CHAPTER VI

Activities of certain milk enzymes in relation to period of lactation and dietary fat intake. Partial purification of alkaline phosphatase.

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Breast milk has been known to possess a number of enzyme systems such as lipase and esterase (Arshavskii, 1940; Freudenberg, 1951; Jacqmain et al., 1953), acid and alkaline phosphatases (Chanda, et al., 1951; Vittu, 1946; Stewart et al., 1958, and Belvady, 1960), lysozyme (Fleming, 1932; Rossenthal et al., 1931) and xanthine oxidase (Belvady, 1960; Owen and Hytten, 1960; Bradley and Gunther, 1960). It has been suggested that enzyme systems normally present in human milk may contribute to the superiority of this food over artificial baby foods.

Concurrent with the investigations outlined in the previous chapters, studies were made of the activities of some of the above enzymes such as lipase, esterase, and acid and alkaline phosphatases which are believed to be involved in fat metabolism. Variation in the same with period of lactation, dietary fat intake, and fat supplementation were also studied. One of these enzymes, viz, alkaline phosphatase was partially purified and characterised.

EXPERIMENTAL

A total of 135 subjects were used in these experiments.

The samples were collected by the procedure described in Chapter II and analysed immediately on arrival in the laboratory. Several samples were pooled together for the purification studies.

Estimation of enzymes in milk

Lipase: Lipase was estimated by essentially the same method as that of Biosonnas (1948). The assay mixture consisted of : Tween 20, 1.5 ml; phosphate buffer, pH 6.8, 200 µmoles; milk sample, 0.2 ml; phenolphthalein (in alcohol), 0.04%, 0.3 ml; and water to 5.0 ml. This mixture was incubated at 37° for two hours. The blank contained boiled enzyme in place of the fresh enzyme. The reaction was stopped by adding 5.0 ml of absolute alcohol, the mixture warmed in a water bath, and titrated against 0.01N NaOH.

A unit of enzyme activity is defined as the amount of enzyme which under the described assay conditions, liberates an amount of free fatty acids that would be neutralised by 1.0 ml of 0.01N NaOH.

Esterase: Esterase was estimated by essentially the same method as that of Harrer and King (1941). The assay mixture consisted of ethyl butyrate, 2.0 ml; phosphate buffer,

pH 7.2, 100 µmoles; milk sample, 0.2 ml; phenolphthalein in alcohol (0.04 %), 0.3 ml; and water to 5.0 ml. This mixture was incubated at 37° for two hours. The blank contained boiled enzyme in place of the fresh enzyme. The reaction was stopped by adding 5.0 ml of absolute alcohol, the mixture warmed in a water bath, and titrated against 0.01N NaOH.

A unit of enzyme activity is defined as the amount of enzyme which under the described assay conditions, liberates an amount of free fatty acids that would be neutralised by 1.0 ml of 0.01N NaOH.

Acid and alkaline phosphatase

Acid and alkaline phosphatases were determined essentially by the method of Morton (1955). The assay mixture consisted of sodium β glycerophosphate, 100 micromoles; either acetate buffer, pH 4.4, 200 micromoles, or borate buffer, pH 8.6, 100 micromoles, the former for the assay of acid phosphatase and the latter for that of alkaline phosphatase; magnesium acetate, 50 micromoles; milk sample 0.2 ml; and water to 5.0 ml. The mixture was incubated at 37° for 20 minutes and the reaction stopped by the addition of 2.0 ml of 30% trichloroacetic acid. The blank was treated with trichloroacetic acid without incubation. After removing the precipitated protein by

centrifugation, the inorganic phosphorus in the supernatant was determined by the method of Fiske and Subbarao (1925).

A unit of enzyme activity is defined as the amount of enzyme which causes the liberation of 1Ø µg phosphorus in 20 minutes.

Partial purification of alkaline phosphatase of breast milk

On centrifugation at 3000 X g and 0° for 30 minutes the milk sample was found to form three distinct layers, a top layer (fatty), a middle layer (supernatant) and a bottom layer (residual). The three layers were separated after centrifugation and tested for enzyme activity. The fatty layer was treated with butanol before testing as the enzyme has been suggested to be present as a lipoprotein complex in the case of cow's milk(Morton 1955). The supernatant fraction (A) was found to show maximum activity (Table 29) and was used for further purification.

