CHAPTER V

DISCUSSION
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During the present investigation it has been observed that alkaline phosphatase level in milk varied from animal to animal and species to species. The average activity values of this enzyme suggested that in case of buffalo milk the enzyme level was about three times low compared to cow milk (Table 1). This type of individual and species variation in the level of alkaline phosphatase was also reported by Kannan and Basu (8). Mohamed and EL-Rafey (12) also found that Egyptian buffaloes milk contained lower alkaline phosphatase activity than the local and Brown Swiss breeds of cow.

The distribution studies by mechanical, chemical and biochemical fractionation of milk into various fractions revealed its varying extents in all the major fractions of milk (Tables 2-5, Fig. 31). Skimming of milk showed that about 40-45 per cent of the enzyme activity was confined to the cream fraction and the rest was distributed in the skim milk phase (Tables 2, 13a, b and Fig. 31). This suggested that the total activity of the enzyme was high in skim milk but the specific activity was high in cream phase. These results concur with those of Morton (9) who observed that 30-40 per cent of the enzyme is adsorbed on the fat globule and the rest is distributed throughout the skim milk in the form of lipoprotein complex. Association of this enzyme with the milk fat phase was also reported by other workers (12-14, 17, 18).
The ultracentrifugal fractionation of whole milk into fat, ultracentrifugal serum, opalescent layer and micellar casein revealed that about 45%, 20%, 25% and 10% of the enzyme activity was present in these fractions, respectively (Tables 3, 13a, b and Fig. 31). Although all these fractions from buffalo milk showed lower alkaline phosphatase activity than the corresponding fractions from cow milk, whereas the distribution pattern was practically identical in the milk of both the species (Fig. 31). These findings are in agreement with the findings of Kitchen et al (182).

The localization of the skin milk phase enzyme in the opalescent layer was also investigated by Gammack and Gupta (19) in bovine milk. They concluded that the lipoprotein particles in the aqueous phase are derived from the same cellular membrane which provides the surface layer of fat globules. In the present investigation it has been observed that the opalescent layer in buffalo milk was half compared to cow milk quantitatively. Qualitatively too it showed lower alkaline phosphatase activity in buffalo milk (Table 3 and Fig. 31). For the low level of this enzyme in buffalo milk Mohamed and EL-Rafey (12) pointed out a probable reason as a possible difference in the masking effect of fat in the different milks. Although such reasoning could only be supported if the difference in the nature of buffalo and cow fat globule membrane is worked out.

The present observation on the considerable alkaline phosphatase activity in acid and micellar caseins (Table 5) and that of Zittle et al (17) and Morton (9) could be due to
the sedimentation of some of the lipoprotein particles. Invariably acid caseins have shown higher alkaline phosphatase activity than micellar caseins (Table 5). This observation is not surprising since Zittle et al. (17) and Morton (9) observed the activity of this enzyme in caseins prepared by acid precipitation, salt fractionation and rennet coagulation. The high activity of this enzyme in acid casein may be due to the coprecipitation of the lipoprotein particles to which the enzyme is associated as suggested by Zittle et al. (17).

The fat globule membrane and the microsomal fractions isolated from buffalo milk showed lower alkaline phosphatase activity compared to cow milk (Tables 6a,b and 7a,b). This suggests that such membrane or microsomal fraction is of poorer quality as regards the activity content of this enzyme in buffalo milk is concerned. Increased activity of alkaline phosphatase in aqueous phase due to butanol and deoxycholate treatment suggested a lipoprotein nature of this enzyme (Table 9).

The level of alkaline phosphatase in the colostrum of buffalo and cow revealed high level of this enzyme in first day colostrum in both species. The activity level sharply decreased thereafter showing minimum activity at 7-9 post-partum followed by a slow increase again (Fig.1a,b). Further, the milk from buffaloes and cows in late lactation showed 300-1500 units of alkaline phosphatase activity which is 5-6 times higher than those in early lactation. Such observations are in accord with the observations of Kannan and Basu (8) and Mohamed and EL-Rafey (12). Similar
observations have been made by Folley and Kay (4) and subsequently by Haab and Smith (11) as well with bovine milk. Morton (9) also found that the bulk milk samples from the cows in late lactation showed high alkaline phosphatase activity. These observations justify the observed variations in the level of alkaline phosphatase in different samples from time to time.

The distribution studies of this enzyme in colostrum through ultracentrifugal fractionation has been attempted for the first time. This study clearly revealed an identical pattern of distribution of this enzyme in various ultracentrifugal fractions of both buffalo and cow colostrum (Fig. 1a, b), buffalo milk having lower activity. Gradual increase in the sedimentable micellar casein up to 5th day post-partum (Fig. 1a, b) proportionately had increased enzyme activity. The different levels of alkaline phosphatase in colostrum and subsequently in milk suggests that the sequential physiological function of the mammary gland might be responsible for the variations in alkaline phosphatase activity throughout the lactation.

