Differences between the various hemins depend upon the nature of the groups attached to the outside of the porphyrin skeleton, and upon whether the iron may or may not change its valence from Fe^{2+} to Fe^{3+}. There is a general similarity of structure between the iron-bearing hemins and the magnesium-bearing chlorophylls. Furthermore, both occur in nature attached to proteins.

Coenzyme I is a dinucleotide consisting of adenine, nicotinic acid amide, 2 molecules of a pentose (ribose), and 2 molecules of phosphoric acid. Coenzyme II is similar, but contains 3 phosphoric acid molecules. The oxidation-reduction reaction occurs with the appearance and disappearance of a double bond in the nicotinic acid amide portion of the molecule, together with the respective loss or gain of 2 hydrogens as indicated by the broken lines in formula (2).



(2) Coenzyme I, oxidized form, showing where the hydrogen enters on reduction.



(3) Riboflavin phosphate, oxidized form, showing the points at which hydrogen enters on reduction.

The skeleton of the flavin nucleotides is isoalloxazine ribitol phosphate. Its formula in the oxidized state is shown in formula (3). The broken circles indicate the points of attachment of the 2 hydrogens, which reduce it with the disappearance of 2 double bonds in the alloxazine. The substance occurs (without the phosphate) in milk and eggs (vitamin B_2). It has a strong yellow color in solution, which led this group of enzymes to be called the "yellow enzymes."

Proenzymes or Zymogens. As mentioned previously, the protein formed by the

parent cell is not always the enzyme itself but may be an inactive precursor that later on is converted to the enzyme. Pepsin, the proteinase of the stomach, and trypsin and chymotrypsin, the two proteinases of the pancreas, are all fabricated as proenzymes. It is thus not necessary for the fabricating cells to store high concentrations of active proteinase. For example, pepsinogen is converted into pepsin by contact with hydrochloric acid of the stomach. Trypsinogen is converted into trypsin by another enzyme of intestinal origin known as enterokinase, as well as by the action of trypsin itself. Therefore, as soon as a little trypsin is formed, the reaction goes very rapidly because the newly formed trypsin attacks, in addition to its normal substrate, what is left of the trypsinogen. Trypsin also converts chymotrypsinogen into chymotrypsin. These conversions are themselves proteolyses, since the proenzyme proteins are partly hydrolyzed to yield the active enzyme.

Formation and Action of Enzymes

Enzymes are not known to be self-duplicating, and one of the most important physiological questions is: How they are formed? Our present notion is that they are formed from something which functions like a pattern, perhaps from the biological units of heredity, called genes (20).

Perhaps the first successful living form accidentally fabricated an enzyme pattern of left-handed instead of right-handed optical configuration. To fit into and carry on the life of the organism, subsequent enzyme molecules would have to be of the same optical variety or they could not function. Thus an organism could develop in such a way that it was committed to the left-handed optical configuration of the whole living world. It could only serve as food for another organism similarly left-handed. This would corner the food supply and assure the exclusive propagation of such an optical variety of life. During geologic time mutations have undoubtedly occurred, but not so many as might be expected. A famous example in which both mutants have survived concerns a species of evergreen trees that produce *dextro*-pinene in France and *levo*-pinene in America.

There is very little definite knowledge about the reactions which enzymes themselves undergo in their role as catalysts. One important concept, however, has emerged from the research of the past forty years, namely, that the enzyme combines with its substrate. This conclusion, first reached by Michaelis and Menten (40), follows from observations of what happens when an enzyme acts on various concentrations of the substrate. The higher the substrate concentration the faster will a given concentration of enzyme split it, but only up to a certain point. Beyond this point (at which the enzyme may be thought of as saturated with substrate) no further increase in velocity is obtained by increasing the substrate concentration. Measurements of the change in reaction velocity with concentration of substrate agree quantitatively with the assumption that a dissociable compound of enzyme and substrate is formed according to the law of mass action, and that the activity of the enzyme at any time depends upon the amount of this dissociable compound present at that moment.

When it is admitted that enzyme and substrate combine with each other, it follows that there must be an "affinity" between them. It is important to have the measure of affinity because it determines the efficiency of the enzyme, since the enzyme-substrate compound is a dissociable one. This affinity is ordinarily expressed as the substrate concentration (in moles per liter) at which the enzyme acts as though half saturated with substrate. This is the concentration at which the velocity is half of the maximum obtainable. The substrate concentration at this point is numerically equal to K_m (the Michaelis constant, also written K_s), which expresses the affinity of an

enzyme for its substrate and may be derived mathematically from the foregoing assumptions.

In some cases it has also been demonstrated that an enzyme has an affinity for the end-products of the reaction it influences. Although this affinity is usually less than that of the enzyme for the substrate, a combination with end-products, after considerable end-product has been formed, is often sufficient to slow down the action of the enzyme. Probably the enzyme continues to combine with end-products because they have some structural resemblance to the original substrate. There thus arises a situation known as "competitive inhibition"—two or more substances competing with each other for a place at the active center of the enzyme. The competing substances need not, however, be end-products of the reaction. Any substance with a configuration suitable for combining with the enzyme and thereby blocking its active surface can serve as a competitive inhibitor. Thus, certain phosphatases are readily inhibited by *dextro*-tartaric acid although not by its optical antipode. Sometimes the affinity between an enzyme and an inhibitor is so strong that the combination occurs with greater readiness than that between enzyme and substrate, and so prevents the formation of the enzyme-substrate complex. Such a combination occurs between the cholinesterase of nerve tissue and diisopropyl fluophosphate, and accounts for the fact that the latter substance is a powerful nerve poison. There are, of course, other forms of inhibition, many of which are due to drastic chemical changes in the enzyme protein (as the removal of a metal atom by cyanide ion). Others probably have no relation to the chemical reactions of the enzyme—enzyme actions are frequently slowed down by the presence of alcohol or considerable concentrations of salts, but recover when the inhibitor is removed.

The rate of a reaction involving enzyme action is always faster when the temperature is raised. The usual rule is that an increase of 10°C. about doubles the speed of reaction. Since enzymes are also less stable and more rapidly denatured at higher temperatures, in most cases when temperatures of over 45°C. are employed the resulting velocity is a compromise between increased speed due to the higher temperature and a decrease in amount of enzyme present due to its more rapid denaturation at that temperature. Thus, over short time intervals, enzyme activity at high temperatures may reach surprisingly high levels, until the enzyme has been destroyed. This is an important consideration in the processing of fresh fruits and vegetables, when it is customary to heat the product in order to destroy enzyme activity that might later cause spoilage. During the process of destruction marked enzymic changes sometimes take place.

Classification of Enzymes

Until recently, the only way of classifying enzymes was by their function. This is still the most generally used system, and will be employed here. However, we are commencing to classify enzymes also by the kind of proteins they are—whether simple, metal-containing, acting with a coenzyme, and so forth.

When classified by function, most enzymes are easily placed in one of four groups, simply because they represent four very common types of biochemical reactions. Of course there are some dubious cases.

1. **Enzymes of Hydrolysis (Hydrolases).** Hydrolytic enzymes catalyze the splitting of their substrates by the addition of water. For an *esterase*:

$$RCOOC_2H_5 + H_2O \rightleftharpoons RCOOH + C_2H_5OH$$

In this manner, *lipases* (see also p. 746) hydrolyze fats. Other *esterases* are tannase, sulfatase, chlorophyllase, phytase, cholinesterase, phosphatase, etc.

Similarly, proteolytic enzymes, or *proteases*, hydrolyze peptide bonds. They include the *proteinases*, which hydrolyze proteins (*q.v.*) to peptides and partially to amino acids, and *peptidases*, which complete the process by hydrolyzing peptides to amino acids but are not known to attack proteins. Although proteinases can split peptides as simple as dipeptides or tripeptides, a given proteinase can act with measurable velocity only on peptides containing certain amino acids. Recently, trypsin, chymotrypsin, and carboxypeptidase, the proteolytic enzymes of the pancreas, were found to hydrolyze the ester linkages of some esters, but not of all. Thus these enzymes have literally two distinct activities, a fact that may later modify our present ideas of enzymic specificity.

Carbohydrases hydrolyze glycosidic bonds in di- and polysaccharides. Thus complex carbohydrates (*q.v.*) are split by several steps to their constituent monoses, each step usually requiring a separate catalyst. (See phosphorylases, however, in item 3 below.) Typical carbohydrases (see also Table I) are cellobiase, maltase, invertase (sucrase), the amylases, the mucolytic enzymes (lysozyme, hyaluronidase), and the pectin-hydrolyzing enzymes. The exact structure of pectin is not known, but it consists mainly of polymerized galacturonic acid units united by glycosidic linkages. A large portion of the carboxyl groups in the polymer are esterified, and exist as methyl esters. Pectin undergoes two types of enzymic hydrolysis by two different enzymes, pectase and pectinase (see Table I).

Hydrases. A few enzymes are known that *add* water to their substrates without causing a split. They are really hydrating enzymes. *Fumarase* is a well-known example. See Table I.

2. Enzymes of Oxidation. There are four types:

(*a*) *Enzymes that combine with and use molecular oxygen* (*oxidases*). They are mostly metal-containing enzymes or else they contain a derivative of alloxazine as a prosthetic group (the flavine enzymes). See Table I and p. 747.

(*b*) *Enzymes that use peroxides.* The best-known examples are the *peroxidases* and *catalase* (see page 747 and Table I). These are porphyrin enzymes. The peroxidases oxidize phenols and some aromatic amines at the expense of any available peroxide. Catalase decomposes hydrogen peroxide into oxygen and water and also oxidizes alcohols to aldehydes, using only hydrogen peroxide.

(*c*) *Dehydrogenases* (see Table I and p. 747). Chemically speaking, the effect of these enzymes is to transfer hydrogen from one substrate to another. Thus they have two substrates. The substrate that is reduced is the hydrogen *acceptor*; the substrate that is oxidized is the hydrogen *donor*. Usually the enzymes are very specific with respect to their donors, but not to their acceptors. In laboratory tests, methylene blue and similar reducible dyes often serve as good acceptors.

(*d*) *Carboxylases* (*decarboxylases*). See also p. 747. These enzymes are of two types, splitting off carbon dioxide from either α-keto acids or amino acids. Actually the substrate undergoes no oxidation; but the reaction is the final step in the production of carbon dioxide by oxidation in **fermentation** (*q.v.*), and so really completes the oxidative cycle for carbon. Thus yeast carboxylase (Table I) acts on pyruvic acid as follows:

$$CH_3COCOOH \longrightarrow CH_3CHO + CO_2$$

With oxalacetic carboxylase (56), which is widely distributed in nature, the reaction is known to be reversible, one direction resulting in the assimilation of carbon dioxide:

$$HOOCCH_2COCOOH \rightleftharpoons CH_3COCOOH + CO_2$$

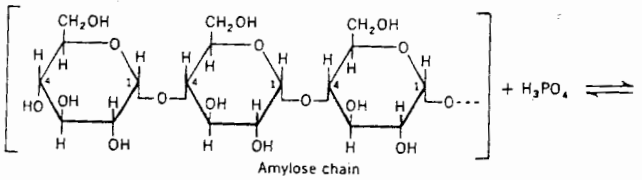
3. Enzymes of Transfer. In a sense all enzymes are transfer agents, and certainly dehydrogenases could be so considered. It is convenient, however, to group some enzymes of transfer together where the transfer itself is what attracts our notice. (See also Table I and *Fermentation*.)

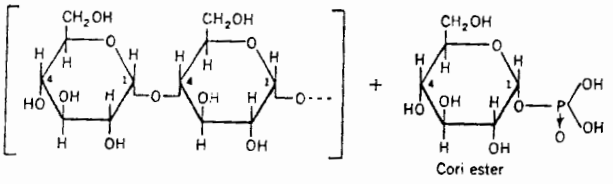
(*a*) *Transfer of amino groups.* The *transaminases* (*aminopherases*) are enzymes that facilitate the reversible transfer of an amino group from an α-amino acid to a α-keto acid, to form a new amino acid and a new keto acid. Glutamic and aspartic acids are known to serve as donors of the amino groups; pyruvic and oxalacetic acids are acceptors. A simple case is:

$$\underset{\text{glutamic acid}}{HOOC(CH_2)_2CHNH_2COOH} + \underset{\text{pyruvic acid}}{CH_3COCOOH} \rightleftharpoons \underset{\alpha\text{-ketoglutaric acid}}{HOOC(CH_2)_2COCOOH} + \underset{\text{alanine}}{CH_3CHNH_2COOH}$$

(*b*) *Transfer of phosphate.* Phosphate transfer is extremely important as a means of carrying energy in biochemical systems, and as a step in the synthesis of starch or glycogen. The addition of inorganic phosphate to adenosine diphosphate (ADP) to form adenosine triphosphate (ATP) requires much energy. (See also *Nucleic acids.*) Such a phosphate bond in ATP is designated a "high-energy phosphate bond" (36). As such, it is frequently transferred to creatine, in animal metabolism, to form creatine phosphate, one of the forms in which chemical energy is stored in animal tissues. In some lower forms of life (for instance in the crabs), high-energy phosphate is stored as arginine phosphate, formed in analogous fashion. High-energy phosphate is retransferred to form ATP when the energy is to be utilized, as in muscle contraction. Transfer of phosphate from ATP to hexose and triose molecules occurs in the course of their oxidation or fermentation. Eventually the phosphate reappears in inorganic form and then repeats the cycle. ATP is such an important constituent of living tissue that there are many enzymes which can handle it as a substrate. A considerable number of phosphate-transferring enzymes are thus known to exist whose action is more specific with respect to their acceptor substrates, for example, hexose + ATP $\rightleftharpoons$ hexose-6-phosphate + ADP, catalyzed by hexokinase. Enzymes of both plant and animal origin are also known that transfer phosphate directly from highly artificial substrates such as nitrophenyl phosphate to primary and secondary alcohol groups, thus synthesizing low-energy phosphate esters. Their function is at present obscure (2).

(*c*) *Phosphorylases* (*transglucosidases*). Phosphorylases in reality transfer glucose. Thus plant phosphorylase separates one glucose unit at a time from an amylose chain by inserting a phosphate group at the bond, and breaking off glucose-1-phosphate (Cori ester). Phosphorylase can thus degrade the amylose portion of starch. The splitting of amylose or other carbohydrate chains by the introduction of a phosphate group that then remains part of the split-products is analogous to hydrolysis, where water is inserted and remains combined. Therefore the reaction is commonly called phosphorolysis, and its catalysts are termed phosphorylases. The reaction is a reversible one; thus phosphorylase separates phosphate from glucose-1-phosphate and adds the glucose unit to the end of the amylose chain. By this mechanism phosphorylase synthesizes amylose. In animals the phosphorylase synthesizes the straight-chain portions of glycogen in an analogous manner. It is noteworthy that in both cases synthesis does not occur unless a glucose chain at least six or seven glucose units long is already present in the system. To demonstrate the reaction it is therefore necessary to include a "primer"—a trace of starch, glycogen, or amylose for the enzyme to build on (49). Sucrose phosphorylase, of bacterial origin, removes phosphate from glucose-1-phosphate and unites the glucose with fructose, when this is present, to form sucrose (16). In an analogous manner glucose may be removed from or added to other sugars.





4. **Mutases** are enzymes catalyzing molecular rearrangements. They appear to be entirely specific. Several are known to be involved in carbohydrate metabolism. For example, glucose-1-phosphate is rapidly changed into its equilibrium mixture with glucose-6-phosphate (where the latter greatly predominates) by phosphoglucomutase (13,14,48). By the use of labeled phosphorus it was found that the phosphate is actually transferred from one position to the other (not split off as inorganic phosphate from position 1 while more inorganic phosphate is attached to position 6) (39).

Table I and the following text go into further details about these and other important enzymes. (See also *Fermentation.*)

Examples of Enzyme Action

LIPOLYSIS

The hydrolysis of fats is an easy reaction to write on paper. Fats are esters of glycerol and the higher fatty acids; the ester groups are hydrolyzed to yield free fatty acids and eventually glycerol. The reaction is reversible.

Enzymic hydrolysis of fats is complicated by several other factors, however. Fats are insoluble in water. Some lipases, such as that of the pancreas, are soluble and other lipases, for example castor bean lipase, are insoluble. At best, lipolysis occurs in a two-phase system, with all the attendant complications. This explains why anything that changes the degree of dispersion of fat in water may be expected to change the rate of lipolytic action. Thus a great variety of unrelated substances either activate or inhibit lipases. So far, the most successful methods of measuring lipase activity have been those in which a variety of impurities, such as bile, egg albumin, and various inorganic salts, are intentionally added. The effect of high concentrations of such impurities is to minimize the relatively small differences in purity between different enzyme preparations.

Lipases have little real specificity, yet they exhibit a preference for some fats over others. Splitting is faster with the esters of fatty acids ranging from C_8 to C_{14} in chain length (6). Other enzymes, usually referred to simply as esterases, split the esters of short-chain fatty acids (butyrates, propionates, and acetates), which are not classified as fats. Esters other than those of glycerol are split by lipases with equal ease. Ethyl and methyl esters of fatty acids appear to act very much as do the glycerides. The benzyl esters are split with even greater rapidity. In the case of glyceride hydrolysis, the fatty acid attached to the middle carbon of glycerol tends to shift if there is a free hydroxyl group at either end of the glycerol molecule. Thus it is quite possible that the hydrolysis of glycerides involves only the splitting of primary esters. Some glycerides can exist in more than one isomeric form, and the preponderance of one form over another depends upon the temperature. This may explain why lipases behave in an unusual manner with respect to temperature. Pancreas lipase is able to split fats with fair rapidity at $-15°$C., and when the system is apparently quite solid (8). Most enzymes would show no appreciable activity under such conditions. On the other hand, pancreas lipase does not attack tristearin until the temperature is raised to about 40°.

The synthesis of fats by lipase has frequently been observed in the laboratory. For this reason it is remarkable that so many plants which lay down large quantities of fat contain very little demonstrable lipase. In the laboratory, when the concentration of water is made low and that of fatty acid and of glycerol high, an equilibrium between hydrolytic and synthetic effects is established.

AEROBIC OXIDATION

Aerobic tissue oxidation occurs in steps, each of which appears to be catalyzed by a particular protein. There are, however, several alternate groups or systems of oxidizing enzymes, so that each system contributes something to the total movement of oxidation. Not all of these systems are equally active in any one tissue. The prominence of the several systems varies in different kinds of cells or tissues.

