Role of Nonantibody Proteins in Milk in the Protection of the Newborn

Bruno Reiter

Since the turn of the century, the antibacterial activity of milk has attracted the attention of scientists in human and veterinary medicine. Medical workers were interested in the spread of milk-borne diseases such as cholera, salmonellosis, and scarlet fever. Veterinarians researched the role of antibacterial factors in the defense of the bovine udder against bacterial infections (mastitis). Dairy scientists worried about the inhibition of lactic streptococci vital for the production of cheese.

Eventually, veterinarians discovered that ungulates are born without immunoglobulins in their blood and depend on the ingestion of colostrum and transmission of colostral antibodies through their permeable gut into the blood. An intestinal role for antibodies was proposed but could only be established after the discovery of secretory IgA. The attention of immunologists has been focused on the role of sIgA in defense against bacterial (and viral) intestinal infections in piglets, calves, and, more recently, human infants. This has resulted in the neglect of other host defense functions of human milk such as lysozyme, lactoferrin (transferrin), lactoperoxidase, etc. These factors can augment antibody action and afford protection in the absence or inefficiency of specific antibodies.

This chapter is concerned with the description, occurrence, and mode of action of these nonantibody factors in vitro. It also deals
with their in vivo activity insofar as the very limited range of animal experiments permit (for reviews, see 1–11).

LYSOZYME (N-ACETILMURAMYLHYDROLASE, E.C. 3.2.1.17)

In 1922, Fleming (12) discovered the "extraordinary bacteriolytic phenomenon" of nasal mucus. He named the agent lysozyme. The wide distribution of lysozyme—in secretions such as milk, saliva, egg white, in tissue extracts (alveolar and intestinal), in "pus," and in blood—led him to believe that it constituted a primary method of destroying bacteria.

The concentration of lysozyme in human colostrum is relatively high (Fig. 1) (4); its concentration declines rapidly with the duration of lactation, but the increased intake of milk by the growing infant ensures an appreciable intake. However, relatively high levels are maintained through the first and possibly second year of lactation (13). In contrast, bovine milk contains little lysozyme (30 μg/100

![Graph](image-url)

**FIG. 1.** Lysozyme in milk: concentration and daily intake. (▲) concentration (mg/100 ml) ± SEM; (○) intake (mg/24 hr) for group B subjects; (△) calculated intake (derived by multiplying milk volumes for group C by the mean concentration of lysozyme). Group B: milk samples provided daily and 24-hr test weighings at each sampling point. Group C: no analysis of milk samples, only 24-hr test-weighing data available. (From ref. 4.)
ml) (14). Unfortunately, we still lack sufficient in vivo evidence to substantiate that the relatively high concentration of lysozyme in human milk compared with bovine milk benefits the breast-fed infant.

**Mode of Action and Antibacterial Spectrum**

Lysozyme is a basic protein, stable at acid pH but unstable at alkaline pH. It hydrolyzes the β,1–4 linkage between N-acetylglucosamine in the peptidoglycan layer of the cell wall of gram-positive and the outer membrane of gram-negative organisms. The susceptibility of an organism to lysozyme depends on the accessibility of the substrate, peptidoglycan, and the origin of the enzyme. In gram-negative organisms, the susceptible substrate is masked by lipoproteins, hence the relative resistance to lysis unless the organisms are first exposed to the action of specific antibodies and complements or treated with agents such as chloroform or ethylenediaminetetraacetic acid (15–17).

Human and bovine milk lysozymes (Tables 1, 2) have about three times the specific activity of egg albumen lysozyme as measured against the very susceptible assay organism *M. lysodeicticus*. The ease of purification of lysozyme from the enzyme-rich egg white and its commercial availability made this lysozyme regrettably the favorite tool for many investigations, notwithstanding the known differences between the lysozymes of different origins.

**Biological Significance**

Rosenthal and Lieberman (18) were the first to suggest in 1931 that lysozyme in human milk has a marked influence on the bacterial flora of the intestine in infants. They showed that the meconium contained no lysozyme; on the third day postpartum, the enzyme appears in the feces of breast-fed infants but not of artificially fed infants. The bacterial flora of the breast-fed infants was found to contain about equal numbers of gram-negative and gram-positive organisms. A suspension of such feces, incubated with complement-inactivated human milk for 24 hr at 37°C, contained few gram-
### TABLE 1. Sensitivity of different bacteria to human milk lysozyme*