Milk alkaline phosphatase level was higher than that of blood serum of the corresponding species (Table 10). These data further suggest that buffalo blood serum contained lower alkaline phosphatase activity than the cow blood serum. These results therefore suggest and strengthen the findings of Folley and White (3) and Folley and Kay (4) that milk alkaline phosphatase is not of blood origin and is a component coming from the mammary gland. The low level of
alkaline phosphatase in buffalo milk could be presumably a species characteristic and the primary reason may be probably confined to the mammary gland which is responsible for the secretion of these metabolic components in the milk.

Purification of alkaline phosphatase from cream and the skim milk phase of buffalo milk has been attempted for the first time. The enzyme from these two fractions of buffalo milk with the use of n-butanol and Sephadex gel filtration could be purified to about 950 and 450 fold, respectively (Tables 12a and 13a). Similar results have been obtained for the cow milk enzyme also (Tables 12 b and 13 b). However, this enzyme from bovine skim milk and cream has been purified to 1000 and 5000 fold by Zittle et al. (26) and Morton (27), respectively. The final preparations of alkaline phosphatase from buffalo milk contained lower activity per unit weight of protein compared to that of cow milk enzyme. This observation strengthens the view that buffalo milk initially contains low alkaline phosphatase activity.

The observed Km values of $6.6 \times 10^{-4} \text{M}$ and $2.6 \times 10^{-4} \text{M}$ for the substrate p-nitrophenyl phosphate for the skim milk and cream phase enzyme, respectively, suggests differential affinities of this enzyme isolated from the two phases of milk (Fig. 4a,b). Barman and Gutfreund (174) observed Km value for p-nitrophenyl phosphate as $4.7 \times 10^{-4} \text{M}$ for bovine cream enzyme, whereas Morton (35) using disodium phenyl phosphate and phosphocreatine calculated the Km values to be $6.1 \times 10^{-4} \text{M}$ and $3.5 \times 10^{-2} \text{M}$, respectively, for these
substrates. Zittle and Bingham (175) found this value to be $1.6 \times 10^{-2}$ M and $1.7 \times 10^{-4}$ M using O-carboxyphenyl phosphate and disodium phenyl phosphate as substrates, respectively, for bovine milk enzyme.

The pH optimum for alkaline phosphatase from the milk of both the species appear to be at about 9.5 (Fig. 3). A wide variation of pH optimum has been observed by various workers for different substrates in different buffer systems. Aschaffenburg (83) found an optimum pH of 10.0 for bovine milk alkaline phosphatase using p-nitrophenyl phosphate in carbonate-bicarbonate buffer system. Mohamed and EL-Rafey (12) reported optimum pH at 9.5 for buffalo and cow milk alkaline phosphatase using disodium phenyl phosphate as the substrate. Horton (29) reported the pH optimum as 9.65 for purified bovine milk alkaline phosphatase using β-glycerophosphate as the substrate. The observed variations in pH optimum of this enzyme could be due to the nature of substrate and the constituents in the buffer used.

The enzyme from both the species hydrolysed various sugar phosphates at comparable rates, but slower than the reference substrate i.e. p-nitrophenyl phosphate (Table 14). The pentose phosphates in general were hydrolysed slower than the hexose phosphates. This type of substrate specificity of buffalo milk alkaline phosphatase is in general agreement with the observations made by Morton (35). This author observed that all the true orthophosphate monoesters were hydrolysed by the purified bovine milk alkaline phosphatase. The enzyme preparations in the
present investigation appeared to contain some pyrophosphatase activity although insignificant. The micellar casein was dephosphorylated at a slower rate than the acid casein by the purified enzyme (Fig. 5a). Maximum dephosphorylation occurred between pH 6-7 (Fig. 5b). These data are in general agreement to those of Zittle and Bingham (47) who observed dephosphorylation of casein to the extent of 80 per cent. The low rate of dephosphorylation observed in the present studies could be mainly due to the differences in the extent of purification and concentration used of the enzyme preparations.

The observed thermal inactivation of the purified enzyme suggests that about 98 per cent of the enzyme activity is lost at 70°C with a holding period of 15 seconds and such thermal inactivation was almost similar for buffalo and cow milk enzymes (Fig. 6). Mohamed and EL-Rafey (12) reported that the enzyme in milk system gets inactivated at 70°C. These are in close agreement with the present data on the purified enzyme. Although the rate of phosphatase inactivation has been reported to vary depending upon the composition of milk product and the pH of the system (50). At the pasteurization temperatures, the enzyme gets completely inactivated (13). Heat inactivation of the enzyme in the bovine milk system has been studied by various workers (14, 49-51), in different ways. It is difficult to compare these published data because of different experimental approaches.

