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COLLABORATIVE REPORT

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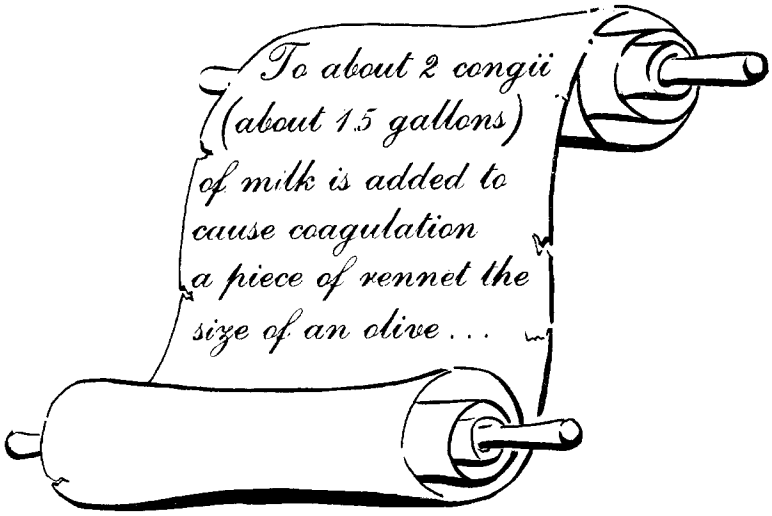



# Commercial Enzymes by Extraction (Rennet)

So reads a cheese recipe from 36 B.C. (*6*). Today's instructions, though, would more likely call for the addition of something like 1 ml. of commercial rennet extract to 1000 pounds of milk to make a batch of cottage cheese. And while the rennet of antiquity was a piece of a suckling animal's stomach, today's rennet is more precisely defined as a highly purified, standardized extract of calves' stomachs, and containing the milk coagulating enzyme rennin.

Rennet extract is today's major enzyme preparation made on a commercial scale and is indispensable to the cheese-making industry. This report deals with the preparation of rennet by one of the handful of commercial enzyme producers in the United States—Paul-Lewis Laboratories, Inc., of Milwaukee, Wis.

## The Commercial Enzyme Industry

Enzymes, the "stuff of life," help make up one of the most unique segments of the chemical processing industry—enzyme manufacturing. This comparatively small, specialty industry is involved in making enzymes for a variety of commercial uses, developing new applications for existing enzymes, and finding new enzyme systems that could be applied on a large scale.

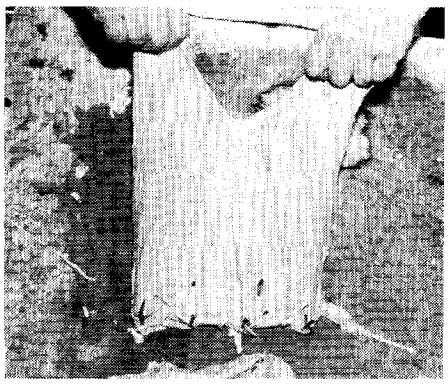
To some extent, every living thing is an enzyme factory. Standard definition of enzymes is that they are catalysts which bring about specific biochemical reactions which make up the processes of living cells. In a sense, enzymes themselves are like living molecules (*4*); that is, they are destroyed or inhibited by the same agents which destroy life—heat, acids, bases, and the like. Nature's catalysts are highly specific in their action and many different enzymes are often required to complete a sequence of reactions taking place in a living cell (*8*).

Commercial interest in enzymes is due to their usefulness in many areas including food processing, textile sizing and desizing, leather bating (softening), some phases of brewing, and pharmaceuticals. Some enzymes are sold for photographic arts and as test reagents. Total enzyme production or sales figures are not available, but the textile industry has been reported (*3*) as the biggest single outlet. As much as 1,500,000 pounds a year of enzymes is used for textile sizing and desizing.