<table>
<thead>
<tr>
<th>Organism</th>
<th>Rate of lysis (Δ% T/min)**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptococcus lactis</td>
<td>0.00</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>0.05</td>
</tr>
<tr>
<td>Sarcine lutea</td>
<td>0.04</td>
</tr>
<tr>
<td>Streptococcus faecalis</td>
<td>0.04</td>
</tr>
<tr>
<td>Bacillus cereus</td>
<td>0.23</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>0.10</td>
</tr>
<tr>
<td>Serratia narcescens</td>
<td>0.00</td>
</tr>
<tr>
<td>Proteus vulgaris</td>
<td>0.00</td>
</tr>
<tr>
<td>Pseudomonas fluorescens</td>
<td>0.08</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>0.21</td>
</tr>
<tr>
<td>Micrococcus lysodeicticus</td>
<td>1.70</td>
</tr>
</tbody>
</table>

*Compiled from ref. 14.

**T is turbidity in phosphate buffer, pH 6.2, determined at 540 nm. For comparison, Δ% T/min for *M. lysodeicticus* by egg white lysozyme is 0.63.

### TABLE 2. Sensitivity of different bacteria to bovine milk lysozyme*

<table>
<thead>
<tr>
<th>Organism</th>
<th>Rate of lysis (Δ% T/min)**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptococcus lactis</td>
<td>0.2</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>0.03</td>
</tr>
<tr>
<td>Sarcine lutea</td>
<td>0.08</td>
</tr>
<tr>
<td>Streptococcus faecalis</td>
<td>0.17</td>
</tr>
<tr>
<td>Bacillus cereus</td>
<td>0.10</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>0.12</td>
</tr>
<tr>
<td>Serratia marcescens</td>
<td>0.10</td>
</tr>
<tr>
<td>Proteus vulgaris</td>
<td>0.06</td>
</tr>
<tr>
<td>Pseudomonas fluorescens</td>
<td>0.08</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>0.30</td>
</tr>
<tr>
<td>Micrococcus lysodeicticus</td>
<td>1.82</td>
</tr>
</tbody>
</table>

*Compiled from ref. 14.

**T is turbidity in phosphate buffer, pH 6.2, determined at 540 nm.
negative organisms. When the feces were incubated with boiled human or raw cow's milk, they observed a relative increase in gram-negative organisms. The researchers attributed this effect of the human milk to its high lysozyme content and suggested that the enzyme may be an important factor in the welfare of infants. The main criticism of the inhibition experiments is that the antibacterial property of milk is now known to be multifactorial.

Recent work confirms the presence of lysozyme in the feces of breast-fed babies (19–21). The feces of infants with diarrhea contain more lysozyme than those of healthy infants. The increase of lysozyme in inflammatory intestinal mucosa parallels the immigration of neutrophilic leukocytes, monocytes, and macrophages, which release the enzyme into the intestinal fluid.

Besides the antibacterial properties of lysozymes, Jolles (22) proposed that lysozyme may have a function as an immunomodulator. Indeed, there exists some support for this hypothesis in the literature. Lodinova and Jouja (21) reported that a lysozyme-containing formula significantly increased the slgA levels in the feces of full-term and preterm babies. The levels of IgM, IgG, and IgA in the serum were not increased. Because of the lack of humoral response, they suggested that the lysozyme activity on bacterial cell walls may stimulate a local immune response.

One may speculate further that the lysozyme content of milk promotes protection through its effect on human leukocytes. Human lysozyme at a concentration of 10 to 400 μg/ml significantly (p<0.001) stimulates phagocytosis of yeast cells by leukocytes in the absence of any serum factors. Albumen lysozyme is without any effect. Lysozyme does not act on the yeast cells but on the leukocytes and cannot therefore be regarded as an opsonin (23).

LACTOFERRIN

The antibacterial activity of this iron-chelating protein was first reported in bronchial mucus and bovine milk (24–26). Lactoferrin has since been shown to be present in the milk of most mammals, albeit at different concentrations, and in the secretion of the non-
lactating bovine mammary gland. The exception so far is rabbit's milk, which contains transferrin, the iron-binding protein in blood. Traces of transferrin are also found in most milks. Lactoferrin also occurs in other exocrine secretions such as saliva, tears, nasal, pancreatic, and intestinal secretions (27,28).

The concentration of lactoferrin (4) in colostrum (Fig. 2) is high, declining rapidly with postpartum age like lysozyme, but the total intake by the infant is considerable, remaining at around 1,000 µg/day for several weeks. Unlike lysozyme, lactoferrin has never been used as a supplement to formula feeds. However, a related protein, conalbumin, purified from egg white has recently been added with claims of a beneficial effect for infants with diarrhea.

