REVIEW OF NONSPECIFIC ANTIMICROBIAL FACTORS IN COLOSTRUM

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Résumé.

LES FACTEURS ANTIMICROBIENS NON SPECIFIQUES DU COLOSTRUM : REVUE BIBLIOGRAPHIQUE. — Les principaux facteurs antimicrobiens non anticorps du colostrum et du lait sont passés en revue dans cet article, en particulier le lysozyme, la lactoferrine, et le système peroxydase. L'auteur souligne que ces mécanismes ont la particularité d'être communs au colostrum et aux leucocytes.

Introduction.

The role of bovine colostrum in providing the newborn calf with maternal immunoglobulins was recognised in the early twenties (Smith and Little, 1922). The dual role of colostrum and postcolostral milk in providing not only circulatory antibodies but also direct protection of the intestinal tract against infection became accepted only after the discovery of secretory IgA (Hanson, 1961; Tomasi and Ziegelbaum, 1963). Previous to that, Roy (1956) had reported in his Ph.D. thesis, that dilute colostrum, given after the small intestine was no longer permeable, had a beneficial effect in reducing scouring and preventing death in calves. He suggested that in the intestine the antibodies of the colostrum acted in the same manner as in the blood. Unfortunately these results were never published because they did not agree with the prevailing concept that protection depended only on antibodies circulating in the blood (see also Davies, 1922; Besredka,

1927 on "coproantibodies" and in this symposium on continuous feeding of colostral antibodies (e.g. Snodgrass and Wells).

Another source of protection which is as yet little explored is the role of non-specific inhibitory systems: lysozyme, lactoferrin, lactoperoxidase, xanthine oxidase (for reviews, see Reiter and Oram, 1967; Reiter 1976 and 1978), vitamin B_{12} and folic acid protein carriers. With few exceptions (e.g. Weiser *et al.*, 1969) textbooks on immunology either neglect or mention only briefly these systems. These non-antibody factors, however, may play an important role in immunity not only by augmenting antibody action but also by offering protection before the immune response becomes effective, thus bridging the immunological gap. It is the purpose of this review to extend the immunological concept for the protection of the newborn calf to include non-antibody factors present in colostrum and postcolostral milk.

Lysozyme.

Occurrence:

Bovine milk contains very little lysozyme compared with human milk—*c.* 13 μg/100 ml instead of *c.* 30 mg/100 ml (Chandran *et al.*, 1964)—. Although the enzyme is regarded as an important factor in the intracellular killing and digestion of phagocytosed bacteria (e.g. a defect in bacterial killing by polymorphs in man was ascribed to the lack of lysozyme (and lactoferrin) Spitznagel *et al.*, 1972), it is completely lacking in bovine polymorphonuclear leucocytes (Padget and Hirsch, 1967).

Lysozyme has been demonstrated not only in secretions (tears, saliva, milk) and tissues (intestinal and alveolar) but also in monocytes, macrophages and Paneth cells (e.g. Syren and Raeste, 1971; Klockers and Osserman, 1974). McClelland and Furth (1975) studied the incorporation of ^{14}C-labelled amino acids into lysozyme and showed that in humans and mice, lysozyme is synthesized *in vitro* in the mucosa of the respiratory and gastrointestinal tract and in lymphoid organs. According to these authors monocytes and macrophages also synthesize lysozyme while granulocytes from peripheral blood contain lysozyme but do not synthesize it.

In man, increased lysozyme levels were obtained in tissues and secretions during inflammation of the gastrointestinal mucosa (Meyer *et al.*, 1948) and in serum with ulcerative colitis (Falchuk *et al.*, 1975). No comparative studies have so far been attempted in the bovine except for the work of Korhonen (1973) who found that the lysozyme concentration increased dramatically in the milk from infected mastitic udders; so far such studies have not been attempted in the bovine intestine.

Table 1.—The lytic effect of bovine milk, human milk and egg white lysozyme on live *Micrococcus lysodeikticus.*

Source of lysozyme	Rate of lysis Δ% T/min*	
	Buffer	+ NaCl (0.1 M)
Bovine milk	1.82	2.1
Human milk	1.70	1.73
Eggwhite	0.63	0.81

Buffer: phosphate buffer pH 6.2.

*Determined in spectrophotometer at 540 mμ.

Compiled from Vakil *et al.*, 1969.

Biological significance:

Only a few bacterial species are killed and lysed by egg white (albumen) lysozyme (e.g. *Micrococcus lysodeicticus, Bacillus subtilis*) but this enzyme can interact with complement and antibody which leads to lysis of Gram-negative organisms (Wardlaw, 1962). Also Hill and Porter (1974) have confirmed that sIgA can bind complement in the presence of lysozyme, making sIgA bactericidal as previously suggested by Adinolfi *et al.* (1966).

Lysozyme splits the 1-4 linkage between N-acetyl muramic acid and N-acetyl glucosamine in the peptidoglycan of the cell wall of Gram-positive and the outer membrane of the Gram-negative organisms. In the latter organisms the peptidoglycan is overlaid by lipoprotein so that the substrate becomes accessible to the enzyme only after extraction with chloroform, treatment with EDTA or after the complement depending bactericidal action of specific antibodies. In Gram-positive organisms the peptidoglycan is more exposed in the cell wall and lysozyme

Table 2.—Sensitivity of different bacteria to bovine or human milk lysozyme under different conditions.

Organisms	Rate of lysis Δ% T/min			
	Bovine		Human	
	Buffer	+NaCl	Buffer	+NaCl
Streptococcus lactis	0.2	0.14	0.00	0.00
Staphylococcus aureus	0.03	0.00	0.05	0.00
Sarcina lutea	0.08	0.05	0.04	0.11
Streptococcus faecalis	0.17	0.06	0.04	0.00
Bacillus cereus	0.10	0.00	0.23	0.22
Escherichia coli	0.12	0.11	0.10	0.06
Serratia marcescens	0.10	0.00	0.00	0.00
Proteus vulgaris	0.06	0.00	0.00	0.00
Pseudomonas fluorescens	0.08	0.00	0.08	0.00
Pseudomonas aeruginosa	0.30	0.36	0.21	0.06

Compiled from Vakil *et al.*, 1969.

can lyse some organisms without previous treatment (e.g. *Micrococcus lysodeicticus*). This depends both on the accessibility of the substrate and the specificity of the enzyme. An extreme example of the specificity of a lysozyme is the enzyme synthesized by bacteriophage infected bacteria. Streptococcal phage lysins lyse only certain serogroups of streptococci (Reiter and Oram, 1963; Oram and Reiter, 1965). Similarly, the lysozyme isolated from milk or other secretions is too often equated with albumen lysozyme. They differ, however, both in their relative lytic activity to *M. lysodeicticus,* the milk enzyme being three times more active, and in their lytic spectrum, many more bacterial species being susceptible to the milk enzyme (Vakil *et al.*, 1969). [Table 1 and Table 2].

Lysozymes are basic proteins and attach, therefore, to the surface of bacteria in the presence of electrolytes (Nakamura, 1923; Reiter and Oram, 1962), but the pH seems to play an important part as well. According to Nakamura (1923) the initial exposure of bacteria to lysozyme at low pH (3-5) and transfer to a medium of high pH (>7.0) appreciably promotes the lytic activity of the enzyme. Hence, it is to be considered whether low pH in the stomach and high pH in the intestines would promote the lytic activity *in vivo*. Lysozyme is quite resistant to digestion (particularly at low pH) and was recovered from breast-fed but not bottle-fed babies (Osserman *et al.*, 1974).

From the foregoing, it appears that we have only scanty knowledge about the possible function of lysozyme *in vivo* and its synthesis in the young calf.

