THE ALKALINE PHOSPHATASE CONTENT OF HUMAN MILK

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The possibility that enzyme systems normally present in breast milk may account for the superiority of this food over pasteurized formulas in promoting the health and nutrition of the newborn infant has been the subject of speculation and study. Of the many enzymes present in milk, some attention has been directed to lysozyme (1, 2), lipase (3, 4), and phosphatase (5-7) as substances which may influence the health and nutrition of the newborn infant.

Clinical observations1 on the feeding of cows milk formulas supplemented with phosphatase indicate that this substance may overcome feeding difficulties of some infants and have led to the present study of the occurrence of alkaline phosphatase in normal breast milk.

The presence and classification of phosphatase enzymes (7) as well as their relationship to the occurrence of phosphorus in various forms (5) have been established for human milk. This work was done on a limited scale and included only the values on milk of the 1st month of lactation.

The present study, in which 199 samples from twenty donors were analyzed, will show that alkaline phosphatase occurs in variable amounts in human milk. Although there appears to be a tendency for phosphatase concentration to be related to the fat content of the milk, there is no apparent correlation with nitrogen content or total solids other than fat. There also seems to be no relationship to age, nationality, or other characteristics of the donor, except that for most of the seven donors from whom samples were taken over a period of 3 or more months a tendency for the phosphatase content of the milk to increase with duration of the lactating period was observed.

EXPERIMENTAL

Selection of Breast Milk Samples Fresh, unpasteurized human milk samples were obtained by special arrangement with the San Francisco Mothers' Milk Bank. Because milk bank donors are limited to mothers who are successfully nursing their infants, this study can be considered as limited to nutritionally normal human milk.

1 Dr. L. Breslow, Chicago, Illinois, personal communication.
In all instances, early morning samples were used, some expressed before and some expressed after nursing the infant. Immediately after expression, the samples were placed in an ice bath until chilled, and then transferred to the refrigerator, where they were held for 1 to 2 hours before collection by the milk bank drivers. At the milk bank a 30 ml. sample was transferred to a screw cap culture tube and held under refrigeration until taken to the analytical laboratory. All samples were analyzed within 4 to 6 hours after expression.

During the course of the study it was determined that under these conditions there was no significant loss in phosphatase activity from the time of expression until the samples were analyzed.

Measurement of Alkaline Phosphatase Activity—Several methods are available for the quantitative estimation of alkaline and acid phosphatase in biological materials (8–21). In all techniques a phosphoric acid ester serves as the substrate, and colorimetric determinations are made on either the inorganic phosphate or the organic moiety of the substrate released by enzymatic hydrolysis. Each of these methods was investigated and a modified procedure based on the Scharer phosphatase test (20) was selected for the following reasons: (1), the rate of hydrolysis of the substrate, disodium phenyl phosphate, is linear for a period of 1 hour; (2), the hydrolysis product, phenol, is readily measured by combining it with the Gibbs reagent (22), 2,6-dibromoquinonechlorimide, to form a blue-green indophenol dye; (3), this indophenol dye can be extracted from milk in a clear solution with n-butyl alcohol; (4), reproducible results are readily obtained.

Methods in which p-nitrophenyl phosphate (12–15) was employed were investigated and, although the hydrolysis product, p-nitrophenol, is a self-indicator, the color could not be extracted into a clear solution for colorimetric measurement.

The Huggins and Tallalay method (11) in which phenolphthalein is the hydrolysis product did not lend itself to the estimation of phosphatase activity in milk because the rate of hydrolysis was not linear and the color could not be clarified by extraction. The Bodansky method (8) was attempted but not used because it was found that phenol could be measured more readily than inorganic phosphate.

The analytical procedure finally adopted for the estimation of alkaline phosphatase in human milk was as follows: (1) Dilute 5 ml. of milk and 10 ml. of 5 per cent MgSO₄·7H₂O solution to 100 ml. with distilled water to make the test solution; (2) prepare the buffered substrate by mixing 6 ml. of 1 m Na₂CO₃ with 4 ml. of 1 m NaHCO₃ and 10 ml. of 0.02 m disodium phenyl phosphate and dilute to 100 ml. with distilled water; (this buffered substrate has a pH of 9.5 at 37.5°); (3) incubate 1.0 ml. of the
test solution with 5.0 ml. of buffered substrate at 37.5° for 1 hour in a test tube; (4) place the tube in boiling water for 5 minutes and then cool the contents to 37.5°; (5) add 0.5 ml. of 0.2 per cent 2,6-dibromoquinone-chlorimide in absolute methanol to the tube, mix, and allow to stand for 15 minutes; (6) extract the blue-green color developed by adding 10 ml. of n-butyl alcohol to the tube, with thorough mixing and centrifuging; and (7) decant a clear n-butyl alcohol layer into a cuvette and measure the optical density at 660 μm.

