Milk Enzymes: Their Role and Significance

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Abstract

Enzymes are delicate organic catalysts produced by living cells, either animal or plant, but work independently from the cells that produce them. They do not necessarily remain at the site of production but are often transported to the site of action through the circulatory systems. Enzymes can be differentiated from other catalysts on the basis of their specificity, chemical nature, and sensitivity. An important characteristic of an enzyme is a strict specificity of the reaction it can catalyze. Enzyme specificity is a vital biological phenomenon for metabolic order and for life itself since, without specificity, every enzyme would degrade haphazardly and indiscriminately every component of the life-giving cell material. Enzymes are proteins, and they function within a narrow pH and temperature range. In general, they are fairly sensitive to heat.

Enzymic activity is determined by measuring either the disappearance of the substrate or the production of the end-products. Some enzymes are named after the specific substrates they act upon; e.g., lactase catalyzes the hydrolysis of lactose. Others are named after the class of substrates they act upon; e.g., proteinase or protease, which works on protein, and lipase which causes hydrolysis of fats. Still others are named after the function they perform; e.g., oxidases which catalyze the oxidation of the substrate. Their names, in general, carry the suffix -ase, the prefix referring to the function or the substrate of the enzyme. However, some of the earlier-discovered enzymes were not named in the above descriptive manner, and some still retain their original names; e.g., pepsin and trypsin.

Certain enzymes are produced in an inactive form and are called zymogens. Such zymogens are then activated before they can function; e.g., trypsinoogen produced by the pancreas needs to be converted to trypsin either by previously formed trypsin or by the enteropeptidase.

Although in the main this paper is concerned with enzymes in foods, and in milk and milk products in particular, it may be well to mention that notable advances in the study and application of enzymes have been made in the medical field. The determination of enzyme activity has been commonly employed as a part of clinical diagnoses of various diseases. In medical enzymology it is assumed that a change in enzyme activity in serum or excretions is due to damage in the cells or to physiological abnormalities. For example, a marked increase in the enzyme level in serum has been observed with the most severe mitochondrial aberrations (87). Of the many theories forwarded to explain this phenomenon, one theory states that due to the death or infraction of the cell the enzymes pass down or are released into the serum or into the extracellular space. However, no direct relationship has been shown between the extent of cell damage and the enzyme concentration in the secretions (4). Catalase and A-esterase levels in milk reportedly increase with udder disease and, therefore, are physiologically important, but no observations have been made concerning the physiological significance of other enzymes in milk.

With advances in the fields of milk and food chemistry and technology during the past few decades has come the development of the field of food enzymology. The measurement of enzymes in foods has been mainly used as an indicator of the state of the foods—their freshness and treatments they may have received. Early workers used tests for amylase enzymic activity in honey, to establish its freshness. Enzymes in general, being heat sensitive, allow determination of the efficacy of processing procedures; e.g., the phosphatase test is widely employed to check the efficiency of pasteurization. Many poisons, preservatives, antibiotics, and insecticides inhibit certain enzymes. The presence of such chemicals, their nature, and their concentration can be revealed by the extent of enzymic inhibition; e.g., relatively low amounts of DDT can be measured by determining the inhibition of added carbonic anhydrase (59). Conversely, use of specific inhibitors may reveal the presence of specific enzymes; e.g., NaF.
inhibits acid phosphatase of milk but not the alkaline phosphatase.

Milk produced under normal conditions, and from normal healthy cows, contains a wide variety of enzymes. While normal milk contains substrates for most of these enzymes, very little is known concerning the significance of these enzymes in milk. It is believed that these enzymes are normally present in tissues or cells and are spilled into milk during the milking process. Dowben and Brunner (26) believe that all milk enzymes originate in the secretory epithelial cells of the mammary gland. It is also believed that nature has provided these enzymes in milk for the benefit of the young, which has a rather incomplete digestive system and is incapable of producing its own enzymes for digestion of milk.

Thus far, at least 19 enzymes have been reported to be found in normal milk (Table 1). In this table the milk enzymes have been classified according to suggestions of the Committee on Enzymes of the International Union of Biochemists (29). The different classes are hydrolases, transferases, oxidoreductases, and so on. Each class is then divided into subclasses, each subclass divided into sub-subclasses and, finally, each sub-subclass contains several enzymes. For example, the serial classification number for lipase is 3.1.1.3. In this number the first digit 3, from the left, represents the class of hydrolases; the second digit 1 represents subclass enzymes acting on ester bonds; the third digit 1 represents sub-subclass of carboxylic ester hydrolases, and the final digit 3 presents the enzyme glycerol ester hydrolase, the systematic name for lipase.