Incubation of alkaline phosphatase with different
concentrations of urea inactivated the enzyme to varying extents (Fig. 7a, b). This could be due to the denaturation of the enzyme molecule. The irreversible inhibition of alkaline phosphatase with urea suggests an irreversible denaturation of the enzyme (Table 15). Such studies have been carried out on the alkaline phosphatase from liver, kidney and intestine (173). The results of present investigation relate the milk and intestinal alkaline phosphatase as regards the irreversible inactivation of the enzyme. The inactivation effect of 2-mercaptoethanol was found to be reversible after dialysis (Table 15). This inhibition may be because of the inhibitory effect of the reagent or it may be due to the cleavage of the -S-S- linkages of the enzyme molecule.

Loss of the enzyme activity due to blocking the N-terminal amino groups of the enzyme molecule may account for either the involvement of this group in the catalytic action or it may be due to a simple inhibitory effect of the added FDNB (Table 16). The influence of amino-group modifying reagents has been studied by Ghosh and Fishman (170) for the rat intestinal alkaline phosphatase where they observed a considerable effect of O-methylisourea, acetic anhydride, formaldehyde and others. Morton (172) also suggested ε-amino group of lysine could well be in the active site. On the basis of the present experimental results, metal, thiol and amino groups can be implicated in the mechanism of catalysis of hydrolysis of monophosphoric acid esters by milk alkaline phosphatase.
The action of rennet and trypsin on the purified alkaline phosphatase suggested that these two enzymes did not alter the active site of milk alkaline phosphatase (Table 17). The observed stimulatory effect of trypsin could be due to the presence of some activating contaminant in the trypsin powder used.

The purified enzyme from both buffalo and cow milk was activated by Mg\(^{2+}\), Mn\(^{2+}\) and Ca\(^{2+}\) ions (Fig. 9 and Table 20). Such observations were also reported by Morton (29). EDTA inhibited the enzyme and addition of Mg\(^{2+}\) ions could reverse the inhibition (Fig. 9). The observations of Anderson (39) lend support to the present findings. These results therefore suggest that like bovine milk enzyme, the buffalo milk alkaline phosphatase also requires metal ions like Mg\(^{2+}\) for its optimal activity.

Inhibition of the enzyme by the addition of p-chloromercuribenzoate (Fig. 11) suggested the involvement of -SH group of the enzyme in its catalytic action. A similar conclusion is drawn from the inhibition of the enzyme by N-ethylmaleimide (Fig. 12). The participation of free -SH groups of alkaline phosphatase from other sources has been well established (170). Addition of cysteine upto a concentration of 0.05 mM stimulated the enzyme activity and beyond this concentration inhibition was observed (Fig. 10). Morton (35) reported similar data on bovine enzyme. However, the stimulatory effect of cysteine at low concentration is not surprising since Ghosh and Fishman (170) observed this type of stimulation and
inhibition of the rat intestinal alkaline phosphatase at low and high concentrations. Although no explanation could be offered for this except a possible role of providing the thiol groups to the enzyme molecule. Inhibition of alkaline phosphatase at high concentration of cysteine may be possibly due to the metal site or metal chelation by cysteine (35, 170, 171).

The molecular sieving of the whole cream extract (2nd butanol step) on Sephadex G-200 column resolved into two enzyme peaks namely peak I (PI high molecular weight) and peak II (PII low molecular weight), (Fig.13). The enzyme preparation from the skim milk phase resolved to give only one peak corresponding to the PII of the cream phase enzyme (Fig.13). These observations suggested that the cream phase contained at least two multiple forms of the enzyme and the PI (high molecular weight) enzyme appeared to be missing in the skim milk phase. Copius Peereboom (30) found that the cream phase enzyme resolved to give two isozyme spots on Sephadex gel by permeation chromatography. Lefran and Han (69) reported that alkaline phosphatase preparations from the bovine butter milk resolved into two enzymes (A and B) on DEAE-cellulose column. The B enzyme was of high molecular weight. Brunia and Martin (70) also reported that the milk of cross bred animals showed the presence of four isozymes on Sephadex gel filtration whereas Romanian pure bred cows milk contained less multiple forms of this enzyme.

The enzyme purified from the milk of both buffalo and cow contained bound sialic acid (Fig.14 and Table 18,19)
and it was found to be high in the enzyme preparations from cream phase (Table 19). The findings of Copius Peereboom (30) lend support to these observations who found the release of sialic acid from alkaline phosphatase of milk by neuraminidase. An alteration in the electrophoretic mobilities of alkaline phosphatase due to such action of neuraminidase was also observed. Association of sialic acid with alkaline phosphatases of other sources like kidney, human placenta and sheep brain was also reported by Moss et al (176), Ghosh et al (177) and Saraswathi and Bachhawat (178), respectively.

