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Bacteriostasis of *Escherichia coli* by milk. III. The activity and stability of early, transitional and mature human milk collected locally

By PAULINE HONOUR and JEAN M. DOLBY
The Clinical Research Centre, Harrow, Middlesex, HA1 3UJ

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SUMMARY

Milk from 150 local mothers has been assayed for bacteriostatic activity for milk-sensitive and milk-resistant indicator strains of *Escherichia coli*. Activity is greatest in colostrum which is active directly against all strains of *E. coli*. One week after delivery of the baby, milk is active against the milk-sensitive strain and becomes active against the milk-resistant strain in the presence of physiological amounts of bicarbonate and iron-binding protein. This activity decreases within 2–4 days on keeping milk unheated at 4 °C but is preserved for at least 4 months and often up to 2 years in milk heated to 56 °C then stored at 4 °C or in milk frozen, unheated, at −28 °C provided it is not repeatedly thawed and frozen. Later lactation milks are usually indistinguishable in activity from 1-week post-partum milk but may be less stable on storage particularly if frozen. Lyophilization *in vacuo* preserves activity of early-lactation milk for at least 6 months.

Heating milk to above 65 °C causes a progressive loss of activity which can be partially restored by adding bicarbonate and iron-binding protein. Iron abolishes the activity of milk and reduces that of colostrum.

INTRODUCTION

The antibacterial activity of milk as measured *in vitro* depends on several systems (Reiter, 1978); their relative importance in protection *in vivo* is not known. We have been concerned with the bacteriostatic system active against Enterobactericeae, particularly *Escherichia coli*.

This system involves a milk protein, lactoferrin which has bacteriostatic activity alone because of its ability to bind iron and deprive the bacteria (Oram & Reiter, 1968); this activity is blocked by ferric iron (Aisen *et al.* 1967). The bacteriostatic activity for *E. coli* of lactoferrin from human milk is augmented by horse serum IgG (Bullen, Rogers & Leigh, 1972; Rogers, 1976), and by human milk IgA (Rogers & Synge, 1978; Spik *et al.* 1978). Bacteriostatic activity involving lactoferrin in milk is potentiated by sodium bicarbonate (Reiter, Brock & Steel, 1975; Griffiths & Humphreys, 1977) which facilitates the transfer of iron to lactoferrin. Bicarbonate is particularly necessary in the presence of citrate in milk, which competes with lactoferrin for the available iron.

Dolby, Stephens & Honour (1977) showed that strains of *E. coli* vary in their

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sensitivity to the bacteriostatic action of milk; those which are inhibited directly have been called milk-sensitive but those susceptible only in the presence of bicarbonate (i.e. in the potentiated system) have been called milk-resistant. Additional iron-binding protein increases the potentiation. This variation in milk-sensitivity is not related to pathogenicity since strains of enteropathogenic serotypes from children with gastroenteritis can be of either kind (Dolby, Honour & Valman, 1977). Miles & Khimji (1975) found that enterochelin upon which milk-resistance may depend was present in virulent and avirulent strains.

To stand any chance of being effective in inhibiting the growth of *E. coli* generally, milk must therefore be active against both sensitive and resistant strains. At the site at which milk has to be effective *in vivo* to be protective, namely the small intestine, bicarbonate and extra iron-binding protein are present even in the neonate (Dolby, Stephens & Honour, 1977) so that testing expressed breast milk *in vitro* in the presence of bicarbonate and added transferrin as a source of extra iron-binding protein is an attempt at mimicking in-vivo conditions. That this is so is borne out by the 'found and expected' ratios of sensitive and resistant strains in babies fed on different diets (Dolby, Honour & Valman, 1977; Dolby, Stephens & Honour, 1977).

This paper describes the in-vitro bacteriostatic activity of human breast-milk alone and with added bicarbonate and transferrin against sensitive and resistant strains, and the stability on storage of this activity under conditions employed in the storing of breast milk for milk banks.