These enzymes, found to be inherently present in milk, could also be grouped as follows: a) Hydrolytic group of enzymes which hydrolyze the main constituents of milk, such as lipases and esterases which hydrolyze fats and cause rancidity, and proteases which hydrolyze proteins and cause certain changes in dairy products; b) enzymes which may have a physiological role, like catalase which appears to be associated with udder infection, and lysozyme which might be associated with the immune properties or disease conditions; c) enzymes associated with the microsomal particles of milk, like xanthine oxidase, cytochrome C reductase, and alkaline phosphatase; and d) enzymes whose role has not yet been defined conclusively; e.g., ribonuclease, salolase, rhodanese, and carbonic anhydrase.

In addition to the 19 enzymes, lactose synthetase has been recently partially purified from milk (9). Of course, there might very well be many more enzymes present in milk, and their detection, determination, and isolation must await further research. Of the known milk enzymes, only a few have been either purified and isolated or crystallized. However, crystallization does not necessarily reflect the homogeneity of the protein, since crystalline urease has been shown to contain several different protein moieties (45). Peroxidase, xanthine oxidase, and ribonuclease have been isolated from milk and crystallized; and lipase, lysozyme, alkaline phosphatase, acid phosphatase, and protease have been isolated from milk in a highly pure form.

A comprehensive review of all the milk enzymes has not been attempted in this paper. Only lipase, phosphatase, lysozyme, and ribonuclease have been discussed in some detail, and a few other enzymes rather briefly. The order in which these enzymes are discussed does not necessarily imply the order of their importance to the dairy industry but, rather, the extent of

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Classification no.</th>
<th>Enzyme</th>
<th>Classification no.</th>
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<tbody>
<tr>
<td>Aldolase</td>
<td>4.1.2.7</td>
<td>Lysozyme</td>
<td>3.2.1.17</td>
</tr>
<tr>
<td>(\alpha)-amylase</td>
<td>3.2.1.1</td>
<td>Peroxidase (incl. lactoperoxidase)</td>
<td>1.11.1.7</td>
</tr>
<tr>
<td>(\beta)-amylase</td>
<td>3.2.1.2</td>
<td>Phosphatase (acid)</td>
<td>3.1.3.2</td>
</tr>
<tr>
<td>Carbonic anhydrase</td>
<td>4.2.1.1</td>
<td>Phosphatase (alkaline)</td>
<td>3.1.3.1</td>
</tr>
<tr>
<td>Catalase</td>
<td>1.11.1.6</td>
<td>Protease (quite specific)</td>
<td>3.4.--</td>
</tr>
<tr>
<td>Cytochrome C reductase</td>
<td>1.9.3.1</td>
<td>Rhodanese</td>
<td>2.8.1.1</td>
</tr>
<tr>
<td>Diaphorase</td>
<td>1.6.4.3</td>
<td>Ribonuclease</td>
<td>2.7.7.16</td>
</tr>
<tr>
<td>Esterase</td>
<td>3.1.1.1</td>
<td>Salolase (arylesterase)</td>
<td>3.1.1.2</td>
</tr>
<tr>
<td>Lactase(^a) ((\beta)-galactosidase)</td>
<td>3.2.1.23</td>
<td>Xanthine oxidase</td>
<td>1.2.3.2</td>
</tr>
<tr>
<td>Lipase</td>
<td>3.1.1.3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) May be the same as Cytochrome C oxidase.
\(^b\) Galactose synthetase could have the same classification number as \(\beta\)-galactosidase (3.2.1.23), since it also catalyzes the galactose transferase reaction.
work done on them. Attempts were made to present as far as possible recent developments in this field. For additional information, the reader is referred to several recent and excellent reviews and chapters on milk enzymes (20, 24, 48, 50, 81, 103, 110).

**Lipases and Esterases**

One of the significant aspects of lipases in the dairy industry is that they produce undesirable rancid flavors in milk and milk products. They may also be essential for development of desirable flavors in certain raw milk cheeses. Also, biologically they are an important group of enzymes, since they are associated with fat metabolism. However, no definitive information is available concerning the nutritional or physiological significance of these enzymes in milk.

The presence of lipases and esterases in milk has been known for a long time. Lipase activity has been found in milk of cow, goat, sheep, and human also (17, 101). An examination of the dairy literature reveals that considerably more work has been done on the lipolytic enzymes of milk than on any other milk enzyme. Just recently, in 1964, two comprehensive reviews on milk lipase were published (20, 50). Continued interest in the field is evidenced by the fact that several dairy researchers are actively engaged in this field.