Lactoferrin (LF)

Occurrence:

This iron binding protein can be isolated from various animal secretions and from leucocytes. Bovine milk contains very little LF (20-200 μg/ml) compared with human milk (>2 mg/ml) (see monograph of Masson, 1970) or sow's milk (0.5 mg/ml); bovine colostrum, however, contains appreciable amounts of LF (2-5 mg/ml) (unpublished data). So far, it is not known whether LF is also synthesized in the intestine but this appears to be quite likely for the following reasons. During chromatography of either sIgA lysozyme or LF, the "purified" proteins are frequently contaminated by some of the other proteins—they "travel together". Since it has been shown that lysozyme *and* sIgA are intestinally synthesized (McClelland and Furth, 1975) it is likely that LF is also synthesized *in situ.*

Table 3.—Effect of citrate and bicarbonate on the bacteriostatic activity of dialysed colostrum.

HCO_3^- (μm/ml)	Citrate (μm/ml)			
	0	0.1	1	10
0	0.7*	1.9	2.6	2.4
0.7	0.7	0.9	2.5	2.5
7.0	1.1	1.1	1.1	2.4
70.0	1.0	0.7	0.7	0.8

*Increase in $\log_{10}$ viable count after 6 hours incubation.

Reiter *et al.* (1975).

Mode of action.

The bacteriostatic activity of lactoferrin has been demonstrated independently in human bronchial mucus by Masson *et al.* (1966), and in bovine colostrum and secretion of the non-lactating udder by Oram and Reiter (1966, 1968). In its natural state this iron binding protein is 25-35% saturated with iron and complexes iron thus making it unavailable to bacteria which have high metabolical requirements for iron. On addition of iron, LF becomes saturated and the inhibition is reversed. The bacteriostatic properties of LF closely resemble those of conalbumin (Feeney, 1951). Since *E. coli* has a high iron requirement (Waring and Werkman, 1942) while lactic streptococci have an extremely low iron requirement (Reiter and Oram, 1968) it is not surprising that *E. coli* is inhibited by LF while lactic streptococci are not. It is reasonable to extrapolate from the low iron requirements of the streptococci to lactobacilli which normally colonize the intestines because of the similarities in metabolism of lactic acid bacteria.

Bullen *et al.* (1972) reported that bovine colostrum becomes bacteriostatic for *E. coli* when the pH is adjusted to 7.4. However, it has since been show that this is not a pH effect because adjustment of the pH with phosphate or tris buffer fails to make the colostrum inhibitory; only when bicarbonate is used as buffer does the colostrum inhibit *E. coli* (Reiter *et al.*, 1975). This was not surprising because it was known that LF binds iron and bicarbonate mole for mole (Masson and Heremans, 1968). Besides bicarbonate, the citrate of the milk has an important effect on the inhibitory activity of LF. It competes with LF for the iron and thus makes it available for the multiplication of the bacteria. Dialysis of colostrum removes the citrate and makes it bacteriostatic without the addition of bicarbonate, the traces of bicarbonate dissolved in the colostrum being sufficient. The inverse relationship between citrate and bicarbonate is illustrated in Table 3 (Reiter *et al.*, 1975; Bishop *et al.*, 1976; Griffith and Humphrey, 1977).

Although specific antibodies were shown to increase the bacteriostatic activity of LF (Bullen *et al.*, 1972), good inhibition of *E. coli* can be obtained in the absence of antibodies

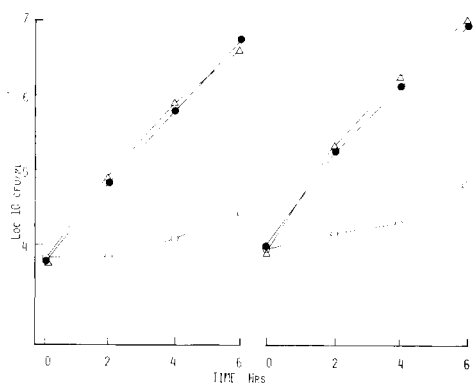

Fig. 1.—Inhibition of *E. coli* in a synthetic medium by lactoferrin.
●——● control
○——○ plus 2.5 mg/ml LF
Δ——Δ plus 2.5 mg/ml LF saturated with Fe^{++}
a) *E. coli* serotype 0101 (serum resistant)
b) *E. coli* serotype 0111 (serum susceptible)
(Law and Reiter, 1977.)

Table 4.—Bacteriostatic activity of bovine colostrum after adjustment of pH to 7.4 with bicarbonate against enteropathogenic (EPEC) and non-enteropathogenic isolates (NEPEC) of *E. coli* from baby faeces.

Isolates	No of strains tested	Bacteriostasis*	
		<10 fold	>10 fold
EPEC	50	34	16
NEPEC	50	39	11

*Average increase of inoculum after 6 h incubation at 37°.

Average increase after saturation with iron ov. 400 fold: (200-850).

Table 5.—Bactericidal effect of bovine colostrum diluted 1:8 in complement containing milk, against enteropathogenic (EPEC) and non-enteropathogenic (NEPEC) isolates of *Escherichia coli* from baby faeces.

Isolates	No of strains tested	Bactericidal*	Growth**
EPEC	50	24	26
NEPEC	50	10	40

*Bactericidal: $\frac{\text{Inoculum cfu}}{\text{cfu after incubation at } 37^\circ} = <1$

**Growth: as above >1.

with purified LF (Fig. 1) [Oram and Reiter, 1968; Law and Reiter, 1977]. This nonspecific bacteriostatic effect is also apparent in the results obtained (unpublished) with the inhibition of isolates of *E. coli* from the faeces of human infants (Table 4). Since all the 50 enteropathogenic and 50 non-enteropathogenic isolates were inhibited by bovine colostrum it is unlikely that the bovine colostrum would contain specific antibodies to all the human serotypes of *E. coli*. Table 5 shows the bactericidal activity of bovine colostrum against the same isolates: only 24% of the enteropathogenic and 10% of the non-enteropathogenic strains were killed by the complement activated bactericidal activity of specific antibodies. Dolby *et al.* (1977 a, b, and pers. comm.) obtained similar results using human milk. A great number of milk samples were tested against various serotypes of *E. coli* of human origin and all the samples inhibited all the strains provided bicarbonate was added. Although purified immunoglobulins increased the inhibitory activity of LF, it is difficult to deduce that any specificity is required because of the universal inhibition.

It was once thought that the iron requirement of a bacterial species depended on its cytochrome content; *Pseudomonas aeruginosa* requires more iron than *E. coli* because of its higher cytochrome content (Waring and Werkman, 1942). More recently Wettstein and Stent (1968), Griffith (1972), and Griffith and Humphrey (1978) have shown that at limiting iron concentration, or in the presence of an iron binding protein (as in bovine colostrum), 90% of the tRNA of *E. coli* was abnormal in that it chromatographed abnormally. The tRNA reverted after the addition of iron to its normal position when chromatographed. This suggested that the low level of normal tRNA is unable to support protein synthesis, causing bacteriostasis which is reversible.

Biological significance.

So far only suckling guinea-pigs have been used for *in vivo* experiments with lactoferrin. The milk of guinea-pigs contains even higher concentrations of lactoferrin than does human milk (Masson, 1970). Bullen *et al.* (1972) showed that the intestines of the suckling guinea-pigs contained higher numbers of *E. coli* and lower numbers of lactobacilli when given daily doses of haematin, compared with the control animals. This suggested an intestinal effect of the lactoferrin but did not prove it because the haematin represents another source of iron. To prove convincingly the *in vivo* effect of lactoferrin, it will be necessary to feed neonates with milk containing iron saturated or unsaturated lactoferrin.

