Alkaline phosphatase of milk.

Phosphatases in general catalyze the hydrolysis of phosphoric acid esters and thereby play an important role in the energy transfer mechanism of the living beings. Demuth (1) was possibly the first investigator to report the presence of a hexose phosphatase in milk, but it remained for Graham and Kay (2) to establish that such phosphatase was a native component of milk system. Folley and White (3) and Folley and Kay (4) showed that milk alkaline phosphatase is derived from the mammary gland rather than from the blood serum. Baile and Morton (5,6) established purified milk enzyme to be the same as that from the mammary gland.

Variation in milk.

Alkaline phosphatase has been studied in the milk of various species. Folley and Kay (4) found that the high level of phosphatase diminished to a minimum at 15-25 days post partum. Aschaffenburg and Neave (7) observed low phosphatase activity during first few weeks of calving. Kannan and Basu (8) studied the alkaline phosphatase in colostrum of various species and reported that the level of this enzyme is high in colostrum of cows, buffaloes and ewes which gradually dropped to a minimum after 4-15 days post partum with a subsequent rise until the end of lactation. They also reported that buffalo milk contained lower alkaline phosphatase than cow. Low level of alkaline
phosphatase in early lactation and high in advanced lactation has been observed by Morton (9) in pooled milk samples of commercial herds. Jacquet and Villette (10) reported high level of phosphatase in secretions like colostrum and mastitis milk. Haab and Smith (11) found that in case of cow, the phosphatase values decreased sharply after the first milking, reached to minimum before the 10th day and then began to increase gradually. Variation due to season was maximum during October to December. No effect of feeding was noticed on the activity of this enzyme in milk. Mohamed and EL-Rafey (12) studied the alkaline phosphatase of Egyptian buffaloes milk and compared with Brown Swiss and local breeds of cow. They reported that the buffalo milk has lower alkaline phosphatase activity than the cow breeds studied. The cow and buffalo milk showed a similar trend of fluctuation throughout lactation.

Nature and distribution of alkaline phosphatase.

Kay and Graham (13) noticed that the enzyme is associated with fat and was released in butter milk by churning. Hetrick and Tracy (14) reported that alkaline phosphatase is believed to be located to a large extent at the fat serum interphase and the whey obtained by acid precipitation of casein from skim milk contained good quantity of the enzyme. Mohamed and EL-Rafey (12) observed that the enzyme content of raw whole milk of buffalo and cow was always higher than the corresponding skim milk.
These studies suggested that the enzyme is associated with fat and also present in the skim milk phase. Zittle and DellaMonica (15) achieved partial purification of alkaline phosphatase from bovine skim milk employing trypsic digestion which assured the presence of enzyme in skim milk phase. The distribution of the enzyme in major milk fractions was, therefore, investigated by Morton (9). It was established that about 30-40% of the enzyme is adsorbed to the fat globules and may be concentrated by separation of cream. The enzyme is released in butter milk by churning cream or by elution with distilled water. The butter fat itself was free of activity. The remaining phosphatase was found to be distributed throughout the milk in the form of lipoprotein complex. The butter milk alkaline phosphatase was also lipoprotein complex. This particulate material could be sedimented together with a portion of casein particles by high speed centrifugation.

The precipitation of casein from separated milk with acids, salts or organic solvents resulted in coprecipitation of lipoprotein particles together with the associated phosphatase. The more specific precipitation of casein with rennin or pepsin permitted some separation of casein and lipoprotein particles. Therefore, the use of n-butanol was suggested to disrupt the lipoprotein complex thereby making the enzyme free.

Subsequently, Morton (16) separated the lipoprotein complex of cows milk and electron micrographs showed that both components occur in milk as discrete particles from
30-300 μm in size. The author reported that the lipoprotein particles contain about 22% total lipid, largely phospholipid, and some other enzyme activities. The lipoprotein particles had properties essentially similar to microsomes from lactating mammary gland and other animal tissues, therefore called "milk microsomes". They are released into milk from the mammary gland during the normal secretory process and occur partially in association with the fat globule.

Zittle et al (17) while studying the fat globule membrane showed that about 50% of alkaline phosphatase activity is recovered in the whey by acid precipitation of casein and similar results were obtained when milk was treated with rennet or pepsin. Approximately 25% of the activity was lost with acid or rennet precipitated casein. About 65% and 67% of alkaline phosphatase activity was recovered in pellet at 105,400 g for half an hour from cream and acid whey of skim milk, respectively. Worseck and Neipel (18) while studying the mass determination of alkaline phosphatase in raw skim milk concluded that it was concentrated at the fat globule membrane in whole milk and was associated with immune globulin of skim milk. Recently, Gammack and Gupta (19) achieved the localization of alkaline phosphatase of skim milk. When such milk was centrifuged at 54,000g for 90 minutes alkaline phosphatase and the phospholipids were concentrated in an opalescent layer above the micellar casein pellet. The residual casein in the opalescent layer was removed by subjecting it to gel filtration on Sagarose SAG-6, using 0.05M Tris-buffer of
pH 8.1 for elution 90% of the activity, phospholipid and less than 10% of the protein content of the opalescent layer was recovered in the particulate fraction. This fraction on analysis for specific activity and phospholipid to protein ratio resembled to the microsomes of fat globule membrane prepared by Morton (16). They concluded therefore that the lipoprotein particles in the aqueous phase are derived from the same cellular membrane which provide the surface layer of fat globules. Since they are present in aqueous phase at the time of secretion of milk, they appear to represent membrane material surplus to that required to coat fat globules. Mohamed and EL-Rafey (12) found that skim milk of cow or buffalo contained low alkaline phosphatase than cream showing association with fat.