**Chemical Properties and Antibacterial Effect of Lactoferrin**

Lactoferrin is a single-chain glycoprotein with a molecular weight of 70,000. It is strongly basic, binding two ferric ions and, syner-

![Graph](image-url)
gistically, two bicarbonate ions; it holds iron at low pH (3.5–4), whereas transferrin loses its iron at low pH.

In the native state, lactoferrin is only partly saturated with iron (8–25%) and can therefore deprive bacteria of essential iron by its chelating power. It had been generally accepted (29–33) that only bacteria with a high iron requirement (e.g., coliforms) are inhibited, whereas organisms with very low iron requirements (e.g., lactic acid bacteria) (34) are not inhibited. Recent research has shown that a wide spectrum of bacterial species, both gram-positive and gram-negative bacteria, are not only inhibited but killed by apolactoferrin (devoid of iron), whereas the bacteriostatic activity of lactoferrin is only temporary and can be reversed by the addition of iron. The bactericidal activity of apolactoferrin is irreversible (35–38).

Although lactoferrin can withhold iron essential for the growth of organisms, the presence of citrate as in milk, 2 to 3 mM in human and 4 to 8 in mM in bovine milk, can make iron available for growth as an iron–citrate complex, which is taken up by bacteria. Bicarbonate, being essential for the chelating of iron by lactoferrin, can overcome this effect (39–42). Bacteria themselves also possess several means of iron chelation in an iron-poor medium or in the presence of lactoferrin: siderophores, siderochromes, secondary hydroxamic acids (e.g., ferrichromes, ferrioxamine B), and phenolic acids (e.g., enterocholin).

**Bactericidal Effect of Apolactoferrin**

Arnold and his collaborators (35–38) demonstrated in a series of papers that apolactoferrin (iron-free) in distilled water had a direct bactericidal effect on a wide range of microorganisms including gram-positive, gram-negative, aerobic, and anaerobic bacteria as well as yeast: *Streptococcus mutans, Streptococcus salivarius, Streptococcus pneumoniae, Escherichia coli* (nonenteropathogenic), *Vibrio cholerae, Pseudomonas aeruginosa, Candida albicans*. However, other morphologically and physiologically similar organisms are completely resistant: *Streptococcus pyogenes, Strep-
tococcus lactis, Lactobacillus casei, Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli (0126,0111—enteropathogenic), Enterobacter cloacae, Salmonella newport, and Shigella sonnei.

The bactericidal effect, within 1 hr, cannot be reversed by iron or magnesium (known to stabilize the inner membrane) and is the result of a two-step process with a log phase of ~15 min. The apolactoferrin needs to remain in direct contact with the cell surface throughout. Viable organisms can be recovered after removal of the protein by washing within 50 min. Slime formation—S. mutans grown with sucrose—or capsule formation—S. pneumoniae passaged through an animal—reduces the bactericidal effect. The process is energy dependent: no killing occurs at 2°C, and organisms harvested in the early phase of stationary growth are markedly more resistant than organisms in the exponential phase. Detailed studies with S. mutans showed that glucose uptake and metabolism are inhibited, as is the incorporation of amino acids and purines.

So far there is no evidence that this bactericidal effect takes place in milk containing native lactoferrin; in vivo it may be of some relevance to the intracellular killing of leukocytes. The observed effect on the metabolism points to a damaging effect on the inner membrane remarkably similar to the effect of the lactoperoxidase system, which has been shown to damage the inner membrane. It is difficult to free all traces of lactoperoxidase from lactoferrin preparations (32). Also, since hemoproteins are known to have peroxidative activity, the bactericidal effect could be attributable either to this reaction or to contaminating lactoperoxidase.

**Biological Significance**

Human colostrum contains high concentrations of lactoferrin, up to 15 mg/ml; the concentration declines with postnatal age but less rapidly than those of sIgA, IgM, and IgG. However, with the increased intake of milk that occurs as the baby grows, the total intake of lactoferrin actually increases after birth and reaches a peak at about 5 days (4,13). This high intake suggests an important role for lactoferrin, but before we can ascribe a biological function, it
has to be shown that lactoferrin escapes digestion and any detrimental effect of the low pH in the stomach. In general, human milk passes rapidly, "undigested," into the duodenum (43).

