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Nonspecific Bactericidal Activity of the Lactoperoxidase-Thiocyanate-Hydrogen Peroxide System of Milk Against *Escherichia coli* and Some Gram-Negative Pathogens

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Two strains of *Escherichia coli* and one strain each of *Salmonella typhimurium* and *Pseudomonas aeruginosa* were killed by the bactericidal activity of the lactoperoxidase-thiocyanate-hydrogen peroxide system in milk and in a synthetic medium. H_2O_2 was supplied exogenously by glucose oxidase, and glucose was produced at a level which was itself noninhibitory. Two phases were distinguished: the first phase was dependent on the oxidation of SCN^- by lactoperoxidase and H_2O_2, which was reversed by reducing agent, and the second phase was dependent on the presence of accumulated H_2O_2, which was reversed by catalase. The latter enzyme could also reverse the first phase, but only when present in excessive and unphysiological levels. The bactericidal activity was greatest at pH 5 and below, and it depended on the SCN^- concentration and on the number of organisms. Since raw or heated milk neutralizes the acid barrier against infection in the stomach, the bactericidal system discussed may contribute to the prevention of enteric infections in neonates.

It is now well recognized that bovine milk has bactericidal and bacteriostatic properties. Some are the same as in blood, and some are peculiar to the mammary or other secretory glands. These antibacterial systems include complement and specific antibodies (22), lactoferrin (2, 14, 19, 21, 23), lysozyme, polymorphonucleated leucocytes (28-30), and lactoperoxidase thiocyanate-hydrogen peroxide (LP-SCN^--H_2O_2) (24, 26, 27) (for a review see [24]). This latter system was shown to kill strains of group A streptococci and temporarily inhibit strains of groups B, D, and N streptococci (15, 26), and it constitutes the milk inhibitor previously observed and referred to in the literature as "lactenin" (9, 10, 11, 20, 35-37).

The end products of the LP-catalyzed oxidation of thiocyanate have no inhibitory effect on streptococci; inhibition appears to be due to an intermediate oxidation product (6, 7, 17, 18, 26).

The inhibitory effect of this system occurring in milk, saliva, and other biological secretions (24, 25, 27) was amply confirmed by the independent investigations of Klebanoff and his coworkers using *Lactobacillus acidophilus, Streptococcus faecalis, Staphylococcus albus, Escherichia coli,* and *Bacillus megatherium* as test organisms (12, 13). Hitherto the LP-SCN^--H_2O_2 system was assayed by different methods: lactic acid production or inhibition of individual enzymes of the glycolytic pathway, pH values, absorbance, oxygen uptake, reduced nicotinamide adenine dinucleotide oxidation, and uptake of labeled amino acids. By all these criteria, the system was considered to be inhibitory.

In this paper the effect of the system on gram-negative organisms is reconsidered. It will be shown that the system is bactericidal for two strains of *E. coli*, one serum sensitive and one serum resistant, and for one strain each of *Salmonella typhimurium* and *Pseudomonas aeruginosa*.

MATERIALS AND METHODS

Bacteria. *E. coli* strains 0111 K_{58}:(B4):H-,NCTC 9703 and 0101:K?H?, *P. aeruginosa* strain 30/70, and *S. typhimurium* strain C143/6, phage type 95, were used in this study. Stock cultures were maintained on nutrient agar slopes and subcultured when required at 37 C overnight in nutrient broth.

Enzymes and chemicals. LP (42 purpurogallin units/mg) was purchased from Sigma Chemical Co. (St. Louis, Mo.). Glucose oxidase (GO) (grade 1) and catalase (39,000 U/mg) were from Boehringer (Mannheim, West Germany). All chemicals were analytical reagent grade, and all solutions were prepared in glass-distilled water.

Buffers and growth media. Assays were determined in acetate buffer, and the pH optimum for LP

was carried out in citrate phosphate buffer.

Milk was obtained aseptically by cannulation from bacteria-free udders; whey was prepared by renneting raw milk and was sterilized by passing through a membrane filter (0.45 μm; Millipore Corp.).

The ammonium salts synthetic medium was prepared as follows: (solution A) KH_2PO_4, 15.1 g; $(NH_4)_2SO_4$, 2.2 g; $FeSO_4 \cdot 7H_2O$, 1 ml of 0.006-g/ml solution; water to 1 liter; (solution B) lactose, 2 g; $MgSO_4$, 0.2 g; water to 100 ml. Solution A was autoclaved at 15 lb for 15 min and solution B at 10 lb for 10 min. Before use, 10 ml of solution B was mixed with 90 ml of solution A.

Assays. (i) LP assay. To 3 ml of buffer containing the enzyme, 50 μg of dianisidine and 50 μg of H_2O_2 were added. The change in extinction at 520 nm was monitored on a Pye Unicam SP800. One unit of activity was defined as the amount of LP required to give an increase in $E_{520}^{1\,cm}$ of 1.0 in 5 min.

