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Effect of storage and heat on antimicrobial proteins in human milk

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SUMMARY Human milk, after storage and pastuerisation at 73°C for 30 minutes at a milk bank, was found to have little surviving IgA, IgG, lactoferrin, lysozyme, and C3 complement. Accurate pasteurisation at 62·5°C produced a loss of 23·7% of the lysozyme, 56·8% of the lactoferrin, 34% of the IgG, but no loss of IgA. Storage by deep freezing at −20°C for 3 months produced no appreciable loss of lactoferrin, lysozyme, IgG, IgA, or C3.

Human milk is thought by many paediatricians to be the best food for low birthweight infants (Davies *et al.*, 1972). In England and Wales five large-scale human milk banks operate primarily for this purpose (Rolles, 1973) and most maternity units make some effort to collect milk for their special care babies. We question whether the final product after storage and processing has the same value for the preterm infant as feeding at the breast has for the mature one. Little is known of the best method of collection, storage, and processing of human milk and this paper is intended to draw attention to two aspects: the effect of heating and storing of human milk on its content of immunoglobulins and other antimicrobial factors.

Methods

Milk samples. Human milk was collected by mothers in their own homes, using glass Woolwich shells to obtain overflow milk. The milk was stored unfrozen in the family refrigerator for up to 48 hours, until collected by the staff of the Human Milk Bank, St David's Hospital, Cardiff. Aliquots of milk as it arrived at the milk bank were analysed either raw, after deep freezing for 3 months at −20°C, after lyophilisation and reconstitution, or after pasteurisation at the laboratory or milk bank.

Pasteurisation. This was achieved by placing bottles of milk into a steam-heated water bath. The holding temperature during the study was found to be 72° to 73°C for 30 minutes, although the temperature aimed for was 65°C. Cooling was achieved by circulating water at room temperature. Laboratory pasteurisation was performed by heating small (2 ml) samples in an accurately regulated water bath and using constant agitation until the holding temperature was achieved, and then held for 30 minutes. Five temperatures (°C) were used, 60°, 62·5°, 65°, 67·5°, and 70°.

Quantitative electroimmunoassay.

Antigen and monospecific antiserum. Lysozyme was prepared by the method of Jollés and Jollés (1967) and lactoferrin prepared from human milk as previously described (Ryley, 1972). Monospecific antiserum to α_1-antitrypsin, IgG, lactoferrin, and lysozyme was raised in rabbits (Ryley, 1972; Ryley and Brogan, 1973; Ryley *et al.*, 1975). Antisera to C3 component of complement and IgA were obtained from Hoechst Pharmaceuticals, Hounslow, England.

Electroimmunoassay. 2 µl volumes of either raw or treated milk were analysed by an electroimmunoassay method against monospecific antiserum in 1% agarose, or in the case of IgG 1% ion agar as previously described (Ryley and Brogan, 1973). Dilution of a laboratory control serum standardised against both a Behring human serum and plasma controls were used for the estimations of α_1-antitrypsin, C3, IgA, and IgG. Dilutions of 1 mg/ml solution of both lysozyme and lactoferrin antigens were used in their appropriate assay. All estimations were carried out on duplicate plates.

Results

Raw milk. Table 1 shows the results of assays on 25 random donations to the milk bank.

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Table 1 *Concentration of 6 proteins in human milk donated to a milk bank (mg/100 ml milk)**

	No. of samples	*Mean ± SE*	*Range*
α_1-antitrypsin	25	3·42± 0·07	1·0–21·2
C3	24	1·77± 0·018	0·7–4·0
IgG	25	0·43± 0·04	0·2–2·4
IgA	25	7·96± 0·12	0·9–20·6
Lactoferrin	25	419± 77	74–1606
Lysozyme	25	5·9± 1·4	0·5–32·5

*Values in Tables 1–4 are given in traditional units, i.e. mg/100 ml; to convert to g/l, multiply by 0·01.

Table 2 *Effect of milk bank pasteurisation on 6 milk proteins (mg/100 ml milk)*

Protein (no. of samples)	*Raw milk (mean ± SE)*	*Pasteurised milk (mean ± SE)**
α_1-antitrypsin (16)	2·38± 0·30	1·47±0·25 (61·8)
C3 (16)	1·35± 0·13	<0·1
IgA (16)	5·5± 1·10	<0·1
IgG (16)	0·42± 0·05	<0·1
Lysozyme (16)	7·3± 2·0	0·17±0·1 (2·3)
Lactoferrin (16)	337±57·3	3±1·1 (0·9)

*Numbers in parentheses are means as % of raw value.

Pasteurisation. There was marked destruction of activity after milk bank pasteurisation (72°–73°C) (Table 2). Only α_1-antitrypsin showed any significant survival.

Table 3 shows the results of well-controlled heating under laboratory conditions for milk lysozyme, lactoferrin, IgG, and IgA at 5 temperatures, 60°, 62·5°, 65°, 67·5°, and 70°C, each for 30 minutes. IgA survived with relatively little loss until the temperature of 70°C was used (33·3% loss). IgG was much more labile, with 65°C producing a loss of 77·2%. Lactoferrin showed a similar pattern of thermolability with a slightly greater loss (85%) at 65°C.

The results for lysozyme showed a wide variation dependent on pH. The mean results are shown in Table 3, but there were 5 samples of pH 6 and 4 samples of pH 7. At 65°C there was a mean loss of 98·7% at pH 7, but only a 27% loss at pH 6.