All the operations to be detailed below were carried out at 0°. To 30 ml of fraction A were added 6.3 g of ammonium sulphate and allowed to dissolve. The mixture was stood for 10 minutes and the precipitate obtained separated by centrifugation at 4,800 X g for 20 minutes. The precipitate was dissolved in .01M borate buffer pH 8.6 to a final volume of 30 ml (Fraction B).

To 25 ml of fraction B were added 7.0 g of ammonium sulphate and the mixture stood for 10 minutes. The precipitate formed was removed by centrifugation at 4,800 X g for 20 minutes and dissolved in .01M borate buffer pH 8.6 to a final volume of 25 ml (Fraction C).

To 20 ml of fraction C were added 5.6 g of ammonium sulphate and the precipitate formed seperated by centrifugation at 4,800 X g for 20 minutes and dissolved in 0.01M borate buffer pH 8.6 to a final volume of 20ø ml. (Fraction D). The different fractions were dialysed for 4 hours against .002M borate buffer and tested for enzyme activity.

The protein content (N X 6.38) of the original enzyme preparation was estimated by the micro-Kjeldahl method and that of the different fractions by the modified method of Warburg and Christian (1952).

RESULTS

The activities of lipase, esterase, and acid and alkaline phosphatases at different stages of lactation are shown in Table 24, from which it can be seen that except in the case of acid phosphatase there is a decline in enzyme activity after the first month of lactation after which the values remain fairly steady throughout the period studied.

Table 24

Activities of lipase, esterase, acid and alkaline phosphatases* in human milk at different stages of lactation

Enzyme	Lactation period in months					!
	Below 1 (10)	1-3 (10)	3-6 (10)	6-12 (10)	Over 12 (10)	
Lipase	6257 ±33	5870 ±29	5643 ±28	5550 ±25	5615 ±26	
Esterase	4028 ±23	3607 ±21	3550 ±23	3490 ±21	3517 ±21	
Alkaline phosphatase	3820 ±21	3440 ±19	3356 ±18	3370 ±19	3368 ±19	
Acid phosphatase	1150 ±14	1118 ±13	1076 ±13	1128 ±14	1137 ±14	

* Expressed in terms of units per 100 ml. The values given are means with standard errors.
! The numbers in parentheses indicate the number of subjects.

Table 25 shows the effect of dietary fat on the fat as well as lipase, esterase, acid and alkaline phosphatase contents of milk. It can be seen from the same that lipase, esterase, and alkaline phosphatase activities increase with the fat content of milk. The product moment correlations between the fat content of milk on the one hand and the concentrations of lipase, esterase, and alkaline phosphatase on the other hand are respectively 0.39, 0.86, and 0.84, all of them being statistically significant.

Tables 26, 27, and 28 show the effects of dietary fat supplementation (studies detailed in Chapter V) on the lipase, esterase, and alkaline phosphatase contents of milk. It can be seen from the same that fat supplementation in the range of 25 - 35 g corresponding to 45 to 55 gm of total intake of fat results in significant increase in the lipase, esterase and alkaline phosphatase activities of breast milk. Thus these studies point to a positive relation between dietary fat and milk fat on the one hand and the activities of lipase, esterase, and alkaline phosphatase on the other.

The yields of alkaline phosphatase and the degree of purification achieved in the different fractions are

Table 25

Activities of lipase, esterase, and alkaline phosphatase* in human milk in relation to dietary and milk fat levels

Classification with regard to fat intake @	Dietary intake g/day		Fat content of milk g/100 ml	Enzyme units per 100 ml		
	Range	Mean		Lipase	Esterase	Alkaline phosphatase
First quartile (16)	8-28	18 ±1.6	4.13 ±0.24	5461 ± ±28	3376 ±21	3280 ±23
Second quartile (15)	28-50	37 ±1.3	4.30 ±0.14	5762 ±24	3551 ±26	3528 ±22
Third quartile (16)	50-72	61 ±1.6	4.86 ±0.25	6037 ±34	3925 ±27	3861 ±23
Fourth quartile (12)	72-115	89 ±6.7	4.72 ±0.28	6032 ±30	4010 ±27	3905 ±24

* The values given are means with standard errors.
@ The numbers in the parentheses indicate the number of subjects in each group.