## Enzyme Manufacturing Processes

Industrial enzymes come from bacterial, fungal, higher plants, and animal sources (*1*). From an engineering point of view, enzymes are made by one of two basic ways: extraction, the subject of

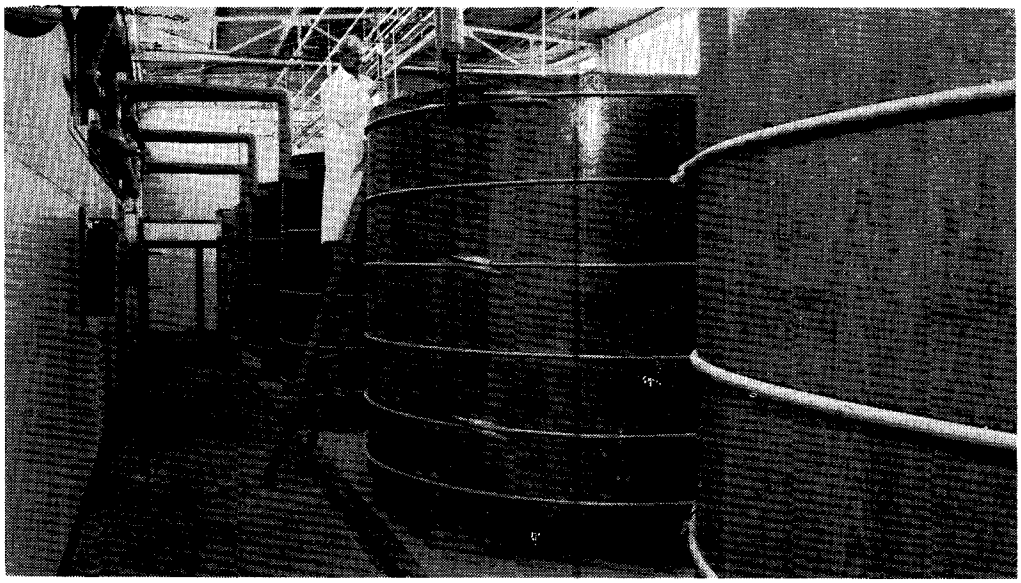
Calves' stomachs come from the slaughter house packed with a lot of salt and are stored this way at −30° F. if not processed immediately (left). After washing with saturated salt solution, the stomachs are stretched, then defatted (right)



this report, and fermentation (microbial enzymes).

Strictly speaking, industrial enzymes are not isolated from their various sources. Isolation is a proposed (*5*) term for those rare instances when a pure enzyme with no chemical contaminants has been obtained. Commercial enzymes are, instead, "purified"—enzymes together with other matter are found in an industrial preparation.

Extraction is the older method of commercial enzyme preparation, and is used to produce enzymes from animal and plant sources. Enzymes by fermentation made their debut around the turn of the century when J. Takamine introduced the commercial process of cultivating enzymes from molds (*9*). Then, in 1917, commercial enzyme preparation from bacterial sources was started. Today, only a relatively small number of microbial enzymes are used commercially. Among these are amylases and proteases of bacteria and molds, invertase from yeast, and pectinases and tannases from mold species.



This battery of wooden vats is used during the extraction process (finishing tanks are similar). Each vat has a capacity of 2000 gallons, holds the equivalent of 10,000 to 15,000 stomachs

## Paul-Lewis Laboratories

The Wisconsin firm was incorporated in 1937 and, almost since the beginning, has concentrated on enzyme production techniques and enzymology. Paul-Lewis' products are divided into two major divisions; dairy and brewing. Specialties round out the product line. Major dairy product is rennet extract. Others include colorants, catalase, and starter cultures. For the brewing industries, Paul-Lewis makes proteolytic and diastatic enzymes for many uses ranging from chill-proofing to actual brewing operations. The long list of specialty enzymes is aimed at the pharmaceutical, meat, and paper industries.

Rennet is a typical industrial enzyme preparation made by extraction and calls for most of the unit operations found at the Paul-Lewis plant. Similar equipment is used to extract many other enzymes; papain and ficin are but two examples.

## Rennin and Rennet Extract

Like all known enzymes, rennin is a protein, as shown by its inactivation by heat, ultraviolet light, formaldehyde, chloroform, bichromate, and other substances. The compound is the main milk-clotting enzyme secreted by the fourth stomach of the suckling calf. Its inactive precursor is prorennin, which is activated into rennin by hydrogen ions.

