Influence of the heat treatment of human milk on some of its protective constituents

Human milk was subjected to heat treatments of graded severity and examined for its content of immunoglobulins, lactoferrin, lysozyme, vitamin B_{12}-and folate-binder proteins, and lactoperoxidase. Holder pasteurization (62.5° C 30 minutes) reduced the IgA titer by 20%, and destroyed the small content of IgM and most of the lactoferrin. Lysozyme was stable to this treatment, but with an increase in temperature there was progressive destruction, to near 100% at 100° C. The same was broadly true of the capacity of milk to bind folic acid and protect it against bacterial uptake; with vitamin B_{12} the binder was more labile at 75° C than at 100° C. The milk contained no detectable lactoperoxidase.

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IT IS WIDELY HELD among pediatricians that the incidence of infectious disease is lower in breast-fed than in bottle-fed infants.[1] Certainly the maternal milk confers specific protection against many pathogenic bacteria and viruses by virtue of its content of immune antibodies. It also contains a variety of nonspecific factors that strongly and selectively influence the growth of different microorganisms in vitro and may play an important part in the nutrition of the infant and in protecting it against infection. These "nonspecific" factors include the iron-binding protein lactoferrin, the proteins that bind vitamin B_{12} and folate, and lysozyme and lactoperoxidase.[1-4]

Expressed breast milk is widely used in the nurturing of small-for-gestational age and premature infants. Some are given milk from their own mothers without any intermediate heat treatment, while others receive milk from donor mothers that has been heat treated to destroy contaminating bacteria. The present study was undertaken to determine to what extent the immunoglobulins and other protective proteins in the milk are stable to such heating.

MATERIALS AND METHODS

Milk samples. Milk was obtained from the Human Milk Bureau at St. David's Hospital, Cardiff. It was a bulked sample representing mainly mature milk from 25 or more donors, and it had been stored unfrozen at about 4° C for one to two days by the time it was received at our laboratory. It was centrifuged at 2° C for one hour at 75,000 g, and the aqueous phase was carefully decanted and filtered through Kleenex tissue. The fat and sediment was discarded.

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See related article p. 167.

Abbreviation used
HTST: high temperature-short time

Heat treatment of milk. Samples of milk (1.5 ml) were sealed into small glass ampules and heated in a bath of detergent maintained at the required temperature ± 0.1° C. They were then cooled in iced water and stored at −30° C until tested.

The times and temperatures of heating were as follows: 56° C 30 minutes; 62.5° C 30 minutes (holder pasteurization); and 65, 70, 75, 80, 85, 90, 95, and 100° C all for 15 minutes. For some of the tests milk was heated by a small-scale laboratory process which closely simulated high temperature-short time pasteurization.[5]

Estimation of immunoglobulins. The radial immunodiffusion technique of Mancini and associates[6] was used

Table I. Immunoglobulin activity (mg/ml) in unheated and heat-treated human milk

Heat treatment	*IgA*	*IgM*	*IgG*
None	0.50	0.10	None detected
56° 30 min	0.48	0.10	None detected
62.5° 30 min	0.39	0	None detected
70° 15 min	0.24	0	None detected
80° 15 min	0.10	0	None detected

Table II. Total lactoferrin and unsaturated iron-binding capacity in unheated and heat-treated human milk

Heat treatment	*Lactoferrin* (mg/ml)*		*Unsaturated iron-binding capacity† (μgFe/ml)*		
None	3.4	3.7	3.4	3.1	3.1
56° 30 min	–		3.0	–	–
Holder pasteurization (62.5° 30 min)	1.2	1.5	1.3	0.7	0.8
65° 15 min	1.0	1.0	0.6	0.4	0.4
70° 15 min	0.2	0.1	0.1	0	0
75° 15 min	0.2	0.2	0	0	0

*Duplicate determinations were made on each test sample.
†Each value represents a different preparation of milk.

with Boehringer low-level ICL immunoglobulin plates (Boehringer, Mannheim GmbH). The antisera were raised against serum immunoglobulins. IgA occurs as "secretory IgA" in milk, but the cross reaction with serum IgA antiserum was sufficient for the purpose of this study. The values are expressed as milligrams of immunoglobulin activity per milliliter of milk.