The agar-gel electrophoresis of the whole cream extract (2nd butanol step) showed the presence of three alkaline phosphatase zones namely 'A' (slow moving), 'B' (fast moving) and 'C' appearing towards the negative electrode (Fig. 15). The skim milk phase alkaline phosphatase preparation isolated from the opalescent layer had only 'B' enzyme zone of cream when subjected to electrophoretic run under the identical conditions (Fig. 15). This observation suggested a differential isozyme pattern of alkaline phosphatase in the two phases of milk of both buffalo and cow. Copius Peereboom (30, 68) also observed the presence of at least three enzyme zones namely Α, Ε and Υ- alkaline phosphatases.

The polyacrylamide disc electrophoresis of the whole cream extract showed the presence of at least three alkaline phosphatase bands namely A₁ (fast moving), A₂ (slow moving) and another 'B' band which did not migrate much from the
point of application of the gel rod. However, it developed an extended patch without any resolution (Fig. 16). These results on buffalo and cow milk alkaline phosphatase were practically identical. Recently, Copius Peerboom (30,68) also observed a similar pattern with bovine cream alkaline phosphatase. The PI enzyme of cream (high molecular weight) obtained through Sephadex column, when subjected to polyacrylamide disc electrophoresis showed the presence of three bands (A1, A2 and the non migrating) of alkaline phosphatase as observed in the original extract before fractionating it on Sephadex column (Fig. 16). The PII (low molecular weight) enzyme did not show any non migrating enzyme protein and resolved into the other two bands (A1 and A2) observed in the PI enzyme, although it showed a high concentration of A1 and A2 enzyme (Fig. 16). These results therefore suggested that the PI enzyme had some additional form of alkaline phosphatase and also revealed that the two peak enzymes were contaminated with each other and hence have shown similar isozymes with different concentrations. The results also revealed that the skim milk enzyme pattern resembled to the PII enzyme of cream phase. The foregoing discussion therefore entail to conclude that the isozyme pattern of buffalo milk alkaline phosphatase closely resembles the bovine milk alkaline phosphatase on the basis of our observations.

On the electrophoretic mobilities and the molecular size, if a faster moving (band B) lower molecular weight enzyme (PII) is named as \( \cdot \)-alkaline phosphatase,
conventionally the slower moving (band A) higher molecular weight enzyme (PI) becomes the β-enzyme. The skim milk phase enzyme may therefore represent the <i>α</i>-alkaline phosphatase since it showed the similar mobility and molecular size as the <i>α</i>-enzyme in cream phase (Fig. 15).

The observed stimulatory effect of milk and milk dialysate (Fig. 17) on the alkaline phosphatase of both buffalo and cow milk provides clear indication that both major and minor constituents are likely to participate in such stimulation. The data on dialysed milk (Fig. 17) further ensure that the stimulatory factors are more likely to reside in the milk dialysate. The stimulation observed due to dialysed milk (Fig. 17) although relatively much less prominent, further indicates the participation of major nondialysable constituents in alkaline phosphatase activation. The more pronounced effect of milk dialysate in case of buffalo could be due to the higher concentration of dialysable constituents in this milk compared to that of cow milk (179).

It is further evident from the study of dialysable constituents on this enzyme (Table 20) that metal ions like Ca<sup>2+</sup>, Mg<sup>2+</sup> and Mn<sup>2+</sup> exhibited dramatic stimulation (3-4 fold in case of buffalo, 1.5-2.0 fold in case of cow) of alkaline phosphatase activity. It was also reported earlier by Kannan and Basu (33) and Morton (29) that these ions do stimulate the enzyme activity. Whereas another milk enzyme namely lipase was observed to be inhibited by these metal ions (180). The inhibition by PO<sub>4</sub> of enzyme supports the
observations by Morton (29) who indicated that PO$_4$ acts as competitive inhibitor of alkaline phosphatase.

The stimulation of enzyme activity by dialysed milk can be explained on the basis of observations on the individual milk proteins (Fig. 18). It appears that the integrity of the casein micelle may not contribute to such stimulation since acid casein, k-casein, $\alpha_s$-casein or $\beta$-casein were effective in such process (Table 21). Shahani and Chandan (180) observed inhibition of milk lipase with $\beta$-casein which further indicates the specificity of milk enzymes towards the effect of milk constituents. The relatively greater stimulation by boiled casein micelle can be attributed to the available Ca$^{2+}$ and Mg$^{2+}$ ions for the enzyme from the casein micelle due to heat treatment.