Samson et al. (44) submitted human colostrum to tryptic and peptic digestion in vitro: tryptic digestion failed to release the iron or abolish the bacteriostatic effect, but peptic digestion or keeping the colostrum at low pH abolished both properties. Indeed, keeping purified bovine lactoferrin at pH 5 or 3 leads to a loss of 25% or 90%, respectively, of its original iron-binding capacity, but this can be nearly restored by keeping the pH at 7.4 for 4 hr (32). These observations, plus the concentration of antitrypsin in milk, which remains relatively high up to 24 days post-partum (falling afterwards below the sensitivity of the assay used) (4), and rapid passage through the stomach indicate that lactoferrin may escape destruction at least in the early life of the newborn.

**In Vivo Antibacterial Activity of Lactoferrin**

The milk of guinea pigs is relatively rich in lactoferrin and transferrin (0.2–2 mg/ml of each). Bullen et al. (30) infected suckling guinea pigs orally with *E. coli* 0111 and killed some at daily intervals. The *E. coli* were colonizing both the small and large intestine, but gradually lactobacilli became the dominant flora within 3 to 5 days in the small intestine and within 3 days in the large intestine. In artificially fed piglets, the *E. coli* 0111 remained the dominant flora throughout, although lactobacilli began to establish themselves, particularly in the large intestine from 4 to 6 days. To prove that the iron-chelating proteins were responsible for the suppression of *E. coli*, a third group of suckling piglets were daily dosed orally with hematin; they were killed after 3 days only. Their intestines were predominantly colonized by *E. coli* 0111, although the large intestine already contained appreciable numbers of lactobacilli.

The suckled animals fed hematin showed an increase in *E. coli* of 4 log cycles in the small intestine and over 2 log cycles in the large intestine compared with the controls (suckled and no hema-
—obviously a striking difference. It is unfortunate that these piglets were killed after 3 days although all the other experiments lasted 6 days; even the artificially fed piglets began to be colonized by lactobacilli between 3 and 6 days (note that the lactobacilli appeared naturally).

The few *in vivo* experiments indicate that lactoferrin can contribute to the suppression of *E. coli* in the intestine. Large-scale experiments have so far been hindered because it proved impossible to develop a technological process to purify sufficient lactoferrin. Although bovine whey is in abundant supply, it contains only low concentrations of lactoferrin. An alternative source of an iron-chelating protein is egg white, which is rich in conalbumin; the antibacterial and iron-binding properties of conalbumins closely resemble those of lactoferrin (40,45,46).

At present, a number of clinical trials are being conducted with conalbumin. Infants with acute diarrhea have been fed formula and conalbumin, 600 to 1,200 mg/day. The therapeutic results appear to be promising (Table 3).

### THE LACTOPEROXIDASE SYSTEM

The bactericidal property of milk was first recognized by Hesse in 1894 (48). Raw milk destroyed "cholerabacillus" within 12 hr at room temperature and within 6 to 8 hr at 37°C. Heating milk to 100°C destroyed this property. He suggested, therefore, that raw milk was not only unlikely to be a carrier for cholera bacilli but

#### TABLE 3. Normalization of bowels of infants with acute enteritis (6 months to 1 year)*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>3 days (n=20)</th>
<th>3 to 6 days (n=20)</th>
<th>6 to 9 days (n=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conalbumin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(3.70 ± 0.36, n=20)</td>
<td>11 (55%)</td>
<td>7 (35%)</td>
<td>2 (10%)</td>
</tr>
<tr>
<td>No conalbumin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(6.15 ± 0.41, n=20)</td>
<td>1 (5%)</td>
<td>11 (55%)</td>
<td>8 (40%)</td>
</tr>
</tbody>
</table>

*U. Cornelli Ricordati (*personal communication*).  
bp < 0.01 in favor of the group treated with conalbumin.
might be used as a prophylactic or therapeutic food. Hanssen (49) attributed the bactericidal power of milk against "bacillus typhosus and paratyphosus" to the peroxidative property of milk (measured by the color reaction with para-phenylendiamine in the presence of $H_2O_2$), which was destroyed by heating at 75°C for 15 min, as was the bactericidal activity. The bactericidal effect lasted only for 4 hr and varied according to season, with summer milk when the cows were on pasture being most effective. In retrospect, these observations describe unwittingly all three components of the lactoperoxidase (LP) system, consisting of lactoperoxidase, thiocyanate, and $H_2O_2$. Thiocyanate concentrations are high in the milk of cows on natural pasture (16), and freshly drawn milk probably contains some $H_2O_2$.

Eventually, lactoperoxidase was identified (50), the need for $H_2O_2$ established (51), and the third component identified as thiocyanate (52,53; for reviews see 5–7,9,11).