In general, it is believed that milk contains a multiple lipase system. On the basis of their studies of the effects of pH, formaldehyde inhibition, hydrolysis of different substrates, and effects of different physical treatments, several workers (33, 92, 93) have suggested that milk contains a major lipase with a pH optimum of 8.5 to 9.0 and two other lipases with pH optima at 6.5 and 7.0 and at 7.9. Tarassuk and Frankel (100) reported that milk contains at least two different lipases—one is plasma lipase, associated with casein, and the other is membrane lipase, adsorbed on the fat globule membrane when milk is cooled. The enzymic activity of both the lipases was determined at the same pH, and it is not known whether they differ in their pH optima. Recently, Gaffney and Harper (34) demonstrated the presence of a lipase in the somatic cells obtained from separator slime. This lipase was claimed to be different from the lipase in skim milk. Also, the presence of acid lipase has been demonstrated in milk (1, 105). Forster et al. (30) have reported the presence of \( \alpha \)-, \( \beta \)-, and \( \gamma \)-esterases in milk. Montgomery and Forster (72) concentrated \( \beta \)-esterase from milk which seems to behave like milk lipase in regard to hydrolysis of milk fat (82). In addition, Korn (63) has purified a lipoprotein lipase from milk.

That the major lipase in milk is either associated with casein or with an individual component of the casein system has been well established. Yaguchi et al. (109) reported that lipase of milk is associated with the \( \kappa \)-casein complex of the milk. Downey and Andrews (27) and Gaffney et al. (35) separated several casein fractions by sephadex filtration and DEAE-cellulose chromatography, respectively, and observed the enzymic activity in several of the fractions. However, none of the fractions was reported to be homogeneous. Recently, Chandan and Shahani (18) isolated a milk lipase in a pure form. In these studies Grade A milk was employed to prepare the clarifier slime, a rich source of lipase and casein. Additional steps in the purification method involved preparation of acetone powder of the slime (which presumably breaks the casein:lipase complex), extraction of the enzyme with water, fractional precipitation with ammonium sulfate, acetone precipitation, and Sephadex filtration. The enzyme thus isolated was found to be electrophoretically and ultracentrifugally pure and homogeneous.

Whether milk actually contains many different lipases or whether these different pH optima and properties were observed because of differences in the research and assay procedures is not known. Comparison and evaluation of data obtained for milk lipase obtained by different researchers is difficult, if not impossible, because of the use of different lipase sources varying in impurities, different substrates, different assay methods, and different units of expression.

Several methods have been used for determination of the lipase activity in milk. They are a) the silica gel method, b) the modified Mojonnier method, c) the use of chromogenic substrate and the determination of the development of color, d) the Warburg technique, e) the spectrophotometric determination of fat emulsion clearance, and f) the pH stat method. The first two methods, used most widely, essentially involve the incubation of an enzyme with a fat emulsion at the optimum pH of the enzyme, extraction of the free fatty acids liberated due to lipolysis, and nonaqueous titration with a standard alkali to a phenol red end-point. These methods have inherent deficiencies, in that they fail to titrate the long-chain fatty acids completely at pH 7.0 to 8.0; e.g., only 20 to 80% of oleic acid is titrated at that pH level. Thus, the observed or apparent titer taken as a measure of lipase activity is far short of the true lipolysis. Recently, in our laboratory a pH stat technique has been adapted to the determination
of the lipase activity of milk (82), which appears to be highly sensitive, accurate, and rapid, and permits measurement of the initial reaction velocity.

Equally confusing is the fact that different studies have reported lipase activity in different terms, such as acid degree, free fatty acid titer, \( \mu \)Equiv fatty acids released per minute, or volume of \( CO_2 \) liberated by the hydrolyzed fatty acids. In order to compare different data, it is felt that a uniform lipase unit should be adopted, namely that defined by the Enzyme Committee of the International Union of Biochemists as the amount of lipase which releases 1 \( \mu \)Mole of acid per unit time under standard conditions (29).

The isolated milk lipase (19) conforms to the definition of a true lipase, in that it hydrolyzes only esters of glycerol in two-phase heterogeneous emulsified systems, but shows almost no activity toward simple esters. It possesses a single pH optimum of about 9.0 and a temperature optimum of 37 C. It is very unstable and is highly sensitive to heat, light, and certain chemicals. However, it can be stored frozen and in the dark for long periods without any appreciable loss of activity.

Chandan and Shahani (18) observed that the purified milk lipase is a typical protein with a sedimentation coefficient of 1.14 S. Its molecular weight, determined by two methods—the sedimentation velocity method and the osmotic pressure method—is about 7,000, indicating that it is probably the smallest enzyme ever isolated (22). Each molecule of the isolated enzyme contains two -SH groups, both of which appear to be essential for its activity. Experimental evidence indicates that one -SH is free and the other one is masked (21). Presumably, such -SH groups in the lipase molecule impart to the enzyme inherent instability and cause inactivation of the enzyme by light, by oxidation, and by interaction with certain reagents.

Various natural milk constituents appear to have a very pronounced effect upon lipase activity. Composite milk solids inhibit the purified enzyme and so do various proteins, like most of the caseins. Many lactalbumins and globulins stimulate the enzyme. Lactose has no effect, but all the salts inhibit the enzyme. The nature of inhibition of the enzyme by milk solids indicates that the process of inhibition follows Freundlich’s adsorption isotherm, and the mode of inhibition of the enzyme by proteins, studied ultracentrifugally, involves adsorption, developing a complex between the protein and some of the active sites of the enzyme molecule (95). Therefore, the observed lipase activity in raw milk is the net result of the inhibitory and stimulatory action of the various milk constituents upon the lipase.