Table 26

Variation in the lipase activity* of milk with increasing fat supplementation

Fat supplement@ per day g	Supplementation groups				Control group
	Fat (5)	Fat +Protein (5)	Fat +Vitamin (5)	Fat +Protein +Vitamin (5)	(No supplementation) (5) **
0	5286 ±27	5054 ±27	5064 ±27	5005 ±25	5229 ±27
5	5578 ±29	5202 ±29	5110 ±28	5178 ±27	5220 ±24
15	5776 ±28	5586 ±27	5442 ±27	5460 ±26	5213 ±25
25	5969 ±28	5748 ±27	5636 ±29	5661 ±27	5205 ±25
35	5968 ±30	5745 ±30	5641 ±28	5698 ±28	5223 ±25
45	5965 ±31	5748 ±30	5642 ±29	5656 ±28	5206 ±26

* Expressed in terms of units per 100 ml.
@ The initial fat intake before supplementation in the subjects was 15 to 20 gm/day. Each level was maintained for a month.
** The numbers in the parentheses indicate the number of subjects in each group.

Table 27

Variation in the esterase activity* of milk with increasing fat supplementation

Fat supplement@ per day g	Supplementation groups				Control group
	Fat (5)	Fat +Protein (5)	Fat +Vitamin (5)	Fat +Protein +Vitamin (5)	(No supplementation) (5) **
0	3233 ±20	3200 ±21	3149 ±22	3004 ±23	3186 ±22
5	3444 ±22	3376 ±21	3362 ±22	3194 ±22	3176 ±24
15	3635 ±25	3585 ±23	3521 ±23	3401 ±20	3183 ±24
25	3825 ±24	3856 ±22	3701 ±24	3694 ±22	3184 ±23
35	3823 ±24	3864 ±24	3718 ±24	3810 ±24	3185 ±24
45	3815 ±25	3860 ±25	3701 ±25	3794 ±25	3167 ±21

* Expressed in terms of units per 100 ml.
@ The initial fat intake before supplementation in the subjects was 15 to 20 g/day. Each level was maintained for a month.
** The numbers in the parentheses indicate the number of subjects in each group.

Table 28

Variation in the alkaline phosphatase activity* of milk with increasing fat supplementation

Fat supplement@ per day g	Supplementation groups				Control group
	Fat (5)	Fat +Protein (5)	Fat +Vitamin (5)	Fat +Protein +Vitamin (5)	(No supplementation) (5) **
0	3073 ±18	3072 ±20	3082 ±21	3039 ±19	3086 ±19
5	3453 ±21	3259 ±20	3187 ±21	3291 ±19	3077 ±20
15	3657 ±21	3484 ±22	3442 ±24	3368 ±22	3086 ±20
25	3843 ±24	3747 ±24	3626 ±23	3615 ±21	3065 ±20
35	3837 ±24	3761 ±23	3644 ±24	3622 ±23	3063 ±19
45	3831 ±24	3755 ±24	3638 ±27	3615 ±24	3062 ±21

* Expressed in terms of units per 100 ml.
@ The initial fat intake before supplementation in the subjects was 15 to 20 g/day. Each level was maintained for a month.
** The numbers in the parentheses indicate the number of subjects in each group.

summarized in Table 30. The specific activity of alkaline phosphatase in fraction D is seen to increase more than 12 fold over that of fraction A, but this increase is accompanied by a loss of more than 27% in total activity.

Studies were made on the effect of varying the following factors on enzyme activity with fraction B as enzyme source.

Enzyme concentration: The effect of varying the enzyme concentration is shown in Table 31 and Fig. 7 from which the enzyme activity is seen to be proportional to enzyme concentration.

Substrate concentration: The effect of varying sodium β glycerophosphate concentration is shown in Table 32 and Fig. 8 from which enzyme activity is seen to increase with sodium β glycerophosphate concentration.

pH: The effect of varying the pH of the reaction mixture is shown in Table 33 and Fig. 9 from which the optimum pH for enzyme activity is seen to be 8.6.

Buffer concentration: The effect of varying the buffer concentration is shown in Table 34 and Fig. 10 from which the enzyme activity is seen to increase with the amount of buffer added upto 100 micromoles.