**Distribution of the Components of the Lactoperoxidase System**

*Lactoperoxidase (LP, donor: $H_2O_2$ oxidoreductase, E.C. 1.11.1.7)*

Lactoperoxidase is a trivial name derived from the source—milk—from which it had been first purified (54,55). It occurs not only in milk but also in saliva, tears, cervical mucus, and eosinophils. Their biochemical reactions—formation of complexes with $H_2O_2$ and oxidation of thiocyanate ($SCN^-$), bromide, and iodide—are identical. Lactoperoxidase consists of a single polypeptide chain with a molecular weight between 77,000 and 100,000; LPs derived from different organs in the same animal are immunologically identical.

Human milk has peroxidative bactericidal activity (11,56–58) (Fig. 3) largely derived from polymorphonuclear leukocytes (59). This enzyme is composed of two subunits, has a molecular weight between 118,000 and 144,000, and is not immunologically identical with LP; it also differs biochemically, because unlike LP it can also oxidize chloride. Although the peroxidative value of human milk is
low, it must be remembered that the human infant is born with salivary LP that supplements the milk peroxidative activity. An important characteristic of LP is its resistance to low pH and digestion. The peroxidative values of both bovine and human milk are preserved up to 2 hr in gastric juice from adults and infants with pyloric stenosis (56).

**Thiocyanate**

The thiocyanate anion (SCN⁻) (60–68) is highly permeable and thus ubiquitous in animal tissue and secretions. It is partly derived
endogenously during the detoxification reaction between thiosulfate (metabolic produce of sulfur amino acids) and cyanide catalyzed by the liver and kidney enzyme rhodanase; principally, however, it is derived after ingestion of the anion, its esters, and other precursor compounds such as nitriles, isothiocyanates, and cyanide. Many plants are rich in glucosinolates (thioglycosides), which on hydrolysis by a thioglucosidase, myrosinase, yield SCN⁻ and/or isothiocyanate and nitriles. The Cruciferae are the most important source of thiocyanate for man and animals. Savoy kale contains from 85 to 500 ppm NaSCN, white cabbage about 32, cauliflower about 100, sauerkraut 33 to 200, and other vegetables from less than 1 up to 5 ppm. Some plants such as cassava, sweet potatoes, millet, sugar cane, bamboo, and lima beans are particularly rich in cyanogenic glucosides that, on hydrolysis, release considerable amounts of hydrogen cyanide, which is converted into thiocyanate. Tobacco smoke is also an indirect source of thiocyanate. The distribution of SCN⁻ in human plasma, saliva, gastric juice, urine, and milk is shown in Table 4. The anion occurs not only in biological secretions but also in synovial fluid, cerebrospinal fluid, and lymphocytes.

The concentration of SCN⁻ is influenced by the diet, at least in animals. The SCN⁻ content in bovine milk can be increased by feeding, for instance, kale (up to 13 ppm) or when the cows graze on natural pastures containing a varied flora (including clover, which contains cyanide). There are so far no data on whether the diet (or smoking) of the lactating mother influences the SCN⁻ content of her milk.

**Hydrogen Peroxide**

It is generally assumed that H₂O₂ is absent from milk. Theoretically, however, H₂O₂ can be generated by xanthine oxidase, sulfhydryl oxidase, or Cu²⁺ and ascorbic acid provided free O₂ is present.

Recently, we have shown (69) that milk leukocytes generate detectable amounts of H₂O₂ (nanomolar) in milk. Leukocytes phagocytize casein micelles (and fat globules) (70,71), a process that is known to produce an acceleration in metabolic activity generating...
H_2O_2. Since milk contains both catalase and peroxidase, the H_2O_2 can only be detected after inactivation of the enzyme by sodium azide. In vivo, lactic acid bacteria are a source of H_2O_2. These organisms rapidly colonize the intestinal tract of newborns and are found to be H_2O_2 producing in the milk-fed calf (6,72). No data are so far available for the human newborn, but it can be expected that the same conditions prevail.

The Oxidation Products of Thiocyanate

Lactoperoxidase in the presence of H_2O_2 oxidizes SCN\(^-\) to a number of short-lived intermediate oxidation products with antibacterial activity (52,53). These are now recognized to be hypo-thiocyanite (OSCN\(^-\)), hypohthiocyanous acid (HOSCN\(^-\) at low pH), and higher oxyacids such as cyanosulfurous acid (H_2OSCN) and cyanosulfuric acid (H_3SCN); of these oxidation products, only OSCN\(^-\) has been chemically synthesized. The end products of the oxidation are carbon dioxide, sulfate, and ammonia, all of which are inert, having no antibacterial effect (73). It has now been confirmed that

