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Bile Salt-Stimulated Lipase and Esterase Activity in Human Milk after Collection, Storage, and Heating: Nutritional Implications

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Summary

In a study of human milk obtained in the first month of lactation, lipase and esterase activity were assayed. Bile salt-stimulated lipase (BSSL) and bile salt-stimulated esterase (BSSE) activities in colostrum were similar to corresponding enzyme activities in transitional milk and in mature milk. BSSL and BSSE were significantly ($P < 0.001$) correlated to one another, which suggests that lipase and esterase activities in milk are due to the same enzyme. When milk was allowed to stand at room temperature, in a refrigerator, or subjected to freezing and thawing, wide fluctuations were observed in lipase and esterase activities, but there was no systematic tendency for enzyme activity to increase or decrease. Heating milk to various temperatures between 40–55°C resulted in progressive loss of enzyme activity. The activation energy for the process which inactivates the enzyme was found by linear regression to the Arrhenius plot to be $2 \times 10^5 J \cdot mole^{-1}$. Our findings suggest that lipase and esterase activity in human milk which is donated to hospitals and stored frozen can make a valuable contribution to fat digestion in the newborn infant, but pasteurization destroys the enzyme.

Abbreviations

BSSE, bile salt-stimulated esterase
BSSL, bile salt-stimulated lipase

It is well known that human milk contains two different lipases. A serum-stimulated lipase, which has properties similar to lipoprotein lipases, is found in milk fat globules whereas BSSL occurs in the aqueous fraction of milk (19). These enzymes have been characterised (4, 12, 13, 21, 22), and it seems that BSSL could make an important contribution to fat digestion in the newborn infant (14). BSSL has properties which suggest that it is active in the small intestines of newborn infants. This enzyme is stable at pH values as low as 3.5 and is not affected by trypsin, provided that bile-salts are present. The two primary bile-salts, cholate and chenodeoxycholate can, at relatively low concentrations that are known to occur in the intestines in early infancy, activate BSSL.

In the present study, esterase and lipase activities were determined in milk specimens donated by mothers who gave birth at our hospital. We investigated possible differences between colostrum, transitional milk, and mature milk. The relationship of lipase to esterase activity was studied because these two activities in milk may be due to the same enzyme. Human milk donated to hospitals is stored frozen in milk banks after pasteurization. This milk is fed to preterm infants in special care nurseries. Further studies were, therefore, undertaken to investigate the effects on lipase and esterase activity of standing milk at room temperature or in a refrigerator, freezing and thawing milk, and heating milk.

MATERIALS AND METHODS

Milk samples were donated during the first month of lactation by mothers who had given birth at term, at St. Mary's Hospital. Milk samples were collected after the babies were breast fed, and immediately after collection the milk was centrifuged at 20,000 *g* for 30 min at 4°C to separate the cream fraction from skim milk, which was used as the source of enzyme. Triolein, sodium taurocholate, *p*-nitrophenyl acetate, each 99% pure, and gum arabic were purchased from Sigma London Chemical Co. Ltd., Poole, Dorset, England. DEAE Sephadex A50 and Sephacryl Sepharose 300 were purchased from (Pharmacia Ltd., Hounslow, Middlesex, England).

Esterase activity. We have established that the esterase activity of skim milk, as measured by the hydrolysis of *p*-nitrophenyl acetate, is largely due to the BSSL (see later). Esterase assays were performed under conditions where the sensitivity to changes in substrate concentrations and effects of product were small. Reproductivity in the assay was very good, standard deviation in replicate assays being about 4%. Hydrolysis of *p*-nitrophenyl acetate was determined spectrophotometrically (7, 13). *p*-Nitrophenyl acetate (2 mM) was prepared from a stock solution of *p*-nitrophenyl acetate in acetone (200 mM) by dilution (1:100 v/v) in distilled water. Phosphate buffer pH 7.0 (0.2 M, 500 μl), with or without sodium taurocholate (0.8 mM), and *p*-nitrophenyl acetate (2 mM, 500 μl) were mixed in a cuvette. Skim milk (20 μl) was added and the absorbance change at 400 nm was followed for 3–5 min using a Cary 118C UV-Vis spectrophotometer. Reaction rates were calculated from absorbance changes using an ϵ_{400} value of 9124 $M^{-1} \cdot cm^{-1}$. One esterase unit is defined as the amount of enzyme that releases 1 μmol of *p*-nitrophenol per min.