METHODS

Source of milk

Milk from about 100 mothers delivered in Northwick Park Hospital 4–7 days previously was expressed by hand or by pump into bottles which were collected within 1–2 h by the laboratory. Five specimens of colostra were also collected. (Approval for the requests for milk was given by the Northwick Park Hospital Ethical Committee.) Milk from 25 mothers in the Amersham and Harrow area at home with older babies, was expressed by hand or collected as drip-milk and the milk stored up to 1 month at -10 to -20 °C or up to 24 h at 4 °C before transfer to the laboratory; six of these mothers gave us specimens throughout the lactation period of 4–7 months. For comparison, small samples of pooled or individual mature, drip-milk from the human milk bank at St David's Hospital, Cardiff (Evans *et al.* 1978) were tested.

Only small quantities of 5–20 ml were being handled. Specimens were stored in sterile glass or plastic bottles and generally either transferred immediately to -28 °C or heated at 56 °C for 30 min (see below) and stored at 4 °C on cooling. Deviations from this procedure are indicated in the text.

Thawing of frozen specimens

Specimens were either thawed repeatedly or once only for small samples, at room temperature or in cold water at room temperature; milk was returned to the freezer within 1 h or discarded.

Heating of milk

Specimens of 2–10 ml were heated in a water bath with stirrer at 56–65 °C as indicated, for 30 min; 1–2 ml volumes were heated similarly at 70 °C for 20 min, at 80 °C for 10 min and in a boiling water bath for 5 min.

Lyophilization

Volumes of 0·5 ml were dried from the frozen state achieved by reducing the pressure during centrifugation: they were sealed and stored *in vacuo* at room temperature.

Bacteriostatic test

Volumes were measured with 0·02 ml dropping pipettes into capped glass tubes. To four drops of milk was added one drop of a 10^{-3} saline dilution of a 1% peptone water culture of the indicator strain of *E. coli* after 3 h growth at 37 °C. This gave an initial count of about 10^4 organisms per ml. Where indicated one drop of human-serum transferrin (Sigma, Kingston-on-Thames, England) to give 2 mg/ml and one drop of 0·02 N sodium bicarbonate to give 0·04% final concentrations, were added. The test samples were incubated for 3 h at 37 °C, then diluted ten-fold in saline and the colony counts estimated on MacConkey agar plates. Results are expressed as the number of times the inoculum increased during the test; a less than ten-fold increase was considered bacteriostatic and 11- to 20-fold partially bacteriostatic. Increases in boiled milk and peptone water controls were 50- to 200-fold. Strain V21/1, which was milk sensitive, and either VB11/2 or VB71/1 which were milk-resistant, isolated from the stools of a mother and two breast-fed babies, were the standard indicator strains.

Titration of the bacteriostatic activity of milk

Human milk held in 0·5–1 ml volumes in a boiling water bath for 5 min to inactivate, and called 'milk 100°' has been used as diluent and the milk-sensitive strain V21/1 as the indicator strain.

Since iron-binding protein is limiting below 0·2–0·5 mg/ml (Spik *et al.* 1978) and a 1/2 to 1/4 dilution of milk was enough to abolish bacteriostatic activity, 2 mg/ml of transferrin was added to every tube of milk dilution in 'milk 100°' to determine activity due to other than iron-binding protein. Colostra were diluted as well, without the addition of transferrin to determine the titre of iron-binding protein.

RESULTS

The bacteriostatic activity of individual milks during the lactation period

The 'initial' column of Table 1 shows the typical bacteriostatic activity of colostrum and milk collected from 4 days to 17 weeks *post partum* for the milk-sensitive indicator strain (S), a milk resistant strain (R), and for the milk-resistant strain in milk potentiated by 0·04% sodium bicarbonate and 2 mg/ml transferrin

(R/P). Milk tested either untreated in any way, within 1 h of collecting, or within 1 week after being frozen at −28 °C, not longer than 1 h after collecting, was similar in activity.

