Like many other body fluids, human milk contains a large number of functionally active enzymes. The activity of different enzymes in the milk may be influenced by different factors, including genetic makeup (ethnic groups), nutritional status, and pathological conditions of the donors. The determination of enzyme concentrations in body fluids (particularly in blood serum or plasma) has become an indispensable diagnostic aid. In many illnesses, enzyme determination has superseded other diagnostic methods. Like that of other body fluids, the enzyme activities of milk may be used efficiently in clinical chemistry. The possible effects of milk enzymes on digestion of food in infants' digestive system is not yet elucidated. Some of these enzymes could withstand the proteolytic hydrolysis in gastrointestinal (G.I.) tract to a great extent and may help in the digestion of food materials in infants' diet. During storage of milk (for example in human milk banks) these enzymes may act on the respective substrates present in the milk and thereby change the nutritive and organoleptic properties of the milk. Some of these enzymes may be assayed so as to evaluate the pasteurization quality of the milk as alkaline phosphatase activity is assayed for determination of correct pasteurization of bovine milk.

In the present investigation the activities of different enzymes were determined in milks of normal healthy Indian (Bengalee) mothers. No attempts were however made to study the enzyme excretion pattern in different pathological conditions.
because that was beyond the scope of this investigation. The average normal values for the excretion of these enzymes in the milk of healthy Bengalee mothers were determined for the comparison with the values available in the literature for mothers of different other ethnic groups. The thermal stability of the enzymes were determined for their preliminary characterization.

Alkaline phosphatase of the milk is a particle bound enzyme (Shahani et al., 1973). The exact biochemical role of this enzyme which is a glycoprotein (Steck & Wallach, 1970) is yet to be elucidated. Like other phosphatases, this enzyme too may have some role in phosphate turnover in the body and in calcification of bones. Though this enzyme has been detected in human milk long before (Kon, Nawson, 1950; Stewart, Platon & Kelley, 1958), its effect on children's nutrition and physiology, if any, is not known yet. Alkaline phosphatase is detected in different tissues or cells in different molecular forms characteristic of tissues or cell types (Hamilton, Gornicki, Sussman, 1979). It has been suggested that determination of activity of alkaline phosphatase isoenzyme in blood may be helpful in detection of breast cancer (Coombes et al., 1977). In human breast, alkaline phosphatase is located on luminal surfaces of the epithelial cells of the ducts and acinar glands. During lactation, the milk fat globules pick up the enzyme from these locations (Dowben et al., 1967; Saacke & Heald, 1974; Patton & Keenan, 1975).

The normal value of alkaline phosphatase activity in transition milk of normal Bengalee mothers assayed using phenyl
phosphate as substrate ranged between 3.0 to 10.00 K.A.U. (Average \(^{4.70 \pm S.D. 1.38}\) (Table 6, Table 29). About 70\% of the sample tested contained the enzyme activity in the range of 3.0 - 6.5 K.A.U. A total of 55 samples were grouped according to mode of delivery: normal, forcep and caesarian section. It has been found that mode of delivery has no significant influence on the activity of this enzyme in the milk (Tables 6 & 7). However, presence of some disease may affect the activity of alkaline phosphatase in the milk markedly. A mother (normal delivery) who had an ischemic heart produced milk containing 17.5 K.A.U. alkaline phosphatase activity. Partial gastrectomy in another mother reduced alkaline phosphatase content to 0.8 K.A.U. However at this stage, I could not comment on the possible association of increased or decreased milk alkaline phosphatase activity with any particular disease as number of such samples tested is too small. Alkaline phosphatase activity in colostrum was about 3-4 times higher than that noted in transitional milk (Table 6, Table 29).