Lipase assay. Hydrolysis of emulsified triolein was recorded by continuous titration of released fatty acid to a fixed pH end point, using a Radiometer pH stat. An emulsion of triolein was prepared by sonicating triolein (0.2–1.0 g) in an aqueous solution of gum arabic (5%, 30 ml) for 5 min, using an MSE 100W disintegrator at an amplitude of 7 μ. After adjusting to pH 9.0, sonication was continued for a further 5 min. The emulsion was cooled in ice water during sonication, stored throughout the day on ice, and protected from the light. Fresh emulsion was prepared daily.

Sodium chloride (0.2 M, 3 ml), emulsified triolein (20 mM, 500 μl), and sodium taurocholate (12 ml, 500 μl) were mixed and pH adjusted to 8.0. The addition of titrant, sodium hydroxide (0.05 N) required to maintain a pH of 8.0 was recorded continuously for 3–5 min. Skim milk, 25–100 μl, as added to the incubating mixture and the consumption of titrant was followed for a further 5–10 min. In some experiments defatted bovine serum albumin was added (14 mg/ml, final concentration) as fatty acid acceptor in an attempt to prolong the initial linear rate of proton production. One lipase unit is defined as that amount of enzyme that releases 1 μmol of fatty acid per min. The assay was corrected for the small effect of CO_2 absorption and extensive studies were performed to discover optimal conditions. Reproducibility was acceptable for an assay of this nature and standard deviations in replicate assays was of the order of 8%. Lipase and esterase activities assayed were 1.5 times higher at 37°C than at room temperature (25°C), and in this work measurements were carried out at 37°C unless indicated otherwise.

Enzyme purification. The BSSL was partially purified from skimmed milk by heating at 40°C at pH 4.4, ion exchange chromatography of the whey proteins on DEAE Sephadex, and after concentrating the active fractions using a Millipore 'Pelikan' filter, gel permeation chromatography on Sephacryl Sepharose 300.

Freezing and thawing milk. BSSL, BSSE, and esterase activity without taurocholate were assayed in the skim fraction from an aliquot of whole milk immediately after collection. The remaining milk was frozen at −20°C. Thawing was allowed to take place at room temperature and enzyme activity was assayed in the skim fraction after each freeze and thaw cycle.

Thermal inactivation. Immediately after collection, an aliquot of milk was assayed for BSSL, BSSE, and esterase activity without taurocholate in the skim fraction. The remaining milk was allowed to stand at room temperature (25°C) or in a refrigerator (4°C). At time intervals, aliquots of milk were separated and assayed for enzyme activities. For the higher temperature studies a test tube was heated in a water bath, between 40–55°C, then a quantity of milk was added. Aliquots of heated milk were separated at time intervals, rapidly cooled to room temperature, and assayed for BSSE activity.

Statistics. Linear regression analysis and weighted non-linear regression analysis were carried out as described elsewhere (2, 20). Student's *t* test was used when comparing differences in mean values.

RESULTS

Enzyme activity in colostrum, transitional milk, and mature milk. BSSL and BSSE in milk samples donated by 10 mothers 1–5 d (colostrum), 6–10 d (transitional milk), and after 10 d (mature milk) of lactation showed no statistically significant differences. The mean (± SD) lipase and esterase activities in colostrum were 18.65 ± 4.4 and 23.9 ± 4.3 units·ml^{-1}; in transitional milk, 17.5 ± 5.7 and 24.5 ± 12.1 units·ml^{-1}; and in mature milk, 19.8 ± 5.6 and 24.95 ± 6.05 units·ml^{-1}. Statistical analysis of the data suggested a skew distribution. Sums of squares from curve fitting and coefficients of skew were lower for a log normal distribution than for a normal distribution.

Relationship of lipase to esterase activities. To test the hypothesis that lipase activity and esterase activity are due to the same enzyme, we applied least square regression analysis to the pairs of variables for esterase and lipase activities, both determined on 139 different samples of milk. The data analysed were from untreated skim milk and samples obtained from various stages of purification. The data are displayed in Figure 1 and the best fit line through the origin had a slope of 0.714 (correlation coefficient = 0.834), strongly suggesting a linear relationship ($P < 0.001$). A similar analysis of a subset of the data (115 samples, omitting data from enzyme purifications) had a slope of 0.689 (correlation coefficient = 0.787, $P < 0.001$).