Catalase assay. The production of oxygen from H_2O_2 was measured using an oxygen electrode type E5646 (Radiometer A/S, 72, Emdrupvej, Copenhagen, Denmark).

SCN^- determination. SCN^- was measured spectrophotometrically as $Fe(SCN)_3$ (3).

H_2O_2 determination. H_2O_2 was measured enzymatically with horseradish peroxidase and dianisidine. A 0.1-ml portion of sample was added to 3 ml of solution containing 100 μg of horseradish peroxidase and 30 μg of dianisidine. The extinction at 520 nm was read after 10 min.

Bactericidal activity. Bactericidal activity was measured by determining the viable count over suitable intervals of time as described in detail previously (22).

RESULTS

In preliminary experiments it was established that the milk of individual cows contained 0.75 to 3 U of LP per ml and between 0.015 and 0.15 mM SCN^-. Previously it had been shown (17) that LP catalyzed the inhibition of *Streptococcus cremoris* and that the best way of ensuring an adequate level of H_2O_2 was to add a H_2O_2-generating system such as glucose and GO (12, 17). We found that milk containing less than 0.015 mM SCN^- was noninhibitory for *E. coli* 0111 in the presence of 0.1 U of GO per ml and 0.3% glucose. When the level of SCN^- was raised to 0.225 mM (1) the milk became strongly bactericidal for 6 h and remained bacteriostatic for up to 24 h. These results were reproducible with the milk of different cows. In whey, the bactericidal period continued for the 24-h period tested (Fig. 1).

Since milk contains catalase as well as LP, it was thought that the catalase which is reported to be associated with milk microsomes, casein or whey lactoglobulin (30), eventually terminates the bactericidal activity by decomposing the H_2O_2, whereas the continued bactericidal activity of the whey might result from a reduced level of catalase in it. However, when the various milk samples and their wheys were tested, it was found that the whey samples contained as much catalase as the milk, so that this enzyme could not be responsible for the difference in bactericidal activity.

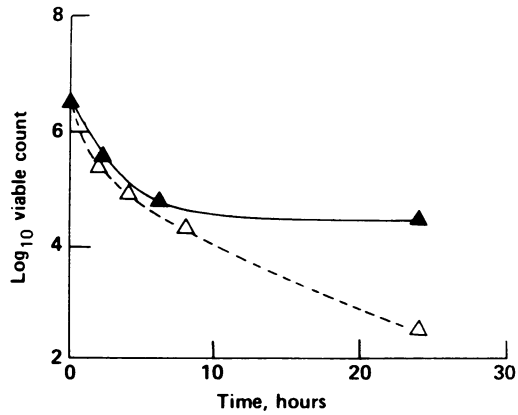

FIG. 1. *Bactericidal activity against E. coli 0111 in milk (▲) and whey (△) containing 0.225 mM SCN^-, 0.10 μg of GO per ml, and 0.3% glucose levels.*

Since milk is a complex medium it was decided to continue the investigation of the LP system in a synthetic medium (see above).

Level of H_2O_2 generated by GO in the absence and presence of E. coli. It was first necessary to establish that the concentration of H_2O_2 produced by GO and glucose was nonbactericidal in the synthetic medium; 0.1 U of GO per ml generated H_2O_2 at an appreciable rate (in the absence of bacteria) at pH 7.0, and the medium contained almost 1 mM H_2O_2 at 6 h (Fig. 2A); at this level H_2O_2 was known to be bactericidal for *E. coli*. In contrast, in the presence of 10^6 colony-forming units (CFU) of *E. coli* per ml less than 0.1 mM H_2O_2 could be detected throughout the same period. Since *E. coli* is known to produce catalase extracellularly, it was likely that the enzyme would decompose all or nearly all of the H_2O_2 generated by the GO. Consequently it was to be expected that the organisms would multiply at the same rate as the control (in the absence of GO) (Fig. 2A). When GO was increased to 0.5 μg/ml the bacterial catalase appeared to be unable to prevent the buildup of free H_2O_2, which caused considerable bactericidal activity (Fig. 2B).

Bactericidal activity of the complete LP-SCN^--H_2O_2 system against E. coli 0111. To study the whole system, LP (1.5 U/ml) and SCN^- (0.225 mM) were added to the synthetic medium containing 0.1 U of GO per ml. The numbers of organisms and the concentrations