### Table 4. Concentration of thiocyanate (as ppm NaSCN) in human body fluids

<table>
<thead>
<tr>
<th>Fluid</th>
<th>Nonsmoker</th>
<th>Smoker</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>1.9 ± 0.74</td>
<td>6.7 ± 2.3</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>(0.96–3.55)</td>
<td>(3.4–11.6)</td>
<td></td>
</tr>
<tr>
<td>Saliva</td>
<td>37</td>
<td>155</td>
<td>62</td>
</tr>
<tr>
<td>Adults</td>
<td>73 ± 8</td>
<td>186 ± 32</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>(19–60)</td>
<td>(58–270)</td>
<td></td>
</tr>
<tr>
<td>Human infant</td>
<td>23 ± 15</td>
<td>—</td>
<td>56</td>
</tr>
<tr>
<td>Urine</td>
<td>16 ± 6</td>
<td>23 ± 11</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(4–38)</td>
<td>(5–45)</td>
<td></td>
</tr>
<tr>
<td>Gastric juice</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting</td>
<td>73 ± 8</td>
<td>65</td>
<td></td>
</tr>
<tr>
<td>Stimulated</td>
<td>24 ± 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Milk</td>
<td>&lt;6.5</td>
<td></td>
<td>56</td>
</tr>
</tbody>
</table>


*b* Values are given as averages ± standard deviations (ranges in parentheses).
the oxidation of SCN\(^-\) proceeds in stages of 1, 2, and 3 equivalents of H\(_2\)O\(_2\), OSCN\(^-\) being the first to appear (51,73–80). It has been suggested that OSCN\(^-\) exerts the bacteriostatic activity and that the higher oxyacids are bactericidal for organisms such as *E. coli* (81) (Table 5). A recent detailed review of the interaction among LP, H\(_2\)O\(_2\), and SCN\(^-\) has been published by Thomas (80).

**The Mode of Action**

Lactic acid bacteria that are catalase negative metabolize sufficient H\(_2\)O\(_2\) under aerobic conditions to be inhibited in the presence of LP and SCN\(^-\) alone (52,53,73,75,83–85). Catalase-positive organisms such as coliforms, salmonellae, and shigellae, including multiple antibiotic-resistant strains, require an exogenous source of H\(_2\)O\(_2\) (6,85–87) added as a chemical generated by an enzyme system such as glucose oxidase/glucose or in a mixed culture, where a surplus of H\(_2\)O\(_2\) is produced metabolically. Although lactic acid bacteria (gram-positive) are only temporarily inhibited, gram-negative organisms are killed. Inhibition of streptococci affects growth, lactic acid production, and respiration; enzymes of the glycolytic pathway such as hexokinase are completely and glucose-6-phosphate dehydrogenase partly inactivated by the LP system. Some strains of the same species, however, can be completely resistant because they possess a "reversal factor" that reverses the inhibition of glycolysis (Fig. 4); this factor catalyzes the oxidation of NADH\(_2\) in the

**TABLE 5. Oxidation of SCN\(^-\) by LP and H\(_2\)O\(_2\)**

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>H(_2)O(_2) + SCN(^-)</td>
<td>OSCN(^-) + H(_2)O</td>
</tr>
<tr>
<td>H(_2)O(_2) + OSCN(^-)</td>
<td>O(_2)SCN(^-) + H(_2)O</td>
</tr>
<tr>
<td>H(_2)O(_2) + O(_2)SCN(^-)</td>
<td>O(_3)SCN(^-) + H(_2)O</td>
</tr>
</tbody>
</table>

End oxidation products: SO\(_4\)\(^2-\), NH\(_4\)\(^+\), CO\(_2\)

*Compiled from refs. 60, 73, 75, 76, 81, 82.

**Bacteriostatic:** OSCN\(^-\) + R—SH → R—S—SCN + OH\(^-\).

**Bactericidal.**
FIG. 4. Inhibition of the glycolysis of *Strep. cremoris* 972 by LP and KSCN and its reversal by an extract of *Strep. cremoris* 803. KSCN (5 μmoles) (A) and LP (70 units) (B) were added to a suspension of strain 972 in 0.1 M glucose, 10 mM potassium phosphate buffer, pH 6.8, and extract of strain 803 (1.0 ml) (C) was added later: (○) glycolysis; (●) concentration of residual KSCN corrected for volume changes. (From ref. 73.)

presence of an intermediate oxidation product of SCN⁻ [now assumed to be OSCN⁻ but then found to be mimicked by the chemically synthesized sulfurdicyanide S(CN)₂ (73)].