Five specimens of colostrum were strongly inhibitory to milk-sensitive *and* milk-resistant strains of *E. coli* without the addition of bicarbonate and transferrin, e.g. V89, Table 1.

Most of 25 to 30 specimens of 5 to 7-day post-parturition milk were either more active against the milk-sensitive strain than the milk-resistant strain, e.g. V97, or only active against the sensitive strain, e.g. V106, Table 1. These milks were potentiated with sodium bicarbonate and transferrin and then became active against the milk-resistant indicator strain. Two milks in this group were like

Table 1. *The bacteriostatic activity of raw early and late milks fresh or stored at −28 °C for milk-sensitive and milk-resistant strains of* E. coli

Specimen	Stored −28 °C	No. times inoculum increased for strain*		
		S	R	R/P
V89, colostrum	Initial	5	6	3
	6 months	3	7	3
	15 months	2	5	2
V97, 5 day *post partum*	Initial	3	15	6
	6 months	4	25	6
	1 year	2	10	2
V106, 8 day *post partum*	Initial	3	66	9
	10 months	1	30	18
V136, 17 week *post partum*	Initial	8	45	5
	7 months	9	40	20
V41/0·5, 4 day *post partum*	Initial	5	11	5
	20 months	0	71	4
V41/14, 14 week *post partum*	Initial	8	50	10
	17 months	3	37	10

The bracketed specimens are from one mother.

* S, Sensitive strain; R, resistant strain; R/P, R with 2 mg/ml of transferrin and 0·04% sodium bicarbonate, i.e. in potentiated milk.

colostra and did not require potentiation for activity against the milk-resistant strain. Two were less active than the majority and although bacteriostatic for the sensitive strain could not be potentiated by bicarbonate to a milk active against the resistant strain.

Milk from an additional 50 mothers 1 week after delivery was only tested after heating at 56 °C for 30 min. All of these milks were then however as active as V106 (Table 1). From Table 5 it can be assumed that most were unchanged by this heat treatment.

The initial activity of milks collected later than 1 week after parturition was similar to 1 week V106, e.g. V136 and V41/14 (Table 1). Of such milks from 25 mothers, six of whom gave us repeated, active specimens during lactation

of up to 5–7 months, only three specimens were poorly active against the sensitive strain, one at 2 weeks, one at 2 months and one at 3½ months. These three specimens were collected within days of the cessation of breast-feeding. Milk from other mothers near the end of lactation did not exhibit this falling off of activity. All the other milk specimens were like the majority of 5- to 7-day milks, i.e. active against the sensitive strain only but with bicarbonate-potentiated activity for the resistant strain.

Milk from the Cardiff milk bank was less active than most locally collected milks; only 2 of 13 individual 1–5 month post-partum specimens had activity similar to V136 (Table 1). The rest and two pool milks were similar to active milk kept at 4 °C, e.g. V182 stored 5 days (Table 5). We were unable to identify specific collection or storage methods as being responsible for the low bacteriostatic activity.

Table 2. *The bacteriostatic titre of colostrum and milk diluted in 'milk* 100'* *and estimated concentrations of IgA and lactoferrin*

	Reciprocal of titre		Estimated	
	Alone	+TF†	IgA (mg/ml)	LF† (mg/ml)
Colostrum (2 specimens)	50	400–800	30	10–25
Milk, 1 week *post partum* (15 specimens)	2	4–16	0·4	0·4– 1

* Milk which has been held in a boiling water bath for 5 min to abolish bacteriostatic activity.

† Transferrin (TF) was added at 2 mg/ml to augment the natural lactoferrin (LF).

The bacteriostatic activity was measured against the milk-sensitive strain V21/1.

Direct lactoferrin and IgA estimations are not available for most of the milks investigated here. Activity was however quantitatively estimated for a milk-sensitive strain of milk diluted in 'milk 100°' with and without transferrin added. Addition of iron-binding protein ensured that this was not the limiting factor. From the results given by Spik and her colleagues (1978) iron-binding protein was limiting below 0·2–0·5 mg/ml and IgA below about 0·05 mg/ml. Usually only colostrum could be diluted and retain activity in the absence of added transferrin (Table 2).