The *in vitro* and *in vivo* effect of LF has been shown in the secretion and udder of non-lactating cows (Reiter and Bramley, 1975). The secretion of the non-lactating udder becomes more serum-like as the milk

components, casein, lactose and citrate are absorbed, while blood proteins and bicarbonate are transudated into the udder. The lactoferrin concentration in the secretion becomes very high, and in the absence of citrate and the presence of bicarbonate, the conditions for bacteriostasis by lactoferrin become optimal. With the formation of colostrum the conditions are reversed, citrate, the harbinger of parturition (Peaker and Linzell, 1975), appearing again and the bicarbonate concentrations diminishing. The dry secretion was shown to be inhibitory for mastitis producing strains of *E. coli in vitro*. While some strains produce severe mastitis in the lactating udder, which can lead to the death of the cows if not treated in time, the dry udder is quite resistant to an infection, but after infusing iron into the dry udder to saturate the LF, the udder became mastitic. This course of events may be responsible for the fact that coli infections occur most frequently after parturition.

In conclusion, it can be stated that LF with or without antibodies can inhibit coliforms *in vitro* but there is no good evidence that this happens in the intestinal tract. We have shown (Law and Reiter, 1977) that LF does not inhibit *E. coli* at or below pH 6.0. At pH 3 and 5 it loses, within 1 h, 90 and 25% respectively of its iron binding activity but strongly recovers it after several hours when kept at pH 7.0. The pH in the stomach would therefore not necessarily damage the LF permanently, considering the buffering effect of the ingested milk. Although extensive tryptic digestion yields iron binding fragments (Brock *et al.*, 1976, and this Symposium) we need *in vivo* evidence of how resistant LF is both to low pH and digestion, particularly in the early life of the calf. Also the rapid absorption of citrate in the upper duodenum (unpublished) plus the secretion of HCO_3 into the lumen of the intestine and the rapid absorption of LF by bacteria tend to justify further work on the role of LF.

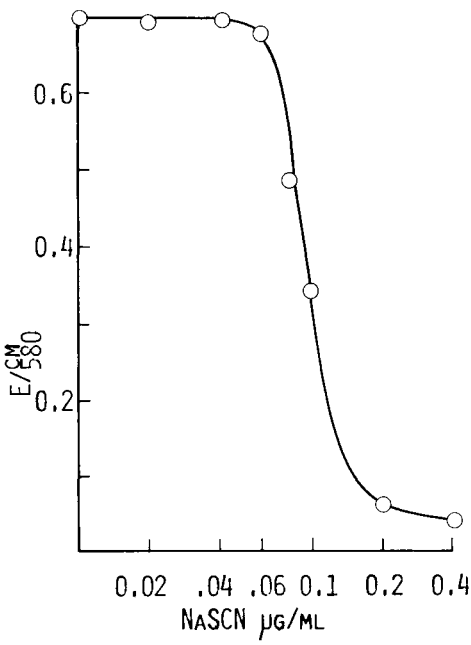

Fig. 2.—Inhibition of *Streptococcus cremoris* 972 in a synthetic medium containing lactoperoxidase and thiocyanate.
(Oram and Reiter, 1966 a.)

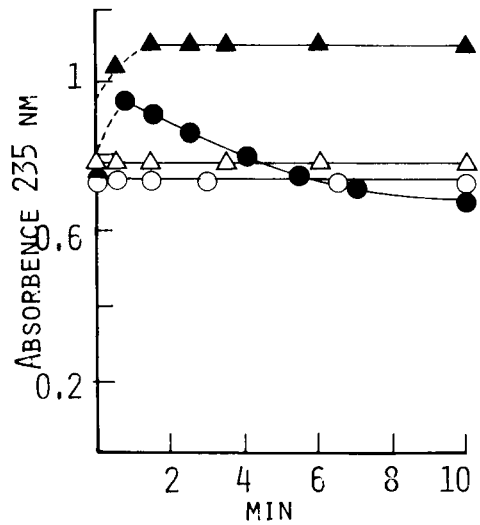

Fig. 3.—Oxidation of thiocyanate by lactoperoxidase and hydrogen peroxide: appearance of intermediate "235" compound.
(Reiter *et al.*, 1964.)
K-phosphate buffer pH 7.4 300 µM
NaSCN 1.92 µM
Lactoperoxidase 60 u
Addition
△——△ distilled water
▲——▲ 1.77 µM H_2O_2
●——● 3.6 µM H_2O_2
○——○ 1.7 µM H_2O_2 without NaSCN.

The lactoperoxidase/thiocyanate/hydrogen peroxide system (LP/SCN $^-$/H_2O_2).

Occurrence of components.

All three components are required (for review, see Reiter and Oram, 1967; Reiter, 1976, 1978) for the system (Fig. 2), which is bacteriostatic at near neutral pH or bactericidal at low pH, in milk and other biological secretions (e.g. saliva, tears, uterine fluid, etc.).

The concentration of lactoperoxidase (LP) in bovine milk is high (*c.* 30 mg/ml, Polis and Shmukler, 1953). The highest level is attained after parturition—between 4-14 days (Kiermeier and Kayser, 1960).

The *SCN*$^-$ concentration in milk depends largely on the feeding regime. Basic low levels of SCN$^-$ are derived from the metabolism of sulphur amino acids. During winter feeding the SCN$^-$ level tends to be low (*c.* 0.025 mM) but is increased on pasture feeding, particularly with natural pastures. Boulangé (1959) detected up to 0.25 mM SCN$^-$ in the milk of cows on natural pasture during the summer months. Increased SCN$^-$ can result from the detoxification of cyanide contained in clover for instance or from glucosides of plants such as Raphani, Brassicaceae; through the activity of rhodanase, glucosides release SCN$^-$ which transudates from the blood into the milk (Virtanen, 1964).

The third component, H_2O_2, can be produced either metabolically by the organisms, e.g. aerobically growing cultures of lactic acid bacteria, or has to be supplied exogenously, particularly if the organisms are catalase positive (e.g. *E. coli*).

Identification of the intermediate oxidation product of thiocyanate.

LP forms a complex with H_2O_2 and oxidizes SCN$^-$ to SO_4^{2+}, CO_2 and NH_3 which are non-inhibitory. The inhibition of Group N streptococci is caused by a short-lived intermediate oxidation product absorbing at 235 nm, the "235 compound" (Fig. 3) [Reiter *et al.*, 1963, 1964]. Some of the inhibitory properties of the "235 compound" were mimicked by the chemically produced sulphur dicyanide $S(CN)_2$ [Wilson and Harris, 1960, 1961]. It was suggested that $S(CN)_2$ was akin to the intermediate oxidation product enzymically produced by LP/H_2O_2 (Oram and Reiter, 1966 a and b). However $S(CN)_2$ is short lived in water; this did not fully coincide with the nature of the inhibitory product because this was found to be dialysable and to remain stable for a few hours, depending on the temperature according to Hogg and Jago (1970 a, b). It was later suggested by the same authors that the antistreptococcal activity might be due to cyanosulphurous or cyanosulphuric acid but HO SCN was discounted. More recently the consensus of opinion (Hoogendorn *et al.*, 1977; Aune and Thomas, 1977) seems to be that SCN$^-$ is peroxidized either to HOSCN directly or via $(SCN)_2$.

$$1)\quad SCN^- + H_2O_2 \xrightarrow{LP} (SCN)_2 + 2H_2O$$
$$(SCN)_2 + H_2O \longrightarrow HOSCN + SCN^- + H^+$$
$$HOSCN \rightleftarrows H^+ + OSCN^-$$

$$2)\quad SCN^- + H_2O_2 \xrightarrow{LP} OSCN^- + H_2O$$

Whatever the identity of the intermediate oxidation product may be, reducing agents in the medium reverse the inhibitory activity, but catalase does not unless the enzyme is added at quite unphysiological levels (150 μ/ml), [Reiter *et al.*, 1976]. It appears that LP has a greater affinity for H_2O_2 than for catalase, hence the LP system is inhibitory in milk which contains both LP and catalase.

Singlet oxygen ($^1O_2$).