Both gram-positive and gram-negative organisms immediately leak potassium and amino acids, which indicates damage to the inner membrane. With gram-negative organisms such as coliforms or pseudomonads, this damage leads to death and eventually lysis (6,7,11). Why the leakage of gram-positive organisms does not lead to death is not clear. The lesser damage to the membrane indicated by lower leakage may be repairable; or the different composition and physical structure of the cell wall may act as a barrier (88). The damage to the inner membrane causes inhibition both of the uptake of nutrients such as glucose, amino acids, etc. and of the synthesis of protein, DNA, and RNA (1,84,89,90).
There are two aspects of the LP system that concern the attachment to brush borders and milk fat globules. It is generally agreed that certain potential pathogens attach themselves to the epithelial surface of the intestine. The intimate attachment favors proliferation of the organisms and reduces their removal within the lumen by peristalsis. Subsequently, enterotoxins are produced, which cause diarrhea. Specific slgA prevents attachment and thus protects the infant.

When *E. coli* possesses K₈₈ or K₈₉ antigens, they attach to porcine or calf intestine, respectively, *in vivo*. Selwood et al. (91) developed an *in vitro* test whereby *E. coli* was found to attach specifically to the host brush border. When these organisms were treated with the LP system, they failed to attach to the brush border in the same number as untreated organisms. The LP system therefore had a similar effect to slgA on attachment to the brush border (7,11). So far this phenomenon has not been confirmed *in vivo*.

Since fat globules are surrounded by a true cell membrane, we considered whether they have the same specific receptors as the brush border. Indeed, it was found that the enteropathogenic bovine strain of *E. coli* possessing K₈₉ antigen only attached to bovine fat globule, and the porcine enteropathogenic strains possessing K₈₈ antigen only to porcine fat globule. Human strains possessing either colonization factor CF1 or CF2 attached to human fat globule but also slightly to porcine fat. The bovine and porcine strains failed to attach to the same degree after treatment with the LP system. The human strains, however, remained unaffected (11). In this context, it is of interest that a high percentage of slgA is attached to the fat globule membrane, incidentally proving that the level of slgA in colostrum and milk may frequently have been underestimated (92). It remains to be seen whether the attachment of slgA to fat globules has any significance for the protection of the newborn during the ingestion of the milk. Since it was suggested that the effect of the slgA is to bind the organisms closer to the bacterial surface, it would therefore be interesting to test the LP system and slgA for attachment.
Biological Significance of the LP System

According to the literature, human milk contains varying and small concentrations of peroxidase activity. If we accept that the peroxidative values represent cell-bound myeloperoxidase, we need to know whether the enzyme is released or not before and after ingestion. The contribution of the salivary LP during suckling appears to be important whatever the milk peroxidative values turn out to be. However, until we have more information available, we can only extrapolate from the results obtained with the calf. The advantages of using the calf as an experimental animal are that (a) it can be separated from the dam without ill effect; (b) as long as it is fed milk only, without any access to roughage, it remains a monogastric animal; (c) feeding host-specific milk avoids digestive and intestinal complications that arise by feeding, for instance, bovine milk to piglets or guinea pigs or infants; (d) it is relatively easy to cannulate the calf throughout the intestinal tract, and it grows slowly, thus allowing continuous experimentation.

We obtained some evidence that the LP system operates in vivo in the calf without an exogenous source of $\text{H}_2\text{O}_2$ (or SCN$^-$) (93–95) (Fig. 5). Calves were infected orally with $E. \text{coli}$ as above and fed heated milk in which the LP had been inactivated ($70^\circ$, 15 min). Nearly all the inoculum could be recovered from the abomasum when sampled at 30 min and 1 hr. Feeding raw milk reduced the number of $E. \text{coli}$ by $\sim99\%$, indicating that the LP system was operating without the addition of an exogenous source of $\text{H}_2\text{O}_2$. The addition of a reducing agent restored the number of organisms to approximately the original inoculum. Complementing the raw milk with a source of $\text{H}_2\text{O}_2$, either glucose oxidase/glucose or magnesium peroxide, further reduced the number of $E. \text{coli}$; we assumed, therefore, that the calves were colonized by $\text{H}_2\text{O}_2$-producing organisms that activated the LP system. Indeed, it was found that $>50\%$ of the lactobacilli colonizing the intestinal tract produced $\text{H}_2\text{O}_2$.

Practical Application of the LP System

When calves are not reared on their dams but under prevailing commercial conditions, they are liable to scouring. Because of
overcrowding and artificial feeding (powdered skim milk plus substitute fats), they are subject to bacterial (and viral) infections. We undertook large-scale trials involving some 300 animals to assess whether the LP system could ameliorate scouring caused by gram-negative potential pathogens and improve their performance measured according to their live weight gains. These trials lasted for 4 years (96).