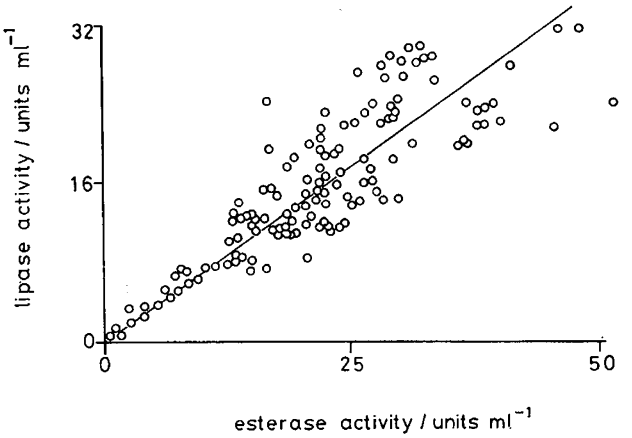

Fig. 1. The relationship between lipase and esterase activities. The plotted points are for both bile salt-stimulated lipase and bile salt-stimulated esterase activities determined in the same sample. The line shown is the best fit regression line through the origin with slope 0.714 (correlation coefficient = 0.834, $P < 0.001$).

Freezing and thawing. The mean (± SD) enzyme activity of BSSL, BSSE, and esterase without taurocholate in 10 milk samples before the freeze and thaw experiments was 8.13 ± 2.88, 5.06 ± 2.33, and 1.22 ± 0.72 units·ml^{-1}, respectively. The milk samples were frozen and thawed eight times and enzyme activities appeared to fluctuate after each freeze and thaw cycle. Each individual set of data on enzyme activity was fitted by non-linear regression analysis to polynomial models of degree 0, 1, and 2, until the F test showed no statistical improvement in fit (18), Table 1. The data were then normalised by dividing by the constant term calculated by regression analysis, which is an estimate of the level of enzyme activity before milk is subjected to freezing and thawing. The means (± SD) of the three sets of normalised enzyme activity in 10 milk specimens are shown in Figure 2. Although the process of freezing and thawing results in large fluctuations in enzyme activity, there is no systematic tendency for this procedure to destroy or to increase enzyme activity. It was shown that this wide variability in response to repeated freezing and thawing was not due to variability in the assays; independent work on the stability of the reagents used and reproducibility of the assay on samples of enzyme stored unfrozen over equivalent periods of time established this.

Room temperature (25°C) and refrigeration (4°C). Mean enzyme activity of BSSL, BSSE, and esterase without taurocholate in 10 milk samples were 14.0 ± 6.63, 7.37 ± 3.45, and 2.27 ± 1.25 units·ml^{-1} before these milk samples were allowed to stand at room temperature or in the refrigerator. Enzyme activities in each milk sample, immediately after collection and at time intervals after standing milk at room temperature or in a refrigerator, were analysed in the same way as the freeze and thaw data previously discussed (Table 1 and Fig. 3 A and B). Random fluctuations in enzyme activities were again observed, but no systematic tendency for the enzyme activity to increase or decrease.

To appreciate the significance of the statistical analysis carried out in Table 1, we emphasize that out of 90 sets of data, 46 showed no statistically significant trend, 27 showed improvement with a linear term, and 17 required a quadratic term. But, 15 of the linear terms were negative and 12 positive whereas nine of the quadratic terms were negative and eight were positive. Using the F statistic at 99% confidence level, we can conclude that there is sometimes a linear trend but only rarely a quadratic trend in the individual milk lipase and esterase activities. The fact that this tendency is about equally often upwards as downwards suggests a wide fluctuation in enzyme activity between

Table 1. *Statistical analysis of freeze/thaw and temperature inactivation**

Treatment	1	2	3	4	5	6	7	8	9	10
Freezing and thawing										
BSSL	−	−	0	0	0	+	0	0	−	0
Esterase without taurocholate	0	+	0	0	+	−	(+ −)	0	0	0
BSSE	0	0	0	−	0	0	(+ −)	−	0	−
Standing at 4°C										
BSSL	0	(+ −)	0	−	−	0	0	0	−	0
Esterase without taurocholate	0	(+ −)	0	0	(− +)	0	(+ −)	+	0	0
BSSE	+	(+ −)	−	0	0	0	+	0	0	+
Standing at 25°C										
BSSL	0	(+ −)	0	(− +)	(− +)	(− +)	−	0	−	0
Esterase without taurocholate	0	0	0	−	(− +)	+	(− +)	+	+	(− +)
BSSE	0	(+ −)	0	(+ −)	(− +)	0	−	+	+	0