For convenience, most of the steps can be grouped into three rather general categories. (*1*) The utilization of atmospheric oxygen to raise certain "permanent" constituents of tissue to their oxidized form. This is the phase of *aerobic oxidation*. (*2*) The reduction of these now oxidized constituents back to their former state occurs when they take hydrogen atoms from the so-called metabolites, such as amino acids, fatty acids, and fragments of carbohydrates which originally come from food. This process is known as *dehydrogenation*. When hydrogen is removed from a metabolite it necessarily becomes relatively richer in carbon and oxygen, and if the process is repeated several times the substance becomes so poor in hydrogen that it resembles in composition (to give a well-known example) an α-keto acid, RCOCOOH. (*3*) The keto acid loses carbon dioxide (*decarboxylation*), and the remaining residue goes through the oxidation and dehydrogenation processes over again.

After the first step, oxygen as such plays no further part in the reaction. What is passed down through the subsequent series of reactions is "oxidation." Through the entire scheme the substances involved are, in effect, carriers of a higher state of oxidation which they transmit to the metabolite that serves as an acceptor of oxidation and a donor of hydrogen. Such carrier substances are often

coenzymes, and the transfer is mediated by the enzyme protein specifically adapted to the particular substrate. Obviously the carriers must be capable of alternate (that is, reversible) oxidation and reduction.

(*1*) After oxygen enters a tissue (in the higher animals oxygen is distributed by hemoglobin), it oxidizes one of the two kinds of enzymes found there, the **oxidases.** All oxidases appear to be proteins connected with an active group (metal-containing or otherwise) on which the oxygen reacts. The more important system, as judged by the proportion of oxygen handled, consists of metalloprotein enzymes of the iron-porphyrin (hemin) class. One enzyme, *cytochrome oxidase* (Table I), is responsible for the introduction in many cells of over 80% of the total oxygen used. This enzyme oxidizes the relatively stable *cytochrome c*, another iron-porphyrin protein. Cytochromes a and b, also present in animal tissues, are so unstable that they have not been studied widely.

In the oxidation of cytochrome c, the iron of the enzyme is reduced while that of the cytochrome becomes ferric iron. The oxidized cytochrome in turn oxidizes other substances, which are apparently limited in number. Cysteine and certain dyes may be oxidized directly, but of more importance is the oxidation of the reduced form of *cytochrome reductase*. The last is a flavoprotein enzyme (a "yellow" enzyme) belonging among the dehydrogenases. Its action is specifically the reduction of oxidized triphosphopyridine nucleotide (TPN, coenzyme II; see p. 741), which is in turn the prosthetic group of other dehydrogenases. This constitutes a link between the cytochrome system and the dehydrogenase systems. There are undoubtedly other links, perhaps not yet as clearly defined.

The metal-carrying cytochrome oxidase is not the only enzyme with which oxygen reacts directly. Another class of such enzymes is connected with oxidizable organic groups that serve as coenzymes. When oxidized, such coenzymes readily lose hydrogen. This hydrogen has been withdrawn from a previous substrate that was thereby dehydrogenated, and it now reacts with oxygen to form hydrogen peroxide and the oxidized (or dehydrogenated) form of the enzyme. Obviously such enzymes are just as much dehydrogenases as they are oxidases, so they are frequently referred to as aerobic dehydrogenases. An example is the D-amino acid oxidase found in most animal organs. It oxidizes the optically unnatural forms of α-amino acids to ammonia and a keto acid. It is noteworthy that the coenzymes are not particularly reactive substances by themselves, but become so when they are attached to an enzyme protein.

(*2*) Nearly all the oxidation of actual tissue metabolites starts with the **dehydrogenases.** These enzymes combine with their substrates in such a way as to activate certain vulnerable hydrogen atoms in the metabolite. The hydrogen reduces the coenzyme of the dehydrogenase, which must of course be in the oxidized state at the start. All dehydrogenases appear to have coenzymes capable of reversible oxidation and reduction, though not all of these have been identified. Many are flavin or pyridine nucleotides. The reactions of glycolysis and fermentation are anaerobic oxidations of this general type for which the details have been worked out most fully at present.

(*3*) Dehydrogenation continues until substances very high in oxygen (organic acids) are produced. These are attacked by **carboxylases** that split off carbon dioxide from the carboxyl group. The remainder of the compound may then be dehydrogenated still further.

Although most, if not all, metabolic carbon dioxide can be attributed to decarboxylating enzymes, the reactions are many, varied, and not all known whereby the intermediate metabolites are transformed into acids suitable for decarboxylation. A very important series of biochemical reactions that ultimately achieves the complete oxidation of a carbohydrate metabolite is known as the "citric acid cycle" of Krebs. Carbohydrates containing glucose are eventually degraded to pyruvic acid, which may be both oxidized and decarboxylated at the same time (oxidative decarboxylation) to yield acetic acid. This acid may in turn condense with oxalacetic acid to give a C_6 acid, aconitic acid, which is changed to isocitric acid. Isocitric acid undergoes dehydrogenation and decarboxylation, and eventually turns up again as oxalacetic acid, when it is ready to condense with more acetic acid and go through the whole cycle again. But in the meantime the original acetic acid has been completely oxidized to carbon dioxide and water.

THE FATE OF HYDROGEN PEROXIDE

While cytochrome oxidase reduces oxygen to water, other enzymes using oxygen reduce it to hydrogen peroxide. Most cells would be poisoned by an accumulation of hydrogen peroxide, and this is disposed of by peroxidases and catalase (see p. 744). *Cytochrome peroxidase* oxidizes reduced cytochrome. Other peroxidases seem to be important in plants but not in animals. *Catalase* is almost

ubiquitous in living tissues and undoubtedly prevents the organism from being poisoned by the hydrogen peroxide from its own oxidations. While catalase, when decomposing hydrogen peroxide to water and oxygen, is not strictly speaking an enzyme of oxidation, it plays a very necessary part in oxidative processes. Catalase also uses hydrogen peroxide to perform certain true oxidations, as the peroxidases do. It conducts the oxidation of a number of common alcohols to their corresponding aldehydes, using hydrogen peroxide (which must apparently be generated slowly) for the process.

Measurement of Enzyme Activity

The assay of an enzyme preparation for its active principle depends upon the measurement of something that has happened to the substrate. (An exception is spectroscopic changes occurring in a coenzyme.) For example, new carboxyl and new amino groups occur on proteolysis, and their increase may be measured by the Van Slyke apparatus or by titration in alcohol or acetone as a solvent, or in the presence of formaldehyde. Another method depends upon measuring the increase in nonprotein nitrogen formed during proteolysis. A convenient modification of this is the determination of the increase in nonprotein tyrosine liberated from a standard preparation of hemoglobin. Still another method takes advantage of the speed with which proteinases coagulate milk, which most of them do very well. In all these methods the substrate must be prepared in a carefully standardized manner.

Blank determinations and control runs are very necessary in any type of enzyme assay. Each enzyme requires methods based on the reaction to be followed. Some of these are outlined in Table II (p. 760), with references to the details, which must be followed scrupulously in practical work. For more complicated cases, especially those involving mixtures of enzymes, many very ingenious procedures have been devised, for which a comprehensive text (73) or the original literature should be consulted.

When a method is known that measures quantitatively the change produced by an enzyme, it is possible to express the amount present in arbitrarily selected units. While the accuracy of most enzyme assays still leaves much to be desired, the results enable us to compare the activity of different preparations of the same enzyme, and also to estimate changes that may take place during purification or otherwise.

The most defensible method of comparing a known with an unknown enzyme preparation is to determine the amount of each that produces exactly the same effect in the same time under exactly the same conditions. This procedure is likely to be laborious, and quicker methods for obtaining approximate values are sufficiently satisfactory.

Enzyme assays are usually based on the principle that the amount of change in a given time is proportional to the amount of catalyst present: this is true in theory, but not always in practice. Inhibition by end-products, actual destruction of catalyst throughout the reaction, and many other factors (among them ignorance of the reaction) lead to deviations from the theoretical value. Nevertheless, most enzyme actions may be approximately measured on this principle, provided it is applied only with "reasonable" limits of enzyme and substrate concentration. Research is always needed to define these limits.

It is frequently an advantage to determine the relationship between enzyme action and time. If a convenient concentration of substrate is decided upon and other conditions such as temperature, pH, and buffer salts are always kept the same, a series of measurements of the change caused by the enzyme may be made at various time intervals, and the observed change plotted against time, as shown in Figure 1. As a rule,

it will be found that the reaction takes one of two courses: a straight line OA (the so-called zero-order reaction), or a curved line OB, which corresponds to a first-order reaction and would be a straight line if the observed changes were plotted logarithmically.

The case of the straight line occurs when the substrate concentration is so high that the enzyme not only is completely saturated with substrate at the start, but remains so even after much substrate has been decomposed. Furthermore, the end-products are not particularly inhibitory. Such a reaction course is represented by the equation $x/t = K$, where x is the amount of substrate decomposed, t is the elapsed time, and K is a constant. On the principle that doubling the quantity of catalyst doubles the effect, the numerical value of K may be taken as the number of units of enzyme present in the system. Thus an enzyme unit may be set up that applies to all other assays carried out in the same way.

Enzyme actions are found very frequently to follow the first-order course (curve OB) when the substrate present is not enough to saturate the enzyme. (It is possible to have one enzyme that follows a straight-line course in a concentrated substrate solution and a first-order course in a dilute substrate solution.) The equation for a first-order reaction is:

$$\frac{1}{t} \log \frac{a}{a - x} = K$$

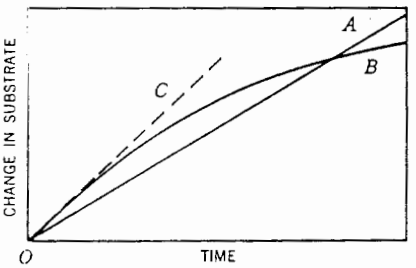


Fig. 1. Hypothetical curves representing zero- and first-order reactions.

where x is the concentration of substrate that has been decomposed at time t, a is the concentration of substrate at the start, and K is a constant. For the same reason as in the previous example, it is often found that the numerical value of K is directly proportional to the concentration of enzyme, and accordingly it (or a more convenient multiple thereof) may be taken as the number of enzyme units in a given volume of the system.

Curves resembling OB are sometimes obtained which on inspection are found not to fit the first-order equation. This may be due to the formation of inhibitory end-products, or sometimes to the reaction's being of the second order. A convenient way to handle such curves is to estimate the slope of the original tangent (OC, Fig. 1). This is usually easy because the first few points generally lie almost in a straight line. The slope of this line is in fact a measure of the initial velocity of the reaction, and is proportional in most cases to the quantity of enzyme present.

As initial velocities must be measured when the reacting system is very young, the actual amount of substrate changed at the time of measurement is relatively small. The success of this scheme obviously depends upon the fact that the reaction in question may be measured with considerable accuracy—which is by no means the rule when enzymes are involved.

When the initial velocity cannot conveniently be determined, the operator may fall back on the common (and generally highly satisfactory) device of constructing an empirical curve relating the amount of substrate decomposed in an arbitrarily selected time interval to the quantity of an enzyme preparation added to the system. Curves vaguely resembling OB (Fig. 1) will again be obtained, but the abscissa will represent milligrams or milliliters of a reproducible enzyme preparation. Curves thus connecting enzyme action with the observed change in the substrate may then be used by arbi-

trarily selecting any convenient quantity on the abscissa as one unit of enzyme. The potency of any other (not too dissimilar) preparation can then be described in terms of these units by ascertaining where it falls on this curve.

In the foregoing completely empirical method, it is obvious that the observed change in the substrate does not need to be directly proportional to the amount of catalyst present. Any function of the amount of catalyst, however complex, will be cared for in the experimentally determined "calibration" curve. It even allows for cases of great mathematical complexity, as when the enzyme becomes more active while the substrate digests. Papain, for example, does this with certain proteins rich in cysteine (18). The weakness of this scheme is that the experimentally determined curves may vary with the purity of the enzyme preparations assayed.

In this method it is also obvious that, even if the enzyme action is not directly proportional to the amount of enzyme present (but is a more complicated function

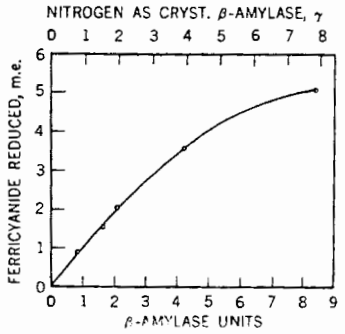


Fig. 2. Relation between β-amylase units and ferricyanide reduced in the method for assay (9). The quantities of β-amylase (crystallized five times) corresponding to the scale of units are shown in micrograms of protein nitrogen.

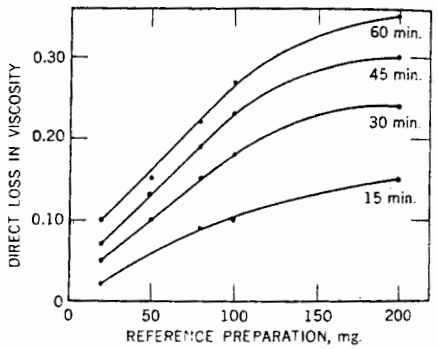


Fig. 3. The relation between milligrams of a reference preparation of wheat proteinase and loss in viscosity of standard gelatin substrate (3).

thereof), it still must be some function of the time of action. This function may also be represented graphically by plotting several curves of observed action versus enzyme quantity, each at a different time interval. Thereafter, if the method is any good at all, it should be possible to check the results of an assay by analyzing the system at each one of the time intervals (which were arbitrarily selected anyhow). Thus the result observed in 10 minutes should check on the 10-minute curve, and the 20-minute result on the 20-minute curve, and so on. To make this clear, two simple examples are given.

(*1*) *The assay of β-amylase* (9). This reaction is not readily analyzed mathematically because the starch molecule does not disappear completely in one step. For our purposes, it remains until the end of the reaction, but it is nevertheless changing all the time, owing to the loss of amylose chains. The observed action of the enzyme is the separation of maltose molecules from starch. Maltose is estimated by its reducing value, as evidenced by the reduction of alkaline copper solution or ferricyanide, or other oxidants. The starch (variety important—usually Lintner-soluble starch) is gelatinized, mixed with dilute acetate buffer (pH 4.6), and acted upon by the enzyme at 30°C. In the method cited here, a sample is withdrawn after 10 minutes' incubation, and its reducing value is determined. A number of these tests with different amounts of crude enzyme from sweet-potato juice

gave a curve similar to that in Figure 2. For reasons of convenience, one unit of β-amylase was selected as that amount of enzyme which liberated one milliequivalent of reducing substance in 10 minutes under the conditions used. Later this enzyme was crystallized and its potency as a substance was determined for the first time. Only then was it possible to estimate the actual weight of β-amylase in the original sweet potato, or to speak of a preparation in terms of the weight of β-amylase in it. Thus the arbitrarily chosen unit can now be redefined as 0.94 microgram (or 0.94 γ) of nitrogen in the form of this particular protein.

(*2*) *Assay of proteinase in wheat flour* (3). This enzyme, a proteinase of the papain type, is present in very low concentration in natural flour. A delicate method of measurement is necessary because preparations, even when purified, are weak and crude. The enzyme is measured by its reduction of the viscosity of gelatin solutions. Since the viscosity of gelatin itself changes with pH and temperature, this test is carried out at pH 5.0 and 35.5°C. Crude extracts of wheat flour (made with 10% NaCl) are used in different amounts with a standard solution of gelatin. Figure 3 shows the drop in viscosity of gelatin observed after 15, 30, 45, and 60 minutes. The weight per milligram of a dry reference preparation was selected as a standard. Samples tested at 15 minutes could be checked by letting them run for 30 or 45 minutes and testing again. Good checks were usually obtained, although there is no simple mathematical correlation between the curves. As long as the assays were run in the same way, different wheat flours could be compared with respect to their extractable proteinase.

On the theory that all enzymes are proteins, the purification of an enzyme may be followed by determining its *specific activity*, the units of enzymic activity found per milligram of protein nitrogen. This value becomes progressively higher as the enzyme protein is successfully purified, until finally the value for the crystalline enzyme is reached.

The concept of enzyme units is of great but latent import to industry. So many enzyme preparations are today articles of commerce that it seems strange many are still sold by weight rather than by units of activity. Many manufacturers, however, are doing their best to correct this situation. Units already defined for lipase, phosphatase, urease, peroxidase, and so on are described by Stern in Jacobs (64).

General Methods for the Preparation of Enzymes

A useful enzyme elaborated by a bacterium, a yeast, or a piece of tissue is often employed without removing it from the cells at all. Thus living *Acetobacter aceti* may be used to oxidize alcohol to acetic acid without first purifying the alcohol oxidase. Yeast ferments sugar to alcohol without extraction of the zymase complex; the many other enzymes in yeast do not interfere because their specific activities lie in other directions.

When the intact organism does not serve the technologist, methods of extraction must be used. These vary all the way from merely grinding some specialized tissue like the pancreas or the liver to form a "brei" to extraction with water, glycerol, dilute salt solutions, or dilute alkalies. An in-between procedure often employed is autolysis, in which the tissue is ground and often also killed by an antiseptic such as toluene. It is then allowed to digest itself by means of its own hydrolytic enzymes and the residual material is either filtered or subjected to one of the extraction methods. In this way "zymase" has been made from yeast cells. An interesting recent development of this scheme is to dissolve the bacteria by an added enzyme: *Micrococcus lysodeikticus* has been dissolved by treatment with lysozyme (p. 744), and crystalline catalase rather easily prepared from the solution (24).

References 70, 73, and 76 discuss in detail the commercial preparation of enzymes

from bacteria, fungi, and other sources. The patent literature on preparation and applications of commercial preparations should also be consulted.

For industrial purposes, enzymes are rarely purified beyond the stage of simple extraction and desiccation of the extract. Some examples of these processes are given below. For malt amylase ("malt diastase"), see *Malt*; for pectases and pectinases, see *Pectic substances*.