Since labelled $^{14}SCN^-$ was not found to be incorporated into the organisms when exposed to the LP system (Marshall, 1978), it is possible that the bacteriostatic and bacteriocidal activity of the intermediate oxidation product of SCN$^-$ is indirect—either through $^1O_2$ or superoxide $O_2^{\bullet-}$, both of these forms of O_2 being highly reactive. These considerations stem from the present concepts of the intracellular systems of polymorphonuclear leucocytes (PMN) which mediate the killing of bacteria. PMN possess a peroxidase, myeloperoxidase (MPO), which oxidizes SCN$^-$ (Sörbo and Ljunggren, 1958) and since PMN produce H_2O_2 when ingesting bacteria or particles (Iyer *et al.*, 1961), it was suggested that PMN inhibit bacteria in a similar manner to the LP system in milk

(Oram and Reiter, 1966a, b). The function of MPO in the PMN has now been well established although the enzyme was not considered to oxidize intracellularly SCN$^-$, but I^- or more recently Cl^- (e.g. Klebanoff, 1975). The oxidation of I^- in milk had been investigated but discounted because the anion was found not to be present at sufficiently high concentrations to inhibit bacteria and was also not as specific as SCN$^-$ in its activity (Reiter *et al.*, 1964). The oxidation of Cl^- was never considered in this context because LP unlike MPO does not oxidize Cl^-.

An interesting hypothesis has now been suggested for one of the intracellular bactericidal systems of PMN (see Klebanoff, 1975 and Johnston *et al.*, 1975); singlet oxygen $^1O_2$ may be produced by the intermediate oxidation product of MPO/Cl^-/H_2O_2 in the following way (Rosen and Klebanoff, 1977):

$$\xrightarrow{\text{MPO}}$$
$$H_2O_2 + Cl^- + H^+ \rightarrow H_2O + HOCl$$
$$OCl^- + H_2O_2 \rightarrow Cl^- + H_2O + {}^1O_2$$

However the occurrence of OCl^- has been disputed by Paul *et al.*, 1970 and Sbarra 1975. According to the latter authors the MPO/Cl^-/H_2O_2 system must be in direct contact with the organisms to be operational and they failed to detect any dialysable product generated by this system. In contrast the LP/SCN$^-$/H_2O_2 system does not need to be in direct contact with the organisms (Hogg and Jago, 1970, a and b; Björck *et al.*, 1975). Thus $^1O_2$ could be produced according to:

$$\xrightarrow{\text{LP}}$$
$$H_2O_2 + SCN^- + H^+ \rightarrow H_2O + HOSCN$$
$$OSCN^- + H_2O_2 \rightarrow SCN^- + H_2O + {}^1O_2$$

This reaction is feasible and would explain why no label of $^{14}SCN^-$ is incorporated and how the highly reactive $^1O_2$ might be generated (see also later). This hypothesis is further strengthened by the report (Piatt *et al.*, 1977) that LP generates $^1O_2$ during the oxidation of Br^- which follows a similar reaction to SCN$^-$ (Hogg and Jago, 1970b).

Superoxide (O_2^-).

This form of O_2 is ubiquitously produced in biological systems and has also been detected in phagocytosing PMN (Babior *et al.*, 1973). According to McCord and Fridovich (1969) and McCord *et al.* (1971) O_2^- leads to the production of the potent oxidizing agent hydroxyl radical (OH') which can be converted by the enzymic activity of superoxide dismutase to H_2O_2. OH' is well known to be damaging to membranes. In PMN it appears that O_2^- is generated by constituents of the leucocyte lysates, presumably through the transfer of electrons from NADH to O_2.

Reiter *et al.* (1964) have shown that the LP system oxidized NADH (Fig. 4) proportional to the H_2O_2 concentration at a constant LP and SCN$^-$ concentration. If it can be proven that this reaction produces O_2^-, the level of superoxide dismutase (SOD) would determine whether this reaction takes place or not.

Mode of action.

It has been shown (Reiter *et al.*, 1963, 1964; Oram and Reiter, 1966a, b) that the LP and

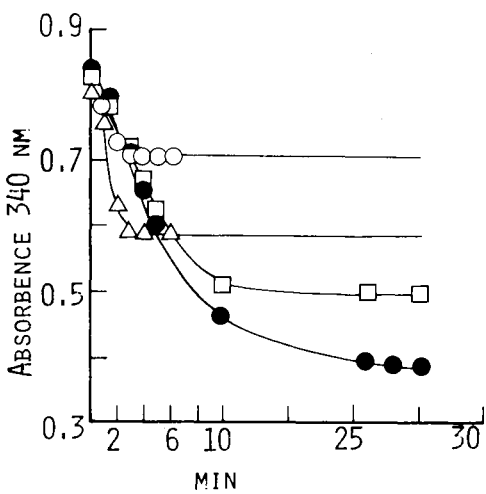

Fig. 4.—Oxidation of nicotinamide-adenine-dinucleotide (NADH) by lactoperoxidase, thiocyanate and hydrogen peroxide.
(Reiter *et al.*, 1964.)
K-phosphate buffer pH 7.4 300 μM
NADH 0.4 μM
NaSCN 0.4 μM
Lactoperoxidase 70 u.
Addition
○——○ 0.30 μM H_2O_2
△——△ 0.60 μM H_2O_2
□——□ 1 μM H_2O_2
●——● 1.5 μM H_2O_2

Table 6.—Uptake of ^{14}C-leucine, ^{14}C-glutamate, ^{14}C-lysine and ^{3}H glucose by *E. coli* 9703.

Absence or presence of system	Labelled substrate	dpm after 2 min of treatment	dpm after 10 min of treatment	dpm after 30 min of treatment
—	leucine	1521	3336	8519
+	leucine	114	151	251
—	glutamate	1079	4734	12972
+	glutamate	189	334	693
—	lysine	1315	3734	9142
+	lysine	304	294	352
—	glucose	990	1913	2525
+	glucose	175	250	173

(Marshall and Reiter, in press.)

SCN⁻ temporarily inhibited the growth and lactic acid production of some strains of Group N streptococci which generate metabolically H_2O_2.

Of the glycolytic enzymes, hexokinase was most strongly inhibited, aldolase and 6-phosphogluconate dehydrogenase were only partly inhibited and several glycolytic enzymes remained unaffected.

Mickelson (1966) postulated the peroxidative conversion of essential enzymic sulphydryl groups because the glyceraldehyde phosphate dehydrogenase is inhibited by the LPs and reversed by reducing agents e.g. cysteine and glutathione (see also Hoogendoorn *et al.*, 1977).

Clem and Klebanoff (1966) demonstrated that a strain of *Lactobacillus acidophilus* was inhibited by the LP system and was also not killed. They also showed that the transport

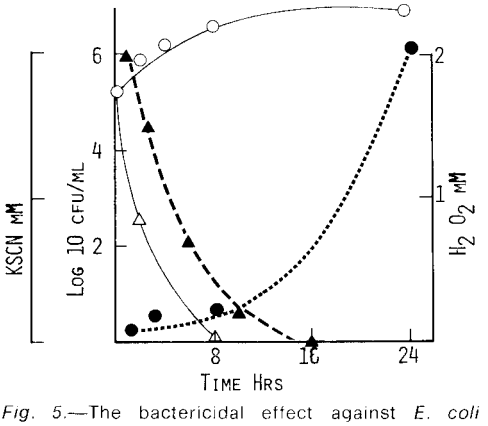

Fig. 5.—The bactericidal effect against *E. coli* serotype 0111 of lactoperoxidase, thiocyanate and hydrogen peroxide*; oxidation of thiocyanate and appearance of free H_2O_2.
○——○ control
Δ——Δ decrease in viable count
▲——▲ oxidation of SCN⁻
●——● H_2O_2 level.
*H_2O_2 produced by glucose oxidase-glucose.