A blank was run for each sample by carrying 1.0 ml. of test solution, inactivated by holding in boiling water 5 minutes, through the entire procedure.

The phosphatase activity was determined as the micrograms of phenol liberated under the conditions of the test and was calculated by using an internal standard in which 10 γ of phenol are treated in the procedure. The following calculation was used: phosphatase activity per ml. of milk = (optical density of test solution X 200)/(optical density of 10 γ of standard).

Several factors influenced the results of the test and precautions were taken to incorporate into the procedure those conditions which tended to give maximal phosphatase activity.

**Mg++ Activation**—The use of Mg++ increased the phosphatase activity approximately 40 per cent. Folley and Kay (16), Motzok (23), and Roche (24) have reported Mg++ as well as other divalent cations as activators for phosphatase. The concentration of 0.003 M Mg++ was found to be optimal in the conditions of this test.

**pH Optimum**—The effect of pH on the phosphatase activity of human milk was tested with use of various buffer systems. The Veronal-acetate buffer system of Michaelis (25) was found to give slightly higher values than the carbonate-bicarbonate buffer system of Delory and King (26) when tested with a variety of milk samples. Both buffer systems showed a pH optimum of 9.4 to 9.5 at 37.5° for alkaline phosphatase. With the Michaelis buffers, a pH optimum of 4.8 to 5.6 at 37.5° was found for the acid phosphatase of human milk. The carbonate-bicarbonate buffer was adopted because of the availability of suitable quality ingredients.

**Maximal Absorption**—With a Coleman model 14 spectrophotometer being used, maximal adsorption of the n-butyl alcohol indolphenol dye solution was found at 660 μm. This maximal absorption point is in agreement with the values reported by Ettinger and Ruchhoft (22).

**Measurement of Other Constituents of Human Milk**—Total solids were determined by evaporating 5 ml. samples in aluminum dishes to apparent dryness on a hot plate, followed by drying under 29 inch vacuum at 70° for 4 hours.
Fat was determined on 5 gm. samples by the Roese-Gottlieb method (27).

Nitrogen was evaluated on 5 gm. samples by the Kjeldahl-Wilfarth-Gunning method (28). The NH₃ was collected in 4 per cent boric acid and titrated to the methyl red end point with 0.1 N HCl.

“Solids-not-fat” represents the difference between per cent total solids and per cent fat.

Results

Samples of milk over varying periods of lactation were obtained from a total of twenty donors. One sample was obtained from six available donors every week and determinations of phosphatase, total solids, per cent fat, per cent nitrogen, and per cent solids-not-fat were made. A total of 199 evaluations of alkaline phosphatase was made over a period of 9 months. The highest value found was 540 units per ml. and the lowest value, 30 units per ml. The average of all these evaluations was 147 units per ml.

The average value of 147 determinations of total solids was 11.40 per cent; of 146 determinations of fat, 2.77 per cent; of 116 determinations of nitrogen, 0.157 per cent; and of 146 determinations of solids-not-fat, 8.65 per cent. The age, nationality, number of children, and the average values obtained for phosphatase, total solids, fat, nitrogen and solids-not-fat are found in Table I.

To determine the existence of an upward or downward trend in phosphatase content with duration of lactation, Table II was constructed. Only those donors (seven) from whom samples were taken over a period of at least 3 months were considered. The tremendous variation in average phosphatase content from individual to individual, plus the fact that in this experiment the weekly samples were collected for varying lengths of time, beginning at different intervals post partum, prohibits any over-all correlation between phosphatase content and duration of lactation. However, from inspection of Table II, it appears that several of the individuals do show a tendency to secrete a higher concentration of phosphatase as lactation proceeds. Actual correlations of phosphatase content with weeks of lactation were made for the samples from each of the seven donors. The correlation coefficients are shown in Table II, four of which are statistically significant at a probability level of 0.01 or less.

Inspection of the individual sets of analyses also indicated a possible correlation between phosphatase and fat content. The correlation coefficient calculated for 143 samples which were analyzed for both phosphatase and fat is 0.57, a value which, because of the large number of
observations, is statistically highly significant \( P < 0.001 \) despite the wide range in values.