Among whey proteins tested (Fig. 19), $\beta$-lactoglobulin was most effective which could be due to the -SH groups provided by this protein to this enzyme. It is logical to think so, since cysteine at low concentration had shown stimulatory effect on this enzyme (Fig. 10). Fishman and Ghosh (170) also observed that cysteine at low concentration stimulates the intestinal alkaline phosphatase. $\kappa$-lactalbumin appears to have no role in this enzyme activity unlike its participation in lactose synthetase activity (118). The stimulatory effect of proteose-peptone on alkaline phosphatase is another example of its stimulatory role as reported in case of xanthine oxidase (181). It is therefore concluded that the higher protein, fat, lactose and ash in buffalo milk compared to that cow milk appear to have no relation with the low alkaline phosphatase activity in buffalo milk.
Studies on the reactivation of buffalo milk alkaline phosphatase have been carried out for the first time and the results have suggested that whole milk samples pasteurised either by high-temperature-short-time or hold method did not show any reactivation of this enzyme in buffalo as well as in cow milk. Such observations are in agreement to those of Wright and Tramer (36,40), O'Sullivan and Shippe (59), Fram (57) and Richardson et al (60). These workers found that reactivation of alkaline phosphatase in bovine milk was possible when it was subjected to temperatures higher than pasteurisation and for a short holding period. Buffalo milk samples when subjected to higher heat treatments ranging from 80°C to 100°C showed reactivation of alkaline phosphatase (Table 22) and its dependence on the way of cooling suggested that a short time exposure to such temperatures was essential (Table 24).

When buffalo milk and the major milk fractions like skim milk and cream were subjected to high heat treatments for shorter time, reactivation of the enzyme was highest (10-13%) in cream and lowest (0.3 to 0.6%) in skim milk (Table 23). These results also revealed that the optimum period for the reactivation of this enzyme in milk or skim milk was 10-12 hours whereas for cream, 14-16 hours optimum period was noted (Fig. 20). These results are in general agreement to the observations made by O'Sullivan and Shippe (59) who found 0.5 to 1.5% reactivation in the milk of individual cows heated at 100°C for 45 seconds and incubated for 18 hours after heating. Fram (56,57) also observed
that the temperature of heating and subsequent storage period for the reactivation varied in milk, skim milk and cream. Eddleman and Babel (58) also made similar observations on the reactivation of bovine milk alkaline phosphatase.

The optimum temperature for the storage of heat-treated buffalo milk, skim milk or cream for the reactivation of this enzyme compared to that of cow suggests that there was not much of variation in the level of reactivation when heat treated samples were stored at temperatures 30°C and 34°C. But storage at 37°C decreased the level of reactivation considerably (Table 25). Such observations have also been made by Fram (57) who found that reaction of enzyme in heat treated cream (80°C for 15 seconds) occurred when stored at 85°F and below and above this temperature, reactivation rate was slowed down. Richardson et al (60) observed that reactivation of the enzyme was optimum at 34°C storage temperature and slight reactivation occurred at 10°C or below. Simonart and Huberlant (61) found that commercially pasteurized cream stored at 26°C to 37°C for 16 hours exhibited reactivation for the enzyme.

Addition of magnesium ions to milk did not increase the level of reactivation, but such addition to the cream doubled the level of reactivation (Table 26, Fig. 21). It appears therefore that in milk probably the naturally occurring magnesium is sufficient to serve the purpose whereas in cream for higher level of reactivation, addition of magnesium is essential. The requirement of divalent cation specially magnesium for the reactivation of alkaline
phosphatase in general and higher levels of reactivation in particular have been observed by most of the workers (23, 36, 54, 59, 60, 66).

Cream phase of either buffalo or cow milk has shown higher level of reactivation compared to that of whole milk or skim milk phase. Buffalo cream phase under the identical conditions of heat-treatment and storage has shown lower level (2-3%) of reactivation compared to the corresponding samples of cow (Table 23). This observation is not surprising since most of the workers on bovine cream have observed higher reactivation compared to the milk (54, 62, 75) and various reasons have been put forth. Lyster and Aschaffenburg (28) found the presence of heat labile inhibitor of reactivation which survived at pasteurization temperatures. Wright and Tramer (54) could not establish any correlation between fat content and the level of reactivation in bovine milk. Paschke (62) found that reactivation occurred in products of high fat content. McFarren et al (76) reported that level of reactivation varied with the fat content of the product and recently, Richardson et al (60) by maintaining a constant enzyme concentration and varying the amount of fat, found that reactivation did not vary with fat content provided the amount of enzyme is held constant. Lyster and Aschaffenburg (28) also observed that the amount of enzyme activity is a governing factor of the level of reactivation. Since buffalo milk invariably contained high fat, but low alkaline phosphatase compared to that of cow milk, the findings of
Lyster and Aschaffenburg (28) and Richardson et al. (60) appear to hold reasonable for the low level of reactivation observed in buffalo milk or cream. Dilution of cream in various proportions with heat treated skim milk reduced the level of reactivation (Table 28). This could be due to the reduction of total enzyme activity in the reactivation system. Recent findings of Copius Peereboom (29) have revealed the presence of \(\alpha\) and \(\beta\)-alkaline phosphatases in cream phase. \(\beta\)-alkaline phosphatase capable of reactivation, had been shown to be present exclusively on the fat globule membrane.

Addition of whole casein to cream fraction of buffalo milk did not show any effect on the reactivation of alkaline phosphatase (Table 29), but its addition to the purified enzyme increased the level of reactivation (Fig. 25). It appears therefore that the amount of casein residing in the cream phase is sufficient in the reactivation process and hence extra addition of casein did not show further effect.