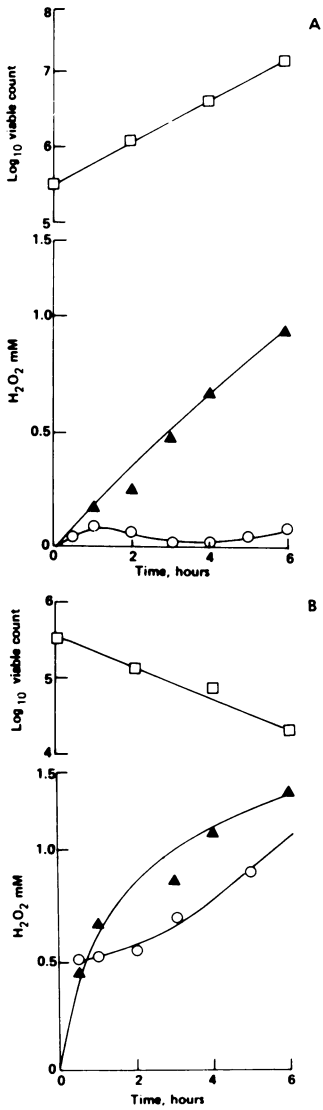

FIG. 2. *H_2O_2 levels produced by GO in a synthetic medium in the absence of E. coli and its effect on the organisms. (A) H_2O_2 produced by 0.1 μg of GO per ml without E. coli (▲) and with E. coli (10^7 CFU/ml) (△); growth of E. coli (□) (control). (B) H_2O_2 produced by 0.5 μg of GO per ml without E. coli (▲) and with E. coli (10^7 CFU/ml) (○); bactericidal effect (□).*

of SCN^- and H_2O_2 were determined throughout the incubation period of 24 h (Fig. 3). The SCN^- was rapidly oxidized in the first 6 h, and the numbers of organisms were reduced from the initial inoculum of about 10^6 CFU/ml to less than 10^4 CFU/ml. The continued bactericidal activity of the system can be attributed to the increased concentration of H_2O_2. A similar experiment was carried out in whey with almost identical results.

It appeared, therefore, that there were two phases: the first bactericidal phase depended on the oxidation of SCN^- by LP and H_2O_2, and the second phase depended solely on the accumulation of H_2O_2. This concept was confirmed when reducing agents (sodium dithionite, cysteine, or reduced nicotinamide adenine dinucleotide) or catalase were added to the synthetic medium containing the complete LP system. Figure 4 shows that the reducing agent partly or wholly reversed the bactericidal activity, and that 75 U of catalase per ml reversed the second bactericidal phase only, without affecting the first phase, but 150 U/ml partly reversed the first bactericidal phase. When the catalase concentration was further increased to 300 U/ml, the bactericidal activity due to the oxidation of SCN^- by LP was completely abolished (data not shown); heated catalase had no effect.

From the foregoing it seemed likely that the initial bactericidal phase would depend on the level of SCN^- originally present in the medium. Figure 5 shows that 0.015 mM SCN^- was sufficient to initiate the bactericidal phase in synthetic medium when ~10^6 CFU of *E. coli* were used as the inoculum. The bactericidal phase lasted for only 4 h, after which the surviving organisms started to multiply. Increasing the SCN^- concentration to 0.15 mM reduced the number of organisms about one-half log cycle more at 4 h (compared with the lower SCN^- concentration), and the bactericidal phase continued afterwards. The surviving organisms at the lower level of SCN^- were presumably capable of synthesizing sufficient catalase to prevent the accumulation of a bactericidal concentration of H_2O_2; hence the commencement of growth after 4 h. When the inoculum was increased about 10-fold (Fig. 5), to

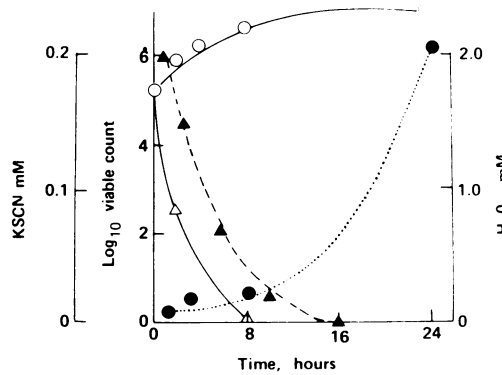

FIG. 3. *Bactericidal effect against E. coli 0111 of LP (1.5 μl/ml), SCN^- (0.225 mM), and GO (0.1 μl/ml) in a synthetic medium. Control, growth in medium only (○); bactericidal system decrease in viable count (△); oxidation of SCN (▲); H_2O_2 level (●).*

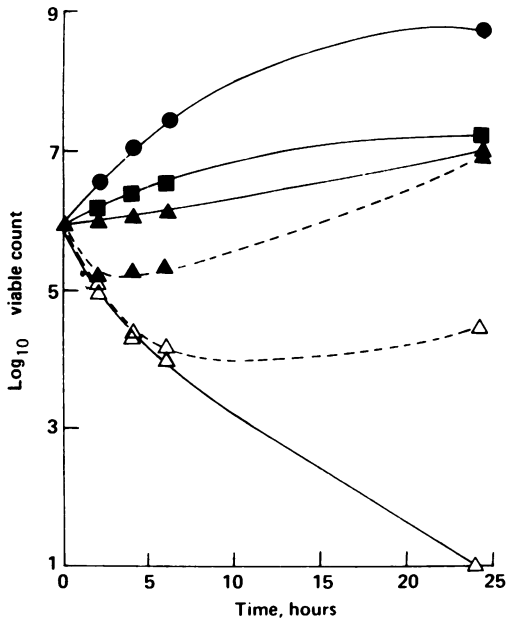

FIG. 4. *Reversal of bactericidal effect of LP system (conditions as in the legend to Fig. 3) against E. coli 0111 by various reducing agents and catalase. Control, no reducing agents (△——△); 75 U of catalase added per ml (△- - -△); 150 U of catalase added per ml (▲- - -▲); 1 mM reduced nicotinamide adenine dinucleotide added (▲——▲); 10 mM cysteine added (■); 1 mM $Na_2S_2O_4$ added (●).*