**Table I**

*Comparative Levels of Alkaline Phosphatase and Other Constituents of Human Milk*

<table>
<thead>
<tr>
<th>Donor</th>
<th>Nationality</th>
<th>No. of children</th>
<th>No. of determinations</th>
<th>Phosphatase*</th>
<th>Per cent total solids</th>
<th>Per cent fat</th>
<th>Per cent nitrogen</th>
<th>Per cent solids-not-fat</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. M.</td>
<td>Scotch-Irish</td>
<td>30</td>
<td>5</td>
<td>3.11.54</td>
<td>3.282</td>
<td>3.0.168</td>
<td>3.8.65</td>
<td></td>
</tr>
<tr>
<td>P. M.</td>
<td>Irish</td>
<td>27</td>
<td>4</td>
<td>3.11.47</td>
<td>3.234</td>
<td>3.0.219</td>
<td>3.9.13</td>
<td></td>
</tr>
<tr>
<td>L. H.</td>
<td>Norwegian</td>
<td>33</td>
<td>3</td>
<td>3.11.50</td>
<td>3.4.33</td>
<td>3.8.23</td>
<td>3.9.13</td>
<td></td>
</tr>
<tr>
<td>E. E.</td>
<td>Caucasian</td>
<td>22</td>
<td>2</td>
<td>5.12.07</td>
<td>5.3.31</td>
<td>5.0.187</td>
<td>5.8.76</td>
<td></td>
</tr>
<tr>
<td>D. R.</td>
<td>Russian</td>
<td>19</td>
<td>2</td>
<td>5.12.18</td>
<td>5.1.84</td>
<td>5.8.84</td>
<td>5.7.68</td>
<td></td>
</tr>
<tr>
<td>M. P.</td>
<td>Caucasian</td>
<td>19</td>
<td>1</td>
<td>5.12.18</td>
<td>5.1.84</td>
<td>5.8.84</td>
<td>5.7.68</td>
<td></td>
</tr>
<tr>
<td>M. H.</td>
<td>German-English</td>
<td>35</td>
<td>2</td>
<td>5.12.18</td>
<td>5.1.84</td>
<td>5.8.84</td>
<td>5.7.68</td>
<td></td>
</tr>
<tr>
<td>C. M.</td>
<td>Japanese</td>
<td>30</td>
<td>2</td>
<td>5.12.18</td>
<td>5.1.84</td>
<td>5.8.84</td>
<td>5.7.68</td>
<td></td>
</tr>
<tr>
<td>L. K.</td>
<td>Polish-German</td>
<td>20</td>
<td>1</td>
<td>5.12.18</td>
<td>5.1.84</td>
<td>5.8.84</td>
<td>5.7.68</td>
<td></td>
</tr>
<tr>
<td>J. S.</td>
<td>Scotch-Irish-English</td>
<td>19</td>
<td>1</td>
<td>5.12.18</td>
<td>5.1.84</td>
<td>5.8.84</td>
<td>5.7.68</td>
<td></td>
</tr>
<tr>
<td>E. A.</td>
<td>German-French</td>
<td>26</td>
<td>1</td>
<td>5.12.18</td>
<td>5.1.84</td>
<td>5.8.84</td>
<td>5.7.68</td>
<td></td>
</tr>
<tr>
<td>Y. M.</td>
<td>French</td>
<td>23</td>
<td>3</td>
<td>5.12.18</td>
<td>5.1.84</td>
<td>5.8.84</td>
<td>5.7.68</td>
<td></td>
</tr>
<tr>
<td>Y. B.</td>
<td>Caucasian</td>
<td>24</td>
<td>2</td>
<td>5.12.18</td>
<td>5.1.84</td>
<td>5.8.84</td>
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</tr>
<tr>
<td>J. P.</td>
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<td>5.12.18</td>
<td>5.1.84</td>
<td>5.8.84</td>
<td>5.7.68</td>
<td></td>
</tr>
<tr>
<td>R. A.</td>
<td>Irish</td>
<td>26</td>
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<td>5.12.18</td>
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<td>5.8.84</td>
<td>5.7.68</td>
<td></td>
</tr>
<tr>
<td>B. C.</td>
<td>Irish-English</td>
<td>28</td>
<td>1</td>
<td>5.12.18</td>
<td>5.1.84</td>
<td>5.8.84</td>
<td>5.7.68</td>
<td></td>
</tr>
<tr>
<td>S. V. L.</td>
<td>German-English</td>
<td>32</td>
<td>4</td>
<td>5.12.18</td>
<td>5.1.84</td>
<td>5.8.84</td>
<td>5.7.68</td>
<td></td>
</tr>
<tr>
<td>V. T.</td>
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<td>4</td>
<td>5.12.18</td>
<td>5.1.84</td>
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<td>5.7.68</td>
<td></td>
</tr>
<tr>
<td>E. P.</td>
<td>&quot;</td>
<td>26</td>
<td>1</td>
<td>5.12.18</td>
<td>5.1.84</td>
<td>5.8.84</td>
<td>5.7.68</td>
<td></td>
</tr>
</tbody>
</table>