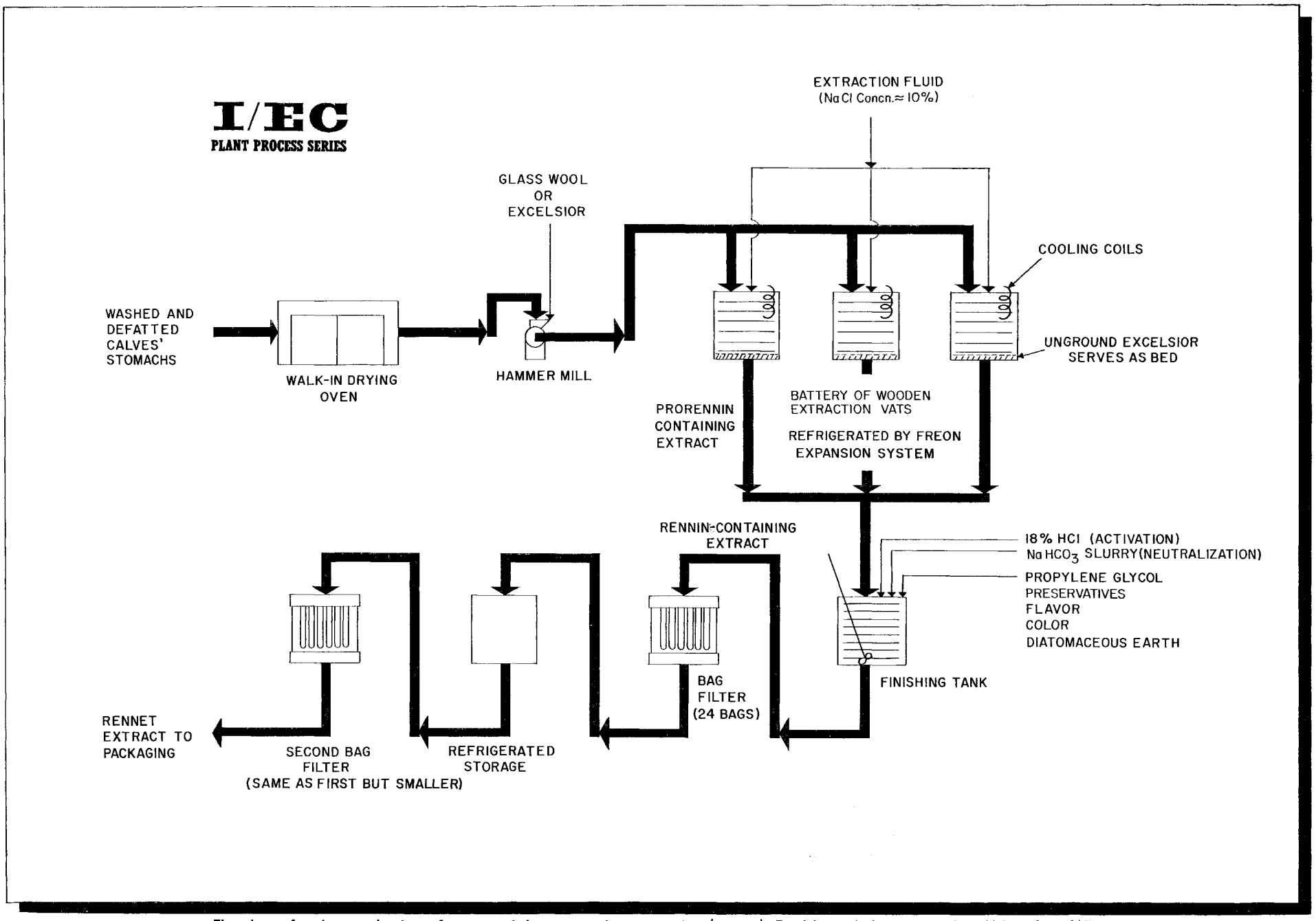
Pure, crystalline rennin has been prepared, but chemical and physical properties of the material have not been thoroughly studied (*7*). All that is known so far is that the pure material has a low solubility and that both crystalline form and enzymatic activity are retained on drying. Isoelectric zone, the pH range at which an enzyme exhibits its lowest degree of solubility is pH 4.45 to 4.65. The enzyme is inactivated when exposed to a temperature of 50° C. and pH 6.9 ± 0.1 for 14 minutes.