Table 29

Alkaline phosphatase activity in different fractions of milk

	Enzyme units	Percent activity
Whole milk	4105	100
Supernatant	2875	70
Fat layer (After butanol treatment)	1010	24

Table 30

Partial purification of alkaline phosphatase

Purification step	Total volume ml	Total protein (mg)	Total units	Specific activity units/mg protein	Yield
Fraction A	30	492.0	840.0	1.70	100
Fraction B	30	219.0	798.0	3.30	94
Fraction C	30	72.0	720.0	10.00	86
Fraction D	30	27.6	615.0	22.0	73

Table 31

Effect of enzyme concentration

Enzyme added (mg protein)	Activity units
1.80	6.52
3.61	12.83
7.22	24.80
9.00	30.50
10.80	35.30

Table 32

Effect of substrate concentration

Sodium β Glycerophosphate added (micromoles)	Activity units
50	13.40
100	26.00
150	33.40
200	36.20
225	38.30

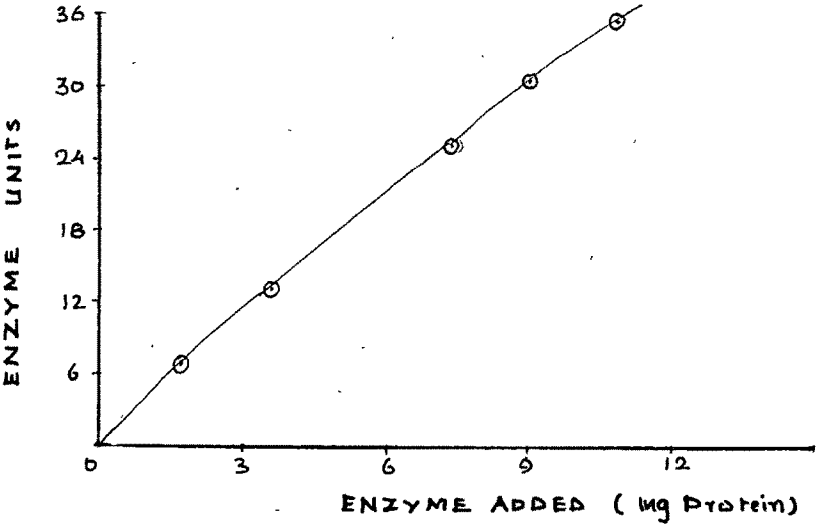

Fig. 7 Effect of enzyme concentration on glycerophosphate breakdown.

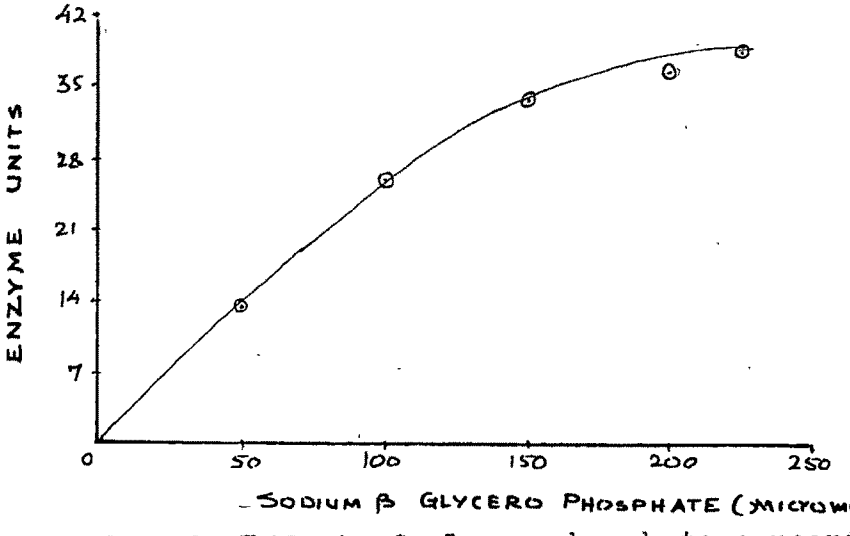

Fig. 8 Effect of glycerophosphate concentration on glycerophosphate breakdown.