Pepsin, the proteinase of the stomach, is used where proteolysis must be conducted in a strongly acid medium (pH 1.5–3.0). The only enzymes in the commercial preparation appear to be proteinases, that is, pepsin itself and an enzyme extremely active with respect to the liquefaction of gelatin. The mucosa of the hog or beef stomach (preferably the former) is minced and extracted at pH 2–3 with two to three times its volume of dilute hydrochloric or phosphoric acid. The mixture is stirred for about two days at 38 °C., after which it is cooled and the undigested tissue filtered off. The filtrate is dried in a vacuum dryer which may run fairly warm (40 °C.) because pepsin is fairly stable at this temperature. Further purity may be achieved by fractional precipitation of the filtrate with alcohol or acetone, followed by low-temperature drying. Commercial pepsin is used in making peptones and in the photographic industry to recover silver from used film.

Rennin is the milk-coagulating enzyme of the mucosa of the fourth stomach of young calves. The mucosa may be minced as usual. The rather thin "brei" is adjusted to pH 2–3 with hydrochloric acid and incubated at 42 °C. to convert the zymogen (prorennin) to rennin. It is next adjusted to pH 5.5 with sodium phosphate. In the presence of the phosphate the mixture becomes fluid, and is dried in a vacuum and powdered. The product contains some fat, which is removed from the dry powder by solvent extraction. Another method of manufacturing rennin comprises drying and grinding the calf stomach. The powder is then stirred for several days with a solution of sodium chloride containing a preservative. The extract is separated from the undissolved tissue, and acidified with sufficient hydrochloric acid to precipitate the mucin. The enzyme is next precipitated by the addition of enough sodium chloride to saturate the solution. The precipitate is filtered off and dried at room temperature. The product contains considerable salt, but less protein impurity than that obtained by the first method. Large quantities of rennet are used in making cheese (see Vol. 4, p. 807), and smaller amounts in making rennet casein (see Vol. 3, p. 225), junkets, etc. (see also p. 756).

Pancreatin is a mixture of the enzymes of the pancreas gland. See Table I. It contains pancreatic amylase (an α-amylase), lipase, carboxypeptidase, and the two pancreatic proteinases, trypsin and chymotrypsin. The pancreas of hogs or cattle is used as starting material. For some purposes the glands are merely dried, powdered, and sieved to remove fibers. More powerful preparations are made by mincing the glands with water and enough acid to give pH 4–5. A preservative such as chloroform or boric acid may be used. The mixture is incubated for several hours or is kept at room temperature for a day, while the zymogens, trypsinogen and chymotrypsinogen, are hydrolyzed to their respective enzyme proteins by the trypsin present. The solid particles of tissue are then removed in a filter press, often with the help of a filter-aid. The filtrate which contains the enzymes may be dried to a powder in a vacuum dryer or a current of cold air. Another method of obtaining a potent mixture of the pancreas enzymes is to freeze the glands and slice them while frozen. The slices are placed on racks over shallow trays in a cold room (+5 °C.). Some autolysis occurs as the tissue thaws, and an exudate drips into the trays, which is collected and dried. It is necessary to use pancreas that has been frozen for some time until it is active, otherwise the yield of exudate is small (68). Pancreatin has many industrial uses, among them the tanning of leather (p. 756), the degumming of silk (p. 757), and the manufacture of gelatin, protein hydrolyzates, certain types of glue, and peptones for bacteriological media.

Trypsin (see also Table I) as a commercial product is also made from the pancreas, but in such a way that most of the amylase and lipase are removed, leaving only the proteolytic enzymes. The initial steps resemble those already described for pancreatin. The aqueous filtrate or exudate is then treated to remove the unwanted enzymes. One method formerly practiced abroad made use of adsorbents. The aqueous extract was neutralized with magnesium oxide, and the precipitate formed, containing the amylase, was filtered off. The filtrate was next acidified with phosphoric acid and reneutralized with chalk. The precipitate of calcium phosphate adsorbed the lipase, which was removed by filtration. The final filtrate could be dried directly, or else the enzyme proteins were precipitated by alcohol or acetone and then dried (at low temperature) after being filtered out. Today,

most trypsin is probably made from aqueous extracts of pancreas by fractional precipitation with acetone or an alcohol at as low a temperature as practical.

Papain (see also Table I) is the only plant proteinase now in extensive use. The brownish commercial product is simply the dried and powdered latex of the papaya tree (*Carica papaya*), which grows in tropical and subtropical regions. The latex is obtained by making a series of longitudinal cuts in the skin of the mature but green fruit while it hangs on the tree. In a few minutes the latex ceases to flow from the cuts, but the operation may be repeated at daily intervals for a week or more, depending somewhat upon the rainfall. As the fruit ripens, however, the yield of latex lessens. Ripe fruit yields no latex and apparently contains no papain. The latex is frequently dried by very crude means, but drying in a vacuum gives a more active preparation of a lighter color. However, the enzyme is unusually resistant to heat. A more active and stable product may be made by mixing salt with the fresh latex and drying only until the material has a pasty consistency. Papain is used in the U.S. for the "chillproofing" of beer (p. 757), for tenderizing meat, and in the tanning industry (p. 756).

Enzymes similar to papain have been obtained from the latex and sap of figs (ficin) and milk weed (asclepain) and from the juice of pineapple (bromelin). Ficin has been used as an anthelmintic.

FURTHER PURIFICATION OF ENZYMES

For scientific purposes it is often desirable to purify an enzyme preparation, particularly to remove from it all enzymes but the one desired. Because apparently each enzyme and tissue requires a different treatment, no single method can be followed. The methods employed usually consist of fractional precipitation of the proteins by a salt such as ammonium sulfate, dialysis, and precipitation of still more foreign protein by mild heating, acid, or moderate concentrations of alcohol or acetone. Another procedure, in growing use for purifying enzymes, employs the process of *adsorption*. Enzymes, like other proteins, are often readily adsorbed on inorganic substances like colloidal iron or hydrated aluminum oxides. As all proteins are not adsorbed equally well under the same conditions, it is often practical to select (usually by trial and error) a set of conditions under which the enzyme protein is adsorbed more or less specifically. Frequently the adsorbed material may be washed with water or some appropriate salt solution, thus removing still more impurities. Finally the enzyme may be recovered from the adsorbent by elution with dilute alkali or with a solvent that reacts with the surface of the adsorbent. Thus an enzyme protein adsorbed on aluminum oxide may often be disengaged and brought into solution by treating the adsorbate with a phosphate solution. It was by the use of such methods that Willstätter made his pioneer discoveries on the detailed action and properties of enzymes. One discovery was that enzymes behave quite differently toward various adsorbents, some adsorbents being much more specific than others. Adsorption was always best at some particular pH level, and elution was best at another, usually more alkaline. In this way it has been possible to separate a great many closely related enzymes and examine their properties after isolation.

Theoretically, an enzyme should adsorb specifically and readily on its substrate, provided of course that the substrate is insoluble in the enzyme solution used. This idea has not had much practical application, probably because the enzyme is also constantly freeing itself as well, owing to the decomposition of the substrate. Recently, however, good success has attended the adsorption of α-amylase on starch from a solution containing so much alcohol (40%) that the enzyme is inactive therein. The principle should be applicable to many other cases.

When an enzyme has been greatly purified by one or several of the foregoing methods, it is usually possible to precipitate the enzyme protein in crystalline form from its solution by the addition of salts. It is necessary to know the most favor-

able pH, concentration of protein, and temperature of crystallization. Ammonium sulfate is used most widely for this purpose. Generally a protein may be successfully crystallized when it is over 50% pure, but small amounts of sugars, gums, or glycosides often prevent crystallization of much purer proteins. When seed crystals have once been obtained, the process can be quite simple. Recrystallization is probably the most satisfactory method of freeing one enzyme protein from other accompanying enzymes. Several recrystallizations are usually necessary. Other impurities besides enzymes make relatively little difference because they are inert substances, except in cases in which measurements such as molecular magnitude are to be made.

Crystalline enzymes are now believed by many workers to represent the pure catalytic protein substance. Their potency sometimes surpasses understanding. For example, a single molecule of catalase decomposes over 2,000,000 molecules of hydrogen peroxide per minute. On the other hand, one molecule of α-amylase from malt breaks apart only 20,000 bonds in the starch molecule per minute.

Technological Applications of Enzymology

In general the applications of enzyme chemistry to technology are of three kinds: (*1*) removal of an enzyme that produces an unwanted result; (*2*) measurement of the enzyme content of a given material as an index of the way the material has been processed in the factory; and (*3*) production of a wanted and serviceable change by an enzyme either occurring naturally or intentionally added.

REMOVAL OF UNWANTED ENZYMES

Unfortunately the removal of unwanted enzymes is not always simple, but the problem is an important one in many branches of the food (*q.v.*) industry. To eliminate the action of an unwanted enzyme, physical removal, destruction by heat, or inhibition by chemicals or temperature control all serve the purpose. The simplest example is removal by physical means. Thus, the germ of wheat is removed in milling flour because it is rich in proteolytic and esterolytic enzymes that later affect the quality of the bread. Similarly, it is advantageous to cut off the tops of freshly harvested sugar canes because the tops are especially rich in invertase (see Table I). If not removed, the invertase diffuses into the body of the canes and inverts some of the cane sugar, thus decreasing markedly the yield of sucrose from the cane.

Heat is the usual method by which unwanted enzymes are destroyed. It is not a perfect method, because enzymes are frequently so heat-resistant that harmful heat effects occur in the product. Fruits and vegetables to be stored in the frozen state are first blanched (heated for a precise time either in a current of steam or by some other convenient means). The purpose of the heating is to destroy at least partially the enzymes that on subsequent storage cause the development of bad flavors, dark colors, and sometimes unpleasant odors in the product. The temperature of blanching varies, of course, with the time of exposure. The details vary with each product and probably with the opinion of each factory operator. In the case of orange juice, the flavor is imparted mainly by small particles in suspension. However, a pectin-digesting enzyme (pectinesterase) is present in orange juice which causes these particles to aggregate as a precipitate of calcium salts of the partially de-esterified pectin. To eliminate this enzyme, higher temperatures are necessary than are usually used in blanching. Yet high temperatures tend to give the juice a burnt taste, and so the

general compromise is to heat the juice for a very few seconds to a relatively high temperature (at least that of boiling water) and then cool it with equal rapidity.

Other methods of inhibiting enzyme action than heating have had only occasional application because as a rule they require the addition of inhibitory chemicals. This leads to legal as well as other complications.

Much may be achieved in the preservation of natural products like fruit by keeping them at low temperatures above freezing. In fruit the oxidation of carbohydrate is a continuous process accompanied by the evolution of heat and carbon dioxide. Fruit therefore tends to heat up in storage; the higher the temperature rises the faster the respiratory enzymes act; and, in turn, the rate of heating increases. Often when fruits or vegetables are shipped, they are chilled at the start. The low initial temperature causes the rate of heating to be slow, and a long time may elapse before the fruit is warm enough to spoil. This precooling results in a saving of refrigeration (*q.v.*), which obviously will be greater when the bulk of the shipment is large, as in a cargo vessel. A further development of the principle of inhibiting enzyme action during the transport of fruits or vegetables is the use of solid carbon dioxide. This is useful not only as a refrigerant, but the high content of carbon dioxide in the atmosphere acts as an inhibitory end-product on some of the respiratory enzymes.

DETERMINATION OF ENZYME DESTRUCTION

One of the best methods for determining how successful a heat treatment has been in destroying unwanted enzymes is to assay the heated material for some selected enzyme and determine how much of the enzyme is left. Usually the enzyme selected for the test is one that is simple to assay. It is advisable also that it be at least moderately heat-resistant, for the unknown enzymes of spoilage may be more heat-resistant than the test enzyme. Recently, peroxidase, a relatively heat-stable enzyme, has been much used as a test enzyme for fuits and vegetables; the amount of peroxidase remaining after heat treatment has been fairly well correlated with the keeping quality of the heated product.

The pasteurization of milk (see Vol. **4,** p. 788) may be fairly accurately tested by determining the amount of phosphatase remaining in the milk after heating (45). Since the amount decreases as pasteurization becomes more rigorous, underpasteurization is readily indicated by the test.

PRODUCTION OF DESIRABLE CHANGES BY ENZYMES

Fermentation (*q.v.*). Alcoholic fermentation is a good example of a process in which enzymic changes occur both on addition of an enzyme and on addition of a living organism, yeast. The starchy grain is first heated to gelatinize the starch, and then malt (containing diastatic enzymes) is added to convert the starch to fermentable sugar (maltose). If the desired product is alcohol, yeast is then added. The use of amylase as malt (*q.v.*) is without doubt the greatest industrial use of enzymes (see Vol. **1,** pp. 259, 291; Vol. **2,** p. 384), but the action of these amylases is not entirely known. When grain is fermented by ordinary methods the equivalent of 85–90% of the starch present is recovered as alcohol, and although some of the remainder could doubtless be obtained by improvement of the method, equipment, etc., a considerable loss of starch appears to occur in an unexplained fashion. An important industrial problem centers about this loss and how to prevent it. There are many other fermentations of industrial importance brought about by other organisms. *n*-Butyl alcohol (*q.v.*) is produced

from sugars by one organism, and lactic acid (*q.v.*) by another. The manufacture of vinegar (*q.v.*) from alcohol is an enzymic process in which a living microorganism (*Acetobacter aceti*) is used; since the alcohol is oxidized to acetic acid with atmospheric oxygen, the process involves forced aeration. The enzyme that catalyzes the oxidation, when isolated from the bacteria, produces the same effect, but it is much more economical to employ the intact living cells. (See also such articles as *Citric acid*; *Glycerol.*)

Tanning (see *Leather*). The hair and excess flesh are first removed from the hides and then the latter are bated so that they become swollen, more or less porous, and permeable to the various tanning agents in which they are later soaked. Bating has always been effected by enzyme action. As late as 1915, soaking the hides in water containing quantities of dog dung was rather widely used. When it was found that the plumping effect was produced largely, if not entirely, by proteolytic enzymes, commercial preparations of many proteinases were proposed for this purpose. Crude pancreatin (see page 752) is one of the oldest and best-known bating materials. The lipase content of pancreatin may also play some role, since the conditions are usually correct for its action (the hides are alkaline), and castor bean macerates which contain a great deal of lipase have also been suggested as an adequate bate. Papain and enzymic bates derived from microorganisms have also been proposed. The quantity of enzyme material used in the tanning industry probably represents the second-greatest industrial use of enzymes.

Cheese-Making (see *Dairy products*). The essential step in preparing cheese from milk is the coagulation of casein (*q.v.*), which is then processed into cheese. The casein may be coagulated by the addition of acid or of proteolytic enzymes with high milk-clotting powers. Most cheese, however, is made by coagulating the casein with rennin (see page 752). Rennin, though essentially a coagulating enzyme for casein, probably also has a very weak proteolytic action, which could be expected to go on in the cheese. The use of rennin produces an elastic curd from which the whey is easily squeezed out, but which still retains calcium combined with the protein. Rennin is not the only proteinase used in cheese-making. Mixtures of rennin with pepsin have been employed. The use of papain has also been reported, and in this case continued proteolysis during ripening of the cheese would seem to be assured. It is reported that, in the Balkan countries, the juice of figs (which is rich in the proteolytic enzyme ficin) is used in preparing the curd. The character of the cheese can be varied considerably by different coagulating enzymes.

Bread-Making (see Vol. **2,** p. 278, and references 58 and 64). The role played in bread-making by the enzymes found in flour is a controversial matter. Raw flour contains relatively small amounts of many enzymes, including a proteinase of the papain type (page 751), which is believed by some to soften the dough. Like all enzymes of the papain type, the proteinase of flour is inactivated by oxidation, a fact regarded by various chemists as explaining the action of certain substances used for bleaching flour (for example, chlorine) as well as other substances (such as bromates) employed as bread improvers. All these are energetic oxidizing agents and also make firmer doughs. This view of the relationship between bread improvers and the inhibition of proteolysis is, however, a matter of active dispute at present. In any event, the amounts of these oxidizing agents required in bread-making are so minute that it seems reasonable to suppose they affect a catalyst in the flour rather than a constituent

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TABLE I. Some Important Enzymes with References to More Detailed Accounts.

PROTEASES (see also page 744)

Proteinases. Their substrates include all proteins except the keratins. Hydrolysis is usually very slow with native proteins. Proteinases also split simple peptides, but as a rule very slowly. Higher degradation products of proteins are split rapidly. Amino acids may be liberated by proteolytic action.

Pepsin. Usual source is the stomach of higher animals. Also occurs in the digestive tract of chickens. Formed as a proenzyme, pepsinogen, which is changed to pepsin by hydrochloric acid. Pepsin clots milk and hydrolyzes proteins, probably first denaturing them. From casein it liberates large quantities of free tyrosine. Characterized by a very acid pH optimum, about 1.5 for protein digestion and about 5.3 for milk clotting (68). See also pages 742 and 752.

Pepsinlike enzymes occur in the digestive tracts of many fish. Action very similar to animal and bird pepsins.

Rennin occurs in the fourth stomach of young calves. Proteolytic action, if any, is very weak. It is a powerful milk-clotting agent (pH optimum about 5.4) (65). See also pages 752 and 756.

Trypsin (see also page 752) occurs in pancreas. Hydrolyzes proteins, probably in very specific fashion. Occurs first as the zymogen, trypsinogen. Trypsin digests trypsinogen to trypsin, chymotrypsinogen to chymotrypsin (68). See pages 742 and 744. Recently trypsin has been found to act also as an esterase (see "Esterases" below) on certain esters of amino acids, as toluenesulfonylarginine methyl ester (46). Similar enzymes are found in other animal tissues.

Chymotrypsin (68) occurs in pancreas, together with trypsin. Hydrolyzes proteins but does not activate any known zymogens. Its specific activity is less than that of trypsin. It is a powerful milk-clotting agent, with a pH optimum of 7. Chymotrypsin may also act as an esterase. It splits, among other esters, tyrosine ethyl ester (29). See page 744. Trypsin or chymotrypsin-like enzymes exist in snake venoms.

Papain (73). See also pages 753, 756, and 757. Found in the latex of green papaya fruit. It is typical of many enzymes found throughout the plant world, such as ficin from fig latex, asclepain from milkweed, and bromelin from pineapple. A characteristic is their content of SH groups, on which the activity of the enzyme apparently depends. After mild oxidation the enzyme is inactive, but may be reactivated by reducing agents such as cysteine, HCN, and others, including any SH groups in the proteins being digested by partly active enzyme. Papain is relatively resistant to heat and may be employed at temperatures of 50–60 °C., where the rate of proteolysis is very rapid. The enzyme clots milk readily. Living organisms are rarely acted upon by proteinases, but papain attacks certain intestinal worms (*Ascaris*) while they are alive. Papain splits hippuryl amide with the liberation of ammonia.