More recently (*2*), work on crystalline rennin showed that at least four components could be separated electrophoretically. This poses the possibility that more than one of these components has milk-clotting activity. Also, further fractionation should achieve a considerable increase in specific activity.

Just how long rennin or, more precisely, rennet has been used in cheese making is a moot question. Very likely it was already in widespread use during prebiblical days. The enzyme's clotting activity may first have been observed when milk was stored in goatskin bags (actually goat stomachs), widely used in that era. Milk so contained clotted after a short time leading to the observation that animal tissue can coagulate milk.

Thus stumbling upon cheese, the ancients began to make it by immersing animal stomachs in milk. To conserve their milk supply, these early cheese makers probably first soaked the stomachs in whey (the liquid portion remaining after milk is clotted), then

Washed and defatted stomachs are hung on dowels and dried in this air circulating oven at 110° F. for 20 hours




Flowsheet for the production of commercial enzymes by extraction (rennet), Paul-Lewis Laboratories, Inc., Milwaukee, Wis.

dumped the mixture into the next day's milk.

Commercial production of rennet extract began in Europe sometime in the nineteenth century, and in the United States about 75 years ago. Paul-Lewis started manufacturing the material in 1950.

Currently, about 300,000 gallons of 100% commercially standardized rennet extract is used in the United States. World use is estimated as being equivalent to 1,000,000 gallons of American commercial rennet extract. About 1 ml. of rennet is used to make cottage cheese from 1000 pounds of milk. Different cheeses call for different amounts; for example, cheddar uses 3 to 3.5 ounces. Paul-Lewis and several other producers make about 90 to 95% of all the rennet produced today for United States' consumption. Paul-Lewis has enjoyed a consistently growing share of the market for this enzyme extract.

## Raw Material Preparation

Calves' stomachs at the slaughter house are packed with large amounts of salt in steel drums with polyethylene liners, or in wooden barrels. If not processed immediately, the stomachs are left in these containers and stored at about −30° F. At this low temperature, Paul-Lewis finds no apparent loss of enzyme activity even after a year's storage.

In preparing the tissue for extraction, the stomachs are first washed with saturated sodium chloride solution—a less concentrated solution would extract prorennin—to remove the solid salt. The stomachs are then stretched and excess fat is stripped from them. Washed and defatted stomachs are hung on dowels and dried in an air circulating oven at 110° F. for 20 hours.

Quality control begins at the drying stage. Out of a drum of about 700 stomachs, 10 of them are chosen at random (up to 20 from a new supplier) and extracted with salt water. To be acceptable, the crude rennet extract must check in at some arbitrary percentage of a purified standard. The shipment is then graded on the basis of the extract's activity.

Because individual stomachs vary widely in enzyme content (rennin level hinges on a calf's diet), this kind of a check may seem to be rather crude. But statistical comparisons of these preliminary evaluations with actual production yields correlate very closely. This sample extract is discarded after assaying, and the main batch of stomachs is then sent on to the extraction stage.

**In addition to rennet extract, Paul-Lewis also makes these enzymes:**

**Ficin**—protein digestant isolated from the latex of some species of tropical fig trees. Specifically used in brewing, meat tenderizing, leather making, textiles, peptone preparations, waste protein recovery, and as an anthelmintic. Optimum temperature is 30° to 50° C.; rapidly inactivated above 50° C. and by oxidizing agents, heavy metals, and pH values below 3 or above 9.

**Protease P**— soluble active enzymes from papain. Used in baking, brewing, meat tenderizing, grain processing, tanning, and others. High temperature resistant (optimum action at 70° C.). Inactivated at 85° C. for 30 minutes.

**Starch Liquefying Enzymes** (bacterial alpha-amylase)—any industrial application in which starch liquefaction and heat stability are needed. pH optimum is 5.5 to 6.5, completely destroyed at 90° C. for 20 to 30 minutes in presence of starch.

**Lactivase**—trade name for enzyme (a protease and amylase) preparation that prevents formation of oxidized flavor in milk.

**Catalase**—specific catalyst for decomposing $H_2O_2$. Maximum activity at pH 7.0 and 0° to 10° C. Completely inactivated at 65° C. and by HCN, $H_2S$, $NH_2OH$, and some surface active agents.