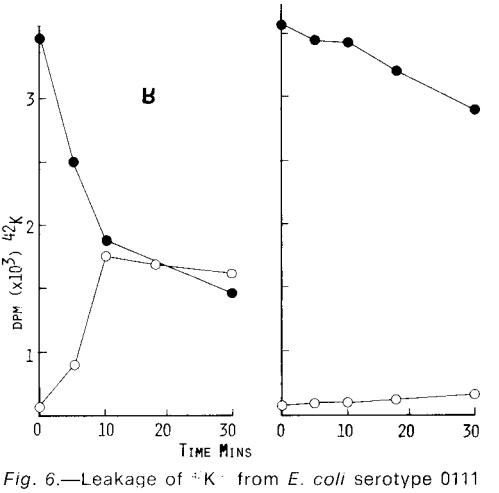

Fig. 6.—Leakage of ^{42}K⁺ from *E. coli* serotype 0111 on treatment with LP-system.
a) LP-treated organism.
b) Control.
○——○ ^{42}K in medium
●——● ^{42}K associated with organisms.
(Marshall and Reiter, in press.)

of glutamic acid was inhibited by the LP system as might be expected since the uptake of amino acid requires energy.

Björck *et al.* (1975) and Reiter *et al.* (1976) have since shown that catalase positive Gram-negative organisms were killed by the LP system provided H_2O_2 is exogenously supplied; e.g. *E. coli* (Fig. 5), *Pseudomonas aerogenes, Salmonella* typhimurium.

More detailed work on *E. coli* showed that the organisms were only killed after exposure to the LPs for 1½-2h and could recover their viability after 30 min of exposure when transferred to a fresh medium without the LPs. Eventually, in the LP medium, the organisms are lysed (Plate 1). Lysis always occurs at the polar end of the bacterial organisms but it has not yet been determined whether the new outer membrane formed after division or whether the old membrane is affected.

In contrast to the slow bactericidal effect, inhibition of some metabolic activities of the organisms, uptake of amino acids and glucose occur very rapidly (Table 6). DNA, RNA and protein synthesis are inhibited within 30 min (Marshall and Reiter, 1976 and in press) being a secondary effect of the damage caused to the inner membrane. This damage is indicated by the leakage of $^{42}K^+$ (Fig. 6), and amino acids and apparently causing the inhibition of the active transport of glucose and amino acids.

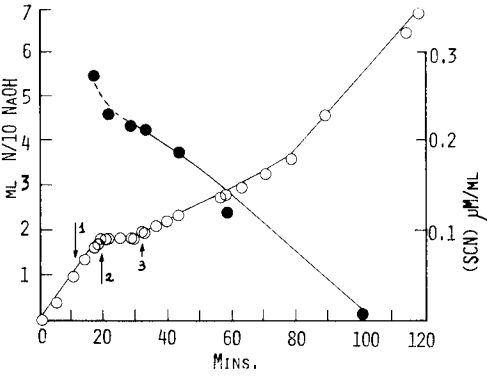

Fig. 7.—Inhibition of *Str. cremoris* 972 and its reversal by a cell free extract of a resistant strain of *Str. cremoris* (803).
(Reiter *et al.*, 1964.)
1. 5.0 μM SCN.
2. 70 u LP.
3. 1 ml 803 extract of resistant organisms.

Table 7.—Bactericidal effect of the LP-system against porcine and bovine serotypes of *E. coli* tested.

09: K "P_{16}", 064: K "V142".
0147: K_{89}, K_{88} a, c; 0149: K_{91}, K_{88} a, c.
0157: K "V17", K_{88} a, c; 0157: K "V17".
0.24:

Resistance to the lactoperoxidase system.

As already mentioned, not all Group N streptococci are temporarily inhibited by the LP system. The sensitive strains are "self inhibited" at the pH of milk (*c* 6.8) and do not require an exogenous source of H_2O_2; they are catalase negative and produce metabolically enough H_2O_2 to activate the LP system. Resistant strains also produce H_2O_2, but possess a "reversal factor" which catalyses the oxidation of $NADH_2$ in the presence of the intermediate oxidation product of SCN^- (the "$NADH_2$-oxidizing enzyme"). Consequently lysates of resistant strains reverse the inhibition of sensitive strains (Fig. 7).

So far we have not detected amongst the coliforms any LP resistant strains or resistant mutants within a culture. *E. coli* was treated with the LP system, and the percentage of kill determined after 6 h; the residual organisms were then subcultured into new medium (without the LP system) and exposed again to the LP system. The percentage of killed organisms was found not to be altered even after repeated subculturing, indicating that no resistant mutants had arisen.

The LP system appeared equally effective against human, bovine and porcine enteropathogenic serotypes of *E. coli* (Table 7). Also multiple antibiotic resistant strains of *Klebsiella aerogenes* isolated from the faeces of infants were found to be susceptible to the LP system. More recently, however, *Sarcina lutea* was found to be resistant to the bactericidal effect of the LP-system (Reiter, Marshall and Phillips, unpublished). This was based on the findings of Krinsky (1974) who reported that human PMN killed a colourless mutant strain of *S. lutea* much more readily than a carotenoid-containing strain. He attributed this resistance to the well known quenching of singlet excited oxygen by carotenoids, thus supporting the suggestion that $^1O_2$ is one of the mediator