Figure 1 illustrates the activity on dilution of 4 1-week post-partum milks against several sensitive strains of *E. coli*. Bacteriostatic activity was lost at 1/4 to 1/16 dilution and milks behaved similarly against two strains. Table 2 gives the range of active maximum dilutions for colostra and 1-week milks with estimated values for IgA and lactoferrin.

Similarly dilution experiments could be carried out in 0·1 % peptone water or 10 % milk in phosphate-buffered saline but not in 1 % peptone water even although the available iron was less than that required to reverse the bacteriostatic activity.

The bacteriostatic activity for the milk-sensitive strain of all milk specimens described above was abolished from milk and considerably decreased in colostra

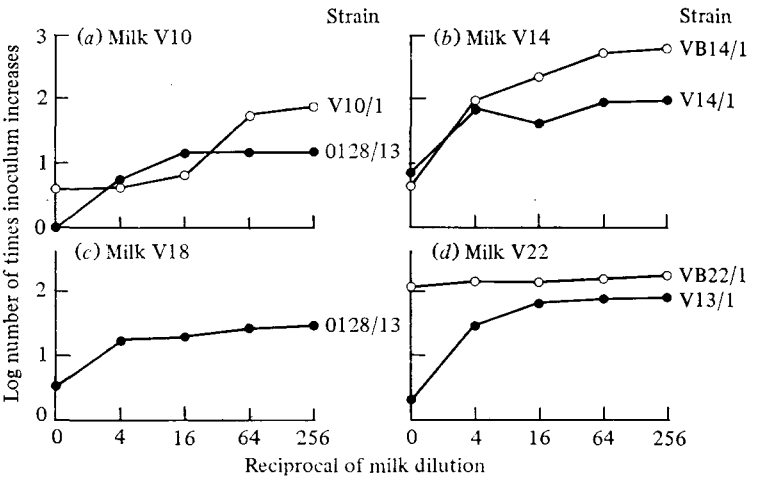

Fig. 1. Bacteriostatic activity of dilutions of milk in 'milk 100°' with 4 mg/ml of transferrin added, against strains of *E. coli*. (*a*) Milk from mother V10 against her own milk-sensitive, commensal strain and a milk-sensitive strain of enteropathogenic serotype. (*b*) Milk from mother V14 against her own and her baby's milk-sensitive strains. (*c*) Milk from mother V18 against the same enteropathogenic strain as in (*a*). (*d*) Milk from mother V22 against a milk-sensitive strain V13/1 and her baby's milk-resistant strain VB22/1.

Table 3. *The iron reversal of the bacteriostatic activity of colostrum, and milk collected 4–6 days after delivery*

	No. times inoculum increased with ferric ammonium citrate (μg/ml)									
	For sensitive strain					For resistant strain				
Specimen	0	20	200	2000	20000	0	20	200	2000	20000
V83, colostrum	4	16	44	36	30	4	10	10	6	6
V119, milk	3	61	40	42	40	27	67	36	17	21
V186, milk	4	34	37	37	4	With Bic/TF*				
V186, milk						4	36	41	19	2

* Bicarbonate was at 0·04% and transferrin at 2 mg/ml.

by the addition of 20–200 μg/ml of ferric ammonium citrate as shown in Table 3. The potentiated activity of milk against the milk-resistant strain was reversed with greater difficulty and not in colostra (Table 3) until the initial very high activity began to deteriorate on storage. Results with higher concentrations of iron salt are not understood; these concentrations in 1% peptone water do not inhibit the growth of *E. coli*.