**Mashing Enzymes**—a proteolytic enzyme preparation used to supplement natural malt enzymes in brewing.

**Urease**—specifically decomposes urea to ammonium salts and carbonic acid in presence of buffers. Use in quantitative analysis of urea in blood and urine. Inactivated by halogens, fluorides, borates, quinones, $H_2O_2$, and formaldehyde.

**Cervase**—standardized blend of purified proteolytic and diastatic enzymes. Removes high molecular weight proteins from beer to effect clarity.

**Sausase**—sausage casing tenderizer consisting of standardized proteolytic enzymes.

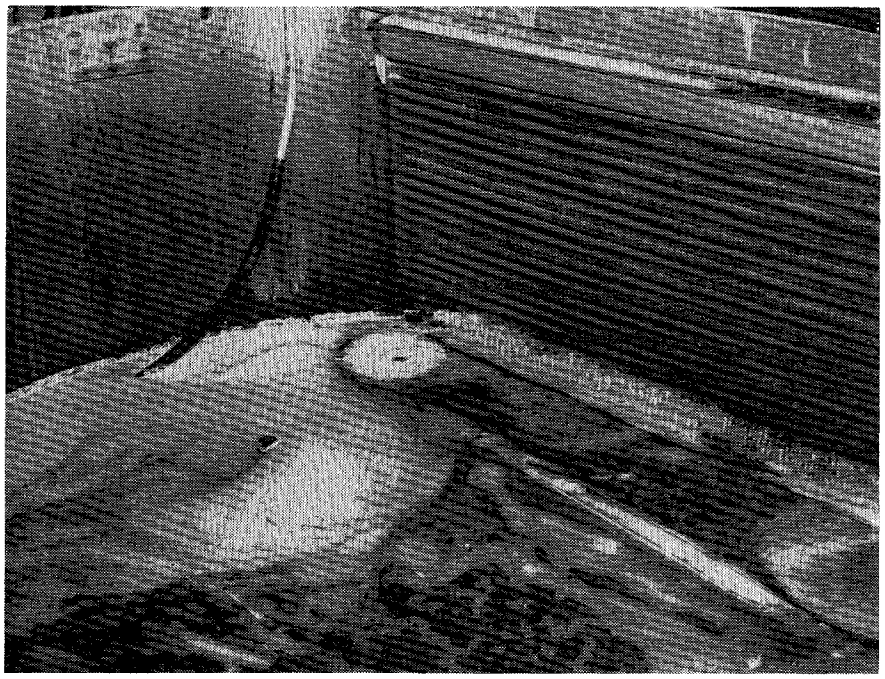
**Liquid Protease**—blend of proteolytic enzymes from various sources used in treating fish wastes which go into high protein feeds.

**Premeasured Amylase Units**—for conversion of starch in the paper industry.

**Protease 177**—increases yields during lard rendering.

**Bromelin** (purified)—supplements ficin and papain in many uses.

**Nonenzymatic Products**—cheese color, L-cysteine hydrochloride, foam stabilizer, and dairy cultures.



Inside the extraction vat, countercurrent extraction goes on for about two days. Prorennin is being extracted here. A portion of the extracting liquid goes over the refrigerator coils (right), kept at 35° to 40° F. by a Freon expansion unit

These Are Typical Classes of Industrial Enzymes (1)

| Class | Type of Activity | Current and Potential Uses |
|---|---|---|
| | Hydrolytic Enzymes and Sources | |
| Amylases (bacterial, fungal, animal, and plant) | Hydrolyze starch, but vary in degree of starch splitting. Some liquify and dextrinize, others saccharify. Differ in thermal stability, pH optima, activity range, and in other ways | Brewing, distilling, baking, making soluble starch, adhesives, textiles, paper |
| Proteases (bacterial, fungal, animal, and plant) | Hydrolyze protein, varying in degree of protein splitting and in specificity for particular peptide linkages. Also differ in thermal stability, pH optima, and the like | Tanning, chill-proofing, baking, dairy and other food uses, dry cleaning, making protein hydrolyzates, fish liver oils |
| Pectic Enzymes (fungal) | Hydrolyze pectin. Split off methyl groups and break polygalacturonic linkages. | Making and clarifying fruit juices and concentrates; making wine |
| Lipases (fungal and animal) | Hydrolyze fats. Rate of hydrolysis depends on mixture of glycerides | Cleaning grease traps, specialty products, cheese |
| | Nonhydrolytic Enzymes and Sources | |
| Glucose Oxidase (mold) | Desugarizes by specifically oxidizing glucose, in the presence of oxygen, to gluconic acid | Stabilizing flavor concentrates. With catalase, stabilizes egg solids, removes oxygen as deteriorant in packaged food |
| Catalase (mold and animal) | Specifically decomposes hydrogen peroxide into water and oxygen | Food preservation, especially dairy foods. Also used with glucose oxidase (see above) |
| Lipoxidase (plant) | Catalyzes autooxidation of unsaturated fats, forming hydroperoxides which oxidize carotene | Whitening bread |