Milk constituents and alkaline phosphatase activity.

An inverse relationship between alkaline phosphatase concentration and milk yield in cows was found (4). No relationship could be established between the fat content of cow milk and its phosphatase content (20). However, a correlation relationship between the phosphorus, thiamine contents and phosphatase activity was observed during the lactation period in case of cows. Caufield and Martin (21) and Kosikowski et al (22) found that addition of sucrose to milk exerted a protective effect on the enzyme at the time of pasteurization. Haab and Smith (11) found that individual cows showed an inverse relationship between milk yield and phosphatase content, however, no relationship could be established between fat percentage and breed with
enzyme activity. Kiermeier et al. (23) found that the activities of acid and alkaline phosphatases were directly related to the changes both in pH and its fat content. Alkaline phosphatase was correlated to protein content of milk.

Purification of alkaline phosphatase.

The recognition of association of bovine milk alkaline phosphatase with lipid like substances (18,24) and the unique ability of butanol to disrupt the lipid-protein complexes (25), initiated Zittle and DellaMonica (26) to purify alkaline phosphatase from bovine skim milk. The method precisely involved removal of casein by rennet clotting, ammonium sulphate precipitation of proteins from the resultant whey, butanol treatment, acetone fractionation and finally addition of Filter-Cel. Through seven steps of fractionation, they could purify the enzyme more than 1000-fold. An electrophoretic examination of this purified enzyme showed that at least three protein components were present. Presence of phosphodiesterase activity was also reported in this preparation.

Morton (27) purified alkaline phosphatase from bovine milk cream. He reported a yield of about 1.5% and about 5600-fold purification compared to whole milk. The method consisted in separation of cream preparation, butanol treatment, overnight preservation at pH 8.5, ether-acetone fractionation, ammonium sulphate precipitation, charcoal adsorption, second ether-acetone fractionation, second charcoal adsorption, second ammonium sulphate precipitation
and finally ether-acetone fractionation. Comparison of the activity with a protein fraction separated electrophoretically indicated that the purified enzyme was probably homogeneous.

Lyster and Aschaffenburg (28) purified alkaline phosphatase from bovine milk cream with the modification of Morton's method (27) by substituting acetone and alcohol fractionation for the steps involving ammonium sulphate precipitation and adsorption of impurities on charcoal and alumina gel. The preparations had specific activities 9-13 times higher than the Morton's (27) purified enzyme.

**Chemical nature and properties of alkaline phosphatase.**

Morton (29) studied the chemical analysis of purified milk and intestinal mucosa alkaline phosphatase, both enzymes were found to be colourless, nonconjugated proteins which were free of phosphorus, nucleotides and other related compounds. The absorption maxima of the purified alkaline phosphatase showed a single absorption maxima at 280 μm. Purified enzyme was reported to contain phosphorus lower than 0.1 per cent, carbohydrates 2 per cent, total nitrogen 16.2 per cent. The analytical data showed that milk enzyme contained 27 tyrosine and 13 tryptophan moles per 10^5 g protein with a ratio of tyrosine to tryptophan = 2:1. Copius Peereboom (30) demonstrated the association of sialic acid and its subsequent release by neuraminidase and acid hydrolysis.

Schramm and Arndt (31) have reported the molecular weight of the enzyme to be 60,000 which was
supported by Morton (29). Copius Peereboom (30) demonstrated the presence of \( \alpha \) - and \( \beta \) - isozymes of alkaline phosphatase of 140,000 and 570,000 molecular weight, respectively.