The partial restoration of bacteriostatic activity to heat-inactivated milk

Pasteurization is commonly practised in the storage of human milk. The loss of activity of a heated 1-week milk pool for a milk-sensitive strain is shown in

Fig. 2. The decrease in bacteriostatic activity after heating at 70 °C or above is partially reversed by adding transferrin at 2 mg/ml but complete reactivation is not achieved and must therefore be dependent on something other than iron-binding protein, presumably IgA. Addition of sodium bicarbonate as well as or instead of transferrin has no effect.

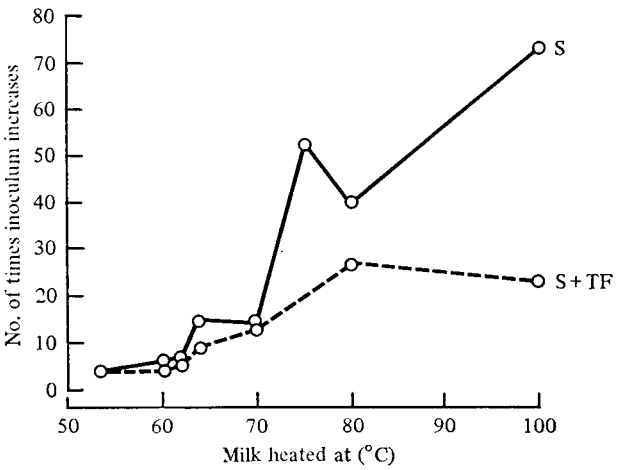

Fig. 2. The effect of heat on the bacteriostatic activity of milk for a milk-sensitive strain (S). Transferrin (TF) was added as indicated at 2 mg/ml.

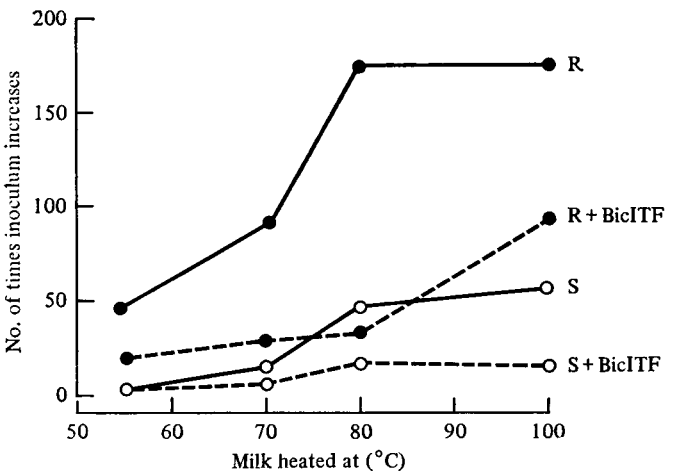

Fig. 3. The effect of heat on the bacteriostatic activity of milk for milk-resistant (R) and milk-sensitive (S) strains compared. Sodium bicarbonate (Bic) was added at 0·04% and transferrin (TF) at 2 mg/ml.

Against the milk-resistant strain, both transferrin and bicarbonate are necessary to achieve some restoration of activity to heated milk as shown in Fig. 3. The effect of transferrin in restoring activity for the milk sensitive strain is shown by the bicarbonate-transferrin curve in Fig. 3.

The effect of 30 min at 56 °C and 5 min at 100 °C on the activity of milk V97, 5-day post-partum, and the activity on storage (see below) can be seen in Table 5. There was an initial loss of activity on heating to 100 °C, but not to 56 °C, but no subsequent change over a 15 month storage was observed.

Colostra heated to 80 °C (further heating caused gelling) became inactive whereas heating at 56 °C had no effect.