Peptidases ("ereptases"). There are apparently many peptidases widely distributed in plants, animals, and microorganisms. Each is characterized by considerable specificity that depends upon the acid or basic nature, as well as the steric arrangement of the amino acids constituting the peptide. These enzymes split synthetic substrates of the proper optical variety (28,38).

Aminopolypeptidase occurs in animal intestines and in yeast. It splits amino acids bearing the terminal amino group from polypeptides.

Leucylpeptidase occurs in yeast and animal intestines. An amino peptidase that splits leucine when bearing a free terminal amino group. It requires magnesium ion for activation.

Carboxypeptidase is found in the pancreas. It splits the terminal amino acid bearing the free carboxyl group from di- or polypeptides. See also page 739.

Dehydropeptidases are widespread in animal tissues. They convert, for example, glycyldehydroalanine to alanine, ammonia, and pyruvic acid (19). They may be important factors in the metabolism of tumors.

Dipeptidases occur in yeast and animal intestines. These enzymes split dipeptides in general. Some are activated by cobalt and magnesium ions.

CARBOHYDRASES

Split off sugar residues from higher carbohydrates (see page 744).

α-Amylases (58). Salivary glands, pancreas (p. 752), molds, bacteria, and malt (page 755) are rich sources. The enzymes split starches (*q.v.*) and/or glycogen into dextrins and thereafter slowly split the dextrins into maltose and traces of glucose. They destroy the branched-chain structure of starch (amylopectin) and glycogen. Almost complete demolition of starch may be obtained in time. Malt α-amylase requires calcium and may be a calcium protein. Animal α-amylase requires chloride.

β-Amylase (58) is found in higher plants; grains are rich sources. The enzyme in grains splits amylose completely and directly to maltose; it also splits amylopectin (the branched-chain part of starch) or glycogen, but stops where the carbohydrate chain branches. When the

TABLE I. Some Important Enzymes (*continued*).

CARBOHYDRASES (*continued*)

branched carbohydrate chains are broken between the branches (in the presence of α-amylase) β-amylase attacks the straight-chain fragments thus produced. Thus the two enzymes together hydrolyze starch more rapidly than either alone.

Pectic enzymes (see also *Pectic substances*). *Polygalacturonase* (*pectinase*) occurs in many microorganisms and perhaps in some higher plants. The true substrate of polygalacturonase is not pectin but pectic acid, that is, pectin from which many of the methyl ester groups have been removed. *Pectinesterase* (*pectase*) (see below under "Esterases") catalyzes this demethoxylation of pectin, and thus facilitates the action of polygalacturonase. The latter enzyme is important in fruit juice clarification (pages 754 and 757), and is used in many instances for the removal of pectic constituents from plant extracts.

Phosphorylases (49), found in muscle and potato, split glucose from amylose in the presence of inorganic phosphate to form glucose-1-phosphate. They also synthesize amylose from glucose-1-phosphate. See also pages 744 and 745.

Glycosidases (44) (β-glycosidases) are widespread in plants. β-Glucosidase splits glucose from β-glucosides. Yeast invertase (sucrase), a β-fructosidase, splits sucrose to glucose and fructose (see Vol. 4, p. 335). The emulsin enzymes (from almonds, cassava) are glycosidases. Hydrogen cyanide is often liberated from cyanogen-containing glucosides by crude preparations of these enzymes. (See also *Glycosides*.)

ESTERASES (see page 743)

Lipases (50) occur in gastric mucosa, pancreas, and castor beans. They split glycerides and other esters of higher fatty acids to equilibrium mixtures. The reactions are reversible (see p. 746).

Phosphatases (10) are very widespread—obtained from animal (bone, muscle, etc.) and many plant sources. Bone phosphatase, pH optimum about 9, splits primary and secondary esters of phosphoric acid. Most other phosphatases split primary esters only. Tertiary esters are not split by any known phosphatase.

Trypsin and chymotrypsin occur in the pancreas. These proteinases (see "Proteinases" p. 758) also hydrolyze particular esters, as toluenesulfonylarginine methyl ester by trypsin, and tyrosine methyl ester by chymotrypsin (29,46).

Pectase (*pectinesterase*) hydrolyzes the methyl esters that occur in pectin, and so forms free carboxyl groups in the polymerized galacturonic acid units. Pectin is thus progressively and greatly demethoxylated to pectic acid. The enzyme is widespread in the plant kingdom. The reaction is industrially important in the partial de-esterification of pectins to produce "low methoxyl" pectins, and in facilitating the action of polygalacturonase in decomposing the polygalacturonic acid chains (see "Pectic enzymes" above).

SOME OTHER ENZYMES OF HYDROLYSIS

Arginase (34). Liver and jack beans are good sources. It hydrolyzes arginine to ornithine and urea, and is activated by manganese and/or cobalt.

Urease (72). The jack bean is a rich source, although the enzyme is widespread in bacteria and plants. Urease, the first enzyme to be crystallized, hydrolyzes urea to carbon dioxide and ammonia. Urea is the only known substrate.

HYDRASES (see page 744)

Fumarase (53) occurs in microorganisms and liver. It catalyzes the formation of the equilibrium mixture between fumaric and *levo*-malic acids. Fumarase seems to possess absolute specificity.

Glyoxalase (53) is found in yeast. It adds water to methylglyoxal, forming lactic acid. The enzyme requires glutathione, which probably combines first with the methylglyoxal and is then split off again with the addition of water.

ENZYMES OF OXIDATION AND FERMENTATION (see also pages 744 and 746)

Catalase (72) apparently occurs in all tissues except in a few bacteria. It decomposes hydrogen peroxide to water and oxygen and oxidizes some alcohols with hydrogen peroxide (p. 747).

Peroxidases (57), which occur principally in roots and milk, oxidize *o*- and *p*-substituted phenols or amines (also hydriodic acid) at the expense of peroxy compounds, including hydrogen peroxide and potassium persulfate. They are iron proteins. See p. 747.

Polyphenol oxidase (31) is found in potatoes. It uses oxygen directly to form quinones from its substrates, the polyphenols. It is a copper protein.

Ascorbic acid oxidase (37) is usually found in plant tissues high in ascorbic acid. Cauliflower is a good source. The enzyme oxidizes ascorbic acid with oxygen to form dehydroascorbic acid. It is a copper protein.

Cytochrome oxidase (21) is very widespread throughout nature, but the usual laboratory source is muscle. An important enzyme of first attack in tissue oxidation (see p. 747). It oxidizes the cytochrome from the ferrous to the

TABLE I. Some Important Enzymes (*concluded*).

ENZYMES OF OXIDATION AND FERMENTATION (*continued*)

ferric stage, using atmospheric oxygen. It is a hemin protein.

D-*Amino acid oxidase* (12,23,51) occurs in the kidney. It oxidizes only D-amino acids to keto acids and ammonia, thus destroying amino acids which have an unnatural optical form. The action is accelerated by small quantities of L-amino acids.

L-*Amino acid oxidase* (12) occurs in the kidney and liver. It oxidizes L-amino acids or their corresponding hydroxyl derivatives.

Carboxylases are found in yeast, animal tissues, and bacteria. Yeast carboxylase (32) splits carbon dioxide from α-keto acids, producing aldehydes. Thus pyruvic acid yields acetaldehyde plus carbon dioxide. Diphosphothiamine (phosphorylated vitamin B_1) acts as coenzyme. Bacterial carboxylase (17) splits amino acids to keto acids and carbon dioxide; its coenzyme is pyridoxal phosphate (which has the properties of vitamin B_6). See also p. 747.

Alcohol dehydrogenase (*acetaldehyde reductase*) occurs in yeast (41). The last enzyme involved in the production of ethyl alcohol from sugar, it reduces acetaldehyde to alcohol by using hydrogen which is transferred from reduced cozymase (coenzyme I). See also p. 747.

Aldolase is found in yeast and muscle (52). It splits hexose diphosphate to 2 moles of triose monophosphate, an important intermediate step in the fermentation of a hexose.

Luciferase (22,27), occurring in fireflies and certain marine animals, causes the oxidation of luciferin by oxygen, thus producing bioluminescence.

ENZYMES OF "TRANSFER REACTIONS" (see page 744)

Transaminases (25), widespread in both animal and plant tissues, are often obtained from heart muscle. They transfer amino groups from certain amino to keto acids, and vice versa.

Phosphorylases. See p. 759.

Hexokinase (11,33) is found in yeast and probably in all glucose-fermenting tissues. It is the enzyme of first attack in the fermentation of a hexose, transferring phosphate from adenosine triphosphate to hexose. It requires magnesium ions.

TABLE II. Some Commonly Used Methods for Enzyme Assays.

Proteinases

(*a*) Partial breakdown of hemoglobin; determination of the nonprotein tyrosine liberated (43).

(*b*) Determination of newly formed amino or carboxyl groups: by titration (35,55); by formol titration (42); by Van Slyke method (30).

(*c*) Milk-clotting power (5).

(*d*) Decrease in viscosity of a gelatin solution (3).

Amylases

(*a*) Viscosity changes in starch (28a).

(*b*) β-Amylase—increase in reducing value owing to formation of sugars (26a,43).

(*c*) α-Amylase—decrease in color of a starch digest on addition of iodine (43,47).

Glycosidases

Changes in optical rotation (an important technological method for invertase, etc.).

Esterases (including *Phosphatases* and *Lipases*)

(*a*) Increase in acid formed on hydrolysis of esters, measured by continuous titration at constant pH (37a). The method is useful in practically every instance in which a nonvolatile acid is formed as one of the reaction products of an enzyme action, as in the production of gluconic acid from glucose by glucose oxidase.

(*b*) For lipases—titration of fatty acid liberated (7,72).

(*c*) For phosphatases—estimation of increase in inorganic phosphate or of increase in free phenol arising from hydrolysis of a phenyl phosphate (2).

Catalase

(*a*) Decrease of hydrogen peroxide (4).

(*b*) Evolution of oxygen (57).

Peroxidase

(*a*) Color intensity of an oxidized phenol (54).

(*b*) Decrease of hydrogen peroxide (57).

Dehydrogenases

(*a*) Uptake of oxygen (coenzyme required) (15).

(*b*) Decolorization time of methylene blue in anaerobic solution (26).

Direct Oxidases

Oxygen consumption (Warburg technique) (15).

Invertase

Calculation of reaction velocity from polarization readings (1).

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* Added by the Editors.

A. K. BALLS

EOSIN, EOSINE, $C_{20}H_8Br_4O_5$. See "D&C Red Nos. 21, 22, and 23" under *Colors for foods, drugs, and cosmetics; Indicators; Xanthene dyes.*

Food Chemicals Codex
Fourth Edition 1996

Microbial Limits:

Aerobic Plate Count Proceed as directed in chapter 3 of the *FDA Bacteriological Analytical Manual*, Seventh Edition, 1992.

Coliforms Proceed as directed in chapter 4 of the *FDA Bacteriological Analytical Manual*, Seventh Edition, 1992.

Salmonella Proceed as directed in chapter 5 of the *FDA Bacteriological Analytical Manual*, Seventh Edition, 1992.

Staphylococcus aureus Proceed as directed in chapter 12 of the *FDA Bacteriological Analytical Manual*, Seventh Edition, 1992.

Yeasts and Molds Proceed as directed in chapter 18 of the *FDA Bacteriological Analytical Manual*, Seventh Edition, 1992.

Packaging and Storage Store in tight containers in a cool place.

Enzyme Preparations

DESCRIPTION

Enzyme Preparations used in food processing are derived from animal, plant, or microbial sources (see CLASSIFICATION, below). They may consist of whole cells, parts of cells, or cell-free extracts of the source used, and they may contain one active component or, more commonly, a mixture of several, as well as food-grade diluents, preservatives, antioxidants, and other substances consistent with good manufacturing practice.

The individual preparations usually are named according to the substance to which they are applied, such as *Protease* or *Amylase*; such traditional names as *Malt*, *Pepsin*, and *Rennet* also are used, however.

The color of the preparations—which may be liquid, semiliquid, or dry—may vary from virtually colorless to dark brown. The active components consist of the biologically active proteins, which are sometimes conjugated with metals, carbohydrates, and/or lipids. Known molecular weights of the active components range from approximately 12,000 to several hundred thousand.

The activity of enzyme preparations is measured according to the reaction catalyzed by individual enzymes (see below) and is usually expressed in activity units per unit weight of the preparation. In commercial practice (but not for *Food Chemicals Codex* purposes), the activity of the product is sometimes also given as the quantity of the preparation to be added to a given quantity of food to achieve the desired effect.

Additional information relating to the nomenclature and the sources from which the active components are derived is provided in the *General Tests* section under *Enzyme Assays*, Appendix V.

Functional Use in Foods Enzyme (see discussion under CLASSIFICATION, below).

CLASSIFICATION

Animal-Derived Preparations

Catalase (bovine liver) Partially purified liquid or powdered extracts from bovine liver. Major active principle: *catalase*. Typical application: manufacture of certain cheeses.

Chymotrypsin Obtained from purified extracts of bovine or porcine pancreatic tissue. White to tan, amorphous powders soluble in water but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *chymotrypsin*. Typical application: hydrolysis of protein.

Lipase, Animal Obtained from two primary sources: (1) edible forestomach tissue of calves, kids, or lambs, and (2) animal pancreatic tissue. Produced as purified edible tissue preparations or as aqueous extracts. Dispersible in water; insoluble in alcohol. Major active principle: *lipase*. Typical applications: manufacture of cheese; modification of lipids.

Pancreatin Obtained from porcine or bovine (ox) pancreatic tissue. White to tan, water-soluble powder. Major active principles: (1) α-*amylase*, (2) *protease*, and (3) *lipase*. Typical applications: preparation of precooked cereals, infant foods, protein hydrolysates.

Pepsin Obtained from the glandular layer of hog stomach. White to light tan, water-soluble powder; amber paste; or clear amber to brown aqueous liquids. Major active principle: *pepsin*. Typical applications: preparation of fish meal and other protein hydrolysates; clotting of milk in manufacture of cheese (in combination with rennet).

Phospholipase A_2 Obtained from porcine pancreatic tissue. Produced as a white to tan powder or pale- to dark-yellow liquid. Major active principle: *phospholipase* A_2. Typical application: hydrolysis of lecithins.

Rennet, Bovine Aqueous extracts made from the fourth stomach of bovine animals. Clear, amber to dark-brown liquid or white to tan powder. Major active principle: *protease* (pepsin). Typical application: manufacture of cheese. Similar preparations can be made from the fourth stomach of sheep or goats.

Rennet, Calf Aqueous extracts made from the fourth stomach of calves. Clear, amber to dark-brown liquid or white to tan powder. Major active principle: *protease* (chymosin). Typical application: manufacture of cheese. Similar preparations can be made from the fourth stomach of lambs or kids.

Trypsin Obtained from purified extracts of porcine or bovine pancreas. White to tan, amorphous powders soluble in water but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *trypsin*. Typical applications: baking; tenderizing of meat; production of protein hydrolysates.

APPENDIX V: ENZYME ASSAYS

A list of the enzymes covered by the general monograph on *Enzyme Preparations*, is shown in the accompanying table. Also incorporated in the table are the trivial names by which each is commonly known, as well as the systematic names of the major components or of the enzyme for which the preparation is standardized, in accordance with the *Recommendations (1992) of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology on the Nomenclature and Classification of Enzymes*.

Enzyme Preparations Used in Food Processing

TRIVIAL NAME	CLASSIFICATION	SOURCE	SYSTEMATIC NAMES (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. (7) *Bacillus stearothermophilus* (8) *Bacillus subtilis** d-*Bacillus megaterium* (9) *Bacillus subtilis** d-*Bacillus stearothermophilus* (10) *Bacillus licheniformis** d-*Bacillus stearothermophilus*	1,4-α-D-glucan glucanohydrolase	3.2.1.1
β-Amylase	carbohydrase	(1) barley malt (2) barley	1,4-α-D-glucan maltohydrolase	3.2.1.2
Bromelain	protease	pineapples: *Ananas comosus* *Ananas bracteatus* (L)	none	3.4.22.32 3.4.22.33
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 longibrachiatum* (formerly *reesei*)	Endo-1,4-(1,3;1,4)-β-D-glucan 4-glucanohydrolase	3.2.1.4
Chymosin	protease	(1) *Aspergillus niger* var. *awamori** d-calf prochymosin gene (2) *Escherichia coli* K-12* d-calf prochymosin gene (3) *Kluyveromyces marxianus** d-calf prochymosin gene	cleaves a single bond in *kappa* casein	3.4.23.4
Chymotrypsin	protease	bovine or porcine pancreatic extract	none	3.4.21.1
Ficin	protease	figs: *Ficus* sp.	none	3.4.22.3
α-Galactosidase	carbohydrase	(1) *Mortierella vinacea* var. *raffinoseutilizer* (2) *Aspergillus niger*	α-D-galactoside galactohydrolase	3.2.1.22
β-Glucanase	carbohydrase	(1) *Aspergillus niger* var. (2) *Bacillus subtilis* var. (3) *Trichoderma longibrachiatum*	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. (4) *Rhizopus niveus*	1,4-α-D-glucan glucohydrolase	3.2.1.3