Measurement of iron-binding capacity. Total lactoferrin was measured by the radial immunodiffusion method of Fahey and McKelvey,[7] using rabbit antiserum against a purified preparation of lactoferrin which contained only trace amounts of lysozyme.[4]

Unsaturated iron-binding capacity was determined by titrating the milk against a standard solution of ferric nitrilotriacetate.[8]

Measurement of vitamin-binding capacity. The capacity of the milk to bind added vitamin was measured by adding excess of [G-^{3}H] cyanocobalamin or [G-^{3}H] folic acid to a sample of the test preparation, and then separating free and protein-bound vitamin by gel filtration.[3, 9]

Growth tests with *Escherichia coli* 0 101:K?:H? (NCTC 9703). The milk samples were first dialyzed against buffer of pH 7.0 containing 0.05M phosphate and 0.01M $NaHCO_3$, and then tested as described by Reiter and colleagues.[10]

Table III. Influence of different heat treatments of human milk on its capacity to bind added [^{3}H] cyanocobalamin and [^{3}H] folic acid

Heat treatment	*Unsaturated binding capacity (ng/ml)*	
	Cyanocobalamin	*Folic acid*
None	43.4	21.9
HTST pasteurization	26.2	–
62.5° 30 min	22.6	19.7
65° 15 min	18.4	16.4
70° 15 min	14.4	14.2
75° 15 min	14.0	11.5
80° 15 min	15.0	10.2
85° 15 min	18.4	4.2
90° 15 min	21.6	2.4
95° 15 min	22.8	1.5
100° 15 min	18.6	1.5

Table IV. Influence of different heat treatments of human milk on its lysozyme activity

Heat treatment	*Lysozyme activity (μg egg white lysozyme equivalent/ml milk)**	
None	98	99
HTST pasteurization	103	107
62.5° 30 min	103	105
70° 15 min	62	68
80° 15 min	35	37
90° 15 min	7	7
100° 15 min	2	3

*Each value represents a different preparation of milk.

Tests on bacterial uptake of vitamins. The milk preparations were tested for their capacity to inhibit uptake of [G-^{3}H] cyanocobalamin in *E. coli* (NCTC 9703), and of [G-^{3}H] folic acid in *Bifidobacterium bifidus* (NCDO 1452), as described by Ford.[3]

Determination of lysozyme activity. Lysozyme activity was assessed from the rate of lysis of a suspension of dried cells of *Micrococcus lysodiecticus,* as described by Litwack.[11] Crystalline egg white lysozyme (Worthington Biochemical Corp.) was used as the standard.

Determination of lactoperoxidase. Lactoperoxidase was measured by following the oxidation of o-dianisidine by H_2O_2 at 37° C.[12]

RESULTS

Immunoglobulins. Table I shows the effects of heat treatment. The content of IgA—the predominant class in

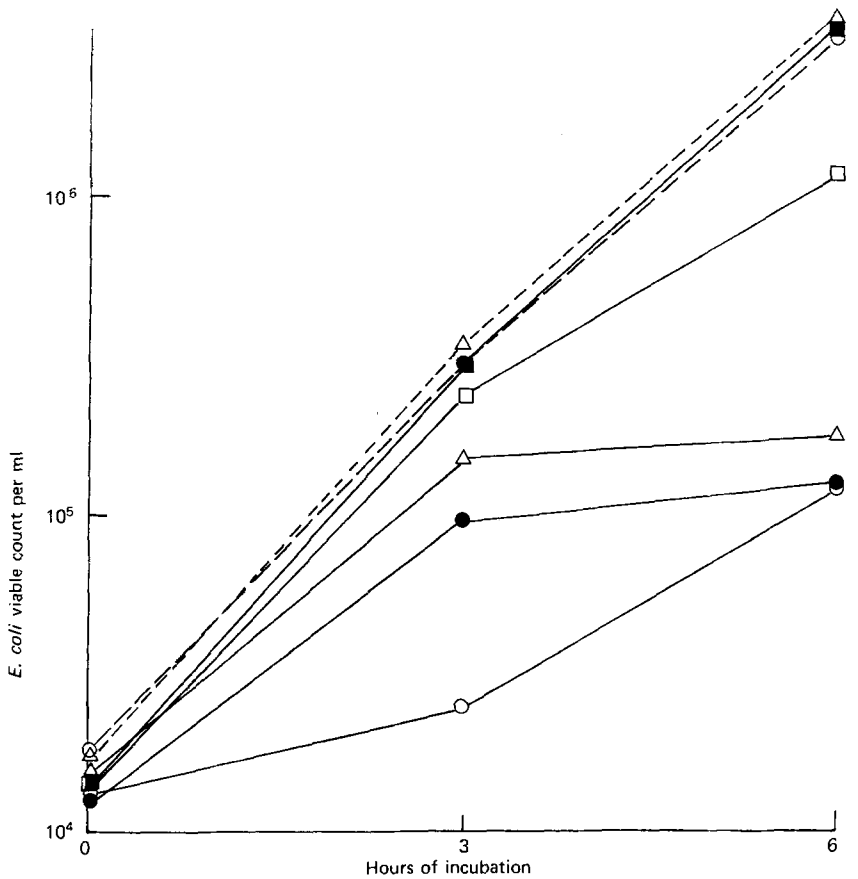

Fig. 1. Influence of heat treatment of human milk, and the effect of adding iron, on growth of *Escherichia coli.* —o— = Raw milk; ---o--- = raw milk + Fe; —●— = 62.5° C 30 minutes; —Δ— = HTST; —□— = 70° C 15 minutes; —■— = 80° C 15 minutes; ---Δ--- = 80° C 15 minutes + Fe.