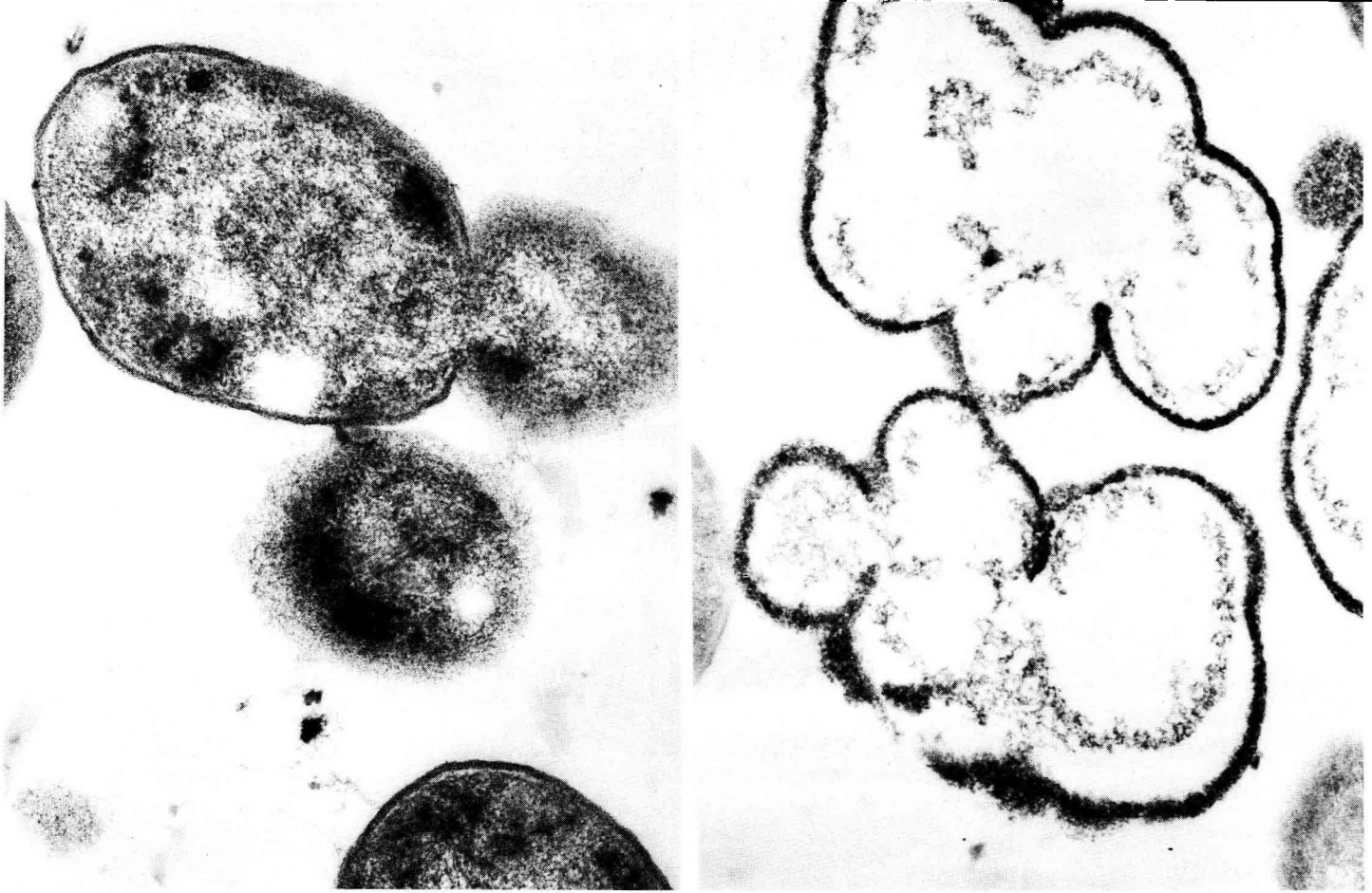

Plate 1.—Damage and lysis to *E. coli* 9703 after exposure to LP system. Left: 1 ½ h. Right: 4 h.
Cells were collected on a Millipore filter (0.22 m pore size), covered with a thin layer of agar and fixed for 1 h in 0.2 M cacodylate-HCl buffered 3% gluteraldehyde. The filter was washed in buffer, fixed for 1 h in 1% osmium tetroxide and *en bloc* stained with 1% uranyl acetate for ½ h. After dehydration in a graded series of alcohol-water mixtures, it was embedded in araldite. (By courtesy of B.E. Brooker and D.E. Hobbs, NIRD.)

of the intracellular bactericidal action in PMN. Fig. 8 shows that the white mutant was easily killed by the LP-system while the pigmented strain was quite resistant. Since carotenoid pigments are located in the inner membrane of an organism, often associated with quinones, it is possible that the LP-system, by damaging the membrane, affects the electron transport-mechanisms.

Biological effect.

For some time now it has been recognised that enteropathogenic strains of *E. coli* (and other intestinal pathogens) associate intimately with the epithelial surface of the intestine. This attachment favours proliferation and reduces their removal by peristalsis, the subsequent production of enterotoxins causes diarrhoea. Certain plasmid controlled surface antigens like K_{88} in the piglet (Ørskov *et al.*, 1961; Smith and Linggood, 1971; Jones and Rutter, 1972) play a vital role in the process of attachment and pathogenesis. *In vitro* this surface antigen haemagglutinates erythrocytes and adheres to brush border cells (for review see Jones, 1975) [Plate 2]. Some workers (Schrank and Vervey, 1976) regard the motility as a virulence factor because it enables the organisms *(Vibrio cholerae)* to traverse the continuous blanket covering the intestinal villi and reach the epithelial cells. Other workers (Jones *et al.*, 1976) dispute that motility matters because non-motile mutants seemed to attach equally well. No equivalent data are available for enteropathogenic strains of *E. coli*.

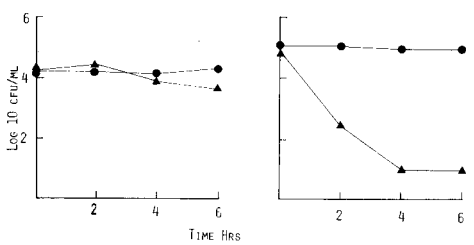

Fig. 8.—The bactericidal effect of the LP-system against a white mutant of *Sarcina lutea* and its failure to kill the pigmented (carotenoid) wild strain of *S. lutea*.
a) wild pigmented strain
b) white mutant.
●——● control
▲——▲ LP-treated.

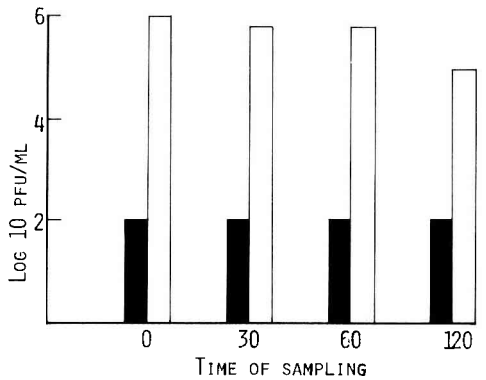

Fig. 9.—The bactericidal effect against *E. coli* 9703 of abomasal fluid after feeding raw milk:
Black column: Bactericidal effect after addition of glucose oxidase/glucose as source of H_2O_2: *E. coli* cfu/ml in abomasum.
White column: Reversal of bactericidal effect after addition of dithionite ($Na_2S_2O_4$): *E. coli* cfu/ml in abomasum.

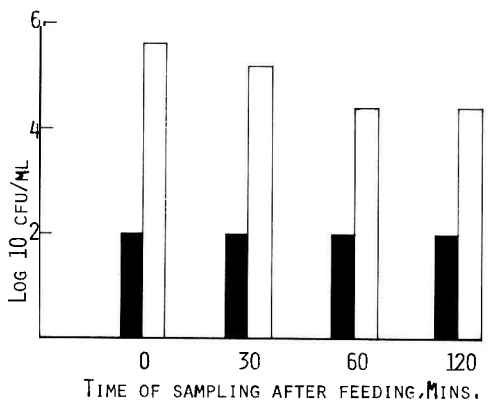

Fig. 10.—The bactericidal effect against *E. coli* 9703 of feeding H_2O_2 producing lactobacilli as source of H_2O_2 to activate the LP-system
Lactobacillus casei 10^5 cfu/ml.
E. coli 10^5 cfu/ml.
Black column: *E. coli* in abomasal fluid after feeding fresh milk containing *E. coli* and *L. casei*.
White column: As above but after addition of $Na_2S_2O_4$ to reverse bactericidal effect.

However, it was of interest to determine whether the LP system affected the motility of *E. coli* since glucose metabolism was shown to be affected (see above) and it had previously been found that the motility of spermatozoa was inhibited (Reiter and Gibbons, 1964). Indeed, a motile strain of *E. coli* became immediately non-motile after exposure to the LP system. At the same time, attachment to isolated brush border cells was prevented *in vitro* (Reiter, Turvey and Cole, unpublished) [Plate 3]. It remains to be established whether this inhibition of attachment is caused by inhibition of the motility or by a surface charge in the organisms caused by the LP system.

In vivo bactericidal activity.

The object of the *in vivo* experiments was to determine (1) the ability of the LP system to play a part in reducing the coliforms in the abomasum and the duodenum, and (2) the possibility of designing a milk substitute containing an active LP system (Reiter and Marshall, unpublished).