Besides the requirement of an emulsion medium, the lipases have specificity for substrate and for position of fatty acids in the glyceride molecule. The purified milk lipase acts not only on milk fat but also on olive oil and various simple and mixed glycerides. The relative activity of the lipase toward various triglycerides seems to vary inversely with the chain length of fatty acids in the triglycerides (54). Also, milk lipase appears not to differentiate between short- and long-chain fatty acids attached to the primary positions of the same triglyceride, indicating that it lacks intramolecular specificity. However, it possesses intermolecular specificity, in that in a mixture of triglycerides it hydrolyzes the triglycerides containing butyrate at a faster rate (50, 53, 55).

Concerning the positional specificity, Jensen and his coworkers (50, 51, 53) observed that, like pancreatic lipase, crude or purified preparation of milk lipase preferentially releases fatty acids from the primary or \( \alpha \), \( \alpha' \) positions of a triglyceride, and hydrolyzes triglycerides at the fastest rate, followed by diglycerides and monoglycerides in decreasing order. However, several lipases of plant and bacterial origin are either specific for the \( \beta \)-position of glycerides (2, 106) or lack positional specificity.

Although only one pure milk lipase has been isolated with a pH optimum of 9.0, several workers have reported in milk the presence of multiple lipase systems with pH optima ranging between 6.5 and 9.0. Also, the difficulties experienced by previous workers in purifying milk lipase might have stemmed from the fact the enzyme is highly reactive with milk constituents and is highly unstable. In Table 2 are presented comparative data concerning isolated pure milk lipase and the unpurified lipase or lipase activity of milk. The chemical and enzymic properties of the purified milk lipase and the effect of several physical and chemical treatments upon its activity, in general, parallel the properties and characteristics of the gross milk lipase activity and of unpurified milk lipase, as reported by numerous workers. There is reason to believe, therefore, that there may not be as many different lipases present in milk as has been suggested by several workers. Since in their studies milk, milk powder, or crude extracts were employed as the enzyme source, the observance of slightly different characteristics might have been due to the influences of milk constituents, or so-called impurities, upon one lipase.
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TABLE 2
Comparative data on purified milk lipase and the unpurified lipase activity of milk

<table>
<thead>
<tr>
<th>Isolated lipase</th>
<th>Unpurified lipase system of milk*</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Data</td>
</tr>
<tr>
<td>Optimum pH</td>
<td>9.0</td>
</tr>
<tr>
<td>Optimum temperature</td>
<td>37 C</td>
</tr>
<tr>
<td>Optimum substrate conc</td>
<td>10%</td>
</tr>
<tr>
<td>Effect of</td>
<td></td>
</tr>
<tr>
<td>Oxygen</td>
<td>Inhibitory</td>
</tr>
<tr>
<td>Light</td>
<td>Inhibitory</td>
</tr>
<tr>
<td>Heat</td>
<td>Inhibitory</td>
</tr>
<tr>
<td>Certain salts</td>
<td>Inhibitory</td>
</tr>
<tr>
<td>Glutathione</td>
<td>Stabilizing</td>
</tr>
<tr>
<td>p-chloromercuribenzoate</td>
<td>Inhibitory</td>
</tr>
<tr>
<td>N-ethylmaleimide</td>
<td>Inhibitory</td>
</tr>
<tr>
<td>Iodoacetate</td>
<td>Inhibitory</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>Inhibitory</td>
</tr>
<tr>
<td>Substrate specificity</td>
<td></td>
</tr>
<tr>
<td>Milk fat</td>
<td>Lipolyzed</td>
</tr>
<tr>
<td>Vegetable oils</td>
<td>Lipolyzed</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>Lipolyzed</td>
</tr>
<tr>
<td>Simple esters</td>
<td>Not lipolyzed</td>
</tr>
<tr>
<td>Positional specificity</td>
<td>1,3 position</td>
</tr>
</tbody>
</table>

*Unpurified lipase studies involved the use of milk, milk powder, or crude enzyme extract of milk as the enzyme source.
* Dealt with the esterase activity in milk.

Finally, information has been accumulating concerning an apparent reactivation of lipase activity during storage. Harper and Gould (40) and Nilsson and Willart (79) observed that milk subjected to high-heat treatments, which supposedly inactivate the lipase, showed a slight lipase activity. Also, certain milk samples in market channels or during storage, particularly those pasteurized by the high-temperature methods, have shown an increase in the free fatty acid content. Recently, such a phenomenon was observed in sterilized, concentrated milk products, including ice milk mix and egg nog drink (43, 61). Whether such an increase in the free fatty acid content is due to the spontaneous hydrolysis of milk fat, to reactivation of the lipase system, or to the heat-stable acid lipase is not known and provides an interesting avenue of research.