**Effect of metals.**

Perry and Doan (34) studied the effect of copper, nickel, iron, aluminium and tin as these metals always come in contact with milk during the processing. They used seven concentration levels of these ions from 0 to 10 p.p.m and found no change in the enzyme activity at any level. Zittle and DellaMonica (16) found that cow milk alkaline phosphatase was inhibited by sodium tetraborate to the extent of 50 per cent at 0.09 M which has been also supported by Morton (35). Wright and Tramer (36) studied the effect of \( \text{Mg}^{2+}, \text{Zn}^{2+}, \text{Fe}^{2+}, \text{Ni}^{2+} \) and \( \text{Sn}^{2+} \), the results indicated that \( \text{Mn}^{2+}, \text{Zn}^{2+} \) and \( \text{Fe}^{2+} \) had activating effect whereas \( \text{Ni}^{2+} \) and \( \text{Sn}^{2+} \) inhibited the enzyme. They observed inactivation of phosphatase by EDTA at concentration \( 3 \times 10^{-2} \text{M} \) and it was confirmed that a metallic ion is essential for alkaline phosphatase activity since after neutralization of EDTA with ions such as \( \text{Ca}^{2+}, \text{Mg}^{2+} \) or \( \text{Zn}^{2+} \) no such inactivation of phosphatase occurred. Using whole milk as an enzyme source, Haab and Smith (32) showed that added \( \text{Mg}^{2+} \) did not increase phosphatase activity. They concluded that natural magnesium ion concentration of milk was not a limiting factor. Kannan and Basu (33) reported that addition of \( \text{Mg}^{2+}, \text{Mn}^{2+} \) and \( \text{Ca}^{2+} \) ions to the phosphatase concentrates of milk activated alkaline phosphatase activity, whereas
the addition of \( \text{Zn}^{2+} \) higher than \( 10^{-6} \text{M} \) inhibited. Morton (29) showed that maximum activity of the purified enzyme was obtained with \( 10^{-2} \text{M} \) Magnesium chloride. Beryllium and Zinc salts both caused considerable inhibition. Morton (35) subsequently reported inhibition of enzyme by iodine and suggested probably due to the iodination of phenolic hydroxyl groups of the enzyme. Sodium phosphate, arsenate and arsenite were found to be competitive inhibitors of this enzyme.

**Effect of organic phosphorus and other compounds.**

A number of organic-pyrophosphates, phosphates, phosphites, phosphonates and fluorophosphonates were found to cause slight inhibition of milk and intestinal mucosa alkaline phosphatase (35).

The effect of 15 amino acids studied by Kannan and Basu (33) revealed that cow and buffalo milk alkaline phosphatase was not activated by any of the amino acid. At pH 10.0, however, only sheep milk phosphatase was activated by glycine and methionine. Morton (35) reported that cysteine was an effective inhibitor of alkaline phosphatase at \( 10^{-3} \text{M} \) concentration. The inhibition possibility pointed out was the metal chelation. Alanine was able to show some increase in enzyme activity up to \( 5 \times 10^{-2} \text{M} \) concentration. Whereas glycine did not show any marked effect. Inhibition of alkaline phosphatase by lysine and glutamic acid has been reported by Zittle and DellaMonica (37).

While studying the effect of aliphatic alcohols on
alkaline phosphatase, Zittls and DellaMonlea (38) reported that ethanol, methanol and isopropanol 5-20% inhibitory to bovine intestinal alkaline phosphatase whereas the milk alkaline phosphatase was not affected by alcohols at pH 9.7 and was stimulated at higher pH values. Studies over a range of pH values, concentration of ethanol and substrate, suggested that ethanol by reducing the dielectric constant of the medium influences the enzyme substrate interactions both directly and through the dissociation of charged groups involved in phosphatase activity. Differences in the degree of dissociation of the specific charged groups could account for the different properties of two phosphatases.

Alkaline phosphatases of serum, bone, intestinal mucosa, and milk were inhibited to the extent of 70-100 per cent by adrenaline, adrenochrome and O-dihydroxyphenols with substitution at C4, and by other O-quinones (39). Chelating agents like 8-hydroxy-quinoline and EDTA were also found to be strong inhibitors, but the type of inhibition differed from that of dihydroxyphenols and quinones in that it could be neutralized by addition of Mg^{2+}. Whereas it had no effect on the inhibition by phenols and quinones. The inhibition by the chelating agents was obviously concerned with the metal complex of the enzyme.

Wright and Tramer (40) found that penicillin had no significant effect on the substrate hydrolysis. Morr (41) studied the effect of penicillin and aureomycin and
no relationship could be obtained between the enzyme activity and aureomycin level. Penicilline treatment showed about 22 per cent inhibition which could be restored completely by increasing the substrate concentration.

Perry and Doan (34) studied irradiation effect on pasteurized milk and found that there was no significant effect. Mohamed and El-Rafey (12) showed that sunlight as well as ultraviolet radiation had no effect on the phosphatase activity in milk. Glew (42) reported that the enzyme was completely inhibited after electron radiation of 25 meads in fresh raw milk.

**Optimum pH, temperature and incubation period.**

Kay and Graham (13) reported that for maximum hydrolysis, the pH of the buffer system must be 9.2. Aschaffenburg and Mullen (43) using p-nitrophenyl phosphate as substrate found maximum hydrolysis between pH 9.8 to 10.2. Kannan and Basu (33) reported optimum pH as 10.0 for cow and buffalo milk alkaline phosphatase. Sanders et al (44) found the optimum pH as 9.8 to 10.0. Haab and Smith (32) found optimum pH to be at 10.0 whereas Morton (29) for the purified enzyme noted pH optimum at 9.65 using β-glycero-phosphate as substrate.

Aschaffenburg and Mullen (43) found an optimum temperature at 37°C. Sanders et al (44) reported an optimum between 32°C to 37°C whereas Haab and Smith (32) found maximum hydrolysis at 36.5°C.