$>10^7$ CFU/ml, 0.015 mM SCN^- was insufficient to effect any bactericidal activity, and after an initial bacteriostatic phase of 6 h growth began at 8 h (5×10^7 CFU/ml; data not shown). Increasing the SCN^- concentration to 0.225 mM killed the higher inoculum at the same rate as the lower inoculum, indicating a direct relationship between the bactericidal effect and the concentration of the oxidizable substrate (SCN^-).

Effect of pH on the bactericidal activity of the LP system. The bactericidal activity of the LP system was also highly dependent on the initial pH of the medium. At pH 7.5 there was very little change in count over 8 h, but at lower pH values the bactericidal activity rose sharply (Fig. 6). Since the LP enzymatic activity as assayed by the dianisine method was found to be optimal at pH 5.0 (Fig. 7), it was not surprising that the killing of the organisms occurred very rapidly at lower pH values. However, at pH 3.0 the enzyme activity was found to be greatly diminished (Fig. 7), although the killing was even more rapid at that pH than at pH 5.0 (Fig. 6). This appeared to be due to the ability of *E. coli* to multiply at pH 5 but not at pH 3 (see growth without LP system at pH 5 and pH 3 in Fig. 6). The decreased enzyme activity at pH values above 5.0 could be compensated by higher enzyme concentrations which increased the bactericidal activity. It is therefore clear that the bactericidal activity is determined by the concentration of the enzyme, enzyme activity, SCN^- concentration, numbers of organisms (inoculum), and their rate of multiplication.

Bactericidal activity of the LP system against E. coli 0101, S. typhimurium, and P. aeruginosa. So far the LP system has been shown to be bactericidal for a serum-susceptible strain of *E. coli* (0111); its effect on a serum-resistant strain of *E. coli* (0101) was next tested.

Figure 8 shows good bactericidal activity in the presence of 0.15 mM SCN^- and increased activity when the SCN^- concentration was doubled. *P. aeruginosa* was less susceptible to the LP system, but increased bactericidal activity was also observed when the SCN^- was doubled. *S. typhimurium* was also shown to be affected by the system.

DISCUSSION

The LP-SCN⁻-H_2O_2 system which was known to be inhibitory to *E. coli* (13) has now

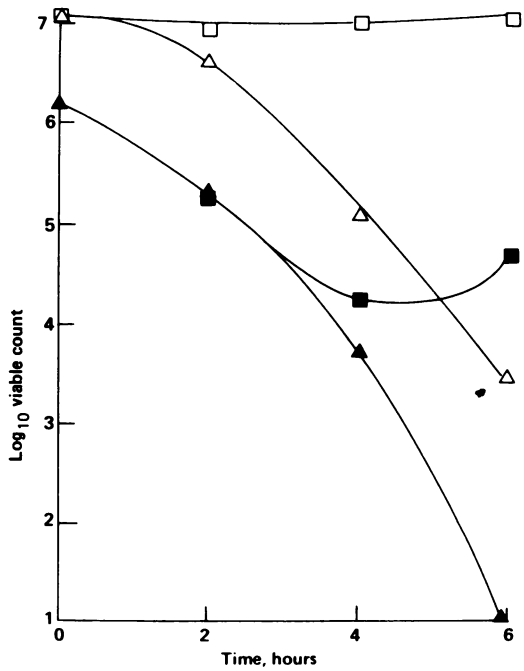

FIG. 5. *Effect of SCN^- concentration and level of inoculum of E. coli 0111 on bactericidal activity. Inoculum ~10^6 CFU/ml with (a) 0.015 mM SCN^- (■) and (b) 0.15 mM SCN^- (▲). Inoculum of ~10^7 CFU/ml with (a) 0.015 mM SCN^- (□) and (b) 0.225 mM SCN^- (△).*