human milk—was not significantly reduced by heating at 56° C for 30 minutes. Pasteurization by the holder process (62.5° C 30 minutes) reduced the content by 20%, but at higher temperatures the percentage of destruction increased sharply. The content of IgM was very low; it survived heating at 56° C but not at 62.5° C. IgG was not detected in the milk.

Iron-binding capacity. Table II shows the content of lactoferrin and the saturated iron-binding capacity in some of the milk preparations. Holder pasteurization involved considerable loss both of lactoferrin and of unsaturated iron-binding capacity, and heating at 70° C caused virtually complete destruction.

Inhibition of *E. coli*. Fig.1 shows the influence of heat treatment of milk, and of supplementation of the milk with iron, on its capacity to support growth of *E. coli.* Growth in raw milk was very poor, the viable count increasing by only one log cycle in 6 hours. Addition of iron (5 μg/ml) to raw milk caused a marked increase in growth, as did heating the milk at 80° C for 15 minutes. Addition of iron to this heated milk gave no further growth increment. In milk pasteurized by the holder or HTST process there was more growth at three hours than in the raw milk, but by six hours there was little difference.

Vitamin-binding capacity. Table III shows the effects of heat treatment of milk on its capacity to bind added cyanocobalamin and folic acid.

B_{12}-binding capacity declined progressively with an increase in processing temperature, to a minimum at 75° C. Further increase in temperature reversed this trend, so that treatment at 95° C was less destructive than at 65° C.

The situation with folate binder was different. Holder pasteurization destroyed 10% of the unsaturated binder, and with increase in processing temperature there was a progressive increase in the loss, to about 90% after 95° C 15 minutes.

Inhibition of vitamin uptake in bacteria. Fig. 2 shows the influence of heat treatment of milk on its capacity to inhibit the uptake of cyanocobalamin by *E. coli.* In the absence of milk, uptake of cyanocobalamin was nearly

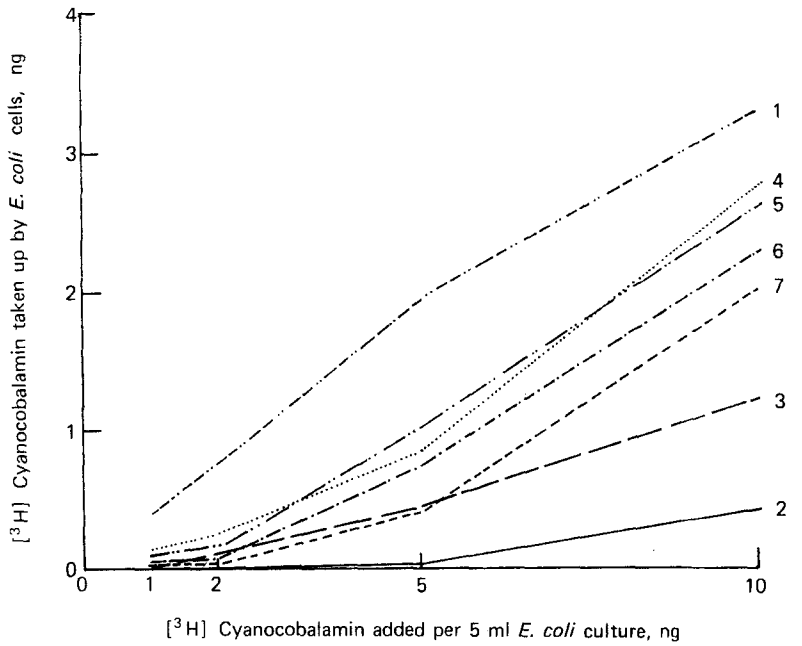

Fig. 2. Influence of heat treatment of human milk on its capacity to inhibit uptake of cyanocobalamin in *Escherichia coli.* To 5 ml portions of bacterial culture were added graded amounts of [^{3}H] cyanocobalamin with: (1) no further addition; (2) 0.2 ml milk unheated, or (3) heated 62.5° C 30 minutes, or (4) 70° C 15 minutes, or (5) 80° C 15 minutes, or (6) 90° C 15 minutes, or (7) 100° C 15 minutes. After incubation for 30 minutes at 37° C the bacterial cells were harvested by centrifugation and analyzed for [^{3}H] cyanocobalamin.