Aschaffenburg and Mullen (43) concluded that up to first 30 minutes incubation, the rate of hydrolysis increased.
linearly with time and decreased at higher incubation period. Mohamed and EL-Rafey (12) using milk as an enzyme source from cow and buffalo found optimum activity at pH 9.5 at 40°C incubation temperature which were identical for both cow and buffalo.

Substrate specificity of milk alkaline phosphatase.

Folley and Kay (4), Roche and Thoi (45) have shown that the alkaline phosphatase hydrolyses a number of different phosphate esters in case of crude and partially purified preparations. Morton (35) investigated the substrate specificity of milk and intestinal mucosa alkaline phosphatases using wide range of compounds under different experimental conditions. It was observed that all the true orthophosphate monoesters, orthophosphoamides, phosphocreatine and phosphoenol pyruvate were hydrolysed by both enzymes. Sodium pyrophosphate, sodium hexameta phosphate, diphenyl pyrophosphate, ATP, ADP and DPN were not hydrolysed ensuring absence of pyrophosphatase activity. Morton (46) subsequently using again milk and intestinal mucosa alkaline phosphatase found that both enzymes quantitatively converted TPN into DPN. DPN was not hydrolysed by either enzyme. These results confirmed that the third phosphate group of TPN is esterified to ribose, since the action of both purified enzymes is restricted to phosphomonoesters and amides.

Zittle and Bingham (47) studied the action of purified milk alkaline phosphatase on casein and phosphoserine. The alkaline phosphatase preparation had
no proteolytic action on casein but split inorganic phosphate from casein and phosphoserine. The optimum action on phosphoserine was found to be at pH 9.5 or higher and with casein the enzyme was most reactive at pH 6 to 7. Both calcium sensitive \( \kappa \)-casein and whole casein were dephosphorylated at comparable rates.

**Heat inactivation of alkaline phosphatase.**

Kay and Graham (13) studied the stability of the enzyme at various temperatures ranging from 50 to 70°C. The enzyme was found to be destroyed instantaneously at 75°C and above. Prucha and Corbett (48) found the phosphatase to be inactivated by an instantaneous exposure at 71.1°C for 35 to 40 seconds. Anderson *et al.* (49) investigated the rate of destruction of phosphatase in milk at temperatures ranging between 60 and 80°C and concluded that the rate of heat inactivation was not a reaction of first order. These observations were confirmed by Hetrick and Tracy (14). Sanders and Sager (50) worked out the time temperature combinations to inactivate alkaline phosphatase. For whole milk 37.5 minutes at 61.5°C, 30 minutes at 62°C, 24 seconds at 71.1°C and 15 seconds at 72°C, were sufficient to inactivate the enzyme. Phosphatase inactivation temperature for skim milk was 0.05°C lower than that of whole milk at any holding time, whilst 0.5°C higher for cream and 2.4°C higher for ice cream mixes. Lear and Foster (51) found that heat inactivation of phosphatase to be non-molecular reaction, the 'Z' value from the temperature log time curve being 4.9°C. Mohamed
and EL-Rafey (12) found that cow and buffalo milk alkaline phosphatase showed same behaviour towards heat treatment and pasteurization.

Reactivation of alkaline phosphatase.

The ability of alkaline phosphatase to resume activity after high temperature short time or ultra high temperature processing of milk and milk products, has been termed reactivation. The enzyme responsible for this activity appeared to be identical with alkaline phosphatase which normally occurs in raw milk, in respect of heat stability, specificity of substrate and rate of hydrolysis of a given substrate at various pH values (40).

Although a positive phosphatase reaction was observed in butter and cream stored after proper pasteurization by earlier workers (52,53), but the reactivation phenomenon in milk was first established by Wright and Tramer (35,40, 54,55). These workers made a systematic approach and first noted reactivation in a commercially sterilized milk and were able to duplicate the phenomenon in laboratory. It was observed that neither HTST processing (72°C for 15 sec.) nor holding type processing (83°C for 30 min.) nor laboratory pasteurization (62.8°C for 35 min.) resulted in the reactivation of phosphatase. They found that reactivation was enhanced by aging of milk prior to pasteurization, the absence of air, the presence of reducing conditions, pasteurization at high temperature for short time and the addition of magnesium and calcium. Whereas laboratory pasteurization, rapid cooling, storage of
Pasteurized milk at low temperature and cold storage of raw milk before pasteurization reduced the rate of reactivation.

Presence of Cu, Co, Ni and the absence of Ca and Mg and the addition of EDTA and PCMB completely prevented reactivation of enzyme. They could not establish any correlation with fat content and initial alkaline phosphatase level with reactivation. No effect on the degree of reactivation was observed when ascorbic acid and penicillin were added separately.

Fram (96) studied reactivation in milk, skim milk, and cream. Reactivation occurred in commercially pasteurized cream with two hours incubation at room temperature. Skim milk and whole milk, however, required longer storage period and higher pasteurization temperatures. They provided convincing evidences to demonstrate that reactivated phosphatase was not of bacterial origin. Subsequently, Fram (97) attempted to find out the optimal conditions for reactivation of cream alkaline phosphatase when 20 per cent cream samples pasteurized at 169°F, 180°F and 210°F for 16 seconds and stored all at different temperatures for 2 to 4 hours, concluded that increased pasteurization temperature and storage period increased reactivated alkaline phosphatase. The optimum storage temperature was found to be 85°F.