* Milk from 10 different individuals was analysed for lipase and esterase activities after eight cycles of freezing and thawing and during standing at 4°C and 25°C. Each of the 30 sets of data was fitted successively by polynomials of degree 0, 1, and 2 using a computer program to find the set of coefficients minimising the sum of weighted, squared residuals. Then the F statistic was calculated to see whether a higher order model was justified at 99% confidence level. The symbol 0 means a constant model was best, one sign indicates a linear model (+ signifies an upward, − signifies a downward trend) and a double sign in parenthesis is where both linear and quadratic terms (of signs indicated) were statistically significant.

Abbreviations: BSSL, bile salt-stimulated lipase and BSSE, bile salt-stimulated esterase.

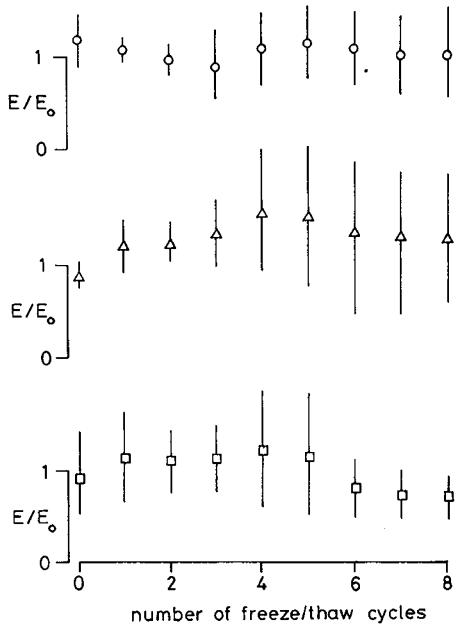

Fig. 2. Effects of repeated freezing and thawing on the fractional enzyme activity (E/Eo). Each data set was normalized by dividing by the constant coefficient obtained by regression analysis. The normalised mean and standard deviations of enzyme activity were calculated for each freeze and thaw cycle. The vertical bars represent ±1 SD, and the means of 10 milk samples are indicated by the following symbols: O, BSSL activity; Δ, esterase activity without taurocholate; and □, esterase activity with taurocholate (bile salt-stimulated esterase).

individual milk specimens, rather than a systematic tendency for the enzyme activity to increase or decrease when subjected to freeze and thaw operations, or standing at room temperature or in a refrigerator.

Heat treatment. Data on esterase activity in milk samples immediately after collection and after heating in a water bath were normalised. The remaining fraction of enzyme activity as a function of time is shown in Figure 4a. The data was fitted by non-linear regression analysis to a single exponential decay model. The regression equation is $V/Vo = Ae^{-kt}$ (V/Vo is the remaining fraction of enzyme activity after heat treatment, A is a pre-exponential factor, e is a constant, k is the rate constant, and t is time). Loss in enzyme activity is always taking place, and that is what A and k measure. The rate constants for this decay are given in the legend to Figure 4b together with the energy of activation for the decay process calculated from the Arrhenius plot (Fig. 4b).

DISCUSSION

The observation that BSSL activity in milk does not appear to vary with the period of lactation is in keeping with other reports (10, 13, 15), but the observed esterase activity also does not appear to vary with lactation. This observation together with the positive correlation between lipase and esterase activities suggest that these two activities in milk are due to the same enzyme. There are at least two advantages in preserving BSSL in human milk donated to hospitals for feeding preterm infants. Fat digestion and absorption is improved in the presence of BSSL, and the esterase activity of this enzyme appears to have a role in the digestion of retinol esters, which are a source of vitamin A for the infant (7, 8).