Addition of three antibiotics (at three different levels) did not effect the reactivation phenomenon of alkaline phosphatase in the cream from both buffalo and cow milk (Table 27). Such observation therefore provides convincing evidence to believe that the reactivated alkaline phosphatase in milk is not of bacterial origin. Wright and Tramer (40) by adding penicillin to the heat treated milk samples also reported similar data. Although the possibilities of bacterial phosphatase production have been reported (71-74), depending upon the thermostability of milk.
and bacterial phosphatase methods have been devised to differentiate the enzyme from these two sources (53, 74, 75).

Since cream fraction was found to give high level of reactivation compared to that of milk and skim milk, it was therefore thought to isolate and purify the enzyme from the reactivated cream. The purification data (Table 30) suggested that the procedure of purification adopted for the raw cream enzyme could also be applied for the reactivated cream. The specific activity data revealed that buffalo cream phase reactivated enzyme had two times higher specific activity than the skim milk phase enzyme and about half to that of raw cream phase enzyme (Tables 12a,b, 13a,b and 30).

Whereas in case of bovine cream reactivated enzyme, the specific activity was about 5 times less than the raw cream enzyme and half to that of skim milk phase enzyme. These observations further showed the differences in the degree of purification of this enzyme isolated from the raw and reactivated cream phase of both buffalo and cow milk.

While studying the kinetics and the properties of the enzyme isolated from the reactivated cream of both buffalo and cow milk, it was observed that the reactivated enzyme hydrolysed p-nitrophenyl phosphate at comparable rates to that of raw enzyme. The Km value (6.6 x 10^{-4} M) was identical to that of the skim milk phase raw enzyme (Fig. 4b). The pH optimum (about 9.5) for the reactivated enzyme was also similar to that of raw enzyme (Table 32). The reactivated enzyme was activated by the addition of magnesium and was inhibited by EDTA (Tables 33, 34).
Thermal inactivation pattern also revealed an identical nature of the raw and reactivated alkaline phosphatase (Table 3). Such results on the similarity of raw and reactivated alkaline phosphatase are in agreement with the observations of Wright and Tramer (40). They observed that the reactivated enzyme (reactivated milk) behaved similarly to that of raw enzyme as regards the optimum pH, substrate specificity, rate of substrate hydrolysis and heat stability. Such similarities in the properties of the enzymes in reactivated milk lead them to conclude the identical nature of the enzyme in raw and reactivated forms.

The thermal inactivation rate of raw and reactivated alkaline phosphatase (Table 3) further establishes that the reactivated enzyme is not of bacterial origin. Barber and Frazier (53) reported that bacterial alkaline phosphatase could withstand heat treatment as high as 76.7°C for 30 minutes while milk alkaline phosphatase was inactivated in 30 minutes at 62.8°C. Similarly Tittsler et al. (75) found that milk alkaline phosphatase was destroyed completely at 70°C for 1.5 minutes whereas microbial phosphatases were generally not inactivated appreciably at 70°C for 5 minutes holding period.

The Sephadex G-200 gel filtration pattern of reactivated alkaline phosphatase of buffalo milk revealed that unlike raw alkaline phosphatase it resolved to give only one enzyme peak corresponding to the Peak II alkaline phosphatase of raw cream (Fig. 22). This further suggested that the reactivated enzyme was identical in its molecular
size to that of raw enzyme of skim milk phase isolated from the opalescent layer (Fig. 13).

The agar gel electrophoretic pattern of the raw and reactivated alkaline phosphatase isolated from the cream phase indicated that the reactivated enzyme preparation gave only one alkaline phosphatase band which had mobility identical to the \( \alpha \)-alkaline phosphatase of the raw cream (Fig. 13). The protein staining results showed that presence of at least four bands, two corresponding to the A and B \( \alpha \)-alkaline phosphatases and the other two bands had higher mobilities than these two protein bands. These results differ from the observations of Copius Peereboom (67) who found that the \( \alpha \)-enzyme (A, as designated here) was capable of reactivation.

The polyacrylamide disc electrophoresis of the enzyme isolated from the reactivated cream did not show the non-migrating enzyme protein at the point of application and resolved into two fast moving enzyme bands (Fig. 16). The enzyme positive peak II (Fig. 16) obtained through Sephadex gel filtration also revealed the presence of similar enzyme bands. The enzyme pattern of the reactivated cream resembled closely to those observed by Copius Peereboom and Beekes (198) who described these as the degradation products of the \( \beta \)-enzyme present exclusively on the fat globule membrane. It appears from these results that the isozyme patterns of raw and reactivated cream differ greatly both on the agar gel and polyacrylamide gel electropherograms and such differences can be utilized for recognising the enzyme in raw and reactivated forms.
The reactivated enzyme on the basis of per unit protein content contained higher sialic acid whereas on enzyme activity basis, it showed less sialic acid compared to that of raw alkaline phosphatase (Table 35). Such differences are likely to be due the variations in the degree of purity of the enzymes isolated from the raw and reactivated cream. The results revealed further that the sialic acid content of the reactivated peak II enzyme was about half to that of peak I enzyme of raw cream. This may however suggest the degradation of peak I during heat-treatment, incubation for reactivation or during isolation and therefore may give rise to peak II, although this remains to be confirmed.