Phosphatases

Phosphatases catalyze the hydrolysis of phosphoric acid esters. A great variety of phosphatases exists in nature—phosphomonooestersases, phosphodiesterases, phosphorylases, pyrophosphatases, nucleotidases, and phytases. Several types of phosphatases reportedly exist in normal milk. However, all the detailed work done has been on two different monophosphate esterases only—alkaline phosphatase with a pH optimum of about 9.6, and an acid phosphatase with a pH optimum of 4.6 to 4.8. In addition, Brunner (11) has reported the presence of ATP-ase, a pyrophosphatase, in the fat globule membrane.

Although it is known that phosphatases are associated with the energy transfer mechanisms, no information is available concerning the physiological significance of phosphatases in milk. Nevertheless, a great amount of work has been done on the alkaline phosphatase, because it is known to be significant for both technological and public health reasons.

Alkaline phosphatase. Batch pasteurization or high-temperature, short-time pasteurization inactivates the alkaline phosphatase and, therefore, the presence of phosphatase activity in pasteurized milk indicates either improper pasteurization or post-pasteurization contamination with raw milk. Testing milk for phosphatase for regulatory and quality control purposes has been a common practice for nearly 30 yr. Since adoption of the phosphatase test as an indicator of proper pasteurization, several modifications for the detection of phosphatase have been developed which possess a high degree of sensitivity and accuracy.

Numerous substrates have been employed for determination of phosphatase activity. Disodium phenylphosphate, the most commonly employed substrate, upon hydrolysis releases phenol which is determined with 2,6-dibromoquinonechlorimide by the spectrophotometric method. Phenolphthalein phosphate and p-nitrophenylphos-
Acid phosphatase. Bovine milk contains an acid phosphatase, with a pH optimum of 4.75,
but in a much lower concentration than the alkaline phosphatase. Since the activity of this enzyme in cream is twice as much as that in skim milk, and since ultracentrifuge fractions of buttermilk contain, besides acid phosphatase, considerable amounts of alkaline phosphatase and xanthine oxidase activities (5), acid phosphatase also might be associated with the microsomal particles of the fat globule membrane. Bingham and Zittle (7) reported that in addition to aromatic phosphates and pyrophosphates, acid phosphatase hydrolyzes casein, which provides a natural substrate for this enzyme in the milk medium.

The acid phosphatase is perhaps the most heat-stable enzyme of milk (49). It is a highly basic protein and has been purified, 40,000-fold, using repeated chromatography on Amberlite IRC-50 resin and acetone precipitation. Manganese strongly activates the enzyme. Magnesium, which activates alkaline phosphatase, has no effect upon acid phosphatase activity. Fluoride inhibits acid phosphatase but not alkaline phosphatase (7).

Milk appears to contain substances inhibitory to acid phosphatase, since Bingham and Zittle (7) observed that during the initial steps of purification the total phosphatase activity doubled in amount. This is not surprising, because normal constituents of milk often inhibit enzyme activity, as was discussed in the case of lipase.

Although considerably less work has been done on the acid phosphatase than on the alkaline enzyme, the acid enzyme may have more significance from the dairy technology viewpoint. First, it is more heat-stable than alkaline phosphatase and is not inactivated completely upon pasteurization. Secondly, it shows greater activity toward casein than does alkaline phosphatase (7). The phosphatase releases the phosphate which presumably is attached to the serine or threonine moiety, thereby releasing hydroxyl groups in the casein molecule and, therefore, possibly making the dephosphorylated casein more reactive and oxidizable. Such a phenomenon may have some bearing upon spoilage of milk when it is stored for long periods. Besides, Zittle (110) indicates that release of the acid phosphate molecule from the casein may increase its isoelectric point and thereby affect the processes involving coagulation. Also, physiologically this acid phosphatase may be more significant than the alkaline phosphatase, because of its possible activity in the low pH intestinal surroundings.

**Lysozyme**

Lysozyme is an enzyme which lyses certain bacteria by hydrolyzing the $\beta$-linkage between muramic acid and glucosamine of mureopolysaccharide of the bacterial cell wall. It was first isolated from egg-white, a very rich source of this enzyme. It is also widely distributed in many physiological fluids and plant and animal tissues. A recent review by Jolles (57) provides comprehensive information concerning the enzyme, chemical, and physical characteristics of lysozymes of several different sources. It has been reported to be present in the milk of the cow, donkey, mare, bitch, sow, cat, rabbit, llama, monkey, and human (15, 23, 96); only traces, or no lysozyme activity at all, is found in the milk of goat, sheep, and guinea pig (15).

It has been established now that lysozyme is inherently present in bovine milk, although until recently its presence in milk as a native ingredient was in question. Lack of agreement between different reports concerning whether bovine milk contains lysozyme may be due to deficiencies in the assay methods employed in studies with bovine milk, which is very low in the lysozyme content. The procedures of Smolellis and Hartsell (97), Litwack (68), and Shahan et al. (96) lack sensitivity. The modified methods used by Rhodes et al. (88) and Parry et al. (82) appear to be more sensitive and rapid and permit measurement of the initial reaction rate.