Eddleman and Babel (98) studied reactivation in separated milk and whole milk at various time temperature combinations with subsequent storage at 5°C and 27.8°C
and observed for reactivation after every 24 and 48 hours. No reactivation could be observed in skim milk up to 138°C pasteurization temperature. Heating to 140°C showed reactivation on storage for 24 to 48 hours. In whole milk also similar trend was observed. When milk and cream mixed in the ratio of 1:1 sufficient reactivation occurred. They concluded that reactivation in case of milk and cream increased with temperature to a certain extent and then gradually decreased.

O'Sullivan and Shipe (59) observed 0.5 to 1.5 per cent reactivation in the milk of individual cow when heated at 100°C for 45 seconds and incubated for 18 hours. Addition of EDTA prior to incubation completely inhibited reactivation and it was only 30-50 per cent when added after incubation. Mg²⁺ had maximum effect when added prior to incubation. Milk dialysed against distilled water did not show reactivation and when dialysed against low level of Mg²⁺ increased reactivation. Aging of milk at 4°C showed a progressive decrease in reactivation.

Richardson et al (60) using a continuous flow HTST laboratory pasteurizer established optimum conditions for phosphatase reactivation. The optimum being at a pasteurization temperature between 220 and 230°F for cream, and 220 and 250°F for whole milk with 3 seconds holding time, 34°C as storage temperature and a MgCl₂ concentration of about 1.5 per cent for cream and 2.0 per cent for whole milk.

To avoid the interference due to bacterial phosphatase
effect of preservatives was also studied. Preservatives used were chloroform, toluene, mercuric chloride, potassium dichromate, formaldehyde and aureomycin. The samples containing galactose and aureomycin showed essentially the same degree of reactivation as a sample containing no preservative. Samples containing combination of toluene and chloroform inhibited reactivation to less extent but potassium dichromate reduced into half whereas mercuric chloride entirely prevented reactivation.

Simonart and Huberlan (61) observed that commercially pasteurised cream when stored at 26 to 37°C for 16 hours showed reactivation. They confirmed that reactivated phosphatase was not of bacterial origin and thought the phenomenon to be of physico-chemical nature.

Paschke (62) found that reactivation occurred in products of high fat content. No reactivation was observed in pasteurized milk, sterilized cream and sour cream.

Freeman et al (63) made a survey of phosphatase reactivation in the butter prepared from cream pasteurized by HTST commercial plants and found that most of the samples gave positive phosphatase reactions. Subsequently, these workers (64) investigated the effect of heat treatment and storage temperature on reactivation of phosphatase in butter. Treatment at 93°C for 10 seconds resulted in the large number of positive reactions. No positive reaction resulted in 77°C pasteurized samples. Lowest reactivation occurred at 4.4°C storage temperature. It was observed that lowest incidence of positive reactions occurred during
spring and the highest during fall season.

Lyster and Aschaffenburg (28) studied reactivation of alkaline phosphatase using a model system consisting of purified alkaline phosphatase, $\beta$-lactoglobulin, $\beta$-glycerophosphate and a divalent cation, kept in a boiling water bath for 45 seconds. It was observed that 10-30 percent of the original activity appeared after incubation at $37^\circ$C for 4 hours. The necessity of the presence of a divalent cation preferably Mg$^{2+}$ which could be replaced by Ca$^{2+}$ or Zn$^{2+}$ with a severe loss of reactivation, was demonstrated. Salts of Hg, Zn and Cd, were found to inhibit reactivation of the enzyme.

Presence of an inhibitor and activator of reactivation was brought out. The inhibitor was dialysable and heat labile, though stable to $63^\circ$C for 30 minutes whereas the activator was heat stable and non-dialysable.

Kresheck and Harper (65) investigated the role of individual milk constituents on the reactivation of purified alkaline phosphatase. It was found that whole casein, whey proteins and deproteinized whey, when added individually to a mixture of pure phosphatase and MgCl$_2$, the degree of reactivation was much less than that obtained with whole milk and skim milk. Maximum reactivation was provided by a combination of whole casein, $\beta$-lactoglobulin, sodium acetate and deproteinized whey. Lyster and Aschaffenburg (28) also found that addition of $\beta$-lactoglobulin and Mg$^{2+}$ enhanced reactivation. They observed further that $\beta$-lactoglobulin could be replaced by very small amount of
boiled milk but not by \(\alpha\)-lactalbumin or cysteine and reduced glutathione. Citrate was found to be an inhibitor of reactivation.

**Mechanism of Reactivation.**

Rechardson *et al* (60) proposed that the inactivation of the enzyme by heat treatment might be caused by a transfer of \(\text{Mg}^{2+}\) ions from the enzyme into a colloidal solution. In this concept the enzyme was thought to survive the pasteurization in a structurally unchanged form, which was only enzymatically inactive because of lack of these ions. During the reactivation process \(\text{Mg}^{2+}\) ions might gradually become accessible again to the enzyme and which would then regain its original enzymatic properties.