The pH activity profile (Fig. 1) of human milk alkaline phosphatase is almost the same as that of bovine milk enzyme assayed under identical condition. The optimum pH of activity for both cases was recorded between pH 9.8 - 10 (carbonate/bicarbonate buffer). However the human milk enzyme is more heat stable than bovine milk enzyme (Fig. 2). Alkaline phosphatase of cow milk is inactivated within the temperature range 70-75°C. Thus this property of the enzyme may be used for the testing of adequate pasteurization. But human milk after heating at 75°C
for 15 min retains enough alkaline phosphatase (5-12%) to give false positive test indicating incomplete pasteurization even after proper pasteurization. To assay this enzyme as a test for adequate pasteurization of milk from human donors may not be helpful. It is interesting to note that the presence of heat stable alkaline phosphatase (resistant to heating at 56°C for 30 min) was reported earlier in normal human placentae and in serum of pregnant woman (Elder, Bonello & Ellul, 1971). The present study indicates the possibility of the presence of such heat stable alkaline phosphatase isozyme in serum and milk during lactation. However Hamilton, Gornicki & Sussman (1979) reported earlier that milk contains alkaline phosphatase isoenzyme similar to that present in liver which is different from placental alkaline phosphatase.

Human serum lipase is almost exclusively of pancreatic origin and lipolysis of milk fat by this enzyme is studied by several authors (Jensen et al., 1963; Jensen et al., 1964b). The estimation of this enzyme may be used as diagnostic aid in cases of different pancreatic diseases (Varley, 1975). Lipolysis by human milk is caused by a mixture of milk enzymes out of which bile salt stimulated lipase is predominating. This enzyme is conspicuous by its stability in acidic environment in stomach. Human milk lipase can hydrolyse most of the milk glycerides and vitamin A esters reasonably quickly in simulated stomach and intestinal environments of the newborns (Hernell, 1975; Hernell & Olivecrona, 1974a, 1974b; Olivecrona & Hernell, 1976). The digestive power of the newborn is not fully developed at
the initial stages of life so this lipase in the human milk may be very helpful in digestion of milk fats by the newborn (Royer, 1978). This is probably only milk enzyme to which some role in infants' nutrition may be attributed at present. The major lipolytic activity of human milk is not fully expressed in absence of bile salts, therefore it does not pose problems such as development of rancidity or alteration of nutritional qualities during storage (Hernell, Gebre Medhin & Olivecrona, 1977). Nutritional status of the donors affects the milk lipase content. The secretion of this enzyme in the milk is quantitatively different depending on the ethnic origins of the mothers (Hernell, 1977).

The major lipolytic activity in Bengalee mothers' milk is stimulated in the presence of detergent. The activity was found, on the average, 5-7 times more when tween 20 was the substrate instead of olive oil (Tables 7 & 8). The detergent property of tween 20 is responsible for the stimulation in milk lipase activity. Low molecular weight organic ester (ethyl acetate) was also hydrolysed by human milk efficiently. The rate of ethyl acetate hydrolysis was faster than that of glyceride hydrolysis in the milk. It is not possible to state definitely at present whether ester and glyceride hydrolysis were carried out by two different enzymes or the lipase in the milk has low specificity and could act on organic acid esters containing very short fatty acid component. No significant difference was found in the lipase activities of colos­trum, transitional or mature milk (Table 8, Table 29) but
<table>
<thead>
<tr>
<th>Enzymes studied</th>
<th>* Enzyme activity in units/ml</th>
<th>Stage of Lactation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Colostrum</td>
<td>Transition</td>
</tr>
<tr>
<td></td>
<td>Mean ± S.D. Range of activity**</td>
<td>Mean ± S.D. Range of activity**</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>13.38 ± 1.81 9.76-17.00</td>
<td>4.70 ± 1.38 1.94-7.46</td>
</tr>
<tr>
<td>Lipase (with water soluble substrate)</td>
<td>35.30 ±12.68 10.0-50.6</td>
<td>33.88± 5.70 22.48-45.28</td>
</tr>
<tr>
<td>Lipase activity (water insoluble substrate)</td>
<td>-</td>
<td>4.93 ± 1.08 2.77-7.09</td>
</tr>
<tr>
<td>Esterase activity</td>
<td>8.13 ± 0.76 6.61-9.65</td>
<td>5.87 ± 0.84 4.19-7.55</td>
</tr>
<tr>
<td>Amylase activity</td>
<td>3.39 ± 0.94 1.51-5.27</td>
<td>4.27 ± 0.86 2.55-5.99</td>
</tr>
<tr>
<td>Peroxidase activity ***</td>
<td>5.66 ± 0.84 3.98-7.34</td>
<td>3.05 ± 0.61 1.83-4.27</td>
</tr>
</tbody>
</table>