## Extraction Is Heart of Process

Before extraction, the stomachs are ground in a hammer mill together with either excelsior or glass wool. Ratio of tissue to glass wool or excelsior is about 3 to 1. There are several reasons for using the glass wool or excelsior. First, the materials serve as inert supports, akin to filter aids in a filtration medium. Other types of supports would absorb some active material from the tissue, while glass wool and excelsior absorb very little. Another reason for using a support is mucin (a glycoprotein). If the stomachs were to be extracted without an inert support, mucins ropy characteristics would keep the extracting fluid from percolating. Moreover, mucin makes the extracting mass so thick that it cannot be stirred or centrifuged.

The ground mixture from the hammer mill is conveyed into wooden extraction vats. Paul-Lewis uses a series of such vats. Each vat has a 2000-gallon capacity and holds the equivalent of 10,000 to 15,000 stomachs. About 10 inches off the bottom of the vat is a wooden lattice covered with unground excelsior, which serves as a bed. The powdered tissue–glass wool (or excelsior) mixture is then put into the vat.

A solution of rennet extract obtained from previous rennet washing operations is used to cover the vat contents. This wash cover has a salt concentration of about 10% and a pH of 5.95 to 6.05. Countercurrent extraction is started and continues for two days if the salt concentration is at its optimum (10%). Extraction takes longer if sodium chloride concentration is more than 10%.

A Freon expansion system keeps the extracting mixture at 35° to 40° F. A portion of the circulating liquid goes over the refrigerator coils, temperature of which is thermostatically controlled. A circulating pump moves the extracting liquid, which is assayed daily for milk-clotting activity.

When activity fails to increase, the fluid is drawn off to finishing tanks, also made of wood. Here, prorennin in the extract is activated to rennin with an 18% hydrochloric acid solution. Acid is added with agitation until the extract's pH reaches 4.6; about 2 hours. Activation requires 14 to 36 hours, depending on how long it takes all of the prorennin to become converted to rennin.

When there is no further activity increase, excess acid is neutralized by adding a stoichiometric amount of sodium bicarbonate as a water slurry. This treatment raises the pH to about 5.7. During neutralization, stirring is continued to drive off the carbon dioxide formed and helps replace it with air. Next, propylene glycol is added to a concentration of about 5%. The glycol

acts as a protein stabilizer (or solubilizer) and holds down bacterial growth. Caraway flavor and caramel color are added, as are sodium propionate (2%) and sodium benzoate (0.1%). Diatomaceous earth is also added at this stage to help in the filtering operation.

Contents of the finishing tanks undergo two filtrations. First, the material is pumped to a bag filter unit built to Paul-Lewis specifications. The filter set-up consists of 24 stockinglike filter bags held in a vertical position. These bags are made of canvas and are lined on the inside with muslin.

About a third of the bags is filled with extract at the start of the filtering step. Initial liquid which filters through the bags is pumped back through the filter. Then, as the clear filtrate begins to come through, it is pumped from the bottom of the filtration apparatus to a refrigerated storage tank in a cooler. When the filtration begins to slow down, another third of the bags is filled.

The filtrate from this first filtration is assayed, then blended to standard strength. Quality control steps include enzymatic activity, pH, salt concentration, color, and clarity. If the extract passes all of the tests, it is given a temporary okay.