TRIVIAL NAME	CLASSIFICATION	SOURCE	SYSTEMATIC NAMES (IUB)[a]	IUB NO.[a]
Glucose Isomerase	isomerase	(1) *Actinoplanes missouriensis* (2) *Bacillus coagulans* (3) *Streptomyces olivaceus* (4) *Streptomyces olivochromogenus* (5) *Streptomyces rubiginosus* (6) *Streptomyces murinus* (7) *Microbacterium arborescens*	D-xylose ketoisomerase	5.3.1.5
Glucose Oxidase	oxidoreductase	*Aspergillus niger* var.	β-D-glucose: oxygen 1-oxidoreductase	1.1.3.4
β-D-Glucosidase	carbohydrase	(1) *Aspergillus niger* var. (2) *Trichoderma longibrachiatum*	β-D-glucoside glucohydrolase	3.2.1.21
Hemicellulase	carbohydrase	(1) *Aspergillus niger* var. (2) *Trichoderma longibrachiatum*	(1) α-L-arabinofuranoside arabinofuranohydrolase (2) 1,4-β-D-mannan mannanohydrolase (3) 1,3-β-D-xylan xylanohydrolase (4) 1,5-α-L-arabinan arabinanohydrolase	3.2.1.55 3.2.1.78 3.2.1.32 3.2.1.99
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. (4) *Candida pseudotropicalis* (5) *Kluyveromyces marxianus* var. *lactis*	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. (5) *Rhizomucor miehei* (6) *Candida rugosa*	(1) carboxylic-ester hydrolase (2) triacylglycerol acylhydrolase	3.1.1.1 3.1.1.3
Maltogenic Amylase	carbohydrase	*Bacillus subtilis** d-*Bacillus stearothermophilus*	1,4-α-D-glucan α-maltohydrolase	3.2.1.133
Pancreatin	mixed carbohydrase, protease, and lipase	bovine and porcine pancreatic tissue	(1) 1,4-α-D-glucan glucanohydrolase (2) triacylglycerol acylhydrolase (3) protease	3.2.1.1 3.1.1.3 3.4.21.4
Papain	protease	papaya: *Carica papaya (L)*	none	3.4.22.2 3.4.22.6
Pectinase[b]	carbohydrase	(1) *Aspergillus niger* var. (2) *Rhizopus oryzae* var.	(1) poly(1,4-α-D-galacturonide) glycanohydrolase (2) pectin pectylhydrolase (3) poly(1,4-α-D-galacturonide)lyase (4) poly(methoxyl-L-galacturonide)lyase	3.2.1.15 3.1.1.11 4.2.2.2 4.2.2.10

TRIVIAL NAME	CLASSIFICATION	SOURCE	SYSTEMATIC NAMES (IUB)[a]	IUB NO.[a]
Pepsin	protease	porcine or other animal stomach tissue	none	3.4.23.1 3.4.23.2
Phospholipase A_2	lipase	animal pancreatic tissue 2-acylhydrolase	phosphatidylcholine	3.1.1.4
Phytase	phosphatase	*Aspergillus niger* var.	(1) *myo*-inositol-hexakisphosphate-3-phosphohydrolase (2) orthophosphoric-mono ester phosphohydrolase	3.1.3.8 3.1.3.2
Protease (general)	protease	(1) *Aspergillus niger* var. (2) *Aspergillus oryzae* var (3) *Bacillus subtilis* var. (4) *Bacillus licheniformis* var.	none	3.4.23.18 3.4.24.28 3.4.21.62
Pullulanase	carbohydrase	*Bacillus acidopullulyticus*	α-Dextrin-6-glucanohydrolase	3.2.1.41
Rennet	protease	(1) fourth stomach of ruminant animals (2) *Endothia parasitica* (3) *Rhizomucor miehei* (4) *Rhizomucor pusillus* (Lindt) (5) *Bacillus cereus*	none	3.4.23.1 3.4.23.4 3.4.23.22 3.4.23.23
Trypsin	protease	animal pancreas	none	3.4.21.4

[a] *Enzyme Nomenclature: Recommendations (1992) of the Nomenclature Committee of the International Union of Biochemistry*, Academic Press, New York, 1992.
[b] Usually a mixture of pectin depolymerase, pectin methylesterase, pectin lyase, and pectate lyase.
*The asterisk indicates a genetically modified organism. The donor organism is listed after "d-."

The following procedures are provided for application as necessary in determining compliance with the vendor's declared representations for enzyme activity. For all of the procedures use filtered, ultra-high purity water with a resistivity of 16 to 18 megohms.

ACID PHOSPHATASE ACTIVITY

Application and Principle This procedure is used to determine acid phosphatase activity in preparations derived from *Aspergillus niger* var. The test is based on the enzymatic hydrolysis of *p*-nitrophenyl phosphate, followed by the measurement of the released inorganic phosphate.

Reagents and Solutions

Glycine Buffer (0.2 *M*, pH 2.5) Dissolve 15.014 g of glycine (Merck, Catalog No. 4201) in about 800 mL of water. Adjust the pH to 2.5 with 1 *M* hydrochloric acid (consumption should be about 80 mL), and dilute to 1000 mL with water.

Substrate (30 m*M*) Dissolve 1.114 g of *p*-nitrophenyl phosphate (Boehringer, Catalog No. 738 352) in glycine buffer, and adjust the volume to 100 mL with the buffer. Prepare fresh substrate solution daily.

TCA Solution Dissolve 15 g of trichloroacetic acid in water, and dilute to 100 mL.

Ascorbic Acid Solution Dissolve 10 g of ascorbic acid in water, and dilute to 100 mL. Store under refrigeration. The solution is stable for 7 days.

Ammonium Molybdate Solution Dissolve 2.5 g of ammonium molybdate [$(NH_4)_6MoO_{24}.4H_2O$] (Merck, Catalog No. 1182) in water, and dilute to 100 mL.

1 M Sulfuric Acid Stir 55.6 mL of concentrated sulfuric acid (H_2SO_4) (Merck, Catalog No. 731) into about 800 mL of water. Allow to cool, and make up to 1000 mL with water.

Reagent C Mix 3 volumes of *1 M Sulfuric Acid* with 1 volume of *Ammonium Molybdate Solution*, then add 1 volume of *Ascorbic Acid Solution*, and mix well. Prepare fresh daily.

Standard Phosphate Solution Prepare a 9.0-m*M* phosphate stock solution. Dissolve and dilute 612.4 mg of potassium dihydrogen phosphate (KH_2PO_4) (dried in desiccator with silica) to 500 mL with water in a volumetric flask. Make the following dilutions in water from the stock solution, and use these as standards.

(a) The additive is prepared by reacting soybean oil in toluene with hydrogen peroxide and formic acid.

(b) It meets the following specifications:

(1) Epoxidized soybean oil contains oxirane oxygen, between 7.0 and 8.0 percent, as determined by the American Oil Chemists' Society (A.O.C.S.) method Cd 9–57, "Oxirane Oxygen," reapproved 1989, which is incorporated by reference in accordance with 5 U.S.C 552(a) and 1 CFR part 51. Copies are available from the American Oil Chemists' Society, P. O. Box 3489, Champaign, IL 61826–3489, or may be examined at the Division of Petition Control (HFS–215), Center for Food Safety and Applied Nutrition, Food and Drug Administration, 1110 Vermont Ave. NW., suite 1200, Washington, DC, or at the Office of the Federal Register, 800 North Capitol St. NW., suite 700, Washington, DC.

(2) The maximum iodine value is 3.0, as determined by A.O.C.S. method Cd 1–25, "Iodine Value of Fats and Oils Wijs Method," revised 1993, which is incorporated by reference in accordance with 5 U.S.C. 552(a) and 1 CFR part 51. The availability of this incorporation by reference is given in paragraph (b)(1) of this section.

(3) The heavy metals (as Pb) content can not be more than 10 parts per million, as determined by the "Heavy Metals Test," Food Chemicals Codex, 3d ed. (1981), p. 512, Method II (with a 2-gram sample and 20 microgram of lead ion in the control), which is incorporated by reference. Copies are available from the National Academy Press, 2101 Constitution Ave. NW., Box 285, Washington, DC 20055, or may be examined at the Division of Petition Control (HFS–215), Center for Food Safety and Applied Nutrition, Food and Drug Administration, 1110 Vermont Ave. NW., suite 1200, Washington, DC, or at the Office of the Federal Register, 800 North Capitol St. NW., suite 700, Washington, DC.

(c) The additive is used as a halogen stabilizer in brominated soybean oil at a level not to exceed 1 percent.

Dated: June 14, 1995.

Fred R. Shank,

Director, Center for Food Safety and Applied Nutrition.

[FR Doc. 95–15349 Filed 6–23–95; 8:45 am]

BILLING CODE 4160–01–F

21 CFR PART 184

[Docket No. 84G–0257]

Enzyme Preparations From Animal and Plant Sources; Affirmation of Gras Status as Direct Food Ingredients

AGENCY: Food and Drug Administration, HHS.

ACTION: Final rule.

SUMMARY: The Food and Drug Administration (FDA) is affirming that certain enzyme preparations derived from animal and plant sources are generally recognized as safe (GRAS) for use as direct food ingredients. This action is a partial response to a petition filed by the Ad Hoc Enzyme Technical Committee (now the Enzyme Technical Association). The following enzyme preparations derived from animal sources are affirmed as GRAS in this final rule: Catalase (bovine liver), animal lipase, pepsin, trypsin, and pancreatin (as a source of protease activity). The following enzyme preparations derived from plant sources are affirmed as GRAS in this final rule: Bromelain, ficin, and malt.

DATES: Effective June 26, 1995. The Director of the Office of the Federal Register approves the incorporation by reference in accordance with 5 U.S.C. 552(a) and 1 CFR part 51 of a certain publication listed in 21 CFR 184.1024(b), 184.1034(b), 184.1316(b), 184.1415(b), 184.1443a(b), 184.1583(b), 184.1595(b), and 184.1914(b), effective June 26, 1995.

FOR FURTHER INFORMATION CONTACT: Laura M. Tarantino, Center for Food Safety and Applied Nutrition (HFS–206), Food and Drug Administration, 200 C St. SW., Washington, DC 20204, 202–418–3090.

SUPPLEMENTARY INFORMATION:

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I. Introduction

In accordance with the procedures described in § 170.35 (21 CFR 170.35), the Ad Hoc Enzyme Technical Committee (now the Enzyme Technical Association), c/o Miles Laboratories, Inc., 1127 Myrtle St., Elkhart, IN 46514, submitted a petition (GRASP 3G0016) requesting that the following enzyme preparations be affirmed as GRAS for use in food:

(1) Animal-derived enzyme preparations: Catalase (bovine liver); lipase, animal; pepsin; rennet; rennet, bovine; and trypsin.

(2) Plant-derived enzyme preparations: Bromelain; malt; and papain.

(3) Microbially-derived enzyme preparations: *Aspergillus niger*, var. (lipase, catalase, glucose oxidase, and carbohydrase); *Bacillus subtilis*, var. (carbohydrase and protease mixtures); *Rhizopus oryzae* (carbohydrase); and *Saccharomyces* species (carbohydrase).

FDA published a notice of filing of this petition in the **Federal Register** of April 12, 1973 (38 FR 9256), and gave interested persons an opportunity to submit comments to the Dockets Management Branch (HFA–305), Food and Drug Administration, rm. 1–23, 12420 Parklawn Dr., Rockville, MD 20857. The petition was amended by notices published in the **Federal Register** of June 12, 1973 (38 FR 15471), proposing affirmation that microbially derived enzyme preparations (carbohydrase, lipase, and protease) from *A. oryzae* are GRAS for use in food; in the **Federal Register** of August 29, 1984 (49 FR 34305), proposing affirmation that the enzyme preparations ficin, obtained from species of the genus *Ficus* (fig tree), and pancreatin, obtained from bovine and porcine pancreas, are GRAS for use in food; and in the **Federal Register** of June 23, 1987 (52 FR 23607), proposing affirmation that the enzyme preparation protease from *A. niger* is GRAS for use in food. In the June 23, 1987, notice, FDA also noted the petitioner's assertion that pectinase enzyme preparation from *A. niger* and lactase enzyme preparation from *A. niger* are included under carbohydrase enzyme preparation from *A. niger*, and that invertase enzyme preparation from *Saccharomyces cerevisiae* and lactase enzyme preparation from *Kluyveromyces marxianus* are both included under carbohydrase enzyme preparation from species of the genus *Saccharomyces*. The agency further noted that, therefore, pectinase enzyme preparation from *A. niger*, lactase enzyme preparation from *A. niger*,

invertase enzyme preparation from *S. cerevisiae*, and lactase enzyme preparation from *K. marxianus* were to be considered part of the petition. Interested persons were given an opportunity to submit comments to the Dockets Management Branch (address above) on each amendment.

After the petition was filed, the agency published, as part of its comprehensive safety review of GRAS substances, two GRAS affirmation regulations that covered three of the enzyme preparations from animal and plant sources included in the petition. These two regulations are: (1) § 184.1685 *Rennet (animal derived)* (21 CFR 184.1685), which was published in the **Federal Register** of November 7, 1983 (48 FR 51151) and includes the petitioned enzyme preparations rennet and bovine rennet; and (2) § 184.1585 *Papain* (21 CFR 184.1585), which was published in the **Federal Register** of October 21, 1983 (48 FR 48805). The agency concludes that rennet, bovine rennet, and papain are already affirmed as GRAS and listed in existing regulations and need not be addressed further.

In letters to FDA (Refs. 1 and 2), the petitioner asserted that the enzyme preparation malt (amylase) includes extracts from germinated (malted) barley or ungerminated (unmalted) barley. In addition, certain published references (Refs. 3 and 4) submitted by the petitioner describe the enzyme preparation pancreatin as a substance containing the enzymes amylase, lipase, and protease.

In a notice published in the **Federal Register** of September 20, 1993 (58 FR 48889), the agency announced that the petitioner had requested that the following enzyme preparations be withdrawn from the petition without prejudice to the filing of a future petition: (1) Pancreatin used for its lipase activity, (2) pancreatin used for its amylase activity, and (3) amylase derived from unmalted barley extract. In that notice, the agency stated that, in light of the petitioner's request, any future action by FDA on the petition would not include a determination of the GRAS status of these three enzyme preparations.

This final rule is a partial response to the petition and addresses only enzyme preparations from animal and plant sources. Microbial enzyme preparations will be dealt with separately in a future issue of the **Federal Register**. Furthermore, in accordance with the September 20, 1993, **Federal Register** notice, FDA's determination of the GRAS status of the enzyme preparation malt includes only the enzyme preparation derived from malted barley extracts. Likewise, FDA's determination of the GRAS status of the enzyme preparation pancreatin includes only the use of pancreatin as a protease.

II. Standards for GRAS Affirmation

Pursuant to § 170.30 (21 CFR 170.30) and 21 U.S.C. 321(s), general recognition of safety may be based only on the views of experts qualified by scientific training and experience to evaluate the safety of substances directly or indirectly added to food. The basis of such views may be either scientific procedures or, in the case of a substance used in food prior to January 1, 1958, experience based on common use in food. General recognition of safety based upon scientific procedures requires the same quantity and quality of scientific evidence as is required to obtain approval of a food additive and ordinarily is based upon published studies, which may be corroborated by unpublished studies and other data and information (§ 170.30(b)). General recognition of safety through experience based on common use in food prior to January 1, 1958, may be determined without the quantity or quality of scientific evidence required for approval of a food additive regulation, and ordinarily is based upon generally available data and information.

For the enzyme preparations from animal and plant sources that are the subject of this document, the Enzyme Technical Association based its request for affirmation of GRAS status on a history of safe food use prior to 1958. In the preamble to a proposed rule amending § 170.30, which was published in the **Federal Register** of July 2, 1985 (50 FR 27294) (final rule published in the **Federal Register** of May 10, 1988 (53 FR 16544)), FDA stated that general recognition of safety through experience based on common use in food requires a consensus on the safety of the substance among the community of experts who are qualified to evaluate the safety of food ingredients.

III. Background

A. Enzymes

Enzymes are proteins or conjugated proteins,[1] produced by plants, animals, and microorganisms, that function as biochemical catalysts (Ref. 5). Further, most enzymes are very specific in their ability to catalyze only certain chemical reactions; this high degree of specificity and strong catalytic activity are the most important functional properties of enzymes (Ref. 6). The practical applications of enzymes used in food processing include the conversion of starch to sugars in brewing, the tenderizing of sausage casings and meat, and the partial hydrolysis (breakdown) of proteins that would otherwise form a haze when beer is chilled (Ref. 7).

B. Enzyme Nomenclature

Enzymes were originally known principally by their trivial (common or historical) names. These trivial names typically were based on one of two methods of nomenclature: (1) By the addition of "-in" or "-ain" as a suffix to a root indicating the source of the enzyme (e.g., papain from papaya or pancreatin from pancreas); or (2) by the addition of the suffix "-ase" to a root indicating the substrate (specific reactant) for the enzyme (e.g., lactase, which acts on the substrate lactose) (Ref. 8). Some proteases, however, have trivial names that are not based on either of these two methods (e.g., trypsin).

In 1956, the Third International Congress of the International Union of Biochemistry (IUB) organized a Commission on Enzymes to devise a systematic strategy for naming enzymes. The system developed by the Commission on Enzymes combined a naming system and a numbering system (Ref. 8). With the exception of most proteases, the systematic name is derived from the names of the substrate, product, and type of reaction.[2] The systematic number is based on the class and subclasses to which the enzyme belongs. The two classes of enzymes in the numbering system relevant to this document are class 1, oxidoreductases (e.g., catalase), which are active in biological oxidation and reduction; and class 3, hydrolases (e.g., glycosidases (carbohydrases), lipases, and proteases), which catalyze the splitting of chemical bonds by the addition of water.

The following examples illustrate the trivial name, functions, and Enzyme Commission (EC) name and number of enzymes that are components of some of the enzyme preparations that are the subject of this document (Refs. 9 through 11).

α-amylase. Hydrolysis of α-1,4-glucan bonds in polysaccharides (starch, glycogen, etc.), yielding dextrins and oligo- and monosaccharides (1,4-α-D-glucan glucanohydrolase, EC 3.2.1.1).

Catalase. Decomposition of hydrogen peroxide (H_2O_2), yielding water and

[1] A conjugated protein is a protein that contains a nonamino acid moiety such as a carbohydrate.

[2] In general, proteolytic enzymes are not sufficiently defined to apply short systematic names.

b. *Methods of manufacture.* Bromelain is obtained from pineapple juice (pressed from the stems of pineapples that remain after harvesting the fruit) by precipitation with alcohol or ammonium sulfate (Refs. 8, 12, and 13). Ficin is obtained from the latex of a variety of tropical fig trees by precipitation with acetone or alcohol (Refs. 9, 12, and 14).

Malt is produced from germinated barley. The petition describes the following process for the manufacture of malt (Ref. 19). Barley is softened by a series of steeping operations in water at 10 °C to 30 °C until the moisture content of the kernels reaches 40 to 50 percent. The grain is then germinated under controlled conditions for a period of up to 7 days. Reducing substances are added to activate the enzymes. Solids are removed from the extract, which is concentrated, stabilized, and standardized. The resultant syrup is usually a brown, sweet, and viscous liquid with a specific gravity of approximately 1.1 to 1.3 at 25 °C.

c. *Technical effects.* Pre-1958 uses in food of plant-derived enzyme preparations are listed in Table 3, using terminology from the cited reference(s) published before or during 1958.