proportional to the amount of the vitamin added to the bacterial culture, whereas in the presence of 0.2 ml of unheated milk there was no significant uptake until the amount of added vitamin exceeded 5 ng. The raw milk had the property of sequestering 25 to 50 ng of cyanocobalamin per milliliter and protecting it against uptake by *E. coli.* Heat treatment reduced this capacity of the milk to protect cyanocobalamin and, as with the unsaturated binding capacity (Table III), the effect was greatest at the intermediate temperatures.

Fig. 3 shows the results of a similar experiment on folate uptake by *B. bifidus.* Folic acid added alone was taken up quickly and almost completely by the bacteria, as was folic acid added with 0.2 ml of milk that had been heated at 100°C for 15 minutes. In the presence of 0.2 ml of unheated milk there was no uptake of folic acid until the amount added exceeded 2 ng. With increasing severity of heat treatment of the milk its capacity to protect folic acid against bacterial uptake declined progressively.

Lysozyme activity. Table IV shows the lysozyme activity in some of the milk preparations. Holder pasteurization caused no loss of activity but treatment at 70° C and higher temperatures caused progressively increasing destruction of the enzyme. Only about 3% of the activity survived heating at 100° C.

Lactoperoxidase. In the mature milk used in the present study the content of lactoperoxidase was less than 0.05 units per milliliter, the lower limit of sensitivity of the assay method employed.

DISCUSSION

Immunoglobulins. Szöllósy and associates[13] found that treatment of human milk at 65° C for 30 minutes caused no reduction in antibody titer against *E. coli* type 055, whereas we found a 20% fall in IgA and destruction of IgM. They further observed a fall in antibody titer on storage of the milk at 25° C, which they attributed to bacterial enzyme activity. We found no such fall in milk inoculated with a strongly proteolytic strain of *Pseudomonas fluorescens* and stored at 4° C for 47 hours, during which time the viable count increased from 10^3 to 10^7 per milliliter.

The iron and vitamin binders. Human milk, unlike cow milk, is rich in the iron-binding protein lactoferrin, which in its unsaturated form inhibits the growth of several pathogens, apparently by depriving the organisms of iron.[14-16] Breast-fed infants are markedly resistant to infectious gastroenteritis cause by *E. coli,* and Bullen and associates[16] suggest that this resistance is largely contributed by lactoferrin acting in combination with specific antibody. The folate and vitamin B_{12} in human milk, like the iron, are strongly bound to minor whey proteins and,

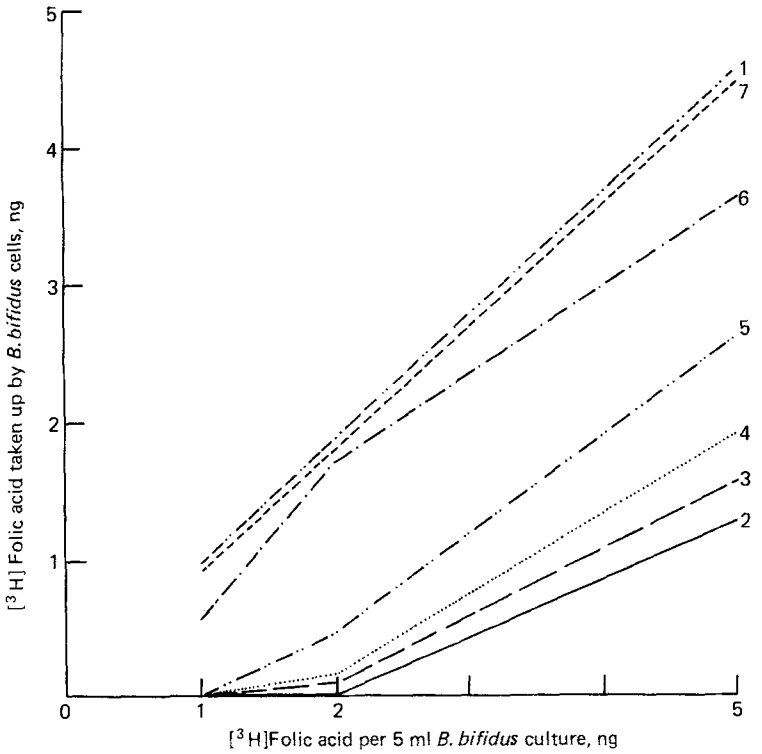

Fig. 3. Influence of heat treatment of human milk on its capacity to inhibit uptake of folic acid in *Bifidobacterium bifidus.* To 5 ml portions of bacterial culture were added graded amounts of [³H] folic acid with: (1) no further addition; (2) 0.2 ml milk unheated, or (3) heated 62.5° C 30 minutes, or (4) 70° C 15 minutes, or (5) 80° C 15 minutes, or (6) 90° C 15 minutes, or (7) 100° C 15 minutes. After incubation for 30 minutes at 37° C the bacterial cells were harvested by centrifugation and analyzed for [³H] folic acid.