Association of sialic acid with alkaline phosphatase of milk and other sources has been demonstrated by various workers (176-178).

Reactivation of alkaline phosphatase in bovine milk and in major fractions of milk like cream, skim milk and milk products (mainly in butter) had been studied by various workers (40, 56, 59, 60, 64). Such studies have definitely established that the occurrence of reactivation varies with time-temperature combinations, nature of the milk system, storage conditions of the heat inactivated system, pH of the system and several other factors. The results of present investigation on the enzyme in buffalo milk system or its major fractions has also elicited similar facts. To prove unequivocally the phenomenon of reactivation, successful attempts have been made to demonstrate the reactivation of purified enzyme from bovine milk in the absence and presence
of milk and its major milk constituents (28, 65).

In the present investigation some preliminary studies have been also made on partially purified enzyme preparations of buffalo milk in comparison to the bovine milk alkaline phosphatase. Maximum reactivation of the enzyme was observed at pH 6.8 and 6 hours of storage at 34°C. Below and above this pH value the level of reactivation was greatly affected (Fig. 23). This suggested that milk pH value is quite conditional for the reactivation of this enzyme. Magnesium ions were not essential for reactivation but addition of such ions up to 20 mM concentration definitely doubled the level of reactivation (Fig. 24) and beyond this concentration concomitant reduction in the level of reactivation occurred. Such inhibition could be due to the high concentration of chloride ion.

Addition of β-lactoglobulin to the enzyme-magnesium mixture increased the level of reactivation by 4-5 fold at 0.05 per cent level, whereas the addition of α-lactalbumin in place of β-lactoglobulin did not increase the level of reactivation (Fig. 27). When β-lactoglobulin was replaced by cysteine or reduced glutathione no satisfactory stimulation of reactivation was observed (Table 38). Micellar and acid caseins from 5-10 mg concentration showed similar effect on reactivation like that of β-lactoglobulin. Although addition of micellar casein showed slightly higher level of reactivation (0.5%) compared to acid casein (Fig. 25). Among casein fractions, κ-casein was found to be equally effective in this respect like that of micellar or acid
casein, but to achieve similar level of reactivation its quantity needed was just double that of acid or micellar casein (Fig. 26). 2-mercaptoethanol treatment of k-casein and its subsequent dialysis before adding to the enzyme solution did not change the behaviour of this protein in relation to the reactivation of alkaline phosphatase. \( \alpha \)- and \( \beta \)-caseins have also been found to increase the level of reactivation but their effect was lower than the whole casein or k-casein at similar concentrations (Fig. 26).

These observations on the reactivation of purified buffalo milk alkaline phosphatase are parallel to those of Lyster and Aschaffenburg (28) and Kresheck and Harper (64), with a main difference in the reactivation levels. Lyster and Aschaffenburg (28) observed reactivation varying from 10 to 30 per cent of the original enzyme activity whereas in the present studies reactivation could be noticed only up to 5 per cent. This observed low level of reactivation appears to be firstly due to the differences in the reactivation systems. Lyster and Aschaffenburg (28) used a system consisting of enzyme with 20 mM magnesium, 20 mM \( \beta \)-glycerophosphate and 0.2 per cent \( \beta \)-lactoglobulin and found that absence of any component from this simple system greatly reduce the level of reactivation. In the present system with buffalo milk alkaline phosphatase, \( \beta \)-glycerophosphate was not used which could be a limiting factor in the extent of reactivation. Secondly, the degree of purification of the enzyme and the amount of enzyme used during heat inactivation could also be a determining factor.
of such low level of reactivation as observed by Lyster and Aschaffenburg (28). How and what roles these milk proteins play is difficult to speculate but the report by Kroshoek and Harper (85) confirm the observed high level of reactivation due to the addition of milk proteins. These workers also reported that addition of whole casein was helpful in achieving high level of reactivation and maximum reactivation was observed when a mixture of whole casein deproteinized whey, whey proteins and sodium acetate was added. These results and those available in the literature indicate that some of the milk constituents specially Mg$^{2+}$ and the protein fractions have significant importance in the phenomenon of reactivation of this enzyme. It appears from these studies that the mechanism of reactivation of alkaline phosphatase in the milk of both the species is similar.

The continued interest of the workers in phosphatase test has resulted either in developing new or modifying the existing techniques so as to rigg up simple procedure with improved sensitivity. Kosikowski (82) added a distinct impact in overcoming precipitation of proteins, filtration and subsequent neutralization in the Aschaffenburg and Mullenb(43) phosphatase test.