TABLE 3.—APPLICATIONS OF PLANT-DERIVED ENZYMES IN FOOD PRIOR TO 1958

Enzyme preparation	Enzyme activity	Food categories	Technical effect or industry application	References
Malt	Amylase	Bread	Baking	7, 14, 15
		Beer	Mashing	14, 15
		Precooked baby cereals	Not reported	15
		Breakfast cereals	Not reported	14, 15
		Distilled beverages	Mashing	15
Bromelain	Protease	Beer	Chillproofing	13, 14, 15
		Condiments	Not reported	15
		Milk	Protein hydrolysis	15
		Evaporated milk	Stabilization	15
		Meat	Tenderizing, softening tissue	13, 14, 15, 20
		Sausage casings	Tenderizing	14, 15
		Fish	Condensing fish solubles	15
Ficin	Protease	Meat	Softening	20

IV. Safety Evaluation

A. Pre-1958 History of Use in Food

Enzymes have been used for many years in the production and processing of food, for example, in the baking, dairy, and brewing industries (e.g., see Refs. 7, 13, and 14). The consumption of food produced using these enzymes has produced no evidence of an associated human health hazard.

The petitioner provided generally available information, including published papers and review articles, showing that the animal- and plant-derived enzyme preparations that are the subject of this document were commonly used in food prior to 1958. For example, the pre-1958 food uses shown in Tables 2 and 3 were documented in articles that were published in or before 1958; the cited references demonstrate that the use of these enzyme preparations in a variety of foods was widely recognized by 1958. Therefore, the agency concludes that the enzyme preparations that are the subject of this document were in common use in food prior to January 1, 1958.

B. Corroborating Evidence of Safety

1. The Enzyme Components

A wide variety of enzymes has always been present in human food. Moreover, many naturally occurring enzymes in the cells of animals and plants used for food remain active after cell death. For example, active enzymes are present in fresh fruits and vegetables and are not inactivated unless the fruits or vegetables are cooked (Refs. 6 and 21).

The enzymes that are the subject of this document are naturally occurring proteins that are ubiquitous in living organisms. They are derived from animals and plants that have been used as sources of food, and are identical or substantially similar[4] to enzymes that have been safely consumed as part of the diet throughout human history.

Issues relevant to a safety evaluation of proteins from food sources are potential toxicity and allergenicity. Pariza and Foster (Ref. 6) note that very few toxic agents have enzymatic properties, and those that do (e.g., diphtheria toxin and certain enzymes in the venom of poisonous snakes) catalyze unusual reactions that are not related to the types of catalysis that are common in food processing and that are the subject of this document. Further, the agency has recently noted, in the context of guidance to industry regarding the safety assessment of new plant varieties, that newly introduced enzymes do not generally raise safety concerns (Ref. 22). Exceptions include enzymes that produce substances that are not ordinarily digested and metabolized, or that produce toxic substances. The functions of the enzymes that are the subject of this document are well known; they split proteins, carbohydrates, lipids, or other substances (e.g., hydrogen peroxide) into smaller subunits that do not have toxic properties and that are readily metabolized by the human body.

The agency is not aware of any reports of allergic reactions associated with the ingestion in food of the enzymes that are the subject of this document. There have been, however, some reports of allergies and primary irritations from skin contact with enzymes or inhalation of dust from concentrated enzymes (for example, proteases used in the manufacture of laundry detergents) (Refs. 23 through 25). These reports relate primarily to workers in production plants (Ref. 24) and are not relevant to an evaluation of the safety of ingestion of such enzymes in food.

[4] Enzymes that have the same function and that are identified by the same name and EC number often differ slightly in structure and properties when they are obtained from different sources. For example, the structure of an enzyme isolated from one tissue (such as the liver) of one animal species, may differ slightly from that of the same enzyme isolated from a different tissue from the same species, or from the liver of another animal species. In part because of this variability, the diet routinely contains many thousands of different enzyme protein molecules. The concept of substantial similarity relative to food safety assessment has recently been discussed by several expert groups. For example a report prepared by an expert group of the Organization for Economic Co-operation and Development (OECD) concluded, in part, "[I]f a new food or food component is found to be substantially equivalent to an existing food or food component, it can be treated in the same manner with respect to safety. No additional safety concerns would be expected." ("Safety Evaluation of Foods Derived by Modern Biotechnology: Concepts and Principles," OECD, 1993, Paris).

Moreover, Pariza and Foster (Ref. 6) note that there are no confirmed reports of primary irritations in consumers caused by enzymes used in food processing.

The 1977 report of the Select Committee on GRAS substances concerning the plant enzyme papain (Ref. 23) supports the view that the ingestion of an active protease at levels found in food products is not likely to affect the human gastrointestinal tract, where many proteases already exist at levels adequate to digest food:

> In common with other proteolytic enzymes, papain digests the mucosa and musculature of tissues in contact with the active enzyme for an appreciable period. Because there is no food use of papain that could result in the enzyme preparation occurring in sufficient amount in foods to produce these effects, this property does not pose a dietary hazard.

In summary, the enzyme components of the preparations that are the subject of this document are identical or substantially similar to enzymes that are known to have been safely consumed in the diet; they do not result in the production of toxic substances; and their use in food for many years has not been associated with reports of allergenicity or primary irritation. Therefore, the agency finds that the presence of the enzyme components does not create a basis for concern about the safety of the enzyme preparations.

2. Enzyme Sources and Processing Aids

The agency has concluded that the enzyme components of enzyme preparations do not raise safety concerns; therefore, the relevant safety issue becomes whether the enzyme preparations contain toxic contaminants. Enzyme preparations used in food processing are usually not chemically pure but contain, in addition to the enzyme component, materials that derive from the enzyme source, as well as from the manufacturing methods used to generate the finished enzyme preparation.

In accordance with § 170.30(h)(1), the enzyme preparations affirmed as GRAS in this document must comply with the general requirements and additional requirements for enzyme preparations in the Food Chemicals Codex, 3d ed. (Ref. 9). When the animal-derived enzyme preparations that are the subject of this document are produced in accordance with current good manufacturing practice (CGMP), they are obtained from animal tissues that comply with applicable Federal meat inspection requirements and that are handled in accordance with good hygienic practices (Ref. 9). Similarly, when produced in accordance with CGMP, the plant material used in the production of enzymes consists of components that leave no residues harmful to health in the finished food under normal conditions of use (Ref. 9).

The enzyme preparations may contain substances, such as salts, preservatives, or stabilizers, that are used in their preparation and purification. When used in accordance with CGMP, these processing aids are substances that are acceptable for general use in foods (Ref. 9). As always, any of these substances that are intended to become or become functional components of the enzyme preparation must be GRAS substances or food additives approved for use in the manufacture of enzyme preparations. Therefore, the agency concludes that the presence of added substances and impurities derived from the enzyme source or introduced by manufacturing does not present a basis for concern about the safety of the enzyme preparations.

3. Dietary Exposure

Because enzymes are highly efficient catalysts, they are needed in only minute quantities to perform their function. When used in accordance with CGMP, the amounts added to food represent only a minute fraction of the total food mass. The history of common use in food for many years of the enzyme preparations that are the subject of this document has produced no evidence of an associated hazard; further, there is no reason to believe that use of these enzyme preparations at levels needed to perform their functions would raise a safety concern. Therefore, the agency concludes that no limits other than CGMP are needed to ensure safe use.

V. Comments

FDA received seven letters in response to the filing notice and none in response to the amendment notices. Three comments concerned microbially derived enzyme preparations, which will be addressed in a separate document. Of the remaining four comments, one came from a food manufacturer, two from trade associations, and one from a consumer group. Three comments supported the petition for GRAS affirmation of the enzyme preparations included in the petition, stating that these enzyme preparations have a long history of use in foods such as cheese, bread, and corn syrup.

One comment asserted that enzyme preparations should not be considered GRAS, and their use should be declared on the label of foods to warn consumers about hazards inherent in their use. The comment stated that enzyme preparations are rarely purified to any significant degree and contain a variety of cellular constituents and metabolic debris. The comment further argued that, although enzyme preparations are used at low levels and are inactivated after the treatment of food, they may elicit allergic reactions and other biological activities which could be detrimental to human health. In support of this statement, the comment cited a published scientific article (Ref. 26) which reported that enzyme preparations from *B. subtilis* caused temporary weight loss and aggravated infection in mice when injected into the abdominal cavity and caused hemolysis and hemagglutination of sheep erythrocytes in in vitro studies. Because this article concerns microbially derived enzyme preparations injected directly into the abdominal cavity, it is not relevant to this rulemaking, which concerns animal- and plant-derived enzyme preparations consumed by mouth.

The agency also notes that under certain circumstances, applicable regulations already require use of an enzyme preparation in a food to be declared on the label, depending upon the nature of the enzyme preparation's use and technical effect in the food. These regulatory requirements are discussed below.

The Federal Food, Drug, and Cosmetic Act (21 U.S.C. 343(i)(2)) requires that all ingredients of multi-ingredient foods be listed on the label of the food. By regulation, FDA has exempted certain ingredients that are used only as processing aids from this requirement. Sections 101.100(a)(3)(ii)(a) and (a)(3)(ii)(c) (21 CFR 101.100(a)(3)(ii)(a) and (a)(3)(ii)(c)) provide an exemption from the ingredient listing requirement for processing aids that are added to a food for their technical or functional effect during processing, but are either removed from the food before packaging or are present in the finished food at insignificant levels and do not have any technical or functional effect in the finished food. Although many enzyme preparations are used as processing aids in food (e.g., the use of amylase preparations in the manufacture of glucose syrup and the use of protease preparations in the manufacture of protein hydrolyzates), other enzyme preparations are not used solely as processing aids in the manufacture of foods (e.g., the use of lipase preparations for flavor production in cheeses and the use of protease preparations in tenderizing meat). In these cases, the enzymes remain active

in the finished food product, functioning as an integral part of the food by enhancing body, flavor, and aroma (49 FR 29242, July 19, 1984). Because such effects in the finished food remove the enzymes from the ingredient listing exemption in § 101.100(a)(3)(ii)(c), the use of such enzymes must be declared on the label. Therefore, whether a label declaration is needed for the use of an enzyme preparation in a food will depend upon its function and effect in the food.

VI. Conclusions

The petitioner has provided generally available evidence demonstrating that the enzyme preparations under consideration were in common use in food prior to 1958. As provided for under § 170.30(a) and (c)(1), FDA has determined that this information provides an adequate basis upon which to conclude that the use of these enzyme preparations in food is generally recognized as safe among the community of experts qualified by scientific training and experience to evaluate the safety of food ingredients.

This evidence of common use in food prior to 1958 without any reported adverse effects from consumption is corroborated by the absence of any reports of toxicity resulting from use of the enzyme preparations in food since 1958, by information that the enzymes themselves and the sources from which they are derived are nontoxic, and by evidence that manufacturing will not introduce impurities that will adversely affect the safety of the finished enzyme preparations. Moreover, the enzyme preparations that are the subject of this document are substantially similar to enzymes naturally present in foods that have been safely consumed in the human diet for centuries.

Having evaluated the information in the petition, along with other available information that related to the use of these enzyme preparations, the agency concludes that the following enzyme preparations derived from animal or plant sources are GRAS under conditions of use consistent with CGMP: Bromelain, catalase (bovine liver), ficin, animal lipase, malt, pancreatin (as a source of protease activity), pepsin, and trypsin. The agency is basing its conclusion on evidence of a substantial history of safe consumption of the enzyme preparations in food by a significant number of consumers prior to 1958, corroborated by the other evidence summarized above.

FDA is therefore affirming that the use of the enzyme preparations that are the subject of this document is GRAS with no limits other than CGMP (21 CFR 184.1(b)(1)). The agency further concludes that the general and additional requirements for enzyme preparations in the Food Chemicals Codex, 3d ed. (1981), pp. 107–110, are adequate as minimum criteria for food-grade preparations of these enzymes.

To clarify the identity of each enzyme preparation, the agency is including in §§ 184.1024(a), 184.1034(a), 184.1316(a), 184.1415(a), 184.1443a(a), 184.1583(a), 184.1595(a), and 184.1914(a), the EC number(s) of the enzyme preparation or of the characterizing enzyme activity(ies) for food use of the preparation[5]. In order to make clear that the affirmation of the GRAS status of these enzyme preparations is based on the evaluation of specific uses, the agency is including in §§ 184.1024(c), 184.1034(c), 184.1316(c), 184.1415(c), 184.1443a(c), 184.1583(c), 184.1595(c), and 184.1914(c) the technical effect and the specific substances on which each enzyme preparation acts, although the data show no basis for a potential risk from any foreseeable use of these enzyme preparations.

VII. Environmental Impact

The agency has determined under 21 CFR 25.24(b)(7) that this action is of a type that does not individually or cumulatively have a significant effect on the human environment. Therefore, neither an environmental assessment nor an environmental impact statement is required.

VIII. Economic Impact

FDA has examined the impact of this final rule affirming the GRAS status of enzyme preparations from animal and plant sources under Executive Order 12866 and the Regulatory Flexibility Act (Pub. L. 96–354). Executive Order 12866 directs Federal agencies to assess the costs and benefits of available regulatory alternatives and, when regulation is necessary, to select regulatory approaches that maximize net benefits (including potential economic, environmental, public health and safety effects; distributive impacts; and equity). The Regulatory Flexibility Act requires Federal agencies to minimize the economic impact of their regulations on small businesses.

The agency finds that this final rule is not a significant regulatory action as defined by Executive Order 12866. The rule requires no change in current industry practice concerning the manufacture and use of these substances. Compliance costs to firms are therefore estimated to be zero. The substances that are the subject of this document pose no health risks to consumers when used as intended. Costs to consumers are therefore also estimated to be zero.

In accordance with the Regulatory Flexibility Act, FDA also has determined that this final rule will not have a significant adverse impact on a substantial number of small businesses.

[5] The EC number is sufficient to define the characterizing activity in the enzyme preparation. Therefore, FDA is not including the EC systematic name in the regulation.

IX. References

The following references have been placed on display in the Dockets Management Branch (address above) and may be seen by interested persons between 9 a.m. and 4 p.m., Monday through Friday.

1. Comments of Ad Hoc Enzyme Technical Committee regarding FDA's draft final regulations, entitled "Enzymes Proposed for Affirmation as GRAS," with a letter dated December 21, 1984, from Roger D. Middlekauff, Ad Hoc Enzyme Technical Committee, to Kenneth A. Falci, FDA.
2. Letter dated September 20, 1985, from Roger D. Middlekauff, Enzyme Technical Association, to Lawrence J. Lin, FDA.
3. Monograph on "Pancreatin," U.S. Pharmacopeia, 21st revision, the United States Pharmacopeial Convention, Inc., Rockville, MD, pp. 777–778, 1985.
4. Monograph on "Pancreatin," U.S. Pharmacopeia, 6th supp., the United States Pharmacopeial Convention, Inc., Rockville, MD, pp. 2595–2597, 1987.
5. Morris, W., editor, The American Heritage Dictionary of the English Language, Houghton Mifflin Co., Boston, MA, p. 438, 1976.
6. Pariza, M. W., and E. M. Foster, "Determining the Safety of Enzymes Used in Food Processing," *Journal of Food Protection*, 46:453–468, 1983.
7. Reed, G., "Industrial Enzymes—Now Speed Natural Processes," *Food Engineering*, 24:105–109, 1952.
8. Scott, D., "Enzymes, Industrial," Encyclopedia of Chemical Technology, Mark, H. F. et al., editors, John Wiley and Sons, New York, 3d ed., 9:173–224, 1978.
9. Monograph on "Enzyme Preparations," Food Chemicals Codex, National Academy Press, Washington, DC, 3d ed., pp. 107–110, and 480–481, 1981.
10. IUB, "Enzyme Nomenclature 1992," Academic Press, New York, pp. 116, 307, 346, 388, 399, 402–403, 1992.
11. IUB, "Enzyme Nomenclature 1964," Academic Press, New York, pp. 66–67, 86–87, 126–131, 136–149, and 170–171, 1965.
12. Tauber, H., "The Chemistry and Technology of Enzymes," John Wiley and Sons, New York, pp. 25–26, 130–131, 140, 145–151, 163–167, 192–193, and 327–335, 1949.

13. Reed, G., "Enzymes, Industrial," Encyclopedia of Chemical Technology, Kirk, R. E. and D. F. Othmer, editors, Interscience Encyclopedia, Inc., New York, 1st supplemental vol., pp. 294–312, 1957.
14. Underkofler, L. A., and W. J. Ferracone, "Commercial Enzymes—Potent Catalyzers that Promote Quality," *Food Engineering*, 29:123, 125–126, 130, and 133, 1957.
15. Underkofler, L. A., R. R. Barton, and S. S. Rennet, "Microbiological Process Report—Production of Microbial Enzymes and Their Applications," *Applied Microbiology*, 6:212–221, 1958.
16. Smythe, C. V., "Microbiological Production of Enzymes and Their Practical Applications," *Economic Botany*, 5:126–144, 1951.
17. Harper, W. J. and J. E. Long, "Italian Cheese Ripening. IV. Various Free Amino and Fatty Acids in Commercial Provolone Cheese," *Journal of Dairy Science*, 39:129–137, 1956.
18. Long, J. E., and W. J. Harper, "Italian Cheese Ripening. VI. Effects of Different Types of Lipolytic Enzyme Preparations on the Accumulation of Various Free Fatty and Free Amino Acids and the Development of Flavor in Provolone and Romano Cheese," *Journal of Dairy Science*, 39:245–252, 1956.
19. Response of the Enzyme Technical Association to the letter dated June 26, 1986, of Lawrence J. Lin regarding GRASP 3G0016, received with a letter dated October 3, 1986, from Roger D. Middlekauff of the Enzyme Technical Association, to Lawrence J. Lin, FDA.
20. "List of Chemicals Approved Under Meat Inspection Act Before September 6, 1958, Which are Exempted from the 1958 Food Additives Amendment of the Federal Food, Drug, and Cosmetic Act," *Food Drug Cosmetic Law Journal*, 13:834–840, 1958.
21. De Becze, G. I., "Food Enzymes," *Critical Reviews in Food Technology*," 1:479–518, 1970.
22. FDA, "Statement of Policy: Foods Derived from New Plant Varieties," 57 FR 22984 at 23005; May 29, 1992.
23. "Evaluation of the Health Aspects of Papain as a Food Ingredient," Select Committee on GRAS Substances, Washington, DC, available through U.S. Department of Commerce, National Technical Information Service, Order No. PB–274–174, 1977.
24. Fulwiler, R. D., "Detergent Enzymes—An Industrial Hygiene Challenge," *American Industrial Hygiene Association Journal*, 32:73–81, 1971.
25. "Enzyme-containing Laundering Compounds and Consumer Health," National Research Council/National Academy of Sciences, National Technical Information Service, Washington, DC, Order No. PB–204–118, 1971.
26. Dubos, R., "Toxic Factors in Enzymes Used in Laundry Products," *Science*, 173:259–260, 1971.