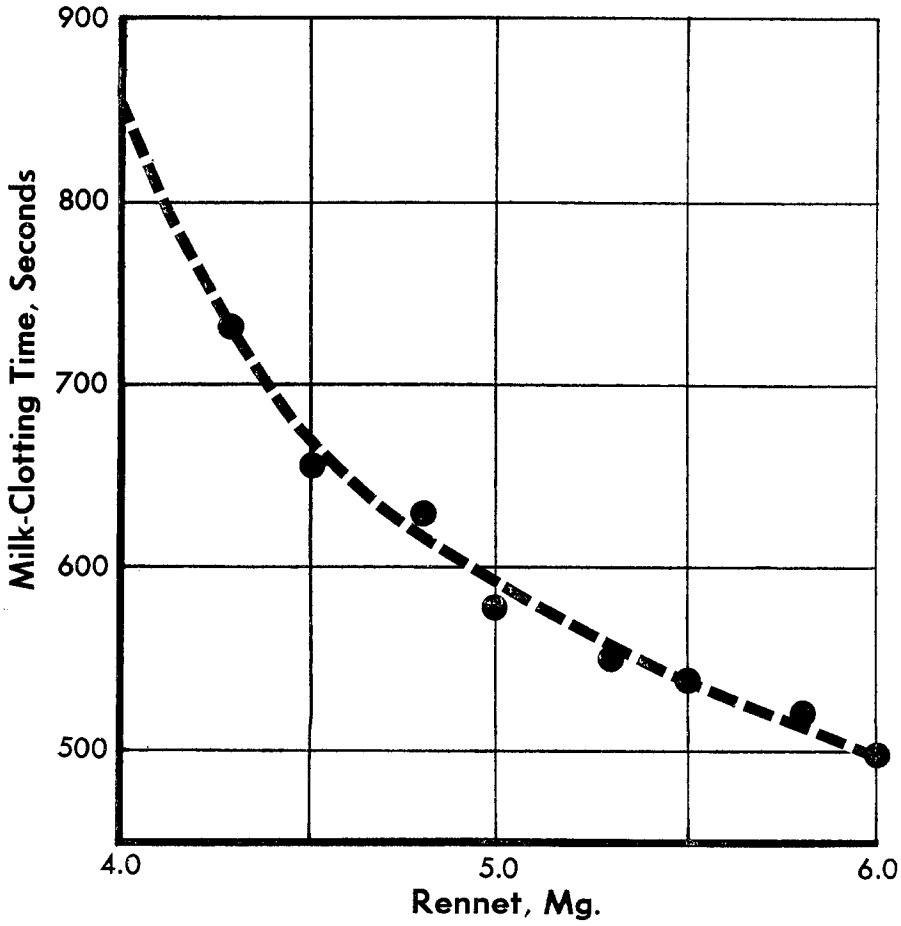
The second filtration is then started. Another filtration unit, similar to that used before except that it has smaller filter bags, is used. The extract from the second filtration goes through the same quality control tests, then is sent to the bacteriology department.

The bacteriological test takes 96 hours and is aimed at determining whether or not any gas-forming bacteria are present. None at all are permitted by Paul-Lewis standards. Neither are molds and yeast. In addition, plate counts must meet certain specifications. All rejected material is reprocessed. The approved product is now ready for packaging.