The storage of raw and heated colostra and milk

As shown in Table 1, colostra and milk collected at different times *post partum* could be stored at −28 °C for over a year without loss of activity. Two milks shown in Table 1 had however lost activity, V136 at 7 months and V41/0·5 at 20 months. There is a great individual variation in stability sometimes dependent on the stage of lactation – the later *post partum* milks tending to be less stable,

Table 4. *The effect of freezing and thawing on the bacteriostatic activity of raw milk stored at −28 °C*

Milk	Stored	Times thawed	No. times inoculum increased in milk for strain		
			S	R	R/P
V93, 1 week *post partum*	3 weeks	2	2	94	28
		13	4	83	62
Pool 4, 4 days *post partum*	5 years	1	2	7	2
	5 years + 6 weeks	2	1	19	6
	5 years + 5 months	5	2	40	5
	5 years + 7 months	2	1	39	9
		7	2	28	7
V182, 5½ months *post partum*	5 days	1	7	66	7
	6 weeks	4	63	34	32

Abbreviations as in Table 1.

e.g. V182 Table 5 – and sometimes on the number of times the specimens have been frozen and thawed as shown in Table 4. Table 5 compares the lack of deterioration in activity of milk heated to 56 °C with that stored raw at −28 °C. Storage of raw milk unfrozen causes at least loss of potentiating activity for the resistant strain within four days; later lactation milks may also become inactive for a sensitive strain. It is therefore possible that milk collected for milk-banks may deteriorate in activity before treatment. There was excellent stability on storage of 56 °C-heated milk at 4 °C (Table 5) which extends at least up to 2 years; 70% of about 50–60 1-week milks had however become non-potentiating after 3 years. A milk which stored poorly at −28 °C (V182, Table 5) retained stability for 3 months in the 56 °C-heated specimen stored at 4 °C.

The high activity of colostrum was better preserved at −28 °C than in the 56 °C heated specimens stored at 4 °C. The loss of activity of raw milk at 4 °C is probably related to bacterial contamination, since two ultracentrifuged, bacteriologically filtered milk pools were kept raw at 4 °C for over 4 years without deterioration, yet activity deteriorated within 48 h at 4 °C in heavily contaminated

Table 5. *Changes in the bacteriostatic activity of* 3 *milks stored under various conditions*

Specimen	Storage time	No. times inoculum increased in milk treated: S	R	R/P	S	R	R/P	S	R	R/P
		Raw, stored −28 °C			56 °C 30 min, stored 4 °C			100 °C 5 min, stored 4 °C		
V97, 5 days *post partum*	Initial	3	15	6	3	10	4	30	34	11
	7 weeks	4	18	2	2	26	3	37	83	20
	4 months	3	20	6	8	20	6	nd	nd	nd
	15 months	4	18	4	8	12	4	85	45	14
		Raw, stored −28 °C			Raw, stored 4 °C			56 °C 30 min, stored 4 °C		
V93, 7 day *post partum*	4 days	1	90	18	5	40	61	2	89	23
	3 weeks	2	94	28	9	59	83	1	83	22
	7 weeks	0	93	26	nd	nd	nd	2	77	24
	4 months	0	77	7	10	40	80	1	32	7
		Raw, stored −28 °C			Raw, stored 4 °C			56 °C 30 min, stored 4 °C		
V182, 5½ months *post partum*	5 days	7	66	7	39	63	30	11	69	5
	3 months	59	60	25	nd	nd	nd	6	32	6

nd, Not done.
Other abbreviations are as Table 1.

specimens. It is also common to pasteurize milk and then store frozen and this is another set of conditions that we have not investigated.

A temperature of 62–63 °C is most commonly used to pasteurize milk. Most of our milk has been investigated after heating to 56 °C only but in comparative experiments the effect of 56 and 63 °C on bacteriostatic activity was similar (Figs. 2 and 3).

Lyophilization

Specimens of colostra active directly against milk-sensitive and milk-resistant strains and specimens of milk collected 1-week *post partum* active against the sensitive strain but potentiating for the resistant strain were thawed from frozen stock and freeze-dried in 0·5 ml volumes. They were of unchanged bacteriostatic activity immediately after drying and for up to 6 months sealed *in vacuo*, stored at room temperature. The activity of milks collected later in lactation was not always preserved on drying but the conditions causing loss of activity have not been defined.