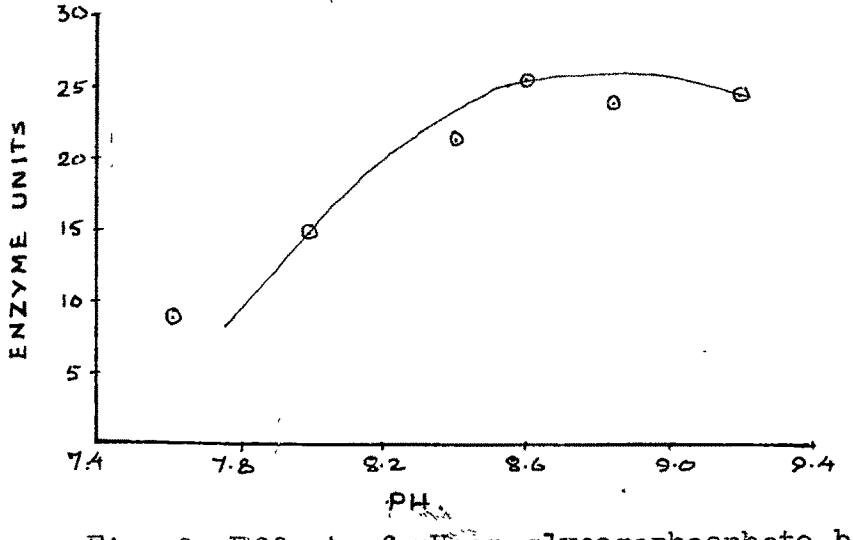

Fig. 9 Effect of pH on glycerophosphate breakdown.

Table 33

Effect of pH

pH	Activity units
7.6	8.73
8.0	14.60
8.4	21.30
8.6	25.80
8.8	24.80
9.2	24.45

Table 34

Effect of buffer concentration

Buffer added (micromoles)	Activity units
25	10.43
50	15.63
100	24.50
150	23.94
200	22.63

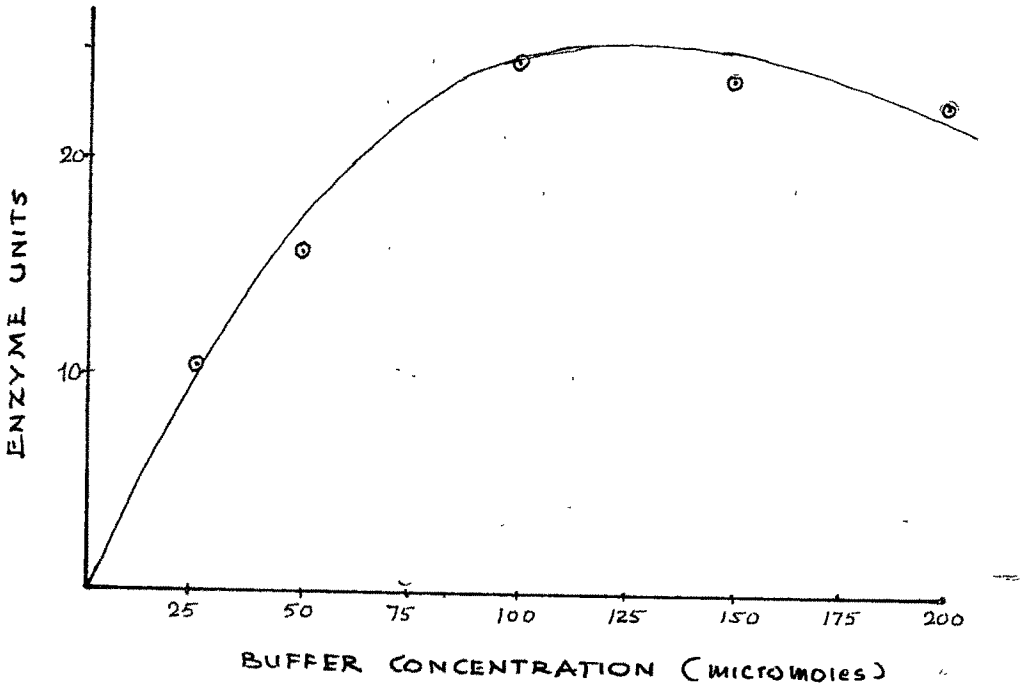

Fig. 10 Effect of buffer concentration on glycerophosphate breakdown.