It is generally accepted that gastric acid secretion is an important bactericidal barrier against enteric infections. (For a review see Gianella *et al.*, 1973.) In the neonate (Hill, 1956) the development of the parietal cells of the stomach and the secretion of acid differ according to whether antibodies are transferred in utero or whether the neonate depends on the ingestion of colostrum. According to Hill the pH in the abomasum of lambs is around 4.4 but in the guinea-pig it is 1.2. The pH in the calf abomasum is somewhat higher than in the lamb—6-7 (Parrish and Fountaine, 1962). In addition, the high buffering capacity of bovine milk neutralises the acid rapidly: bacteria can there-

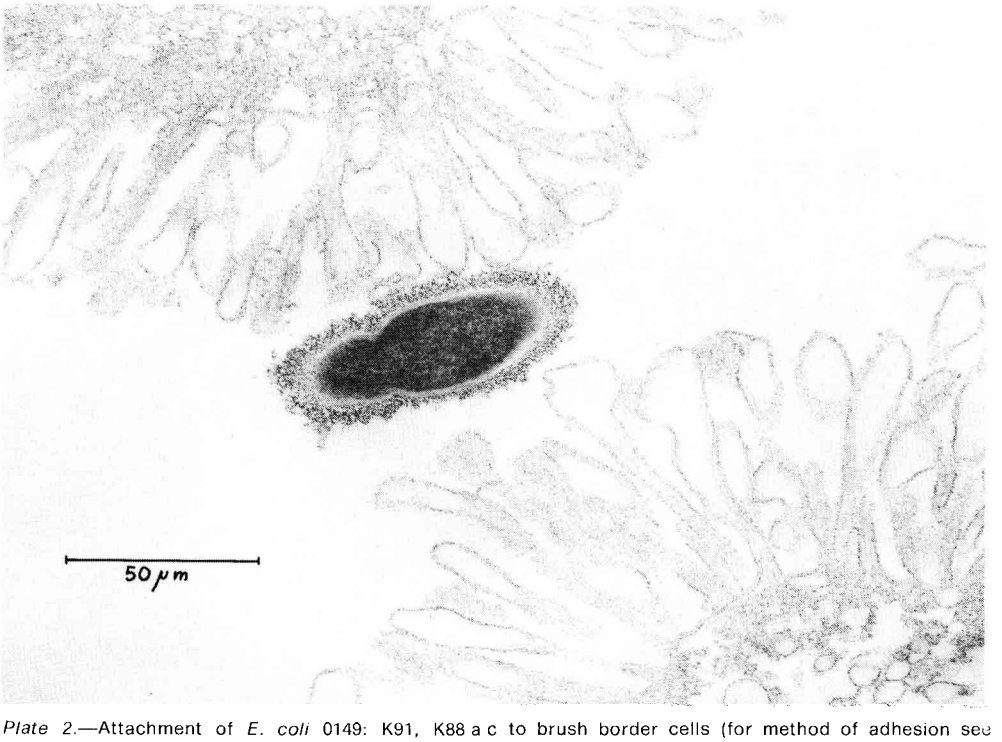

Plate 2.—Attachment of *E. coli* 0149: K91, K88 a c to brush border cells (for method of adhesion see Jones, 1975). Staining: Cells and organisms were exposed to cationic ferritin for ½ hour at room temperature, washed 3 times in phosphate buffered saline, fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer pH 7.4 and post fixed with 1% OsO_4.
(Courtesy of B.E. Brooker and D.E. Hobbs, NIRD.)

fore easily survive and multiply unless there are alternative antibacterial systems in the milk—protective specific antibodies *and* non-specific antimicrobial systems.

In experiments to determine these points, calves were either fistulated in the abomasum or fitted with a re-entry cannula in the duodenum close to the pylorus. They were given fresh milk which always contained LP and in the beginning was complemented with SCN^-. However it was found that the abomasal fluid of calves from 10 days onwards, the beginning of the experiments, contained high concentrations of SCN^-, up to 0.45 mM before feeding, and was diluted to 0.15 mM after the feeding of 2 litre of milk.

As we cannot use animal pathogens for *in vivo* experiments at our Institute, a human serotype of *E. coli* (O111) was used, the same strain as in our *in vitro* experiments. It was shown to be non-pathogenic in conventional reared calves. It was found that feeding fresh milk containing LP and SCN^- made the abomasal fluid bactericidal when glucose oxidase

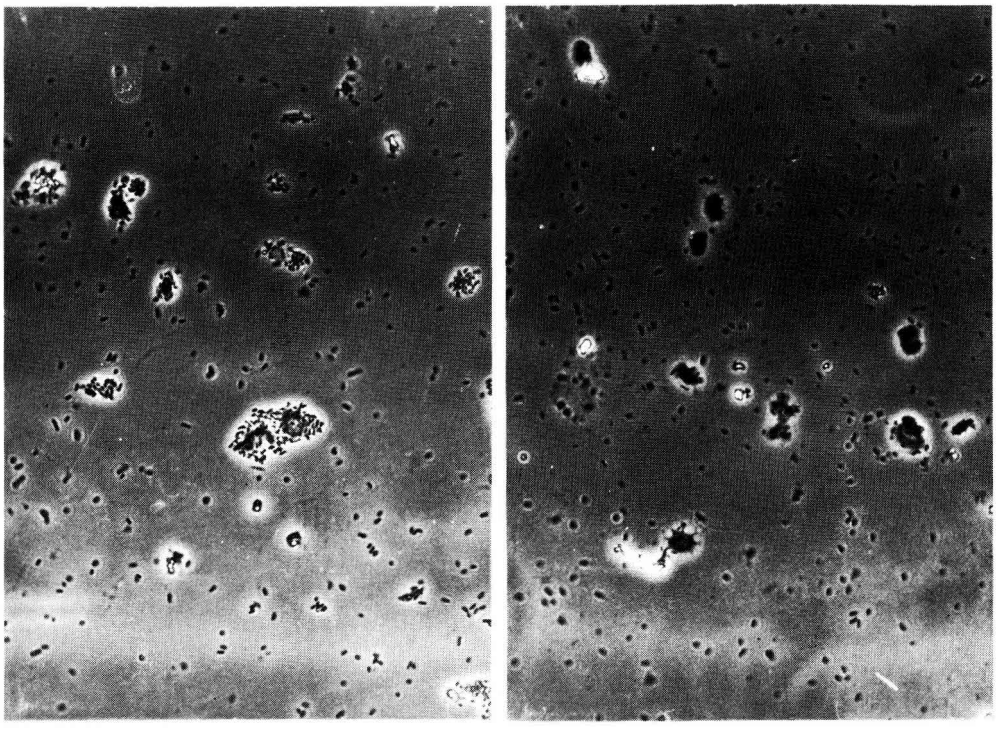

Plate 3.—Attachment of *E. coli* 0149 K_{88} a, c to isolated brush border cells.

a) Left: Attachment of *E. coli.*

b) Right: Reduced attachment of *E. coli* after exposure to LP system.

Porcine brush border cells were washed by centrifugation, and resuspended at approx. 10^6/ml in phosphate buffered saline (PBS). A K_{88}-positive strain of *E. coli* (0149: K91 (B), K88ac (L): H10). Was grown on Nutrient Agar at 37° overnight, harvested in 5 ml PBS, washed and resuspended at approx. 10^8/ml.

Two 0.5 ml aliquots of the K_{88} positive coli were taken and to one was added the complete LP system. Both were left standing at 37°C with occasional stirring for 30 min. The samples were then centrifuged and resuspended in 0.5 ml PBS. 0.5 ml of brush borders were now added to each, and the tubes mixed and allowed to stand for a further 15 min at 37°C with occasional stirring. 0.5 ml of each was then mounted under a cover slip on a glass slide and photographed under phase-contrast conditions.

was added as a source of H_2O_2. To prove that the killing of *E. coli* was due to the LP system, dithionite ($Na_2S_2O_4$) was added to reverse the bactericidal effect. This confirmed as expected that LP resists digestion *in vitro* and *in vivo* (Hultquist and Morrison, 1963; Gothefors and Marklund, 1975).

In other experiments glucose oxidase/glucose was added to milk and fed to the calf. Figure 9 shows that this enzyme also retained its activity in the abomasum and produced enough H_2O_2 to activate the LP-system and to kill *E. coli;* once again the addition of dithionite reversed the bactericidal activity.

Figure 10 shows that H_2O_2 producing lactobacilli can be also used to activate the LP-system. Equal numbers of *L. lactis* and *E. coli* were given to calves in milk and the abomasal fluid tested for the presence *E. coli.* Without addition of dithionite *E. coli* could not be recovered thus proving that the LP-system had become activated.

We have also fed single calves at intervals with glucose oxidase/glucose, dithionite, fresh or boiled milk and observed the variation in the coliform content of the abomasum. It is noteworthy that the lactobacilli were never reduced (Marshall, 1978). Also, we succeeded in colonising some calves with an H_2O_2-producing lactobacillus isolated from a calf and found that the organisms were continuously excreted in the faeces and were detected as having colonized the oesophagus, groove, omasum, abomasum and duodenum. Repeats of these experiments are now in hand.