* Units of enzyme activities are defined in the text.
** Range of enzyme activity is calculated as (x ± 2 S.D.)
*** Specific activity.
esterase activity of colostrum was found significantly higher than that of transitional or mature milk when assayed under identical conditions (Table 11, Table 29). This indicates the possibility of existence of two different enzyme in the milk, one acting on low molecular weight organic esters and other on glycerides. With two different substrates tested, lipase activity in bovine milk was found about 4 times less than that of human milk. I could not detect any methyl acetate esterase activity in bovine milk.

The detergent stimulated lipase activity of human breast milk is heat sensitive enzyme. Heating at 65°C for 15 minutes is fairly sufficient to inactivate this activity almost completely (Fig. 3).

Milk amylase content in normal Bengalee mother does not change significantly during different stages of lactation (Table 12, Table 29). Amylase activity in human milk is very high. When human and bovine milks were assayed for this enzyme, the former showed 40 times more activity than the latter. Human milk amylase is very heat labile enzyme. It is inactivated by heat quicker than milk alkaline phosphatase or lipase. Heating at 55°C for 15 min caused 95% loss in the human milk amylase activity (Table 13, Fig. 4).

Both α- and β-amylase (diastase) hydrolyse (1 → 4) D-glucoside linkage in glucopolymers like starch and glycogen. In serum, amylase activity increases due to acute pancreatitis and different renal diseases (Varley, 1975). However the
physiological role played by human milk amylases is not known at present. The substrate for these enzymes are not present in the milk or in G.I. tract of infants on total breast feeding. Like human milk lipase, human milk amylases can withstand proteolytic degradation in infants' G.I. tract, so it may help in the digestion of supplemented starch or glycogen in infants' diet as pancreatic amylase activity is not fully developed during first year of life (Hadorn et al. 1968, Famon, 1974).

Peroxidase, a typical plant enzyme is also found in milk as lactoperoxidase. The major metabolic function of peroxidases is oxidation of different substrates by $H_2O_2$ produced in the reaction between $O_2$ and reduced flavin coenzymes (Fruton & Simmonds, 1958). Since no such flavin catalysed oxidation is anticipated in milk, the presence of this enzyme in the milk may be considered as fortuitous. The lactoperoxidase activity in guineapig milk increases during the 3-4th week of lactation and increase in the enzyme activity is accompanied by simultaneous increase in the bactericidal activity in the milk. This increase in antibacterial activity is not due to stimulation of lactoferrin activity in the milk and could be counteracted by the conventional lactoperoxidase inhibitors (Stephens, Harkness & Cockle, 1979). The human lactoperoxidase too, may have similar antibacterial activity.

The peroxidase activity in human colostrum is 2-4 times higher than that in transitional milk (Table 14, Table 29). Heyndrickx (1963) reported earlier that the activity of this enzyme in human milk is very low and sometimes not detected.
However all 16 mature milk samples tested in this study for peroxidase content contained peroxidase activity. However the peroxidase activity in the milk of normal Bengalee mothers is about 3-4 time less than that of bovine milk.

Lactoperoxidase of Bengalee mothers' milk is inactivated almost completely by heating at 75°C within 15 min (Table 15, Fig. 5). It is interesting to note that the activity of this enzyme is stimulated by heat pretreatment at 45°C-55°C for 15 minutes. The mechanism of activation of this lactoperoxidase activity by heat could not be explained properly at this stage. The enzyme itself or H₂O₂ acceptor substrate present in the milk may be activated (by dispersion) due to heat treatment, or alternately, the enzyme which may normally exist in the milk aggregated form with lipid or lipoprotein droplets may be released by thermal agitation. No such activation of lactoperoxidase in human milk by heat was studied or reported in the literature previously.