Average................................. 199.147 147.11.40 146.2.77 116.0.157 146.8.65

*Phosphatase activity expressed as micrograms of phenol liberated per ml. of milk.*

No relationship was discernible between phosphatase content and nitrogen content or solids-not-fat content.
PHOSPHATASE OF HUMAN MILK

TABLE II

Effect of Duration of Period of Lactation on Average Phosphatase Levels of Human Milk

<table>
<thead>
<tr>
<th></th>
<th></th>
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<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>weeks</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-5</td>
<td>30 (1)*</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>6-10</td>
<td>95 (4)</td>
<td></td>
<td>85 (3)</td>
<td>164 (2)</td>
<td>120 (4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11-15</td>
<td>132 (3)</td>
<td>121 (2)</td>
<td>184 (5)</td>
<td>127 (5)</td>
<td>91 (3)</td>
<td></td>
<td></td>
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<tr>
<td>16-20</td>
<td>135 (3)</td>
<td>303 (5)</td>
<td>354 (5)</td>
<td>255 (5)</td>
<td>69 (5)</td>
<td></td>
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<tr>
<td>21-25</td>
<td></td>
<td>376 (4)</td>
<td>161 (2)</td>
<td>68 (5)</td>
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<tr>
<td>26-30</td>
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<td>57 (4)</td>
<td></td>
<td>286 (1)</td>
<td></td>
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<tr>
<td>31-35</td>
<td></td>
<td>52 (5)</td>
<td>143 (5)</td>
<td>48 (8)</td>
<td></td>
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<td>36-40</td>
<td></td>
<td>59 (3)</td>
<td>303 (5)</td>
<td>55 (4)</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>41-45</td>
<td></td>
<td>74 (5)</td>
<td>244 (5)</td>
<td>84 (3)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>46-50</td>
<td></td>
<td>128 (1)</td>
<td>250 (5)</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>51-55</td>
<td></td>
<td>225 (4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>56-60</td>
<td></td>
<td></td>
<td>178 (4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>61-65</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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</tr>
</tbody>
</table>

Correlation coefficient...

* The numbers in parentheses indicate the number of observations.
† Probability level 0.01.
‡ Probability level 0.001.

DISCUSSION

The outstanding finding in this study is the wide variation in alkaline phosphatase activity of human milk, both from mother to mother and during the period of lactation of a given individual.

The values obtained for alkaline phosphatase did not differ widely from those reported by Chanda and Owen (5), although samples in the present study were mostly from women who had been lactating for 2 months or more.

The values obtained for per cent of total solids, fat, nitrogen, and solids-not-fat compared favorably with the reported data of other investigators (29-32).

The function, if any, of phosphatase in human milk is unknown. Cranston (33) has proposed that it aids in the absorption of carbohydrate by the infant, but the mechanism is obscure. There may be some theoretical importance to the apparent tendency of phosphatase to increase with fat content, but a correlation coefficient, even when it is much higher than in the present instance, does not in itself imply any cause or effect relation-
ship. One could also conjecture that the phosphatase is linked in some manner to a minor constituent of the milk.

There appeared to be no relationship between the phosphatase values and the age or nationality of the mother, or the number of children in her family, though the number of donors was too small to test these hypotheses adequately. The small number of subjects involved and the variability of the results also make it unwise to draw conclusions about variation of phosphatase content with length of lactating period, even though some of the data of this study suggest that a relationship may exist.

SUMMARY

1. A method of measuring alkaline phosphatase activity of human milk is described.

2. Analyses of 199 samples of human milk gave an average alkaline phosphatase level of 147 units per ml. The range was from 30 to 540 units per ml.

3. Some tendency for high phosphatase levels to be associated with a high fat content was observed. Other than this there appeared to be no relationship of phosphatase values to the other constituents examined.

4. Other than a possible tendency for alkaline phosphatase concentration to increase as lactation progressed, no relationship was found between the level of the enzyme and such characteristics of the donor as age, nationality, or the number of children.

The authors wish to thank the Mothers’ Milk Bank, Inc., and its sponsor, the San Francisco Branch of the American Association of University Women, for their help and cooperation in making this study possible, and Miss Shirley Brazda and Mrs. Judy Pelts for their technical assistance.

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