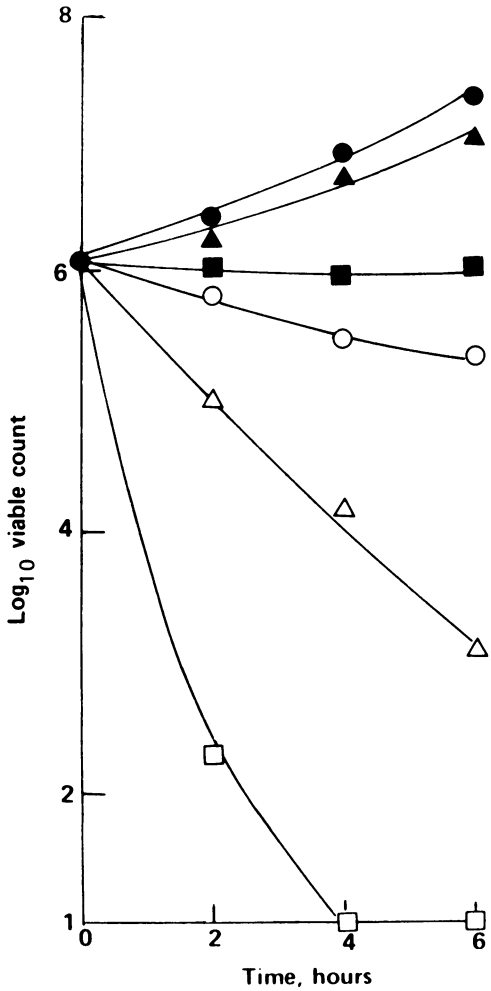

FIG. 6. *Effect of pH on the bactericidal LP system. Controls: growth without the LP system at pH 7.5 (●); at pH 5.0 (▲); at pH 3.0 (■). Bactericidal activity in the presence of the LP system: viable counts at pH 7.5 (○); at pH 5 (△); GO at 0.05 U/ml was used in experiments at pH >5.0, but only 0.02 U of GO per ml was used at pH <5.0 to prevent the buildup of bactericidal levels of H_2O_2, since GO has an optimum pH of 5.0.*

been shown to be bactericidal for *E. coli* and other gram-negative pathogens. The bactericidal effect depends on the level of SCN^-, or rather the intermediary oxidation product that depends on the proportion of SCN^- to the enzymically generated H_2O_2, and is inversely related to the number of organisms. In the present of H_2O_2, generated by GO and glucose, two distinct bactericidal phases appear to be present in whey and in the synthetic medium: the first phase depends on the oxidation of SCN^- and is reversed by reducing agents, whereas the second phase depends on the accumulation of H_2O_2 and is reversed by catalase. Only levels of catalase greater than 150 U/ml reversed the first phase. These observations confirm the finding of Jago and Morrison (8) that only high concentrations of catalase could reverse the inhibition of lactic acid production of lactic streptococci by the LP-H_2O_2 system (the role of SCN^- was unknown at that time). In milk only the first bactericidal phase occurred; after the oxidation of SCN^- the number of organisms remained at the same level, but in whey the second bactericidal phase occurred, as in the synthetic medium. At first it was thought that whey lacked catalase, but direct measurement of O_2 production after addition of H_2O_2 to whey showed that it contained as much catalase as milk. It is therefore not understood why the bactericidal effect differs in milk and whey.

The bactericidal effect was also found to be strongly affected by the pH of the medium. Since the optimal pH for LP activity and the one at which the organisms were able to multiply was found to be 5.0, it was not surprising that rapid killing of the organisms occurred at that pH, apart from the even more rapid killing at pH 3.0, at which the organisms are unable to multiply. Indeed, at pH 7.5 only bacteriostasis was observed unless the enzyme concentration was increased. However, the mechanism be-

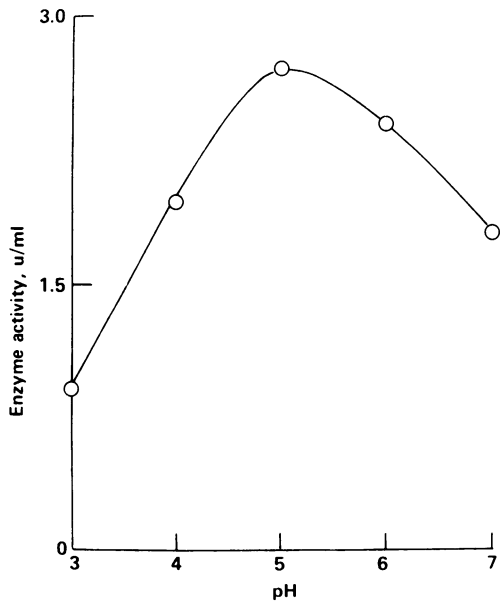

FIG. 7. *Effect of pH on the enzyme activity of LP (for conditions see Materials and Methods) expressed in dianisidine units.*