Promising results were also obtained in an experiment determining the reduction of scouring in calves fed milk containing the complete LP-system by inclusion of SCN^- and glucose oxidase/glucose for the generation of H_2O_2. There was a double control: a) feeding raw milk only; b) adding cystein to raw milk to reverse any bactericidal activity based on the LP-system. The first experiment was carried out in collaboration with M. Knutsson of Astra Ewos, Sweden. Calves (18) from surrounding farms were collected *c* 5 days old and reared under "commercial" conditions. Table 7 shows that the activated LP-system appeared to reduce the number of days the calves scoured by about 50% and showed a surprising weight gain 60% higher compared with the controls. A similar experiment but using calves (120) from the NIRD herd which received a controlled amount of colostrum at birth and under strict supervision, is now being repeated. Preliminary results indicate a weight gain of c 14% to 32% higher when feeding the complete LP-system compared with the control.

Table 8.—The effect of feeding the activated LP-system to calves.

Feed	No. of days calves scoured	Weight gain g/day at 3 weeks
Milk only (6)	12	198
Milk plus cysteine (6)	14	206
Milk plus glucose-oxidase/glucose and SCN^- (6)	7	341

Preliminary experiments feeding newborn piglets on bovine milk were less successful because the milk clot distended the stomach. This was avoided by feeding bovine colostrum (diluted and undiluted). A preliminary experiment at the Agricultural University of Sweden, Uppsala indicates that the LP-system could protect piglets against infection with *E. coli.* Two litters were infected at birth (before taking up colostrum) with *E. coli* $O149K_{88}$ a.e. One litter was placed with the sow and 6 out of 10 piglets died within 48 h. The second litter was divided into controls, fed bovine colostrum only or containing the activated LP-system. Of the controls one died, two had severe diarrhoea and one was unaffected while all the piglets fed the activated LP-system were protected.

Other non-antibody inhibitors.

Properdin, conglutinin, and basic proteins such as β lysin and ubiquitin may occur in colostrum and milk but their significance (with the exception of xanthine oxidase as a source of O_2O_2) is not yet clear (see Reiter, 1976). Vitamin B_{12} and folate protein binders have attracted attention because the free vitamins only are rapidly taken up by bacterial species which colonise the stomach and intestine. Theoretically, therefore, bacteria which cannot synthesize these vitamins should be suppressed in the intestinal tract

(e.g. Ford, 1974; Ford *et al.*, 1975) because denying the vitamins for the growth of vitamin requiring bacteria could be analogous to the withholding of iron by LF.

Discussion.

With a new approach to a problem some aspects which appear at the time important may later prove to be less so. Nevertheless provocative statements may elicit discussions and hopefully more research.

It is apparent that more information is needed about the fate of the protective *in vivo* systems discussed in this review. *In vitro* studies on the resistance of proteins to acids or proteolytic enzymes yield only limited information because it is impossible to reproduce truly physiological conditions. For instance, sIgA is generally accepted to resist acid and proteolysis better than any of the other classes of Igs. Nevertheless there is good evidence that all classes of Igs are excreted in the faeces of babies (e.g. Michael *et al.*, 1971) and Fig. 11 shows the distribution of the Ig classes in the faeces of calves. Two calves were fed colostrum at birth and for the other purposes a "high immunoglobulin milk" (colostrum diluted with milk 1:2). When the faecal extracts were freed from debris by chromatography and assayed by the Mancini technique (Steel & Reiter, unpublished) appreciable amounts of IgM were detected up to day 7.

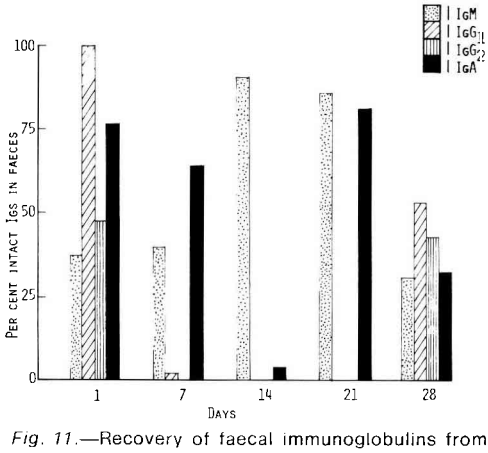

Fig. 11.—Recovery of faecal immunoglobulins from faeces of 2 calves fed dilute colostrum (1:2).

The precipitin reactions of Igs to specific antisera can be misleading because they may retain their precipitin activity but may have lost their antigen binding capacity or other biological functions. Nevertheless, water extract of infant's faeces up to 4 days were shown to possess about 1/10th of the agglutinating and bactericidal properties of the colostrum fed to the babies (Michael *et al.*, 1971).

These *in vivo* results with Igs in the human neonate contrast with the scanty knowledge of the passage of proteins through the intestinal tract of the calf. It is even difficult to cite convincing data on the proteolytic enzymes in the calf during the first 7 to 10 days because such experiments require cannulated calves which precludes experimentation before 7-10 days (e.g. Termouth *et al.*, 1976; Williams *et al.*, 1976 and J.H.B. Roy, NIRD, pers. comm.). Of other protective proteins it is known that lysozyme can survive in the IT of infants because it can be regularly detected in the faeces of breast fed but not bottle fed babies (Hanneberg and Finne, 1974). It is not known whether it can remain active in the IT of the calf and we have no knowledge about the fate of LP in the animal although human milk contains enough LF to detect it in the faeces if it does survive the passage in the IT.

Perhaps our concepts of digestion are too simple: since we know that all the protective proteins are capable of attaching themselves to bacteria it might be better to test the effect of proteolytic enzymes on the bacteria which have been "sensitized" by the attached complement/antibody complex, lactoferrin, lysozyme or lactoperoxidase. This might show whether the activity of these proteins could be impaired once they have complexed with ingested bacteria. Also, as in the piglet, Igs are taken up by the intestinal epithelial cells for 18-21 days after birth without being transported into the blood (Lecce, 1973, a and b). It appears that Igs would not only have a role to play in the lumen of the intestine but be located where the defence was most important (Heremans described the function of sIgA as an antiseptic paint). Since Lecce has also shown that uptake of Igs occurs in rabbits and guinea pigs which receive their Igs *in utero*, it is possible that this process takes place also in the human infant and of

course in the calf. This concept has recently been strengthened by the finding that 10-15% of colostral sIgA is transported into the blood of the infant (Ogra *et al.*, 1977).

This uptake without transport does not seem to be restricted to Ig proteins because it has been shown recently that horseradish peroxidase is also taken up even in piglets infected with *E. coli* (Staley, 1977). This enzyme is easily detectable by histological staining and was used as a marker for this process of uptake. However, it indicates that in analogy the LP from the milk may also be taken up. LP has been isolated from the scrapings of the intestines of pigs (Stelmuszyuska and Zgliczynski, 1971) but it was not clear whether the enzyme was derived from eosinophiles which are packed with LP or whether it is synthesized in the intestines. Since there is now some evidence that sIgA, complement and lysozyme can be synthesized *in vitro* by intestinal tissue (McClelland and Furth, 1975) it is reasonable to ask whether LF and LP are similarly synthesized. This would strengthen the concept that the Igs and non-antibody factors present in colostrum and milk are designed to bridge the immunological gap until the newborn begins to synthesize its own protective systems against infection.

These multiple protective systems occur in secretions which bathe mucous membranes exposed to infection from outside and colostrum and milk contains the same systems to protect the neonate against the entry of microorganisms and establish a favourable ecology in the intestinal tract. Finally, if we accept that leucocytes are the primary defence against invading organisms we could regard the colostrum as liquid leucocytes because so many of the antimicrobial factors are common to both.

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