as with lactoferrin, there is a large excess of unsaturated binder proteins. The free vitamins are taken up avidly by several species of bacteria and it has been suggested that, in protecting the vitamins against bacterial uptake, the binders may strongly influence the ecology of the intestinal microflora and the vitamin nutrition of the suckling infant.[3] Animal experiments indicate that the protein-bound vitamins may be efficiently absorbed from the intestine both before and for some time after the cessation of transport of intact protein across the neonatal gut epithelium.[9, 17, 18]

It is interesting that the B_{12}-binder was most labile at around 75° C (Fig. 2; Table III). The same was true for sow milk, which also is rich in B_{12}-binder. It seems possible that some of the destruction at these intermediate temperatures was caused by a milk enzyme which was itself denatured at the higher temperatures. If so, then prolonged storage of milk in a domestic refrigerator might cause appreciable loss. Assuming a doubling of the rate of the enzymic activity per 10° C rise in temperature, storage at 10° C for 16 hours would be as damaging as heating at 70° C for 15 minutes.

Lysozyme. Human milk is rich in lysozyme, containing about 39 mg/dl, as against only 13 μg/dl in cow milk.[19] It acts directly against many bacteria and it also acts indirectly by potentiating the bactericidal activity of immune antibodies.[20] The purified enzyme is highly stable at acid pH: at pH 4.5, heat treatment for 3 minutes at 100° C causes no loss of activity.[21] This finding has been widely quoted, and so it needs to be emphasized that, in milk at its natural pH, the enzyme is heat labile.

Lactoperoxidase. Cow milk is rich in lactoperoxidase which, in the presence of thiocyanate and peroxide, inhibits a variety of bacteria and viruses.[12, 22, 23] Gothefors and Marklund[24] found that the concentration in human milk was comparatively small; it declined steeply with time after parturition and at six days averaged only 0.5 unit/ml, as against 40 units/ml in cow milk. We found no peroxidase in our milk samples, possibly because of the long-time interval between collection and test. Our samples had been collected over one to two days at 4° C and bulked, whereas Gothefors and Marklund[24] reported on individual samples that had been tested with 4 hours of collection.

General. Most pediatricians would take the view that heat denaturation of the protective proteins in milk is

undesirable, but that in the practical situation it is unavoidable. They may well be correct in this view, and certainly it would be irresponsible to advocate the feeding of raw milk without stringent control of its bacteriologic quality. But we may reasonably wonder whether any heat treatment that is adequate to ensure sterility in milk taken and stored under sanitary conditions might not actually increase the risk of enteric infection in the infant. Even in breast-fed infants the milk contains bacteria,[25, 26] presumably deriving from the mother's nipples and fingers, and it might well be that with careful hygiene in the collection and storage of donor milk the bacterial count could be maintained with acceptable limits. The ideal should be to collect clean milk, with minimal contamination by "skin organisms" and enterobacteria, and to store it immediately in a freezer until it is gently thawed for feeding to the infant. But in many maternity units and breast milk collection centers the practice must fall short of this ideal. Milk from donors living at home may have been collected over a period of 24 hours or longer, with only perfunctory attention to hygiene, and stored in the domestic refrigerator before being brought to the collection center. Under these circumstances extensive growth of psychrotrophic bacteria is to be expected and heat treatment of the milk is clearly necessary, however regrettable it may be. Experience in Hungary[27] led to the recommendation that donor milk should be heated at 65° C for 30 minutes if its bacterial count exceeded a predetermined level. This would involve a large amount of routine bacteriologic testing and it would further prolong the interval between collection and feeding of the milk. But some such attempt to provide a "Grade A" quality of milk for feeding raw to weak and premature babies might be practicable, and it is clearly important to establish whether it would be beneficial. Meanwhile, to the extent that heating of the milk is practiced, it should be no more severe or prolonged than is required for the destruction of likely pathogens. For batch processing of small quantities of milk the holder process (62.5° 30 minutes) would seem to be the method of choice.

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