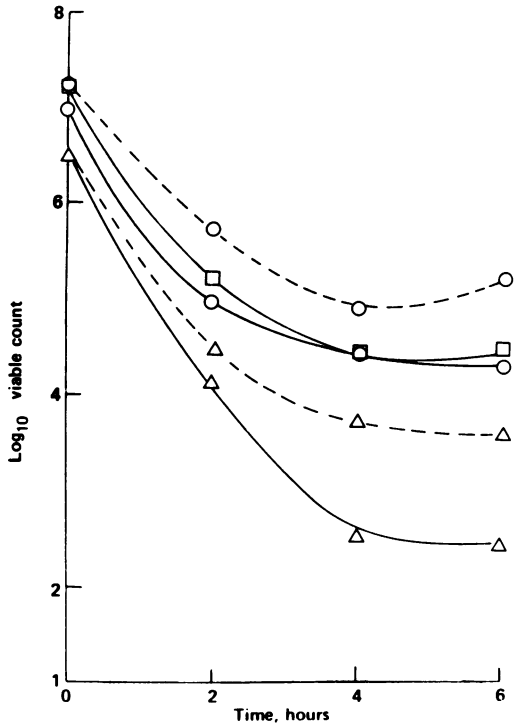

FIG. 8. *Bactericidal effect of the LP system against E. coli 0101 (serum resistant) at a concentration of 0.15 mM SCN⁻ (△---△) and 0.30 mM SCN⁻ (△——△); against P. aeruginosa at 0.15 mM SCN⁻ (○---○); and against S. typhimurium at 0.15 mM SCN⁻ (□).*

hind the pH effect may not depend only on increased or decreased enzyme activity.

It has been previously shown by Hogg and Jago (6, 7) that the enhanced inhibitory effect of lactic acid production of streptococci by the LP system at low pH is independent of whether the antibacterial reaction product (intermediate) was formed at pH 7 and the solution then acidified to pH 4.5 or whether the initial formation took place at the lower pH value. The same workers also demonstrated that the antibacterial substance exists in an acid-base equilibrium with a pK value of 5.1. Recently the use of immobilized GO has enabled us to study the effect of the inhibitory reaction product separate from the enzymatic reaction, and preliminary results confirm that the enhancement of antibacterial effect at a low pH is at least not entirely dependent on the effect of pH on the enzyme system (manuscript in preparation).

In the absence of LP the organisms themselves prevented or reduced the accumulation of H_2O_2 generated by 0.10 μg of GO per ml, presumably because of their catalase production. As a result, little H_2O_2 accumulated and the organisms subsequently multiplied. At 0.5 U of GO per ml the rate of H_2O_2 production was too high to be decomposed microbially, and the concentration became bactericidal. At the lower level of GO there was apparently enough H_2O_2 generated to oxidize added SCN^- in the presence of LP. This may be explained by the fact that LP has a much higher affinity than catalase for H_2O_2 (in the presence of SCN^-?). Substantial concentrations of catalase had to be added to compete significantly with the peroxidase-catalyzed reaction(s).

The system only operates in the presence of all three components. The LP level in individual milk samples varies (0.75 to 3 dianisidine U/ml), and is sufficient for bactericidal activity. The level of SCN^- is far more variable, depending on feeding, and 0.015 mM is insufficient to promote bactericidal activity when more than 10^7 organisms are used as inoculum. SCN^- is derived from the detoxification of cyanide (which occurs, for instance, in clover) by the rhodanase-catalyzed reaction with thiosulphate formed in the liver and kidney or from glucosides present in brassica and raphani (34). The third component, peroxide, accumulates with catalase-negative organisms only under aerobic conditions (8, 20, 36), but with catalase-positive organisms an exogenous supply of peroxide is needed.

In our in vitro system we used glucose oxidase, but in vivo a possible source of H_2O_2 is the "natural" intestinal flora of lactic acid bacteria that colonize the stomach and intestines and are capable of producing H_2O_2; indeed we have evidence that molecular O_2 is present in the abomasum of the calf.

In this context it is of some interest to stress that the serum-susceptible strain was found to be more susceptible to the bactericidal activity than the serum-resistant strain of *E. coli*, which was killed only when the SCN^- concentration was increased. These levels of SCN^- (0.3 mM) are still physiological for milk, since a very recent survey quotes levels of SCN^- of this magnitude (5).

SCN^- can be replaced in theory by iodide, which is known to inhibit streptococci (27) and other bacteria (12, 13), but the concentration of iodide required is far above the levels found in milk or other secretions. Nevertheless, it is of great interest that the bactericidal activity of the myeloperoxidase-iodide-H_2O_2 system depends on the cell wall lipopolysaccharides, like the complement-mediated bactericidal antibody activity of serum, which determines whether an organism is serum susceptible or serum resistant (33). We are therefore now in-

vestigating the susceptibility to the LP-SCN^- system of uridine diphosphate-galactose-4-epimeraseless mutants of *E. coli* and *S. typhimurium* in the absence and presence of galactose, and it appears that the lipopolysaccharides of the envelope influence the susceptibility of the organisms to the system. Besides strain 9703 of the human serotype 0111, we have now tested three more strains of this serotype and three each of serotypes 055 and 0128. Every one of these strains was killed by the LP system. Similar results were obtained with strains of sixteen porcine and five bovine serotypes (manuscript in preparation).