Methyl pentose (fucose) is one of the important microconstituents of no known direct nutritional function in the milk. Fucose was detected first in human body as a component of blood group substances (Watkins, 1966; Ginsburg, 1972). Subsequently this sugar was found in milk oligo saccharides. More than 15 oligosaccharides of different size and structure have been characterised so far (Grollman & Ginsburg, 1967; Kobata, Tsuda, Ginsburg, 1969; Kobata & Ginsburg, 1970; Kobata, 1972) and most of them contain fucosyl residues. It may be suggested that these oligosaccharides in milk are drawn from the blood circulation,
though direct evidence for such suggestion is still lacking.

Gyorgy (1953) and Bezkorovainy et al. (1979) suggested a very interesting and important role of fucosyl oligosaccharides and a fraction of glycopeptide of human milk. The colonization of Lactobacillus bifidus and related organism in infants' large intestine is stimulated by some factor or factors (bifidus factor) present in human milk. The chemistry of bifidus factor is not yet known completely but it is derived from oligosaccharides and also a fraction of glycopeptide containing fucose (Hawk, 1971; Bezkorovainy et al., 1979). Chemical or biological assay method for the estimation of bifidus factor is not available. So the efficiency of the different milks in establishment of indispensable lactic acid producing microflora in infant's could not be measured or compared. In the present study, the estimate of fucose (free or glycosidically bound) was taken as index of bifidus factor content on the assumption that bifidus factor is derived from this methyl pentose. However, only this assumption, bifidus factor content of the milk will be overestimated, because, though fucose is one of the component of bifidus factor, all fucose containing oligosaccharides may not act as bifidus factor. No such correlation between contents of total fucose and bifidus factor in milk is worked out as yet.

The fucose content of the milk may roughly predict at least the potentiality of milk to effect establishment of Lactobacilli in large intestine of breastfed babies. The total fucose content in the milk of Bengalee mothers is fairly high (Table 16). A large part (about 60-70%) of the total fucose
in the milk is present as free sugar or as highly soluble low molecular weight oligomer. The fucose (free or bound) content of the milk is dependent on blood group of the donors. The content of fucose is highest in the milk of donors with AB blood group. B blood group holders also produce milk with fairly high amount of fucose. Fucose or fucosyl oligosaccharides contents in the milk from A or O blood group holders are low.

The human milk may also carry different externus substances which are inadvertently introduced into infants' physiological systems and may produce adverse effects. These externus substances may be introduced into the mothers' metabolic system from environments (from environmental pollutants, like pesticides, fungicides, radionuclides, toxic metal ions etc.), or from substances ingested by the mothers in the form of food or medicinals. To evaluate the quality of breast milk, these externus substances, though these may be present in very small amounts, should also be taken into account.

In the present investigation, excretion in the milk of ampicillin and sulphamethoxazole which are very common medicines used in the hospitals of this region for the treatment of confined mothers with postpartum infections, was studied in detail.

Ampicillin could be detected by microbial assay in the milk, even when a single (250 mg, oral) dose of this antibiotic was given to the mothers. There was a time lapse (lag period) of 2 hour between oral administration and appearance
of ampicillin in the milk. This lag period is sum total of the time taken for absorption of the antibiotic from G.I. tract into circulatory blood and that for transport of the antibiotic across the plasma-milk barrier. The contribution of the former is greater than that of latter, as the time lag remains the same when oral dose is increased two folds but diminishes when antibiotic is given by i.m. injection (Table 17, 18 & 19). Ampicillin could not be detected in milk 12 hour after its single dose oral administration. The excretion of ampicillin in the milk of Bengalee mothers was found less than that reported for other ethnic groups (Takyi, 1970).