List of Subjects in 21 CFR Part 184

Food ingredients, Incorporation by reference.

Therefore, under the Federal Food, Drug, and Cosmetic Act and under authority delegated to the Commissioner of Food and Drugs and redelegated to the Director, Center for Food Safety and Applied Nutrition, 21 CFR part 184 is amended as follows:

PART 184—DIRECT FOOD SUBSTANCES AFFIRMED AS GENERALLY RECOGNIZED AS SAFE

1. The authority citation for 21 CFR part 184 continues to read as follows:

Authority: Secs. 201, 402, 409, 701 of the Federal Food, Drug, and Cosmetic Act (21 U.S.C. 321, 342, 348, 371).

2. Section 184.1024 is added to subpart B to read as follows:

§ 184.1024 Bromelain.

(a) Bromelain (CAS Reg. No. 9001–00–7) is an enzyme preparation derived from the pineapples *Ananas comosus* and *A. bracteatus* L. It is a white to light tan amorphous powder. Its characterizing enzyme activity is that of a peptide hydrolase (EC 3.4.22.32).

(b) The ingredient meets the general requirements and additional requirements for enzyme preparations in the Food Chemicals Codex, 3d ed. (1981), p. 110, which is incorporated by reference in accordance with 5 U.S.C. 552(a) and 1 CFR part 51. Copies are available from the National Academy Press, 2101 Constitution Ave. NW., Washington, DC, or may be examined at the Office of Premarket Approval (HFS–200), Food and Drug Administration, 200 C St. SW., Washington, DC, and the Office of the Federal Register, 800 North Capitol St. NW., suite 700, Washington, DC.

(c) In accordance with § 184.1(b)(1), the ingredient is used in food with no limitation other than current good manufacturing practice. The affirmation of this ingredient as GRAS as a direct food ingredient is based upon the following current good manufacturing practice conditions of use:

(1) The ingredient is used as an enzyme as defined in § 170.3(o)(9) of this chapter to hydrolyze proteins or polypeptides.

(2) The ingredient is used in food at levels not to exceed current good manufacturing practice.

3. Section 184.1034 is added to subpart B to read as follows:

§ 184.1034 Catalase (bovine liver).

(a) Catalase (bovine liver) (CAS Reg. No. 9001–05–2) is an enzyme preparation obtained from extracts of bovine liver. It is a partially purified liquid or powder. Its characterizing enzyme activity is catalase (EC 1.11.1.6).

(b) The ingredient meets the general requirements and additional requirements for enzyme preparations in the Food Chemicals Codex, 3d ed. (1981), p. 110, which is incorporated by reference in accordance with 5 U.S.C. 552(a) and 1 CFR part 51. Copies are available from the National Academy Press, 2101 Constitution Ave., NW., Washington, DC 20418, or may be examined at the Office of Premarket Approval (HFS–200), Food and Drug Administration, 200 C St., SW., Washington, DC, and the Office of the Federal Register, 800 North Capitol St. NW., suite 700, Washington, DC.

(c) In accordance with § 184.1(b)(1), the ingredient is used in food with no limitation other than current good manufacturing practice. The affirmation of this ingredient as GRAS as a direct food ingredient is based upon the following current good manufacturing practice conditions of use:

(1) The ingredient is used as an enzyme as defined in § 170.3(o)(9) of this chapter to decompose hydrogen peroxide.

(2) The ingredient is used in food at levels not to exceed current good manufacturing practice.

4. Section 184.1316 is added to subpart B to read as follows:

§ 184.1316 Ficin.

(a) Ficin (CAS Reg. No. 9001–33–6) is an enzyme preparation obtained from the latex of species of the genus *Ficus*, which include a variety of tropical fig trees. It is a white to off-white powder. Its characterizing enzyme activity is that of a peptide hydrolase (EC 3.4.22.3).

(b) The ingredient meets the general requirements and additional requirements for enzyme preparations in the Food Chemicals Codex, 3d ed. (1981), p. 110, which is incorporated by reference in accordance with 5 U.S.C. 552(a) and 1 CFR part 51. Copies are available from the National Academy Press, 2101 Constitution Ave., NW., Washington, DC 20418, or may be examined at the Office of Premarket Approval (HFS–200), Food and Drug Administration, 200 C St., SW., Washington, DC, and the Office of the Federal Register, 800 North Capitol St., NW., suite 700, Washington, DC.

(c) In accordance with § 184.1(b)(1), the ingredient is used in food with no limitation other than current good manufacturing practice. The affirmation of this ingredient as GRAS as a direct food ingredient is based upon the following current good manufacturing practice conditions of use:

(1) The ingredient is used as an enzyme as defined in § 170.3(o)(9) of this chapter to hydrolyze proteins or polypeptides.

(2) The ingredient is used in food at levels not to exceed current good manufacturing practice.

5. Section 184.1415 is added to subpart B to read as follows:

§ 184.1415 Animal lipase.

(a) Animal lipase (CAS Reg. No. 9001–62–1) is an enzyme preparation obtained from edible forestomach tissue of calves, kids, or lambs, or from animal pancreatic tissue. The enzyme preparation may be produced as a tissue preparation or as an aqueous extract. Its characterizing enzyme activity is that of a triacylglycerol hydrolase (EC 3.1.1.3).

(b) The ingredient meets the general requirements and additional requirements for enzyme preparations in the Food Chemicals Codex, 3d ed. (1981), p. 110, which is incorporated by reference in accordance with 5 U.S.C. 552(a) and 1 CFR part 51. Copies are available from the National Academy Press, 2101 Constitution Ave., NW., Washington, DC 20418, or may be examined at the Office of Premarket Approval (HFS–200), Food and Drug Administration, 200 C St., SW., Washington, DC, and the Office of the Federal Register, 800 North Capitol St., NW., suite 700, Washington, DC.

(c) In accordance with § 184.1(b)(1), the ingredient is used in food with no limitation other than current good manufacturing practice. The affirmation of this ingredient as GRAS as a direct food ingredient is based upon the following current good manufacturing practice conditions of use:

(1) The ingredient is used as an enzyme as defined in § 170.3(o)(9) of this chapter to hydrolyze fatty acid glycerides.

(2) The ingredient is used in food at levels not to exceed current good manufacturing practice.

6. Section 184.1443a is added to subpart B to read as follows:

§ 184.1443a Malt.

(a) Malt is an enzyme preparation obtained from barley which has been softened by a series of steeping operations and germinated under controlled conditions. It is a brown, sweet, and viscous liquid or a white to tan powder. Its characterizing enzyme activities are α-amylase (EC 3.2.1.1.) and β-amylase (EC 3.2.1.2).

(b) The ingredient meets the general requirements and additional requirements for enzyme preparations in the Food Chemicals Codex, 3d ed. (1981), p. 110, which is incorporated by reference in accordance with 5 U.S.C. 552(a) and 1 CFR part 51. Copies are available from the National Academy Press, 2101 Constitution Ave., NW., Washington, DC 20418, or may be examined at the Office of Premarket Approval (HFS–200), Food and Drug Administration, 200 C St., SW., Washington, DC, and the Office of the Federal Register, 800 North Capitol St., NW., suite 700, Washington, DC.

(c) In accordance with § 184.1(b)(1), the ingredient is used in food with no limitation other than current good manufacturing practice. The affirmation of this ingredient as GRAS as a direct food ingredient is based upon the following current good manufacturing practice conditions of use:

(1) The ingredient is used as an enzyme as defined in § 170.3(o)(9) of this chapter to hydrolyze starch or starch-derived polysaccharides.

(2) The ingredient is used in food at levels not to exceed current good manufacturing practice.

7. Section 184.1583 is added to subpart B to read as follows:

§ 184.1583 Pancreatin.

(a) Pancreatin (CAS Reg. No. 8049–47–6) is an enzyme preparation obtained from porcine or bovine pancreatic tissue. It is a white to tan powder. Its characterizing enzyme activity that of a peptide hydrolase (EC 3.4.21.36).

(b) The ingredient meets the general requirements and additional requirements in the Food Chemicals Codex, 3d ed. (1981), p. 110, which is incorporated by reference in accordance with 5 U.S.C. 552(a) and 1 CFR part 51. Copies are available from the National Academy Press, 2101 Constitution Ave. NW., Washington, DC 20418, or may be examined at the Office of Premarket Approval (HFS–200), Food and Drug Administration, 200 C St. SW., Washington, DC, and the Office of the Federal Register, 800 North Capitol St. NW., suite 700, Washington, DC.

(c) In accordance with § 184.1(b)(1), the ingredient is used in food with no limitation other than current good manufacturing practice. The affirmation of this ingredient as GRAS as a direct food ingredient is based upon the following current good manufacturing practice conditions of use:

(1) The ingredient is used as an enzyme as defined in § 170.3(o)(9) of this chapter to hydrolyze proteins or polypeptides.

(2) The ingredient is used in food at levels not to exceed current good manufacturing practice.

8. Section 184.1595 is added to subpart B to read as follows:

§ 184.1595 Pepsin.

(a) Pepsin (CAS Reg. No. 9001–75–6) is an enzyme preparation obtained from the glandular layer of hog stomach. It is a white to light tan powder, amber paste, or clear amber to brown liquid. Its characterizing enzyme activity is that of a peptide hydrolase (EC 3.4.23.1).

(b) The ingredient meets the general requirements and additional requirements for enzyme preparations in the Food Chemicals Codex, 3d ed. (1981), p. 110, which is incorporated by reference in accordance with 5 U.S.C. 552(a) and 1 CFR part 51. Copies are available from the National Academy Press, 2101 Constitution Ave. NW., Washington, DC 20418, or may be examined at the Office of Premarket Approval (HFS–200), Food and Drug Administration, 200 C St. SW., Washington, DC, and the Office of the Federal Register, 800 North Capitol St. NW., suite 700, Washington, DC.

(c) In accordance with § 184.1(b)(1), the ingredient is used in food with no limitation other than current good manufacturing practice. The affirmation of this ingredient as GRAS as a direct food ingredient is based upon the following current good manufacturing practice conditions of use:

(1) The ingredient is used as an enzyme as defined in § 170.3(o)(9) of this chapter to hydrolyze proteins or polypeptides.

(2) The ingredient is used in food at levels not to exceed current good manufacturing practice.

9. Section 184.1914 is added to subpart B to read as follows:

§ 184.1914 Trypsin.

(a) Trypsin (CAS Reg. No. 9002–07–7) is an enzyme preparation obtained from purified extracts of porcine or bovine pancreas. It is a white to tan amorphous powder. Its characterizing enzyme activity is that of a peptide hydrolase (EC 3.4.21.4).

(b) The ingredient meets the general requirements and additional requirements for enzyme preparations in the Food Chemicals Codex, 3d ed. (1981), p. 110, which is incorporated by reference in accordance with 5 U.S.C. 552(a) and 1 CFR part 51. Copies are available from the National Academy Press, 2101 Constitution Ave. NW., Washington, DC 20418, or may be examined at the Office of Premarket Approval (HFS–200), Food and Drug Administration, 200 C St. SW., Washington, DC, and the Office of the Federal Register, 800 North Capitol St. NW., suite 700, Washington, DC.

(c) In accordance with § 184.1(b)(1), the ingredient is used in food with no limitation other than current good

manufacturing practice. The affirmation of this ingredient as GRAS as a direct food ingredient is based upon the following current good manufacturing practice conditions of use:

(1) The ingredient is used as an enzyme as defined in § 170.3(o)(9) of this chapter to hydrolyze proteins or polypeptides.

(2) The ingredient is used in food at levels not to exceed current good manufacturing practice.

Dated: June 14, 1995.

Fred. R, Shank,

Director, Center for Food Safety and Applied Nutrition.

[FR Doc. 95-15239 Filed 6-23-95; 8:45 am]

BILLING CODE 4160-01-P

DEPARTMENT OF EDUCATION

34 CFR Parts 75, 200, 201, 364, 365, 366, 367, 386, 388, 396, 403, 405, 406, 607, 641, 647, and 682

Announcement of Effective Dates

AGENCY: Department of Education.

ACTION: Notice of effective dates.

SUMMARY: Prior to its amendment by the Improving America's Schools Act of 1994 (IASA), section 431(d) of the General Education Provisions Act (GEPA) required that most Department of Education regulatory documents be published in the **Federal Register** for forty-five (45) calendar days, or longer if Congress took certain adjournments, before they became effective. Since future congressional adjournments could not be predicted with certainty when a document was published, the Department could not announce a specific effective date at the time of publication. This notice announces the effective dates for certain regulatory documents subject to the delayed effective date requirement of section 431(d) prior to its amendment.

DATES: For effective dates, see **SUPPLEMENTARY INFORMATION.**

FOR FURTHER INFORMATION CONTACT: Kenneth C. Depew, Division of Regulations Management, Office of the General Counsel, U.S. Department of Education, Room 5112, FB-10, 600 Independence Avenue SW., Washington, DC 20202-2241; telephone: (202) 401-8300.

Individuals who use a telecommunications device for the deaf (TDD) may call the Federal Information Relay Service (FIRS) at 1-800-877-8339 between 8 a.m. and 8 p.m., Eastern time, Monday through Friday.

SUPPLEMENTARY INFORMATION: GEPA section 431(d) was amended by the IASA, Pub. L. 103-382, enacted October 20, 1994. Section 431 was also redesignated as section 437. As a consequence of the new legislation, regulations of the Department are no longer subject to a 45-day delayed effective date. This notice announces the effective dates for those regulations subject to the previous statutory requirement for the delayed effective date. In the future, as a result of the new legislation, it will not be necessary for the Department to publish a special announcement of effective dates.

The effective date provision for each of the regulatory documents included in the notice stated that the effective date would be announced in a notice published in the **Federal Register**. Accordingly, this notice announces the following effective dates:

1. 34 CFR Part 682, final regulations for the Federal Family Education Loan Program, published May 17, 1994 (59 FR 25744).
   DATES: *Effective date:* July 1, 1994.
2. 34 CFR Part 75, final regulations for Direct Grant Programs, published June 10, 1994 (59 FR 30258).
   DATES: *Effective date:* July 25, 1994.
3. 34 CFR Part 386, final regulations for Rehabilitation Training: Rehabilitation Long-Term Training, published June 16, 1994 (59 FR 31060).
   DATES: *Effective date:* July 31, 1994.
4. 34 CFR Part 641, final regulations for the Faculty Development Fellowship Program, published July 1, 1994 (59 FR 34198).
   DATES: *Effective date:* August 15, 1994.
5. 34 CFR Parts 403, 405, and 406, final regulations for the State Vocational and Applied Technology Education Program, National Tech-Prep Education Program, and State-Administer Tech-Prep Education Program, published July 28, 1994 (59 FR 38512).
   DATES: *Effective date:* September 21, 1994.
6. 34 CFR Part 388, final regulations for State Vocational Rehabilitation Unit In-Service Training, published August 5, 1994 (59 FR 40176).
   DATES: *Effective date:* September 21, 1994.
7. 34 CFR Parts 200 and 201, final regulations for the Chapter 1 Program in Local Educational Agencies and Chapter 1—Migrant Education Program, published August 10, 1994 (59 FR 41168).
   DATES: *Effective date:* September 24, 1994.
8. 34 CFR Parts 364, 365, 366, and 367, final regulations for State Independent Living Services Program and Centers for Independent Living Program: General Provisions, State Independent Living Services, Centers for Independent Living, and Independent Living Services for Older Individuals Who Are Blind, published August 15, 1994 (59 FR 41908).
   DATES: *Effective date:* September 29, 1994.
9. 34 CFR Part 607, final regulations for the Strengthening Institutions Program, published August 15, 1994 (59 FR 41914).
   DATES: *Effective date:* September 29, 1994.
10. 34 CFR Part 647, final regulations for the Ronald E. McNair Postbaccalaureate Achievement Program, published August 25, 1994 (59 FR 43986).
    DATES: *Effective date:* November 7, 1994.
11. 34 CFR Part 396, final regulations for Training Interpreters for Individuals Who Are Deaf and Individuals Who Are Deaf-Blind, published October 14, 1994 (59 FR 52218).
    DATES: *Effective date:* November 28, 1994.

Dated: June 21, 1995.

Judith A. Winston,

General Counsel.

[FR Doc. 95-15559 Filed 6-23-95; 8:45 am]

BILLING CODE 4000-01-P

ENVIRONMENTAL PROTECTION AGENCY

40 CFR Part 63

[AD-FRL-5225-9]

National Emission Standards for Hazardous Air Pollutants for Source Categories: Gasoline Distribution (Stage I)

AGENCY: Environmental Protection Agency (EPA).

ACTION: Final rule; correction.

SUMMARY: This document makes clarifications and corrects errors in the regulatory text of the final rule for National Emission Standards for Gasoline Distribution Facilities (Bulk Gasoline Terminals and Pipeline Breakout Stations) which appeared in the **Federal Register** on December 14, 1994 (59 FR 64303).

EFFECTIVE DATE: December 14, 1994.

FOR FURTHER INFORMATION CONTACT: For general and technical information concerning the final rule, contact Mr. Stephen Shedd, Waste and Chemical

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Determining the Safety of Enzymes Used in Food Processing

M. W. PARIZA and E. M. FOSTER

Food Research Institute, Department of Food Microbiology and Toxicology, University of Wisconsin, Madison, Wisconsin 53706

(Received for publication October 18, 1982)

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 beer 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 involving 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 pectyylhydrolase 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 (2) *Endothia parasitica* (3) *Mucor miehei*, *M. pusillus*	None	3.4.23.4 3.4.23.6 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 (*45*). 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. typhimurium* 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 intitated 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 annimals. 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.