We are now investigating whether this system remains active in vivo. It is generally accepted that the gastric acid secretion is an important, or even the most important, "bactericidal barrier" against enteric infections (reviewed by Giannella et al. [4]). Although abundant experimental and indirect evidence exists that enteric infection is promoted in achlorhydric patients, or by ingestion of bicarbonate prior to infusion with salmonellae, shigellae, or cholerae, the neutralization of the gastric hydrochloric acid by ingested milk seems to have been neglected. In preliminary experiments in a fistulated calf we found that ingestion of milk raised the pH in the abomasum from before feeding to ~5.0 after feeding, and the bactericidal activity was immediately abolished. However, when the milk contained the complete LP system, bactericidal activity was restored 1 to 2 h after feeding. Also, when piglets, weaned 2 days after birth, were fed an artificial diet containing the LP system, the number of coliform organisms was appreciably reduced in the stomach and duodenum (manuscript in preparation). Heated milk would, of course, destroy the bactericidal barrier, only because of its buffering capacity, and thus increase the susceptibility to enteric infections in the same way as ingestion of bicarbonate. This may indicate one of the most important differences between feeding of heat-treated milk and suckling of neonates.

It is interesting to note that the analogy between the inhibitory activity of saliva and milk was suggested at the time when SCN^- was demonstrated to be common to both secretions and to represent the third component of the LP-H_2O_2 system (26, 27). In this context the more recent findings of Morrison and Steele (16) are illuminating, because they found that LP was absent from the saliva of the bovine fetus until birth and rose only to 10 to 20% of the adult level in the first postnatal days; adult levels were reached in 6 months. Since the highest concentration of LP in postcolostral milk is reached 1 to 4 days after parturition, after which it declines slowly to a constant level at 14 days, it is likely that the lack of LP in the saliva of the newborn calf is offset by the high level of LP in the milk.

In conclusion, it is attractive to postulate a role for the LP system in the defense against enteric infection in neonates in addition to those specific antibodies, complement (22), and lactoferrin-antibody (2, 23), to provide a further antibacterial factor until the neonate can synthesize its own defense mechanisms.

ACKNOWLEDGMENTS

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ADDENDUM IN PROOF

Since this paper was submitted, a related paper has appeared, as follows: L. Björck, C.-G. Rosén, V. Marshall, and B. Reiter, 1975, Antibacterial activity of the lactoperoxidase system in milk against pseudomonads and other gram-negative bacteria. Appl. Microbiol. 30:199-204.

LITERATURE CITED

1. **Boulangé, M.** 1959. Fluctuation saisonniere du taux thiocyanates dans le lait frais de Vache. C. R. Soc. Biol. 12:2019-2020.
2. **Bullen, J. J., H. J. Rogers, and L. Leigh.** 1972. Iron binding proteins in milk and resistance to *Escherichia coli* infection in infants. Br. Med. J. 1:69-75.
3. **Cosby, E. L., and J. B. Summer.** 1945. Rhodanese. Arch. Biochem. 7:457-460.
4. **Giannella, R. A., S. A. Broitman, and N. Zamcheck.** 1973. Influence of gastric acidity on bacterial and parasitic enteric infektions. Ann. Intern. Med. 78:271-276.
5. **Heeschen, W., H. Wernery, A. Tolle, and H. Korhonen.** 1974. Zur spezifischen und unspezifischen Infektions abwehr der bovinen Milchdrüse. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe A 227:236-248.
6. **Hogg, D. Mc., and G. R. Jago.** 1970. The antibacterial action of lactoperoxidase. The nature of the bacterial inhibitor. Biochem. J. 117:779-790.
7. **Hogg, D. Mc., and G. R. Jago.** 1970. The oxidation of reduced nicotinamide nucleotides by hydrogen peroxide in the presence of lactoperoxidase and thiocyanate, iodide or bromide. Biochem. J. 117:791-797.
8. **Jago, G. R., and M. Morrison.** 1962. Anti-streptococcal activity of lactoperoxidase. Proc. Soc. Exp. Biol. Med. N.Y. 111:585-588.
9. **Jones, F. S.** 1928. The properties of the bactericidal substance in milk. J. Exp. Med. 47:877-888.
10. **Jones, F. S., and R. B. Little.** 1927. The bactericidal properties of cow's milk. J. Exp. Med. 45:319-335.
11. **Jones, F. S., and H. S. Simms.** 1929-30. The bacterial inhibitor (lactenin) of milk. J. Exp. Med. 51:327-339.
12. **Klebanoff, S. J.** 1967. Iodination of bacteria. A bactericidal mechanism. J. Exp. Med. 126:1063-1078.
13. **Klebanoff, S. J., W. H. Clem, and R. P. Luebke.** 1966.