Kresheck and Harper (66) reviewed the available data and gave an interpretation of milk alkaline phosphatase reactivation process. The whole phenomenon has been explained on the assumption of reversible changes in the structure of alkaline phosphatase. They suggested the role of \(-\text{SH}\) groups formed from the enzyme molecule on heat treatment and postulated function of added magnesium in the reactivation. On heat treatment the active enzyme molecule is unfolded and active \(-\text{SH}\) groups are formed resulting into an inactive enzyme. Subsequently under conditions these groups return to an unreactive position thereby again assembling the active enzyme molecule. The magnesium ions possibly through chelation helps to pull portions of the enzyme molecule. It may also assist by binding with negative groups on the surface of the molecule and thus allow
rotation of negative groups towards a positive group so that non-covalent interactions could hold or place the molecule in 'native' confirmation resulting into an active enzyme.

Recently, Copius Peereboom (67) has proposed a theory on renaturation of alkaline milk phosphatase from pasteurized cream. The findings suggested that milk possessed $\alpha$- and $\beta$-isozymes of alkaline phosphatase. The later being strictly present adsorbed on the fat globule membrane in a lipoprotein complex. After pasteurization these lipoprotein particles, together with the $\beta$-isozyme, go into the water phase in a denatured condition. When this water phase is added again to the native membrane structure of the fat globules of raw cream, the denatured $\beta$-isozymes are reincorporated into this structure and recover their original enzymatically active conformation. Owing to this, there is a considerable increase in $\beta$-isozyme content. This so called "$\beta$-effect" is produced by combination of the specific conformation of the $\beta$-isozyme in the three dimensional structure of the membrane component with a factor 'x' which is present in the extract of pasteurized cream. Using Sephadex thin-layer chromatography, the factor was identified as being the denatured phosphatase isozymes and denatured fragments there of which occur in the serum phase of pasteurized cream and milk. As the addition of pasteurized skim milk to raw cream did not produce the $\beta$-effect, the factor 'x' could be identified as the denatured $\beta$-alkaline phosphatase isozyme.
Isozymes of milk alkaline phosphatase.

Copius Peereboom (68) using agar gel electrophoresis found three isozymes namely \( \alpha \), \( \beta \) and \( \gamma \)-isozymes. The \( \gamma \)-isozyme characteristically moved towards the anode side. Through densitometric determinations the average percentages of \( \alpha \), \( \beta \) and \( \gamma \)-forms were reported to be 65, 25 and 10%, respectively. The isozyme pattern of raw and reactivated alkaline phosphatase was found to be different. Subsequently, Copius Peereboom (30) using agar-gel electrophoresis, polyacrylamide disc-electrophoresis and Sephadex thin layer chromatography found that the reactivated cream gave exclusively \( \beta \)-isozyme. This \( \beta \)-isozyme was found to be located only on the fat globule membrane complex and was never observed in the skim milk and skim milk phase of water washed cream, which contained mainly \( \alpha \)-alkaline phosphatase. By studying the isozyme patterns of several samples of reactivated cream it was found that these renatured \( \beta \)-AP patterns may vary considerably. Some samples contained exclusively \( \beta_1 \)-, \( \beta_2 \)-isozyme, other \( \beta_2 \) or a combination of both. A special \( \beta_3 \)-isozyme was identified in reactivated cream but could not be detected in normal "native" isozyme patterns. This \( \beta_3 \)-isozyme had greatest mobility on agar gels. Depending upon their mobilities on Sephadex thin layer chromatograms with respect to the proteins of known molecular weight, it was found that \( \alpha \) and \( \beta \)-isozymes had 140,000 and 570,000 molecular weights, respectively.

Lefrane and Han (69) reported that the alkaline
phosphatase isolates from butter milk resolved into two enzymes, A and B, on DEAE-cellulose chromatography. Fraction A was found to be precipitated at 70-90 per cent saturation with ammonium sulphate and had a molecular weight about 140,000. Fraction B was precipitated at 40-65 per cent ammonium sulphate saturation. Such component had a molecular weight of about 200,000.

Brunia and Martin (70) studied the isozyme pattern of 4 breeds of cow in Rumania and their crosses on Sephadex G-200. The alkaline phosphatase was found to be polymorphic and modified by crossing. Milk from Holstein-Frænsian cow's contained 4 isozymes and milk from other breeds contained less.

Microbial and reactivated milk alkaline phosphatase.

The possibility of phosphatase production in dairy products has been demonstrated by various workers. Palcy (71) and Burgwald and Giberson (72), Hammer and Olson (73) reported bacteria which gave positive phosphatase reaction. Leahy et al (74) found that number of species of bacteria gave positive reaction. However, they believed that for such reaction, the number of organisms present required was too high. Barber and Frazier (53) observed that commercially pasteurized cream developed positive phosphatase reaction after 3-4 days storage at 4°C or 10°C, and the number of bacteria was found to be between 1.48 - 7.8 millions per milliliter of cream. They also found that the bacterial phosphatase could withstand heat-treatment as high as 76.7°C for 30 minutes while milk phosphatase was inactivated in 30 minutes at 62.8°C and thus suggested a way to distinguish
the enzyme from these sources.