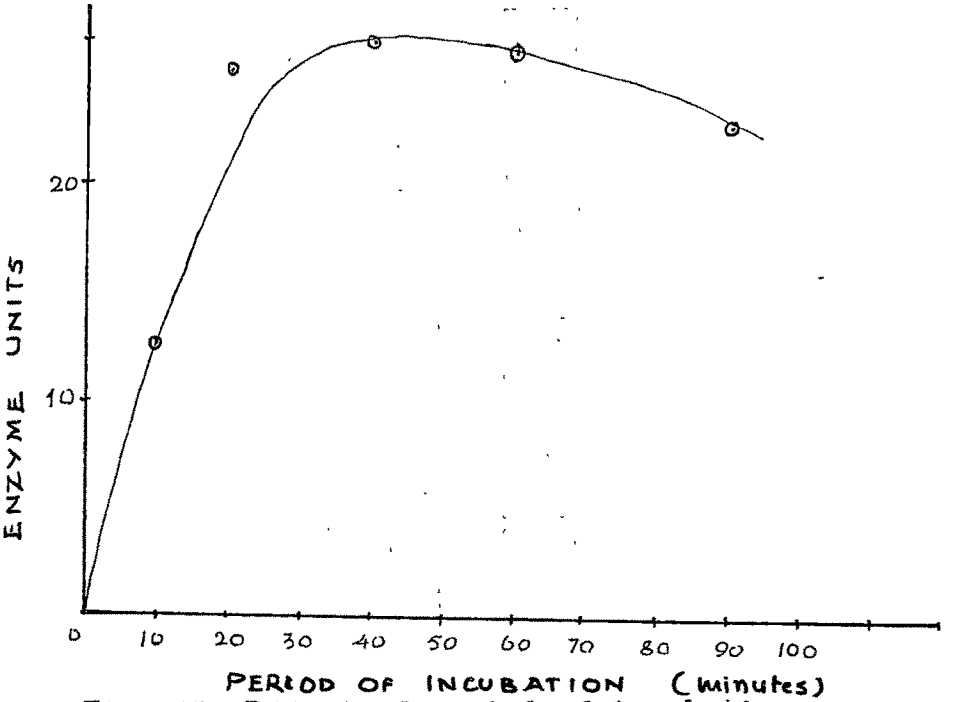

Fig. 11 Effect of period of incubation on glycerophosphate breakdown.

Period of incubation: The effect of varying the period of incubation is shown in Table 35 and Fig. 11 from which the enzyme activity is seen to be proportional upto a period 20 minutes.

Incubation temperature: The effect of varying the incubation temperature is shown in Table 36 and Fig. 12 from which the enzyme activity is seen to have an optimal temperature of 37°.

Magnesium acetate concentration: The effect of varying magnesium acetate concentration is shown in Table 37 and Fig. 13 from which the enzyme activity is seen to increase with the amount of magnesium acetate upto a concentration of 60 micromoles.

Period of storage: The effect of aging on the activity of enzyme was studied by storing it in test tubes at -10°. The tubes were removed at regular intervals and the enzyme activity assayed. The enzyme is seen to be inactivated after 30 days (Table 38).

DISCUSSION

It is of interest to note the presence in breast milk of enzymes which are believed to play a role in the

Table 35

Effect of period of incubation

Period of Incubation (minutes)	Activity units
10	12.70
20	25.20
40	26.10
60	25.80
90	23.70

Table 36

Effect of temperature

Temperature of Incubation ^{o}C	Activity units
25	12.82
30	22.45
37	26.30
45	8.40
50	5.36