Bovine milk contains an average of 13 $\mu$g of lysozyme per 100 ml. Human milk contains 39 $\mu$g/100 ml, or nearly 3,000 times as much as in bovine milk (23). It has been only recently that lysozyme from both bovine and human milk has been isolated in a pure form. Jolles and Jolles (58) were perhaps the first to isolate lysozyme from human milk. Since then, their method was considerably modified in our laboratory and was applied successfully to isolation of both bovine and human milk lysozyme. The method for the isolation of bovine milk lysozyme involves separation of whole milk, preparation of the acid whey from the skim milk, adsorption of lysozyme on to Amberlite IRC-50, fractional precipitation with ammonium sulfate, and then twice-chromatographing on Sephadex G-50. Essentially, a similar pro-
cEDURE is used for isolation of human milk lysozyme, except that the milk is defatted by centrifugation and the lysozyme can be adsorbed on to the cationic resin directly from the skim milk. The bovine and human milk lysozymes thus isolated were found to be electrophoretically and ultracentrifugally pure and homogeneous, the purification for bovine milk lysozyme being 16,300-fold and the specific activity 0.35, and the purification for human milk lysozyme being 195-fold and the specific activity 3.5 (15, 81). The bovine milk lysozyme has a pH optimum of 7.90 and human milk 6.35. Both are highly basic proteins. Their isoelectric points lie near pH 9.5 and 11.0, respectively (16). Both enzymes are fairly heat-stable at acid pH, as is egg-white lysozyme (23, 96). Also, both are activated by several salts. Bovine milk lysozyme exhibits almost no lytic activity in the salt-free system, but the human milk lysozyme does.

Presently, no definitive information is available concerning the nutritional, physiological, or technological significance of lysozyme in milk. Since lysozyme precipitates at 50% saturation of ammonium sulfate, it may be a globulin. Cahn-Bronner (13) and Ferrari et al. (28) report that lysozyme is possibly associated with mechanisms of natural immunity and of local resistance to infection of tissues. Thus, it may be of importance in the defense mechanism of the individual. A variation in the lysozyme content of animal tissues and secretions provides an index for diagnosis of certain pathological conditions. However, no relationship between the presence or absence of mastitis and the concentration of lysozyme was observed (90).

Lysozyme appears in the feces of breast-fed infants but not in those of bottle-fed infants. Cavalieri (14) observed that addition of egg lysozyme to milk results in formation of a softer curd and improves digestibility of the milk. Deragna and Coceiante (25) reported that administration of egg-white lysozyme through food to premature babies reduced their weight loss and gastric infections and resulted in quicker weight increases. A report by Seleste (94) indicates that lysozyme in milk may be associated with its keeping quality. Although certain milk samples possess antibacterial properties, the possible relationship of these properties with the lysozyme content has not been established.

Lysozymes have been purified or isolated from numerous biological systems other than milk, and the voluminous work done on such lysozymes may shed some light upon its physiological significance in milk.

Ribonuclease

Another enzyme present in significant quantities in normal milk is ribonuclease, which catalyzes the hydrolysis of ribonucleic acid. Considerable work has been done on ribonuclease of pancreas and of blood, urine, and other body fluids. Bovine milk has been reported to be a fairly rich source of this enzyme. Milk of various other mammals also contains ribonuclease, but in a much smaller concentration.

Bingham and Zittle (6, 8) have isolated in a pure form two separate enzymes, ribonuclease A and B, from bovine milk. Their isolation procedure involved preparation of an acid whey of milk, ammonium sulfate precipitation, adsorption on Amberlite IRC-50, acetone precipitation, and rechromatography on Amberlite IRC-50, achieving a purification of approximately 2,000-fold. The two nuclease possesses an identical specific activity of 1.16. It was only recently that bovine milk ribonuclease has been crystallized (37).

Like lysozyme, ribonuclease is a low molecular basic protein, is readily adsorbed on Amberlite IRC-50, and is fairly heat-stable at acid pH levels. Its pH optimum is 7.5 and isoelectric point 7.8.

On the basis of the amino acid composition, immunological characteristics, gel electrophoretic mobility, and specific activity, ribonuclease A of milk appears to be similar to ribonuclease A of pancreas. It has been postulated, and it appears convincing, that the pancreatic ribonuclease enter the blood stream through the intestine and is secreted in milk through the mammary glands. No definite information is available regarding the role and significance of ribonuclease in milk, but Brunner (11) has recently isolated from milk its substrate, ribonucleic acid. It has been suggested, however, that the ribonuclease either may be associated in some way with the microsomal component of the fat globule membrane or may affect the nucleic acid component of the membrane and this, in turn, may influence the stability of the fat emulsion.