Tittsler et al (78) tested about 200 samples of microorganisms representing 90 species and 23 genera, for their phosphatase production and thermostability of the bacterial phosphatase. It was observed that only few of these could give positive reaction. The bacterial phosphatase was more thermostable than milk phosphatase. Microbial phosphatases were generally not inactivated appreciably at 70°C for 5 minutes whereas milk phosphatase was destroyed completely at 70°C for 1.5 minutes.

Differentiation of raw and reactivated alkaline phosphatase.

McFarren et al (76) proposed a method for differentiation of reactivated from raw or underpasteurized product on the basis of the fact that a reactivatable product stored with MgCl₂ at 34°C will show about 10-fold increase in phosphatase activity over the same product stored without MgCl₂. In contrast, phosphatase in under-pasteurized products does not exhibit a similar increase in activity with MgCl₂.

Copius Peereboom (30) demonstrated that reactivated alkaline phosphatase samples showed the presence of mainly β-enzyme on agar gel electropherograms, and an additional zone of AP₁ with high intensity appeared on Sephadex gel chromatograms. These characteristic observations on raw and reactivated alkaline phosphatase extracts have been suggested to differentiate the two enzymes.

Phosphatase tests.

Discovery by Kay and Graham (13) that the heat...
sensitive enzyme phosphatase normally present in milk was 
inactivated by exposure to pasteurizing temperatures, 
61.7°C for 30 minutes and or 71.1°C for 15 seconds, lead 
them to develop a test for assessing the efficiency of 
milk processing (77). A multitude of modifications in the 
original Kay and Graham's procedure have made the test 
sensitive, rapid and quantitative (78-81).

Recently, Kosikowski (82) developed a simplified 
phosphatase test that involves the incubation of the 
sample with standard Cornell buffered substrate in a 
standard seamless cellophane tubing immersed in copper 
sulphate solution. During incubation, phenol liberated 
by residual phosphatase enzyme, migrated through the 
membrane in copper sulphate solution. Protein and other 
interesting substances are retained by the membrane, which 
was removed after incubation.

The other most frequently used test is that developed 
by Aschaffenburg and Mullen (43) which chiefly consists in 
utilizing p-nitrophenyl phosphate as substrate in HCl-Na₂CO₃-
sodium veronal buffer. With the action of phosphatase, 
p-nitrophenol is liberated, the colour of which is measured 
after precipitating out the proteins with trichloroacetic 
acid. After filtration colour is developed by adding 
sodium hydroxide. Aschaffenburg (83) subsequently replaced 
the original buffer with carbonate-bicarbonate buffer, 
keeping other conditions of the test same.

Recently O'Brien (84) using disodium phenyl phosphate 
as substrate developed an automated method having an
efficiency of detecting 0.1 per cent raw milk in pasteurized milk. Raynold and Telford (85) using p-nitrophenyl phosphate as substrate developed a continuous automatic method. Kleyn and Lin (86) utilizing phenolphthalein monophosphosphate as substrate devised a test for determining residual phosphatase in pasteurized milk, skim milk and cream.

Lipase.

Because of its role in the flavour development, milk lipase has been extensively studied. Dorner and Widner (87) were the first to report the association of lipase with casein. Subsequently, several reports have appeared (88-92) regarding its association with casein fractions. Recently, Downoy and Murphy (93) have reported the role of lipase in maintaining equilibrium between micellar and soluble casein in milk. Shahani and Coworkers (94-97) obtained an electrophoretically and ultracentrifugally pure and homogeneous lipase with a molecular weight of 9000. While studying the effect of milk constituents it was found that all salts, whey proteins, casein and casein fraction like $\alpha_s$ and $\beta$-caseins inhibited the enzyme; whereas $k$-casein and $\gamma$-globulin stimulated. The specificity of lipase in relation to the fatty acid composition in the glyceride structure was also studied. Jensen et al (98) found that lipase action was inversely proportional to the chain length of fatty acids in the triglyceride molecule.

Fox and Tarassuk (99) obtained ultracentrifugally homogeneous preparation of lipase from skim milk. It is
now known that milk contains two lipases. Plasma lipase associated with casein and the other membrane lipase adsorbed on the fat globule membrane. Kannan and Basu (100) studied lipase content in cow and buffalo milk and reported that buffalo milk contained slightly lower lipase activity than cow.