When the patients were on prolonged therapy and received ampicillin at 6 hourly intervals for 4 days, this antibiotic is excreted steadily in the milk. There is no significant difference in the ampicillin concentrated in the excreted milk during this period except for the milk excreted between 2nd, 4th and 6th hour of the last oral dose. There is a rise in the milk ampicillin level during this time (Table 18, 30).

Ampicillin concentration in the blood plasma and excreted milk were determined simultaneously at a given time (Table 20) and milk plasma ratio (M:P) for this antibiotic was calculated. The ratio is fairly constant except at very low plasma (or milk) ampicillin concentration. Ampicillin concentration in the milk does not reach more than 18% of that in blood plasma, indicating very slow transport of this antibiotic across the milk plasma barrier. Observed M:P for ampicillin in this study was compar-
able to that reported in the literature (Rasmussen, 1959; Knowles, 1965) for benzyl penicillin.

In vitro binding of ampicillin with milk proteins (which may cause diminution of antibiotic activity) was determined (Table 21). Both colostrum and transitional milk could bind ampicillin. The binding by colostrum is significantly higher than that by transitional milk, possibly due to high protein content of the colostrum.

Several adverse effects in normal, healthy babies receiving breast milk containing residue of broad spectrum antibiotic like ampicillin may be anticipated. If the antibiotic concentration in the milk is sufficiently high, the development of helpful intestinal microflora would be impaired and the equilibrium of microfloral composition would be disturbed. The prolonged exposure to low concentrations of antibiotics may help in the selection of microorganism (which may be pathogenic) resistant to that antibiotic in the system. Occasionally even small dose of antibiotic may provoke allergic reactions in susceptible cases. Williams (1976) reported on general candidiasis and diarrhoea in infants originating from ampicillin administered to nursing mothers. In the present study too, it was recorded that the breast fed babies of at least 4 mothers who were on ampicillin therapy had complains of diarrhoea and vomiting. However the possible link between these complains and ampicillin intake by the babies was not studied.

The excretion of ampicillin in the milk when mothers
received this antibiotic at therapeutic dose is very small (Table 30). Total amount of ampicillin an infant may receive through such milk will not be more than few mg even in extreme cases. The small amount of this antibiotic ingested by an infant would be absorbed in its G.I. tract and diluted further by its body fluids. So the effective concentration of ampicillin in the infant's body would be too small to produce any adverse microbiological effect. However the possibility of development of hypersensitivity and allergic reactions in the susceptible babies receiving ampicillin through breast milk could not be ruled out. Such reaction might occur at very low concentration of this antibiotic. In the literature at least one case has been reported on the development of localised fibrile reactions in infant whose mother was on penicillin therapy (Rollier et al. 1967).

Table 30
Excretion of ampicillin in breast milk of Bengalee mothers*

<table>
<thead>
<tr>
<th>Amount of Ampicillin ingested by the mothers mg</th>
<th>2nd hour Ampicillin concentration (µg/ml) in milk after oral dose</th>
<th>4th hour</th>
<th>6th hour</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Range** Mean ±S.D.</td>
<td>Range** Mean ±S.D.</td>
<td>Range** Mean ±S.D.</td>
</tr>
<tr>
<td>250 mg</td>
<td>0.016- 0.027+ 0.04 0.009 0.024- 0.034+ 0.014- 0.025+ 0.007</td>
<td>0.025- 0.027+ 0.047 0.006 0.034+ 0.037 0.007</td>
<td></td>
</tr>
<tr>
<td>500 mg</td>
<td>0.076- 0.095+ 0.13 0.02 0.125- 0.14+ 0.078- 0.089+ 0.08</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* From Table 18.
** Experimental range is mentioned.
The situations arising from sulphamethoxazole residues in the milk are different from those arising from ampicillin residues in this important body fluid. This widely used sulfa drug is absorbed slowly from the human G.I. tract, so even if a small amount of this compound is persistently excreted in the milk, G.I. tract of the breast-fed infants would be continuously flushed with unabsorbed drug residues. This will alter the microfloral composition of infants' colon and help in the selection of sulfa drug resistant bacteria.