Other Enzymes

The other milk enzymes discussed rather briefly are xanthine oxidase, peroxidase, catalase, and protease.

Xanthine oxidase. This is a rather nonspecific enzyme, in that it catalyzes the oxidation of several compounds, such as purines, pyrimidines, and aldehydes. For example, xanthine is oxidized to uric acid and aldehydes to acids. As early as 1902, Schardinger (91) observed the
xanthine oxidase activity in bovine milk. Since then considerable work has been done on this enzyme, resulting in final crystallization of the enzyme by Avis et al. (3). Morton (76) observed that in bovine milk this enzyme is a part of the microsomal particles. Besides being present in cows' milk, it has been observed in the milk of the sheep and goat, but the milk of sow, mare, and human is devoid of this enzyme (71).

Bovine milk xanthine oxidase is a metallo-flavo-protein, containing molybdenum and iron as its metal moieties. Molybdenum forms an integral part of the enzyme, in that there is a relationship between the molybdenum content and its activity. It is a very high molecular weight protein—about 300,000. Its content is variable, being higher in milk obtained toward the end of lactation. The higher the molybdenum content of the forage ingested by the cow, the higher the xanthine oxidase content of its milk.

Regarding the technical significance of xanthine oxidase, there exist conflicting reports concerning its role in the oxidative deterioration of dairy products, and further work needs to be done along those lines. This enzyme is particularly important because it is fairly heat stable, being more stable than lipase and alkaline phosphatase. Contrary to the behavior of various other milk enzymes, the xanthine oxidase activity in milk increases with several heat treatments, homogenization, and by the protease and lipase action. Such treatments presumably release xanthine oxidase from its inhibitors or complexing compounds in milk. It appears to partly survive in the manufacture of low-heat skim milk powder. Furthermore, the reactivation of this enzyme in condensed milk during storage has been observed by Greenbank and Pallansch (36). In several such respects as location and distribution in milk and reactivation phenomena, it bears similarity to alkaline phosphatase. Recently, Whitney (104) has obtained conclusive evidence that milk contains both xanthine oxidase inhibitor and activator. The activator was found to be very heat-resistant. The presence of enzyme activator may have some relationship to the reactivation phenomenon of this enzyme.

Physiologically, milk xanthine oxidase appears to be significant, since it produces antitumor effects when injected in mice with mammary tumors (3).

Peroxidase. Peroxidase, another enzyme associated with the oxidation-reduction system, catalyzes the decomposition of H₂O₂ in the presence of a hydrogen donor. It is perhaps the first enzyme found in milk; its presence in milk has been first demonstrated in 1881. It has been isolated from milk and has been obtained in a pure and crystalline form (73, 74, 86).

Compared to several other milk enzymes, peroxidase is fairly heat-stable. Technologically, therefore, it could be used as an indicator of high-heat treatments of milk. Also, it may be used as an indicator of detecting H₂O₂ added to milk as a preservative. The enzyme contains aromatic groups shown to be prone to oxidative changes, but no definitive information is available concerning whether this enzyme is associated with the flavor deterioration of milk. The physiological significance of this enzyme is not known, although both milk and leucocytes contain this enzyme.

Catalase. Catalase decomposes hydrogen peroxide giving rise to oxygen. The two enzymes, peroxidase and catalase, are distinctly different. While peroxidase needs a hydrogen donor or oxidizable compound for splitting H₂O₂, catalase does not require any such compound.

Catalase has been found to be present in several body organs and fluids, including milk. Since its concentration in milk is related to the leucocyte count, and since its level in colostrum and in mastitic milk is fairly high, its secretion in milk appears to be dependent upon changes in the physiological state of the cow. Nevertheless, it is also found in the milk of the normal or healthy cow. With which constituents it is present in milk is not known, although upon separation it is concentrated in both cream and the slime. Like several other enzymes, it appears to be associated with casein, since McMeekin and Polis (70) have shown that it can be coprecipitated with casein.

Although catalase is one of the principal enzymes in milk, it has not yet been obtained in a pure form. Therefore, little is known about it. Catalases isolated from animal tissues are hematoproteins, and it is believed that milk catalase may also be of the same type of protein. The literature presents conflicting data concerning the heat sensitivity of catalase, but it appears to be more stable than lipase and alkaline phosphatase.

Protease. Protease, as its name suggests, hydrolyzes the peptide linkages of a protein. That bovine and human milk contain protease as a native ingredient has been well established (41, 99). It is associated with casein, with which it can be coprecipitated. Hipp et al. (46) demonstrated that the enzyme could be concentrated with the a-casein fraction, and Zittle (111) reported recently that protease accompanies k-casein almost quantitatively and obtained a 20-fold concentration of the activity.
Its concentration in milk is very low, however. Strangely enough, protease is associated with casein, which itself is a substrate of the enzyme. Raw milk under normal storage does not show spontaneous proteolysis, presumably either because protease is present in a very low concentration, is present in an inactive form, or its action is checked by a naturally present protease inhibitor in milk (62).