**Xanthine oxidase.**

Aurand and Woods (101) reported that the occurrence of spontaneously oxidized flavour in milk was dependent upon the high level of xanthine oxidase and such idea was supported by Smith and Dunkley (102). The ability of this enzyme to express \( \text{H}_2\text{O}_2 \) formation in free fat phase of milk lipid was confirmed recently by Della Monica et al. (103). Avis and Coworkers (104-106) crystallized the enzyme, which was a flavoprotein containing protein, F.A.D: MO: Fe in the ratio of 1:2:(1.3-1.5):8. The molecular weight was found to be 300,000. The amino acid composition has also been investigated. Muraok (107-109) studied the effect of histamine, KCN, and EDTA, whereas Fridovich (110) and Greentee and Handler (111) investigated the effect of urea, guanidinium ions and amino group reagents. Presence of activators and inhibitors of xanthine oxidase has just recently been investigated by Hwang et al. (112).

Besides being present in the milk of cow, it has been observed in the milk of buffalo, sheep and goat but the milk of cow, mare and human was found to be devoid of it (113, 114).
Lactose synthetase.

Babad and Hassid (115, 116) recently demonstrated the presence of lactose synthetase in bovine milk. Ebner and Coworkers (117-121) studied this enzyme extensively. While purifying the enzyme on Bio-Gel P-30 column obtained two protein peaks (A and B), both were enzymatically inactive, and gained activity when combined together. The B protein was finally identified as ç-lactalbumin. ç-lactalbumin isolated from the milk of different species was enzymatically active with the A protein of respective species. The A protein of bovine milk could also synthesize lactose when united with the ç-lactalbumins isolated from milk of different species indicating a nonspecific nature. The A protein has recently been identified by Brew et al. (122) as galactosyl transferase for which N-acetyl glucosamine is an acceptor. This A protein is present in many body tissues and when combined with ç-lactalbumin acts as lactose synthetase.

Catalase.

Kannan and Basu (123) studied catalase content in the milk of various species and reported its concentration in the order as follows: cow > buffalo > sheep > goat. Catalase is reported to be present both in cream and skim milk of bovines. Coprecipitation of this enzyme with casein suggested the association of this enzyme with casein micelles (124). Not much of work has been done on this enzyme but its level has been found to be high in colostrum and mastitis milk.
Peroxidase.

Bovine milk has been reported to contain lower peroxidase than buffalo (125) and also present in the milk of ewe, human and mare (2). Although the enzyme has been obtained in a pure and crystalline form from bovine milk (126-128), its distribution pattern in milk is not known. Its concentration in skim milk was found to be 30.3 mg/litre. It has been proved that the enzyme is a heme protein with an iron content of 0.669 per cent. Morell (129) studied the kinetics of the enzyme.

Acid phosphatase.

Bovine milk contains an acid phosphatase with a pH optimum of 4.75 which is highly basic protein. Mullen (130) studied the variation of the enzyme in cow milk and in the milk of different quarters during the progress of lactation. The enzyme has been purified 40,000 fold by Bingham and Zittle (131). Since its concentration in cream was twice as that in skim milk it appeared to be associated with microsomal particles of fat globule membrane. The enzyme was inhibited by Mg$^{2+}$ and fluorides. Kresheck et al. (132) worked out the kinetics of bovine milk acid phosphatase and reported an optimum pH of 4.9 at 38°C for p-nitrophenyl phosphate as substrate. The $K_m$ was found to be $2 \times 10^{-5}$ M and the activation energy for the enzyme is 9100 cal/mole.

Lysosome.

This enzyme appears to have physiological significance and also associated with the keeping quality of milk. The
enzyme has been extensively studied by Shahani and Coworkers (133-137). The enzyme has been purified from bovine and human milk. The enzyme from both these sources are highly basic proteins with an optimum pH of 9.5 and 11.0, respectively. The enzymes were fairly heat stable as that of egg white lysozyme. Enzyme from both the milks were activated by several salts, but bovine milk lysozyme exhibited no activity in a salt free system unlike human milk lysozyme.

Protease.

It is established that bovine milk contains protease as a native ingredient (138). Warner and Polis (139) found that almost all the proteolytic activity of milk is precipitated with casein. Hipp et al (140) reported that protease was concentrated mainly in \( \kappa \)-casein fraction which could be separated by fractional precipitation with alcohol. Zittle (141), recently observed that protease accompanies \( \kappa \)-casein and could obtain a 20-fold concentration of the activity. Murthy et al (142) observed proteolysis in sterilized milk. This suggested reactivation of protease which was found to be specific for \( \beta \)-casein.

Ribonuclease.

Bovine milk is a fairly rich source of ribonuclease. It appears to be associated with fat globule membrane microsomal fraction and may thus effect the stability of fat emulsion. Bingham and Zittle (143, 144) isolated two separate enzyme ribonuclease A and B from bovine milk.
Groves (145) just recently crystallized the enzyme and found that ribonuclease A of milk depending upon its electrophoretic mobility, immunological characteristics and amino acid composition, appeared to be identical with pancreatic ribonuclease A.

Other enzymes studied or reported to be present in bovine milk are aldolase, \(\alpha\)- and \(\beta\)-amylases, carbonic anhydrase, cytochrome-c-reductase, diaphorase, esterase, arylesterase, rhodanase, sulphhydril oxidase, \(\alpha\)-mannosidase, \(\beta\)-galactosidase, and \(\gamma\)-glutamyl transpeptidase (146).