The amount of sulphamethoxazole residue excreted in the breast milk after a single oral dose is higher than that of ampicillin residue under identical conditions. The time lapse between the oral intake and first appearance in the breast milk of sulphamethoxazole is about 2 hours. Maximum amount of sulphamethoxazole residue in the milk could be recovered between 3-4 hours after its oral intake (Table 20). The excretion of this drug in milk slows down after the 5th hour of its oral intake. When the lactating mothers are on prolonged therapy with sulphamethoxazole (which is normally administered at 12 hourly intervals), it is excreted in milk continuously throughout the treatment period. The concentration of the excreted drug in the milk however changes during the interspace time between two successive doses. It is minimum at the time of fresh oral administration (i.e. 8 or 12 hours after the preceding dose) and reaches a maximum on the 4th hour of its intake (Tables 21 & 22).

Hawking and Lawrence (1951) studied the excretions in the breast milk of two highly soluble sulfa drugs: sulfanilamide
and sulfapyridine which were readily absorbed by the G.I. tract. Milk concentrations of 3-13 mg/100 ml were recorded after 2-3 g oral dose of these drugs. The present investigation showed that the milk concentration did not rise more than 0.5 mg% for sulfamethoxazole (which had a very low absorption rate) after 3 g oral dose of this drug. Milk : plasma (M:P) ratio for sulfamethoxazole recorded in this study was 0.33-0.36 (Table 23), this was again lower than that (1.00) obtained for sulfanilamide or sulfapyridine (Hawking & Lawrence, 1951). A small amount of sulfamethoxazole is immobilized by blood plasma protein. The binding of this drug by milk protein is 8-9 times higher than that by blood plasma protein (Table 24).

During formation and storage in the mammary gland, milk of mothers with normal health is probably microbiologically sterile. But expressed milk from these mothers are always accompanied by different types of bacteria (Williamson et al. 1978; McEnery & Chattopadhyay, 1978). Sources of these bacterial contaminants include, skin, nipple and terminal part of the milk ducts which may retain a small amount of milk before and after the feeding of babies.

The colostrum and transition milks from normal Bengalee mothers contain fairly large bacterial counts (Table 26). The average bacterial count in the transitional milk is slightly more than that in the colostrum but the difference is not significant. Surprisingly, two samples of mature milk tested were found to contain a very small number of viable bacteria. It is
not possible to comment on this result as number of mature milks analysed microbiologically was very small.

Nonpathogenic *Staphylococcus epidermidis* (*S. albus*), well known inhabitant on normal human skin in this region, is detected as most abundant microorganism in the milk samples tested (Table 27). A pathogenic staphylococcus, *S. aureus* was found in the milk from local donors in fairly large number (20-25% of total viable count in most of the cases, 30-35% or even 40% of total viable count in few other cases). Apart from these, a fairly high counts of potentially pathogenic enterobacteria was recorded in the milk of Bengalee mothers. Enterobacteria identified in such milk are *Escherichia coli*, *Klebsiella aerogenes*, *Alcaligenes fecalis* and *Acinetobacter sp.* (Table 27). The presence of enterobacteria in the milk is surprising as these are not the constituent of normal skin microflora. These organisms are part of normal adult intestinal microflora and excreted through feces. Their presence in the milk samples tested indicated the unhygienic living conditions or habits of the donors. It may be mentioned here that enterobacteria were detected largely in the milk with very high bacterial count. Enteric pathogens like *Salmonella* or *Shigella* were not found in the milk samples tested.

The human milk may protect itself from the possible ill effects of potentially pathogenic bacteria at least to certain extent by virtue of its own antimicrobial constituents like lysozyme or ironbinding proteins (Tobinson *et al.*, 1978). *S. aureus* cells are highly sensitive to these antimicrobial milk constituents (Gyorgy, 1971).