There appears to be disagreement in the literature concerning the pH optimum of the enzyme. While several workers (40, 99) report its pH optimum to be 8.5, Kiermeier and Semper (62) observed an optimum of 6.5. Although there may exist two proteases in milk, the difference in the observed pH optima may be due to the difference in the assay methods employed. Differences in, or lack of sensitivity of, the assay methods used by different workers may be responsible also for the discrepancies in the heat lability reported for protease. Kiermeier and Semper observed that a heat treatment of 70°C for 2 min would inactivate the enzyme, but the literature (103) shows that in borax buffer a heat treatment of 80°C for 10 min was required for its destruction. Such a heat treatment is higher than the normal pasteurization heat treatment. Generally, an enzyme in a pure form is more heat-labile than in a crude form. It is not known whether pasteurized or evaporated milk would contain any residual protease. While during short storage periods no proteolysis is observed, upon long storages the residual enzyme, if any, may initiate flavor and texture defects. Although sterilization supposedly inactivates the enzyme completely, Murthy et al. the zymogen form or bound to other constituents. Certain treatments such as agitation or temperature manipulation may be needed to activate these enzymes or to release their active sites which are blocked or attached to other milk constituents, as might be the case with lipase or protease. In some cases possibly the natural substrates present in milk are protected by a nonpermeable membrane which renders them unattackable by the enzymes, and they need to be separated from the protective elements. Homogenization or agitation partially removes the fat globule membrane, rendering the smaller globule more vulnerable to the lipase action (83). In still other cases, nature has provided natural inhibitors in the system for some of these enzymes; e.g., the trypsin inhibitor, the xanthine oxidase inhibitor, and the acid phosphatase inhibitor. While the above conditions may prevail in raw milk in particular, the lack of readily discernible changes in pasteurized or processed milk products due to enzymic action are rare, evidently because such treatments retard or destroy enzymic activity.

As normally conceived, the process of secretion involves ejection of the synthesized milk constituents from the secretory cells of the alveoli into the lumen, movement of milk into the cistern and, finally, elaboration through the teat canal during milking. It is not known whether the secretion of milk results from the rupture of the secretory cell membrane or whether the membrane is permeable enough to let the milk pass through.

Dowben and Brunner (26) suggest that all the milk enzymes originate in the secretory epi-
ported by the fact that during udder disturbances the diseased cells rupture more readily and there results an elevation in the content of the above enzymes. It is surprising, however, that succinic dehydrogenase and cytochrome oxidase, which are cytoplasmic enzymes, do not occur in milk. As pointed out by Morton (76), these two enzymes appear to be associated with mitochondria probably separated from microsomes because of a differential permeability of the upper and lower cell wall. In addition, some milk enzymes may originate in another part of the body and may be transported through the blood to enter milk; e.g., milk ribonuclease A is reportedly similar to pancreatic ribonuclease A (7), and human milk lysozyme appears to be similar to that in saliva and placenta (56).

Based upon available evidence in the literature, it is conceivable that milk enzymes exist in four distinct phases: a) those enzymes which appear to be present in true solution in milk serum, such as catalase, peroxidase, lysozyme, and ribonuclease; b) those associated with the fat or cream phase, such as aldolase (85); c) those associated with the casein particles, like lipase and protease; and d) those associated with the lipoprotein microsomal particles, like alkaline phosphatase. These different enzymes in milk appear to exist in some sort of equilibrium, and treatments that cause any changes in physico-chemical state of milk may cause a shift in their relative distribution; for instance, cooling of milk causes the membrane lipase from the plasma to adsorb irreversibly on to the fat globule (100).

The milk microsomes or the lipoprotein particles isolated from milk have properties and enzyme makeup similar to those of the microsomes separated from the mammary gland and other animal tissues (76). They are probably spilled into milk from the mammary gland during secretion and occur in association partly with the fat globule membrane and partly with casein. Although thus far only xanthine oxidase, alkaline phosphatase, diaphorase, and cytochrome C reductase have been shown to be present in the milk microsomes, other enzymes such as acid phosphatase, cholinesterase, carboxylesterase, glucose-6-phosphatase, and adenosine-5-phosphatase have been observed in the microsomes of other tissues. As suggested by Zittle (116), these microsomes may be pictured as a parcel of enzymes cemented together by phospholipid and nucleic acid. The observation of Tarassuk and Frankel (100), that the lipase system of milk is associated with the membrane as well as with the casein particles, leads us to believe that the lipase also may very well be associated with the microsomal particulate. The observed multiplicity of the enzyme lipase may partly be explained on the basis of the high association of the microsomes with the fat globule membrane and with casein. This phenomenon may also parallel the fact that alkaline phosphatase has been observed in and purified from both skim milk, containing high amounts of casein, and buttermilk, rich in the fat membrane fraction.

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