The basis for suggesting that BSSL makes an important contribution to fat digestion in newborn infants is provided by the results of experimental and clinical studies. When pasteurized milk was used as substrate and skim milk as enzyme source, BSSL hydrolysed all milk triglycerides to free fatty acids and glycerol by 30 min of incubation at pH 6.5, and a taurocholate concentration of 1.5 mM, which is well within the physiologic range (0.02–5.29 mM) in early infancy (13). Another study (10), using fresh milk as source of enzyme and substrate reported that the mean percentage lipid hydrolysed in milks collected in the first 15 d of lactation and, after, were 38.9% and 19.3%, respectively, on incubating for 2 h, which is the estimated transit time from duodenum to jejunum (11). The action of BSSL may also aid the absorption of lipolytic products. Bile salt concentrations in duodenal contents of newborn infants is relatively low, often below that necessary for micelle formation, and as a result fatty acids are absorbed more efficiently than 2-monoglycerides (17). Because BSSL hydrolyses triglycerides to free fatty acids and glycerol (13), whereas the action of pancreatic lipase results in the release of free fatty acids and 2-monoglycerides (5) BSSL is of physiologic importance in this respect. The 2-monoglycerides formed by pancreatic lipase are also hydrolysed by BSSL (16); thus, by complementing the action of pancreatic lipase BSSL may ensure an efficient utilization of milk lipids.

Serial metabolic studies carried out in preterm infants have shown that heat treatment of human milk reduces fat absorption by about a third (1, 24). This is clearly of importance in the nourishment of preterm infants. The reduction of fat absorption can be explained on the basis that pasteurization denatures BSSL

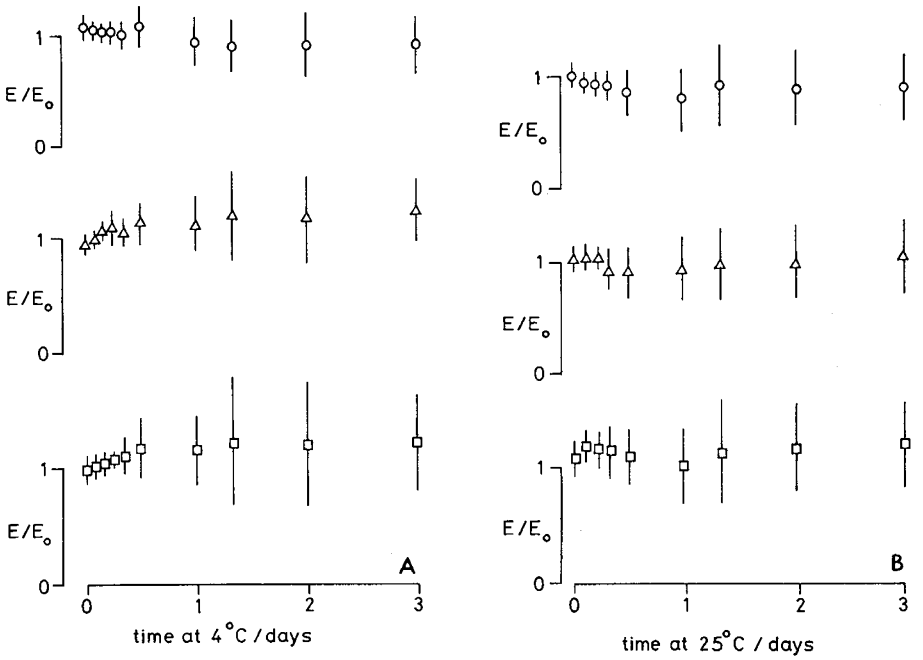

Fig. 3A. Effects of standing milk in a refrigerator (4°C) on the fractional enzyme activity (E/Eo). Each data set was normalized as described in the legend to Figure 2. The vertical lines represent ±1 SD, and the means of 10 milk samples are indicated by the following symbols: O, bile salt-stimulated lipase activity; △, esterase activity without taurocholate; and □, esterase activity with taurocholate (bile salt-stimulated esterase).

Fig. 3B. Effects of standing milk at room temperature (25°C) on the fractional enzyme activity (E/Eo). See legend to Figure 3A for explanation of symbols.