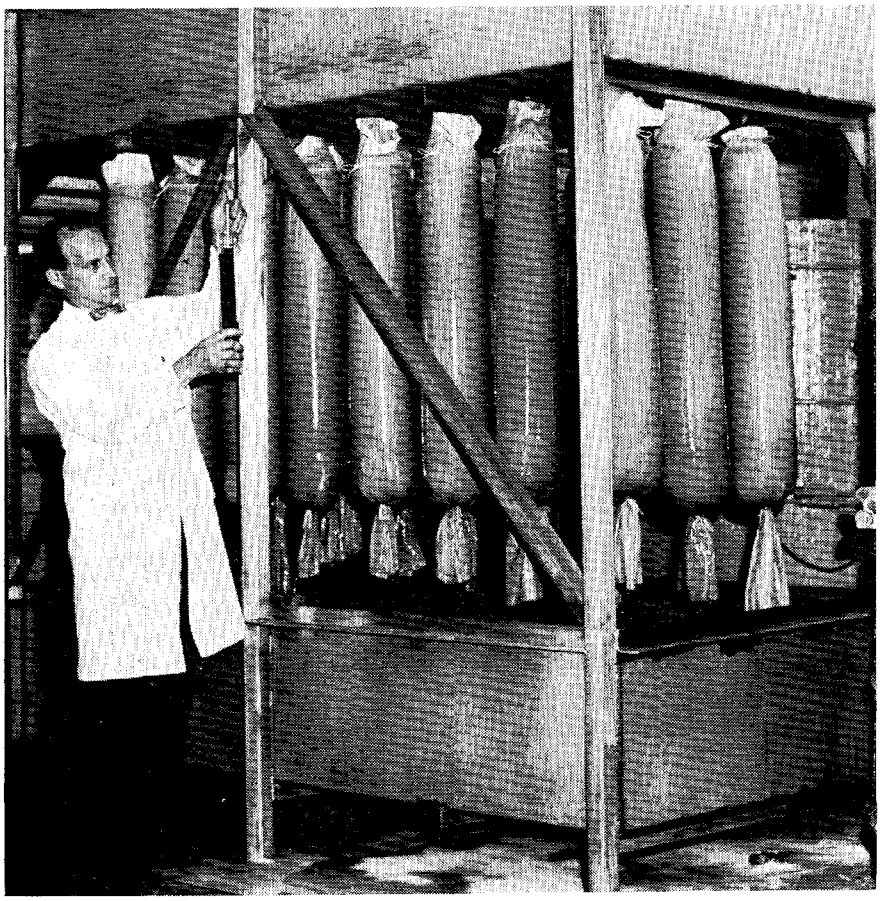
Rennet extract is packed either in polyethylene bags encased in cardboard, or in wooden containers. Steel drums were also used until recently. These became obsolete because of corrosion problems encountered with the high salt concentration of the extract.

The new polyethylene package has several definite advantages over the other containers. It is easy to ship, light weight, convenient to store, and simple to handle. The package comes in two sizes; 1 and 5 gallons. The plastic "balloon" is filled, heat sealed, and the cardboard box then closed. When container reaches the cheese maker, all that needs to be done is to puncture the container with a probe for easy dispensing. The container itself is never opened, thus keeping the contents uncontaminated. Empty con-

Enzyme activity is measured by determining milk-clotting time. The insert is a close-up of an assay showing the end point




Among the various control tests performed on rennet extract is the milk-clotting time. Here, clotting time (in seconds) is plotted against concentration



Bag filters are used to filter the active rennet extract. Bags are made of canvas and are lined with muslin

tainers are discarded by the user, a further convenience.

Paul-Lewis also makes rennet powder, primarily for foreign markets. The powder, approximately four to six times the strength of the liquid extract on a weight-to-volume basis, is made by a salt precipitation technique. A portion of rennet powder production is aimed at home cheese making; rare in the United States, but very common in Europe and other parts of the world. The powder is put up in 25-gram containers for this purpose. For overseas manufacturing plants, rennet powder is put up in packages ranging from 0.5 to 100 kilograms.

## Industrial Enzymes' Future

A large scale growth for the enzyme industry hinges on three developments: the use of fungal amylases instead of malt in distilleries; full-scale commercialization of the glucose-catalase oxidase system for removing oxygen in packaged foods; and FDA approval of catalase in cheese making to decompose hydrogen peroxide. The peroxide, in turn, could eliminate some heating that milk is subjected to during pasteurization. It could also help in the manufacture of those cheeses (like Swiss cheese) which are made from unpasteurized milk.

There is additional interest in using enzymes in many areas ranging from flavor to sewage treatment. But a good many successful enzyme developments await additional results in the research on fundamental behavior of enzymes.

In addition to carrying on its own research program, Paul-Lewis encourages basic enzyme research via the annual Paul-Lewis Award in Enzyme Chemistry. Administered by the American Chemical Society, the award consists of a gold medal and a $1000 cash gift. Winners are chosen by an award committee appointed by ACS, and the medal and money are presented at the Society's spring meeting. Another annual $1000 award, this one for cheese research and administered by the American Dairy Science Association, was instituted by Paul-Lewis in 1959.

As a service to the dairy industry and to schools, Paul-Lewis has prepared a short motion picture depicting rennet manufacture. The movie is available to those two groups as well as to other interested persons.

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