The Dialysis Phosphatase test developed during the present investigation using p-nitrophenyl phosphate substrate has further simplified the procedure in the steps involved in developing the colour. In the proposed method, the post-incubation produces its own chromatogen, p-nitrophenol and does not involve the development of colour and its
subsequent processing as in the method of Kosikowski (82). The effect of substrate concentration, milk volume and incubation period for the assessment of alkaline phosphatase in raw milk is presented in Fig. 28, 29 and 30. Furthermore both the number of steps and reagents have been reduced and thereby the present Dialysis Phosphatase method using p-nitrophenyl phosphate as substrate stands to be economical in time and cost. It offers also promising sensitivity to detect the presence of 0.1% raw milk in pasteurized milk (Tables 40a, b, c).

The observed lower activity of xanthine oxidase in buffalo milk compared to that of cow milk (Table 42) is in agreement with the observations by Krishna Iyengar and Laxaminarayana (154). The distribution pattern in various ultracentrifugal fractions of milk revealed that about 50 and 60 per cent activity of this enzyme is present in fat fraction of cow and buffalo respectively (Fig. 31). The ultracentrifugal whey contained 20-30 per cent of xanthine oxidase and the remaining was found to be located in the opalescent layer. The enzyme appeared to be absent practically from the micellar casein fraction. Although the possibility of the presence of this enzyme in micellar casein could not be ruled out since the high concentration of casein is likely to exert inhibitory effect as observed by Hwang et al (112). These results are not strictly parallel to those observed by Zittle et al (17), and recently by Kitchen et al (182), although a general agreement appears to hold good. The observed high activity (10-20%)
of this enzyme in cream fraction than the reported data by other workers could be due to the method and conditions of fractionation, difference in storage conditions and the sampling variation (individual and bulk).

Buffalo milk showed lower activity of lipase than cow milk (Table 42) and such observations agree well to those of Kanna and Basu (100). The distribution studies showed highest activity of this enzyme in the casein fraction of both buffalo and cow milk which contained about 50 and 80 per cent of the original lipase activity present in buffalo and cow milk respectively (Fig. 31). This suggested that the synthesis of casein and the association of lipase to casein in buffalo milk are negatively correlated since buffalo milk in general contained higher sedimentable micellar casein but lower lipase in it, than cow milk.

The association of lipase with casein has been well documented (87-92). Association of lipase with soluble casein as suggested by Downey and Murphy (93) could also be possible since the opalescent layer and the ultracentrifugal serum also contained measurable lipase activity. These results therefore suggest that the casein fraction of milk can be a suitable material for the isolation of lipase from buffalo milk as has been used in case of cow milk lipase isolation (99).

The peroxidase activity in buffalo milk was also found to be lower than cow milk (Table 42). This observation is deviated from those made by El-Hagarawy (125) who observed higher peroxidase in buffalo milk than cow. The distribution
studies revealed that the enzyme was mainly confined to the ultracentrifugal serum and practically absent from the cream phase (Fig. 31). The striking observation noted was the loss in peroxidase activity in cow milk due to ultracentrifugal fractionation which was not observed in case of buffalo milk.

Buffalo milk showed lower catalase activity compared to that of cow milk (Fig. 31) and such observations has been made earlier also by Kannan (123). The distribution studies revealed that the skim milk phase was the main repository of this enzyme which contained about 70 per cent of this enzyme. The results further indicate that casein fraction contained higher activity of this enzyme compared to the ultracentrifugal serum and the opalescent layer. The high activity of this enzyme in casein may support the view of its association with casein as suggested by McMeekin and Polis (124). However, this particular observation is not in agreement to the recent observations of Kitchen et al (182) who observed low activity of this enzyme in casein and high in the supernatant fraction, although the enzyme distribution levels in cream and the skim milk phase are quite parallel with the present observations. The observed differences in the distribution level of this enzyme in skim milk fractions and specially in casein, could be due to multiple reasons like differential way of fractionation, temperature during fractionation, storage temperatures and the differential sensitivity of the methods used. Although the conditions of fractionation of milk used by Kitchen et al (182) are liable to change the distribution results since in the
present investigation it has been observed that lipase and catalase distribution pattern resembles closely (Fig. 31) and the activity profile of serum and the membrane lipases are greatly effected by cooling, mechanical agitation, homogenization etc. (EQ). It is therefore expected that a long time centrifugation at lower speeds under low temperature conditions are likely to change the distribution pattern if catalase is not firmly bound to the casein and thereby may appear in the serum phase of milk.

It is tempting to conclude from the distribution pattern data of these milk enzymes in buffalo and cow milk that for the isolation of alkaline phosphatase and xanthine oxidase, cream fraction can be the best suitable material whereas for lipase, casein is the main source and for peroxidase and catalase, skim milk phase can be best utilized for these purposes.