ACKNOWLEDGMENTS

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News and Events

9th Annual Food Microbiology Research Conference

The 9th Annual Food Microbiology Research Conference will be held November 2-4, 1983 in Chicago, Illinois. For more information contact: Dr. J. M. Goepfert, Canada Packers, Ltd., 2211 St. Clair Avenue West, Toronto, Ontario, Canada M6N 1K4.

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MEMORANDUM

Date: July 2, 1999

From: Brian Baker

To: National Organic Standards Board

Subject: Amino Acids and Enzymes

The NOSB returned a number of materials to the TAP for further study. Two of the returned materials–amino acids and enzymes–are actually categories of a number of specific materials that are both very broad in scope, entailing a wide range of sources, uses, and applications.

Amino Acids

Amino acids come from a wide variety of sources, have a broad number of uses and applications in both production and handling, and have far-reaching effects on the food system. The petitioners were not specific in which of the over 20 amino acids needed to be considered, and when synthesized, under what category of allowed synthetic they fell.

I have asked the petitioners and manufacturers of the amino acids to specify what amino acids are currently used and for what purposes. They were also asked to provide supporting documentation against both the categories of exemptions in OFPA section 2118 (7 USC 6517(c)(1)(B)). Finally, the petitioners were reminded to provide documentation for the NOSB to consider in the context of the OFPA 2119(m) criteria (7 USC 6517(m)), as was requested in the *Federal Register* notice.

Crops

At present, the single greatest use of free amino acids in organic plant crop production are as chelating or complexing agents for synthetic micronutrients. Certain amino acids are registered with EPA as plant growth regulators. Finally, these may also be sources of nitrogen. Amino acids may be either extracted from denatured proteins, from fermentation organisms, or by the reaction of different chemical feedstocks.

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Those obtained from proteins may be of plant, animal (including human), or microbiological origin. The steps used both to denature the protein and then to extract the amino acids from the denatured protein molecules could reasonably be interpreted to be either synthetic or non-synthetic under the definition in OFPA. Certifiers and state programs have, in the past, considered these sources to be non-synthetic. However, upon closer examination, the chemical steps used to break down the proteins are similar to other reactions and processes that the NOSB has previously voted to be synthetic.

Amino acids may also be manufactured by the reaction of fossil fuels with ammonia. Few would disagree that these are clearly synthetic as defined by OFPA. Finally, a growing number of amino acids are produced from genetically engineered organisms. These would most likely be considered within the definition of GMO products adopted by the NOSB at Indianapolis.

Lysine and glycine are the most-used amino acids as chelating agents in synthetic micronutrient packages. These have usually been considered non-synthetic by certifiers and state programs, as have citric acid and glucose-based compounds such as glucoheptonate. Glutamic acid, along with gamma amino butyric acid, and Aminoethoxyvinylglycine (AVG) are registered with EPA for use as plant growth regulators. Free amino acids can also be taken up as a source of nitrogen by plants, although less readily and efficiently than nitrate or ammonium ions.

The NOSB may want to review amino acids on a case-by-case basis, rather than as a class. Another approach is to distinguish those amino acids that are manufactured from fossil fuels feedstocks, those that are produced by the fermentation of genetic engineered microorganisms, ones that are produced from naturally occurring strains of microorganisms, and those that are derived from plant or animal protein.

Also, concentrated forms of amino acids are used, along with (other) synthetic nutrient sources in fermentation media for microorganisms. This appears to be beyond the scope of the petitions.

Recommendations

1. In crops, the first step will be to determine if those amino acids considered non-synthetic by existing certifiers would also be considered non-synthetic by the NOSB. The TAP would consider only those amino acids extracted from the proteins of non-GMO organisms. The NOSB would then be asked the fundamental question as to whether or not isolation of amino acids from more complex proteins taken from organisms that are not genetically engineered are considered 'synthetic' under the Organic Foods Production Act. After review of several representative processes, the NOSB would vote as to whether or not amino acids produced in such a way would be considered to be synthetic or non-synthetic. If the NOSB determines that these sources are indeed non-synthetic, then such materials may be considered for addition to the prohibited non-synthetics list, or be allowed by default.

2. If amino acids extracted from proteins were to be considered synthetic by the NOSB, then the second step would be to weigh specific uses and applications against the 2119(m) criteria. The only specific applications that appear to qualify for exemption under section 2118 of OFPA are as chelating agents for synthetic micronutrients. That would mean all other uses, specifically as plant growth regulators or nitrogen fertilizer sources, would have to be considered in separate petitions.

Livestock

Amino acids are primarily used to balance feed rations. These also have multiple purposes: lower feed costs, increase conversion efficiency, reduce excretion, and increase production. The essential amino acid that is most often limiting in egg production is L-methionine. Threonine is often limiting in swine, and lysine is limiting in both.

Recommendations

1. In the case of livestock, amino acids used as supplements need to be addressed in the larger context of organic feed formulation. Given a requirement of 100% organic feed, it is unclear how supplements fit. Supplements are usually considered feed. I would request a two-stage vote: first--do amino acids in any way qualify for use in organic livestock feed, or are they categorically prohibited?

2. If they are categorically prohibited, there is no sense conducting further review and the petition needs to be rejected as were others that did not qualify under the 2118(b)(1)(C) list of exemptions.

3. If the NOSB determines that it is possible to add amino acids, and they should be considered on a case-by-case, those that should be first considered for inclusion are L-Lysine, L-Methionine, DL-Methionine, and L-Threonine.

Processing

Amino acids are used for a wide variety of purposes in post-harvest handling and processing, including nutrients, dietary supplements, flavor enhancers, salt substitute, anti-oxidants, preservatives, texturizers, thickeners, and dough conditioners. Several appear to be used as additives in 'functional foods' and digestive aids. They are also used in cosmetics as antioxidants, sun-blockers, emollients, moisturizers, thickeners, and skin and hair conditioners.

The handling application that appears to be the best documented is the use of L-cysteine hydrochloride. This substance has been used in post-harvest handling of carrots and lettuce. It is also used as a dough conditioner and as an anti-oxidant for various dried foods, such as dehydrated potatoes.

Recommendations

1. The NOSB should first clarify how existing NOSB Final Recommendations apply to amino acids. Those used for nutrient supplementation need to be addressed under Final Recommendation Addendum 13, The Use of Nutrient Supplementation in Organic Foods. If a decisive majority of members of the NOSB find allowance of amino acids under this Recommendation objectionable, the NOSB may want to explicitly go on record to say that the policy is limited only to vitamins and minerals, and does not extend to amino acids. Otherwise, that Addendum can be used to support nutritional fortification with amino acids.

2. After reviewing 21 CFR §§172.510, 182.10-182.50 and 184, it appears that none of the amino acids can be considered 'natural flavorings' and therefore none are the subject to Final Recommendation Addendum 14, The Use of Natural Flavors in Organic Foods.

3. In the absence of support for a broad petition for all uses and applications of amino acids in handling, I would suggest that the NOSB focus on the only specific application requested by any petitioner: use of L-cysteine monohydrochloride as an anti-oxidant.

Enzymes

In the case of enzymes, the petitioner requested that all food grade enzymes from all sources that are FDA regulated as food additives should be listed as allowed in organic processing, regardless of their sources, manufacturing processes, uses, or applications. The NOSB demurred in the consideration of such a broad and open petition without further consideration of (a) sources, (b) manufacturing processes, and (c) applications.

Faced with limited information, the NOSB was reluctant to grant blanket approval to enzymes. Instead, the NOSB referred enzymes used for processing to the TAP for a review based on the source organism: Bacterial, Fungal, Plant and Animal. The NOSB also referred enzymes as feed additives to the TAP.

Bacterial

The NOSB recommended that enzymes derived from non-GMO bacteria be added to the National List of materials used in processing and handling at the April 1995 meeting. Those specifically considered are listed in Table 1.

Table 1
Enzymes Explicitly Considered and Recommended for Inclusion on the National List

Enzyme	Source Organism(s)
Carbohydrase / Protease	*Bacillus licheniformis*
Catalase	*Micrococcus lysodiekticus*
Glucose isomerase (insoluble)	*Bacillus coagulans* *Microbacterium arborescens*
Rennet (Milk-clotting enzymes)	*Bacillus cereus*
Urease	*Lactobacillus fermentum*

The actual motion was to allow "Microbial enzymes" but the list put before NOSB were only those enzymes produced by naturally occurring bacteria and some (but not all) unicellular fungi that reproduce asexually. One specific enzyme produced by rDNA techniques--chymosin derived from non-pathogenic *Escherichia coli*--was considered separately and explicitly prohibited at the September 1996 meeting. The other transgenic enzymes included in the original 1995 petition are included in Table 2.

A considerable number of enzymes produced from rDNA organisms have been introduced over the past four years. The petitioner has been cooperative in providing information on what enzymes are produced by genetically modified organisms. The petitioner does not want this to be viewed as withdrawing from consideration those enzymes that are genetically engineered, and still would like those enzymes to be considered for inclusion on the National List.

Added to their working list since 1995 are enzyme source organisms that have had gene-multiplication ("gene-doubling") performed by rDNA techniques. Such organisms are genetically engineered as defined by the NOSB. The petitioner, their members, and their members' customers would like the NOSB to clarify, or possibly reconsider the inclusion of gene-doubling in the definition of genetic engineering, even if transfers between species remains within the scope.

Table 2
Enzymes from Genetically Modified Organisms Petitioned to the NOSB

Enzyme	Source Organism(s)[1]
α-Amylase	*Bacillus subtilis* d-*Bacillus megaterium* *Bacillus subtilis* d-*Bacillus stearothermophilus*
Chymosin[2]	*Aspergillus niger* var. *awamori* d-calf prochymosin gene
	Escherichia coli K-12 d-calf prochymosin gene
	Kluyveromyces marxianus d-calf prochymosin gene
Maltogenic Amylase	*Bacillus subtilis* d-*Bacillus stearothermophilus*
Rennet (Milk-Clotting Enzymes)	*Aspergillus oryzae* d- *Rhizomucor miehei* var. Cooney *et* Emerson

1 d=donor organism.
2 Explicitly recommended to be prohibited for use in organic food

Those produced from plants, animals, and yeast were tabled and returned to the TAP for review. The NOSB recommendation on enzymes from naturally occurring microorganisms is vaguely worded. Table 1 is the most conservative interpretation. There may be a number of enzymes and organisms not included on the list in Table 1 that are allowed under the NOSB's recommendation. Organic food processors are proceeding as if other enzymes are going to be allowed, but a number of those processors and their suppliers are concerned with the remaining uncertainty.

Fungal

Most of the organisms that produce commercial enzymes are considered fungi of some sort. These organisms include the molds *Aspergillus Niger*, *Rhizopus oryzae*, *Rhizomucor meihei*, actinomycetes such as *Actinoplanes* spp., blights such as *Endothia parasitica* and yeasts such as *Candida* spp and *Saccharomyces* spp. The list of enzymes referred to the TAP is not exhaustive. A number of enzymes produced by fungi as well as organisms producing enzymes do not appear in Table 3.

Plant

As with animal-derived enzymes, an increasing number of plant enzymes are being transferred to microorganisms by rDNA techniques. This will enable these enzymes to be produced by fermentation processes. Four plant-derived enzymes were specifically referred to TAP for review: barley malt enzymes (primarily amylase) bromelain, ficin, and papain.

Animal Derived

Animal derived enzymes were not referred to the TAP at the February 1999 meeting. Preliminary investigation finds little current use of these enzymes. The two specific enzymes appear to have generated the greatest interest are bovine rennet and lysozyme from eggs.

Enzymes as Feed Additives

Enzymes can aid animal nutrition by improving the digestibility and palatability of feed. However, enzymes can also be used to stretch low-quality feed. There has been relatively little discussion of this application. As with amino acids, the use of enzymes in livestock production needs to be examined in the broader context of organic feed standards.

Recommendations

1. The TAP and OMRI present the NOSB with a draft set of guidelines that it may choose to recommend. These guidelines will enable the National Organic Program, state programs, certifiers, and others to determine those enzymes that are allowed and prohibited for use in organic processing. These would state which enzymes are acceptable in organic processing and for what purposes based on (a) sources, (b) manufacturing processes, (c) uses and applications, and (d) incidental ingredients. Unless there is a compelling reason to require otherwise, enzymes used in organic feed would need to comply with any policy developed for organic food processing.

2. OMRI develops and presents the NOSB a preliminary and non-exhaustive specific list of enzymes used in processing and as feed additives that would be allowed and prohibited under those guidelines.

Table 3
Enzymes Tabled and Referred to TAP

Enzyme	Source Organism(s)
α-Amylase	Malted barley
ß-Amylase	Malted barley
Amyloglucosidase	*Rhizopus niveus*
Bromelain	Pineapple (*Ananas comosus* and *Ananus bracteatus*)
Carbohydrase	*Rhizopus oryzae*
Carbohydrase / Cellulase	*Aspergillus niger*
Citric acid fermentation	*Candida guilliermondii*
Esterase / Lipase	*Mucor miehei*
α-Galactosidase	*Mortierella vinaceae*
Glucose isomerase (insoluble)	*Actinoplanes missouriensis* *Streptomyces olivaceus* *Streptomyces olivochromogenes* *Streptomyces rubiginosis* *Streptomyces murinus*
Ficin	Figs (*Ficus spp.*)
Lysozyme	Eggs
Papain	Papaya (*Carica papaya*)
Pectinase	*Aspergillus niger*
Rennet (Milk-clotting enzymes)	Bovine *Endothia parasitica* *Mucor pusillus* (Lindt) *Mucor miehei* (Cooney *et* Emerson)

xc: Keith Jones
Kathleen Downey
Zea Sonnabend
Petitioners
Amino Acid and Enzyme TAP Reviewers

OMRI™

1 page total

original to be sent via U.S. Mail: ☒ yes ☐ no

August 6, 1999

Fax to 202-690-3924

To: Keith Jones, USDA National Organic Program
From: Kathleen Downey, Executive Director

Here's our two-week update:

Eleven of the 16 TAP reviews have been sent to the reviewers:
Crops—ethylene and potassium bicarbonate
Livestock—chlorhexidine, glycerine, lanolin, parasiticides, and phosphoric acid
Processing—ethylene, magnesium chloride, phosphoric acid, and wax for fruit

The remaining five (amino acids for crops, livestock, and processing; and enzymes for livestock and processing) will be sent to the reviewers by Tuesday.

OMRI is right on track with its timeline for the TAP reviews.

Organic Materials Review Institute™
Box 11558 • Eugene, OR 97440-3758
Phone 541-343-7600 • Fax 541-343-8971 • E-mail info@omri.org • www.omri.org

Amino Acids
Enzymes

OMRI™

2 pages total

September 20, 1999

Fax to 202-690-3924

To: Keith Jones, USDA/AMS/National Organic Program
From: Kathleen Downey, Executive Director

Brian Baker asked that I fax you this letter. He suggested you may want to review this letter in regards to the original petition and that Rod Crossley may want to see it, too.

Organic Materials Review Institute™
Box 11558 • Eugene, OR 97440-3758
Phone 541-343-7600 • Fax 541-343-8971 • E-mail info@omri.org • www.omri.org

EPL TECHNOLOGIES, INC.

AUG 18 1999

August 10, 1999

Mr. Brian Baker
OMRI
P. O, Box 11558
Eugene, OR 97440-3758

Dear Mr. Baker:

Howard Kravitz has forwarded to me your memo of July 2 to the National Organic Standards Board. This memo expresses your recommendations on a process for determining the organic status of amino acids and enzymes.

I must compliment you on an excellent job of summarizing the status and that you present a very logical approach to the situation.

I have only a couple of comments:

I would hope that the NOSB would not review the amino acids on a case-by-case basis, as this will greatly encumber the process in bureaucracy.

I like your suggestion of distinguishing the amino acids by source. However, you might consider that there are only two categories if one is considering synthetic Vs natural, that is those amino acids derived from fossil fuels (synthetic) and those from other sources. However, they may be other reasons for the four categories, depending on how OMRI or NOSB views products of GMO. The amino acids may be produced naturally in GMO but may not be found there naturally or in some cases the amino acid is found in low concentrations normally but in higher concentrations in the GMO. The process is natural but the source may not be.

Again, thank you for keeping us so well informed. Please let me know if there is anything that I can do to assist you in this process.

Howard Kravitz has left the company and for the time being you can address correspondence to me.

Sincerely,

William R. Romig, Ph.D.
Senior Vice President
Science and Technology

2 International Plaza
Suite 245
Philadelphia, PA 19113-1507
TEL 610.521.4400
FAX 610.521.5985

Pooler , Bob

From: Jim Pierce [jim.pierce@organicvalley.com@i]
Sent: Tuesday, November 14, 2000 11:23 AM
To: Pooler , Bob
Cc: Pam Saunders; George Siemon; Louise Hemstead; Tim Griffin; Dave Engel (E-mail); 'Kelly Shea'
Subject: NOSB Petition List

As Certification Coordinator for Organic Valley/CROPP Cooperative, please allow me to go on record with the following comments regarding the list of materials to be reviewed at the NOSB meeting in Washington, DC on November 15, 2000.

* Animal Enzymes: Should be allowed for use as a non organic ingredient. In the dairy industry, animal enzymes are the oldest, most natural source of enzymes and will produce the highest quality results. They pose no threat to organic integrity. While vegetable-derived enzymes are most commonly used in the organic industry due to consumer demand, there are certain situations where they simply will not perform as well as animal enzymes. In a market where premium quality is as important as organic integrity, animal enzymes are an important tool.

* Periacetic Acid: Should be allowed as a sanitizer in both Livestock and Processing applications. Periacetic Acid is a blend of hydrogen peroxide and vinegar. The acidity of the vinegar makes is very effective as a sanitizer. When it breaks down the hydrogen is released into the air leaving vinegar and water. Periacitic Acid has been approved for use in processing for eight years and should be approved for dairy applications as well. It is a simple, natural effective and environmentally innocuous sanitizer and needs to be available for organic applications.

Thank you for allowing me to represent CROPP/ Organic Valley and to weigh in on these matters. We all hope your meeting is righteous and fruitful.

Respectfully,
Jim Pierce
CROPP/Organic Valley Family of Farms
505 West Main St.
La Farge WI 54639
Phone; 608-625-2602
Fax; 608-625-4177