DISCUSSION

With rare exceptions all the locally collected milk has been bacteriostatic for sensitive and resistant strains of *E. coli* in the presence of bicarbonate and iron-binding protein. The milk which was less active initially or which lost activity

on storage tended to be later lactation milk similar to that collected by milk-banks. Ford *et al.* (1977) reported loss of bacteriostatic activity for *E. coli*, most of the lactoferrin and 20 % of the IgA from the Cardiff milk-bank milk on pasteurization; the loss of lactoferrin was confirmed by Evans *et al.* (1978). Gibbs *et al.* (1977) reported on the instability on storing of frozen drip-milk collected in Oxford. Raptopoulou-Gigi, Marwick & McClelland (1977) were however able to pasteurize early post-partum milk without loss of IgA or lactoferrin and this matches the activity and stability of the 1-week post-partum milks which we have tested.

It is almost certain that what we are measuring in distinguishing colostra (active against all strains directly), milk (active against all strains in the presence of added bicarbonate or bicarbonate and iron-binding protein) and inactive milks, are varying concentrations of IgA and lactoferrin. The concentrations of IgA and lactoferrin estimated from our dilution experiments correlate with those generally found. Figures covering the lactation period and the estimated intake by the babies have been given by McClelland, McGrath & Samson (1978). Various treatments including freezing and heating cause selective degradation of milk proteins involved in bacteriostasis. For instance an initially active milk with only just sufficient IgA and lactoferrin for bacteriostasis will become inactive following only a slight decrease of lactoferrin but can be reactivated by potentiating what is left with bicarbonate.

The experiments of Bullen *et al.* (1972) on the colonization by *E. coli* of suckled guinea pigs given large doses of haematin, and observations in 1-week-old babies on different diets of the ratio of sensitive and resistant faecal *E. coli* (Dolby, Honour & Valman, 1977; Dolby, Stephens & Honour, 1977) suggest that the bacteriostatic system may play some part in the control of the colonization of the small intestine and, as such, is a system that should be monitored in milk banks. There is as yet however, no direct proof of participation of this system in protection of human neonates.

It may be that the only bacteriostatic activity effective *in vivo* is that of colostrum which inhibits sensitive and resistant organisms without participation of bicarbonate and which would have been involved in both sets of observations cited above. Perhaps for various reasons the in-vitro activity conferred by bicarbonate and transferrin is quite irrelevant *in vivo* in spite of their presence in the small intestine.

The reason for feeling cautious about the in-vivo importance of the activity of bicarbonate-potentiated milk is because we have shown that bacteriostatic milk, heated in a boiling water bath to inactivate can be reactivated *in vitro* by bicarbonate and iron-binding protein in concentrations available in the intestines of the neonate; and yet boiled human milk cannot protect against enteritis (Tassovatz & Kotsitch, 1961). Of babies fed on boiled milk during an epidemic of *E. coli* O111, 13 % were infected whereas none of the babies fed at the breast or on raw, expressed breast-milk were. On the other hand in another place at a different time (Svirsky-Gross, 1958) raw, expressed breast-milk protected babies in a similar way to the 1961 study, again against O111, but over 30 % of those

fed a commercial preparation (which cannot be reactivated by bicarbonate and transferrin) were infected. So perhaps the boiled human milk does have some protective effect *in vivo* to match the 'potentiated', in-vitro activity, thus reinforcing evidence for the protective role of bacteriostasis in human neonates.

If this really were so then perhaps more attention should be paid to reserving early, more active, more stable milk for babies particularly at risk from infection, or to minimize the time between expression of the milk and treatment.

We thank all the mothers who contributed specimens of milk and Sister Aikens of Northwick Park Hospital, and Sisters McDonald and D'Cunha of Amersham Hospital for their help in collecting and forwarding most of these. We are particularly grateful to the six mothers, CF, MK, HL, CM, CS, AT for their enthusiastic collection, storage and delivery of specimens over long lactation periods, and to Sister V. M. Lewarne of the Human Milk Bank, St David's Hospital, Cardiff for her generous help in collecting specimens and for the opportunities for discussion.

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