The peroxidase-thiocyanate-hydrogen peroxide antimicrobial system. Biochim. Biophys. Acta 117:63-72.
14. **Masson, P. L., and J. F. Heremans.** 1967. Protides. Biol. Fluids Proc. Colloq. 14:115.
15. **Mickelson, M. N.** 1966. Effect of lactoperoxidase and thiocyanate on the growth of *Streptococcus pyogenes* and *Streptococcus agalactiae* in a chemically defined culture medium. J. Gen. Microbiol. 43:31-43.
16. **Morrison, M., and W. F. Steele.** 1968. Lactoperoxidase, the peroxidase in the salivary gland, p. 89-110. *In* P. H. Person (ed.) Biology of the mouth, vol. 89.
17. **Oram, J. D., and B. Reiter.** 1966. The inhibition of streptococci by lactoperoxidase, thiocyanate and hydrogen peroxide. The effect of the inhibitory system on susceptible and resistant strains of group streptococci. Biochem. J. 100:373-381.
18. **Oram, J. D., and B. Reiter.** 1966. The oxidation of thiocyanate and the nature of the inhibitory compound. Biochem. J. 100:382-388.
19. **Oram, J. D., and B. Reiter.** 1968. Inhibition of bacteria by lactoferrin and other iron-chelating agents. Biochim. Biophys. Acta 170:351-365.
20. **Portmann, A., and J. E. Auclair.** 1959. Relation entre la lacténine L_2 et la lactoperoxidase. Lait 39:147-158.
21. **Reiter, B.** 1968. Grundlegende Probleme der Mastitisforschung. Ostmaerk Milchwirtsch. Ztg. 18 & 19:325-329, 346-351.
22. **Reiter, B., and J. H. Brock.** 1975. Inhibition of *Escherichia coli* by bovine colostrum and postcolostral milk. I. Complement-mediated bactericidal activity of antibodies to a serum susceptible strain of *E. coli* of the serotype 0111. Immunology 28:71-82.
23. **Reiter, B., J. H. Brock, and E. D. Steel.** 1975. Inhibition of *Escherichia coli* by bovine colostrum and postcolostral milk. II. The bacteriostatic effect of lactoferrin on a serum susceptible and serum resistant strain of *E. coli*. Immunology 28:83-95.
24. **Reiter, B., and A. Møller-Madsen.** 1963. Cheese and butter starter: a review. J. Dairy Res. 30:419-456.
25. **Reiter, B., and J. D. Oram.** 1967. Bacterial inhibitors in milk and other biological fluids. Nature (London) 216:328-330.
26. **Reiter, B., A. Pickering, and J. D. Oram.** 1964. An inhibitory system—lactoperoxidase, thiocyanate, peroxide—in raw milk, p. 297-305. *In* N. Molin (ed.), 4th international symposium on food microbiology. Almqvist & Wiksell, Stockholm.
27. **Reiter, B., A. Pickering, J. D. Oram, and G. S. Pope.** 1963. Peroxidase-thiocyanate inhibition of streptococci in raw milk. J. Gen. Microbiol. 33:xii.
28. **Russell, M. W., B. E. Brooker, and B. Reiter.** 1976. Inhibition of the bactericidal activity of bovine polymorphonuclear leucocytes and related systems by casein. Res. Vet. Sci., vol. 20, in press.
29. **Russell, M. W., and B. Reiter.** 1972. Factors affecting the phagocytosis of *Staphylococcus aureus* by bovine polymorphonuclear leucocytes in milk. J. Gen. Microbiol. 71:vi.
30. **Russell, M. W., and B. Reiter.** 1975. Phagocytic deficiency of bovine milk leucocytes: an effect of casein. J. Reticuloendothel. Soc. 18:395.
31. **Shahani, K. M., W. J. Harper, R. G. Jensen, R. M. Parry, and C. A. Zittle.** 1972. Enzyme in bovine milk. A review. J. Dairy Sci. 56:531-543.
32. **Steel, W. F., and M. Morrison.** 1969. The antistreptococcal activity of lactoperoxidase. J. Bacteriol. 97:635-639.
33. **Tagesson, C., and O. Stendahl.** 1973. Influence of the cell surface lipopolysaccharide structure of *Salmonella typhimurium* on resistance to intracellular bactericidal systems. Acta Pathol. Microbiol. Scand. Sect. B 81:473-480.
34. **Virtanen, A. I.** 1961. Über die Chemie der Brassica-Faktoren ihre Wirkung auf die Funktion der Schilddrüse und ihr Übergehen in die Milch. Experientia 17:241-251.
35. **Wilson, A. T., and H. Rosenblum.** 1952. The antistreptococcal property of milk. I. Some characteristics of the activity of lactenin *in vitro*. The effect of lactenin on hemolytic streptococci of the several serological groups. J. Exp. Med. 95:25-38.
36. **Wilson, A. T., and H. Rosenblum.** 1952. The antistreptococcal property of milk. II. The effect of anaerobiosis, reducing agents, thiamine, and other chemicals on lactenin action. J. Exp. Med. 95:39-50.
37. **Wright, R. C., and J. Tramer.** 1958. Factors influencing the activity of cheese starters. The role of milk peroxidase. J. Dairy Res. 25:104-118.