In Sweden, calves were purchased from different farms 5 days after birth. It is known that the incidence of scouring is high under these circumstances. The calves were fed raw milk only and raw milk supplemented with glucose oxidase/glucose and SCN⁻ (at the time we had insufficient data about the likely concentration of SCN⁻ in the gastric juice). Although the days of scouring were only originally reduced from 3 days in the control animals to 2.3 days...
in the animals fed with the LP system, their live weight gain was 63% depressed during the first 3 weeks of the trial. In the following weeks, the difference in live weight gain diminished but was still 23% depressed over the whole 7 weeks of the experiment, after which the calves were weaned. In addition, the "LP calves" had a better appearance (sleek coat), were more lively, and consumed more solid supplement food.

The next three trials were performed at the National Institute for Research in Dairying; the calves were left for 2 days on the dam and then transferred to the calfhouse. Under these circumstances, the incidence of scouring was much less than in the purchased calves. The advantage of feeding the LP system was statistically significant for the first 5 weeks as measured by the live weight gain. Again, the number of days the animals scoured was reduced. The relationship between the percentage of animals scouring and the live weight gain is evident in Fig. 6. The more animals scoured, the higher the gains were to be expected by feeding the LP system.

The LP system has also been shown to be effective for the preservation of cooled and uncooled milk (94,97–99). Psycho-

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**FIG. 6.** Relationships between the effect of the LPS (%) on live weight change and the level of scouring (%) in calves given whole milk only. (From ref. 96.)
trophic organisms multiplying at 4° to 10°, such as pseudomonads (gram negative), are inhibited for 3 days or more, thus preventing the spoilage of milk by their lipolytic and proteolytic activity. The shelf life of uncooled milk was increased by 6 to 12 hr in developing countries under ambient temperatures up to 37°C. Although the control milk (untreated) became quickly sour, the LP system suppressed the lactic acid formation sufficiently to preserve the milk (94).

CONCLUSIONS AND PROSPECTS

It is now generally accepted that colostrum and milk bridge the immunological gap in the newborn, thus protecting it against colonization of the intestinal tract by pathogens until the newborn builds up its own defense systems. The antibacterial activities of the nonantibody protective proteins and their mode of action have been well shown in vitro; there is, however, scanty documentation of their in vivo effects.

Since the milk of each species contains the nonantibody proteins in different concentrations and proportions, the effect of a single factor is difficult or impossible to determine when the newborn is allowed to suckle the dam. Ideally, we need to separate the newborn from the dam as early as possible, milk the dam, and, before feeding, treat the milk so that it contains only one protective protein or none for the control animals. This excludes the employment of conventional small laboratory animals: these are either too immature at birth, or it proves impossible to milk the dam. Alternatively, the newborn can be fed artificially with a formula feed supplemented with one of the protective proteins. Unfortunately, artificial feeding introduces another variant because the feeding of non-host-specific milk creates nutritional and digestive problems that can interfere with the interpretation of the experimental data.

The cannulated calf has proved to be well suited for the study of the lactoperoxidase system. However, a major difficulty in the use of calves or experimental animals is their high cost, elaborate housing, and attendance required. The miniature piglet may be better
suited. The litters are large (average 6), and the piglets can be housed in cages and fed automatically. The milk could be obtained from conventional sows. Cannulation would be more difficult, but the large litter makes the piglets expendable.

The protective role of the LP system appears to be reasonably well established now in the calf, but its possible role in the human infant can only be surmised. Human milk does contain peroxidative activity, at least early in the lactation, but we need to know whether salivary LP supplements the lack of milk enzyme. Thiocyanate secretion occurs as early as the secretion of HCl, but how much do salivary LP and thiocyanate contribute to the gastric juice? The lactic acid bacteria colonizing mouth, esophagus, stomach, and upper duodenum generate H₂O₂. For this purpose, we need to obtain not only samples of saliva under different conditions but also intestinal samples.

Although the protective proteins may be of secondary importance for infants cared for under strict hygienic conditions, they may help to protect the low-birth-weight and premature baby. Research on the proteins may also lead to supplementation of baby feeds whenever breast feeding is not possible and help the development of a cheap antibacterial weaning feed. Such a feed would be helpful in developing countries, where intestinal infections are held down during breast feeding but can become endemic after weaning.

REFERENCES

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52. Reiter B, Pickering A, Oram JD, Pope GS. J Gen Microbiol 1963;33:XII.