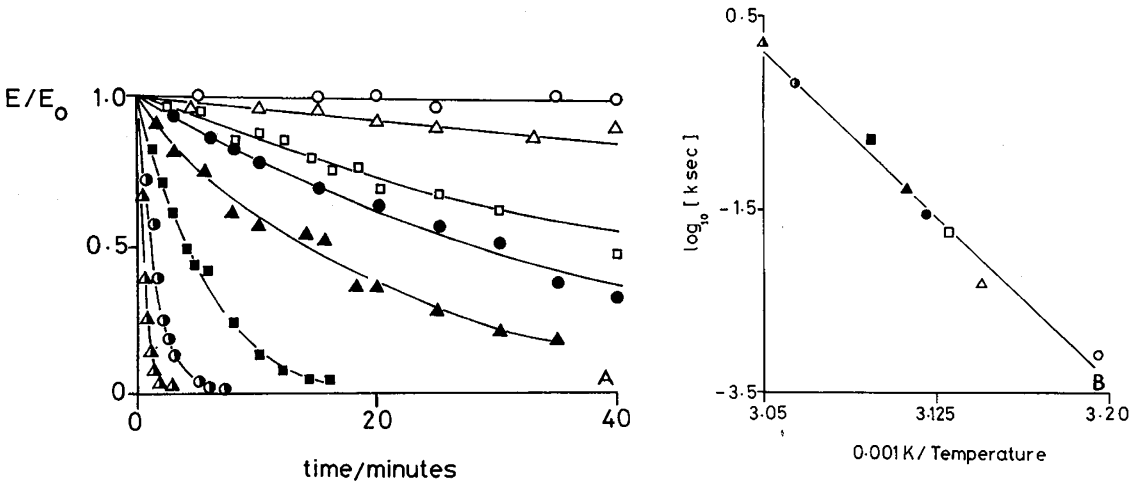

Fig. 4A. Inactivation of enzyme by heating. The lines are for the best fit first order kinetics obtained by non-linear regression analysis to the equation $V/Vo = Ae^{-kt}$. It is presumed that fractional velocity equals fractional enzyme concentration, *i.e.*, V/Vo = E/Eo.

Fig. 4B. Arrhenius plot of the data shown in Figure 4A. The rate constants were as follows:

Temperature 0°C	k/s^{-1}
40.0	1.32×10^{-5}
45.0	7.83×10^{-5}
46.5	3.00×10^{-4}
47.5	4.33×10^{-4}
48.2	9.00×10^{-4}
50.0	3.00×10^{-3}
53.5	1.13×10^{-2}
55	3.17×10^{-2}

Symbols in 4A and 4B indicate the following: O, 40°C; △, 45°C; □, 46.5°C; ●, 47.5°C; ▲, 48.2°C; ■, 50°C; ◑, 53.5°C; and ◭, 55°C.

(3), which our observations suggest takes place at temperatures greater than 40°C.

The contribution of BSSL to retinol ester hydrolyses has been studied in preterm infants, using duodenal contents collected 1 h after a test meal as the source of enzyme (8). It appears that enzyme activity towards retinol esters is three times higher in duodenal contents collected after a test meal of fresh milk than after test meals of pasteurized milk. Feeding pasteurized milk to preterm infants may, therefore, reduce the availability of vitamin A.

Human milk which is stored frozen in hospital milk banks is usually milk which has been pasteurized by heating to 62.5°C for 30 min (holder pasteurization) to eliminate potential bacterial pathogens. Because heating milk destroys BSSL, the effect of freezing without prior pasteurization was investigated, and the results indicate that milk frozen in the raw state retains its BSSL activity. It is, therefore, recommended that all milk received at hospital should be bacteriologically tested to determine whether pasteurization is necessary. If the bacterial count is relatively low and the milk does not contain enterobacteria, the risk of causing infection in newborn infants is not clinically significant (25). These milk specimens should be frozen in the raw state. In addition to retaining its BSSL activity, there are also other advantages in avoiding pasteurization, including preservation of many antimicrobial factors present in human milk (6, 9) and avoiding the loss of essential fatty acids, linoleic acid, and linolenic acid by autoxidation (23). The protective properties and the nutritive value of human milk are maintained for preterm infants.

Human milk which is donated to hospitals is usually collected at home and has to be stored before being taken to hospital. As shown in our study, milk can be stored in a refrigerator, at room temperature, or stored frozen and thawed without a deleterious effect on BSSL activity. From the point of view of BSSL stability, donated milk can be stored at home in a refrigerator or a freezer and allowed to stand at room temperature for short periods, but storage at room temperature is not recommended because this temperature is more likely to favour bacterial growth.

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