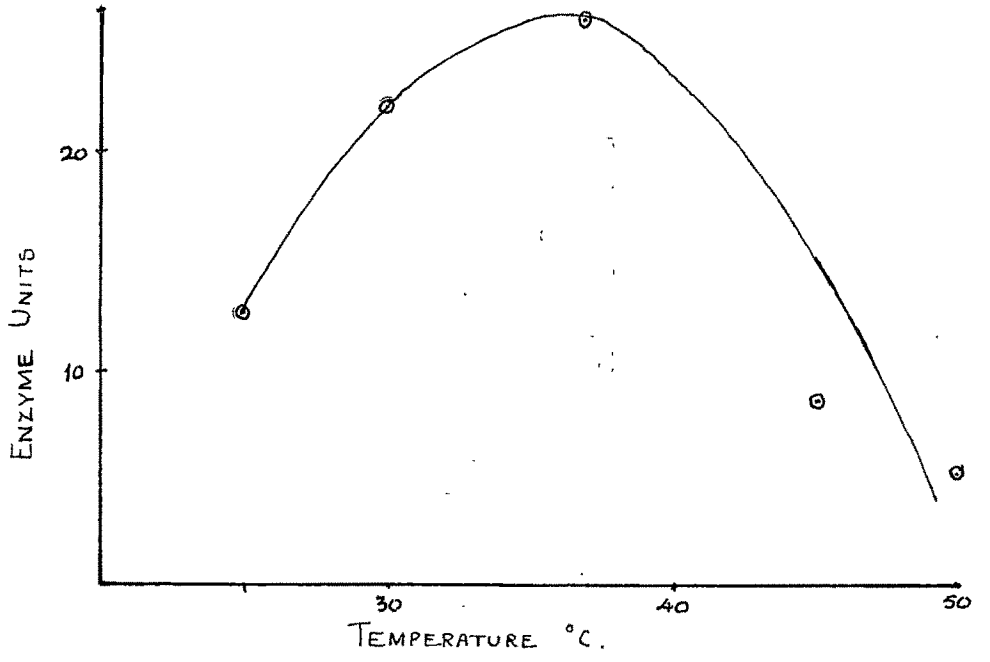

Fig. 12 Effect of temperature on glycerophosphate breakdown.

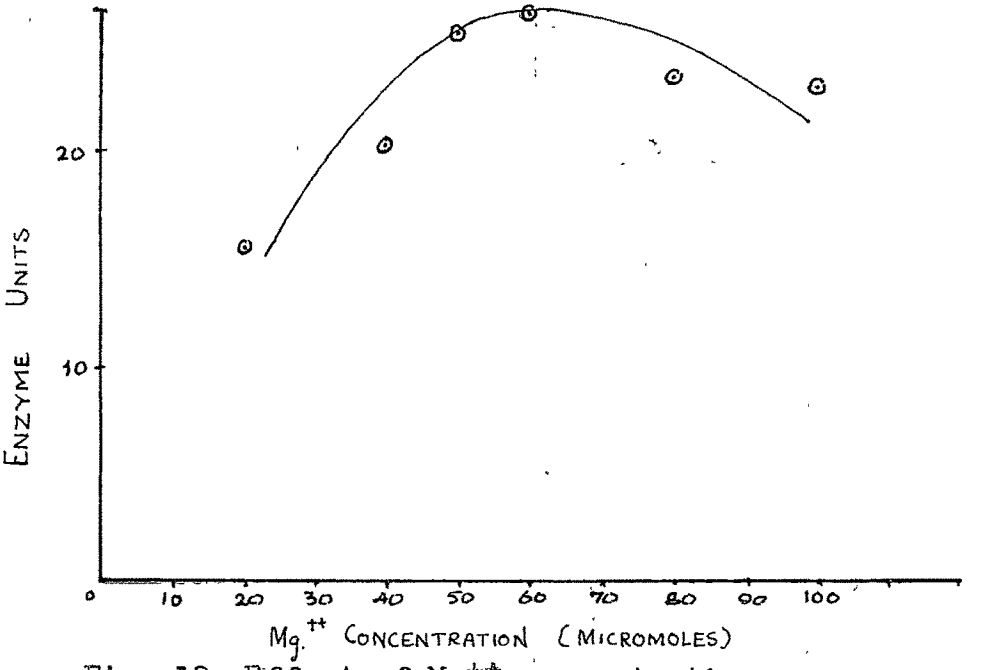

Fig. 13 Effect of Mg^{++} concentration of glycerophosphate breakdown.

Table 37

Effect of magnesium

Magnesium acetate added (micromoles)	Activity units
20	15.60
40	20.30
50	25.80
60	26.60
80	24.20
100	23.90

Table 38

Effect of aging

Period of storage days	Activity units	Loss (%)
0	24.60	0
5	20.42	17
10	14.23	48
15	10.18	59
20	8.40	66
25	5.80	78
30	2.30	99

digestion, assimilation, and metabolism of fat, and their relation to the fat content of milk which may perhaps contribute to some extent towards the superiority of breast milk over other foods.

The observation that alkaline phosphatase activity declines with the progress of lactation is in agreement with that made by Belvady (1960). The positive relation observed between fat and alkaline phosphatase contents of milk is in line with the finding reported by Stewart et al (1958).

In its activation by magnesium, the enzyme resembles that purified from cow's milk by Morton (1953). However, the optimum pH of 8.6 found in the present case differs from the value of 9.65 reported by Morton for cow's milk.

SUMMARY

The activities of lipase, esterase, and acid and alkaline phosphatases in breast milk were studied in relation to period of lactation and dietary and milk fat levels. The activities of lipase, esterase and alkaline phosphatase were found to decline after the first month of lactation and remain steady there after. They were found to show a positive relation to dietary and milk levels of fat.

Alkaline phosphatase from human milk was partially purified and characterised.