Freezing and lyophilisation. Table 4 shows the results of 3 months' storage at −20°C, and of freeze-drying and reconstitution. There was no significant change in lactoferrin, lysozyme, IgA, IgG, and C3, after 3 months' freezing, but a small loss of IgG occurred after lyophilisation.

Discussion

The milk donations were up to 48 hours old, often heavily contaminated with bacteria (a recent study showed that nearly 50% of samples had 10^6 organisms/ml) (C. H. L. Howells and T. J. Evans, unpublished 1975) and from the 'mature' phase of lactation, yet our results for lactoferrin and lysozyme compare favourably with published results (Bullen *et al.*, 1972; Peitersen *et al.*, 1975). Immunoglobulin levels and C3 were, however, quite low compared with the data of others (Mata and Wyatt, 1971; Peitersen *et al.*, 1975), the loss possibly occurring during the 48 hours' storage at the mother's home. Szöllösy *et al.* (1974) showed a marked loss of antibody in human milk during periods of bacterial growth. Also, our IgA values were measured by using a 7S IgA standard and we have not attempted a conversion to secretory IgA as did Peitersen *et al.* (1975), which partly explains the lower values.

We found no published data for α_1-antitrypsin in human milk though Laskowski and Laskowski (1951) measured total antitryptic activity and found it only in colostrum. Bullen *et al.* (1972) postulated that milk antitrypsins could protect lactoferrin from gastrointestinal trypsin and this encouraged us to include it in our studies as it could also help to protect milk proteins from milk proteases (Heyndrickx, 1962) or bacterial enzymes (Moore *et al.*, 1964) during periods of storage *in vitro*.

There is a surprising lack of information on heat stability. Complement was expected to be labile, also expected was the greater survival of IgA compared with IgG. Lysozyme is heat stable at acid pH (Jollés

Table 3 *Effect of pasteurisation for 30 minutes at 60°, 62·5°, 65°, 67·5°, and 70°C on IgA, IgG, lysozyme, and lactoferrin (mg/100 ml)*

		Pasteurisation				
	Raw milk (mean ± SE)	*60° (mean ± SE)*	*62·5° (mean ± SE)*	*65° (mean ± SE)*	*67·5° (mean ± SE)*	*70° (mean ± SE)*
IgG 9 samples	1·05±0·22	0·87±0·45 (82·8)	0·69±0·13 (66)	0·24±0·6 (22·8)	0·1±0·05 (9·6)	0·027±0·022 (2·6)
IgA 6 samples	15·6±2·3	16·3±4·8 (105)	15·8±2·4 (101)	14·3±1·9 (91·9)	13·95±1·7 (89)	10·56±1·0 (67·7)
Lactoferrin 9 samples	565±185	476±171 (84·3)	244±79·7 (43·2)	83·2±29 (14·7)	45·2±16 (8)	32±12·3 (5·7)
Lysozyme 9 samples	3·5±0·94	4·06±0·94 (115·6)	2·68±0·74 (76·3)	1·35±0·45 (38·6)	0·53±0·82 (15·2)	0

*Figures in parentheses are the means as % of raw milk value.

Table 4 *Effect of deep freezing (3 m) at −20°C and lyophilisation of human milk proteins (mg/100 ml milk)*

	Raw milk (mean ± SE)	*Deep frozen milk*			*Lyophilised milk*		
		Mean ± SE	*Mean as % raw*	*P*	*Mean ± SE*	*Mean as % raw*	*P*
α_1-antitrypsin 16 samples	2·38±0·3	1·98±0·2	83·2	<0·05	2·22±0·3	93·3	>0·1
IgA 8 samples	9·55±0·84	9·25±0·83	96·9	>0·1	9·33±0·74	97·7	>0·1
IgG 16 samples	0·42±0·05	0·42±0·04	100	>0·1	0·33±0·04	78·6	<0·05
Lactoferrin 11 samples	332±71·7	338±57·4	102	>0·1	363±79	109·3	>0·1
Lysozyme 11 samples	5·1±1·26	4·6±0·67	90·2	>0·1	4·8±1·19	94·1	>0·1
C3 16 samples	1·35±0·13	1·26±0·11	93·3	>0·1	1·27±0·13	94·1	>0·1

and Jollés, 1961) but very labile at the natural pH of human milk (7·2–7·4) (Chandan *et al.*, 1964), though during storage the fall in pH may aid survival. The apparent increase of lysozyme on heating to 60°C was probably due to release from the often large cellular component of human milk. Storage by deep freezing seemed a very satisfactory procedure and the more expensive lyophilisation showed no advantage.

Although breast feeding is both natural and advantageous to the normal term infant, doubt has been cast on its ability to provide optimal growth for the very preterm infant (Davies, 1977; Fomon and Ziegler, 1977). Autoclaving human milk as practised in some hospitals would also limit its antimicrobial advantage, especially as it has been shown that a breast milk substitute can produce a similar gut flora of lactobacilli (Willis *et al.*, 1973).

We suggest that human milk should be collected in as sterile a manner as possible and deep frozen shortly after collection. If of low bacterial count then its use unheated should be considered. Pasteurisation, if used, should be at the minimum temperature capable of adequate bacterial killing (about 62°C for 30 minutes) (Szöllösy *et al.*, 1974). Unfortunately, there does not seem to be a commercial apparatus available in the United Kingdom capable of dealing with small volumes and achieving uniform and accurate heating.

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