Anti-Streptococcal Activity of Lactoperoxidase III.‡ (27862)

G. R. JAGO† AND MARTIN MORRISON* (Introduced by E. Roberts)
Department of Biochemistry, School of Medicine and Dentistry, University of Rochester, Rochester, N. Y.

It has been known for some time that cow's milk can inhibit the growth of bacteria(1). This inhibitor in the milk has the properties of a protein, being labile to heat, acid and alkali, and non-dialyzable.

Wright and Tramer(2) correlated the peroxidase activity of milk and its inhibitory activity. They found that when milk was treated with sodium azide or hydrogen peroxide, or when it was subjected to heat or to changes of hydrogen ion concentration, peroxidase activity was lost. They further noted that the loss was approximately to the same degree as the loss of the inhibitory activity of milk. On the basis of these results, they suggested that lactoperoxidase had antibacterial properties. This theory was confirmed when purified preparations of the enzyme were shown by Portmann and Auclair(3) to have the inhibitory activity.

Wilson and Rosenblum(4) had previously considered the possibility that the inhibitor present in milk was a peroxidase. They discounted this theory, however, when they found that cyanide, which inhibits peroxidase activity, did not inactivate the inhibitory properties of milk, and that catalase, which would destroy the peroxide necessary for peroxidase activity, also failed to inactivate these inhibitory properties.

Thus, although it would appear that lactoperoxidase does possess antibacterial properties, this activity does not seem to be due to its ability to act as a peroxidase. The present study concerns the mechanism by which lactoperoxidase acts to inhibit *Streptococcal* growth.

Methods. *Streptococcus cremoris,* strain 972, kindly supplied by Drs. Wright and Tramer, was used throughout this investigation. The organism was sub-cultured at monthly intervals in litmus milk and stored at 5°C. Inocula from a freshly coagulated litmus milk culture were placed in tubes and incubated at 30°C until growth was apparent. The culture was then diluted to give the number of cells required for use.

The material to be tested was serially diluted in sterile skim milk which had been autoclaved at 115°C for 10 minutes and adjusted to pH 7.0. Each tube contained 1 ml of the milk containing 0.004% brom-cresol-purple indicator and the test organism. The tubes were incubated at 30°C for approximately 16 hours.

In some experiments, the growth medium for the organism consisted of a whey ultrafiltrate supplemented with amino acids. The amino acid supplement consisted of the following: alanine, arginine, aspartic acid, asparagine, glutamine, glutamic acid, glycine, histidine, hydroxyproline, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophane, tyrosine, and valine. The amino acids were each present in a concentration of 0.5 mg/ml. One ml of this supplement was added to every 3 ml of the whey filtrate.

Various measures of bacterial growth were used, including a change in acid concentration (5), nucleic acid content and optical density at 500 mμ.

Lactoperoxidase was prepared as previously described(6). Horse-radish peroxidase and beef liver catalase were obtained from Worthington, Inc. The concentration of lactoperoxidase was based on the extinction coefficient at 412 mμ(6), while the concentration of catalase and horse-radish peroxidase was determined by measurements at 405 and 403 mμ respectively(7). Peroxidase activity was determined as previously described(6).

* Supported by Senior Research Fellowship of Public Health Service. Present address: Dept. of Biochemistry, City of Hope Med. Research Inst., Duarte, Calif.

† Present address: Dept. of Biochemistry, Univ. of Melbourne, Australia.

‡ This research was supported in part by U. S. Public Health grant.

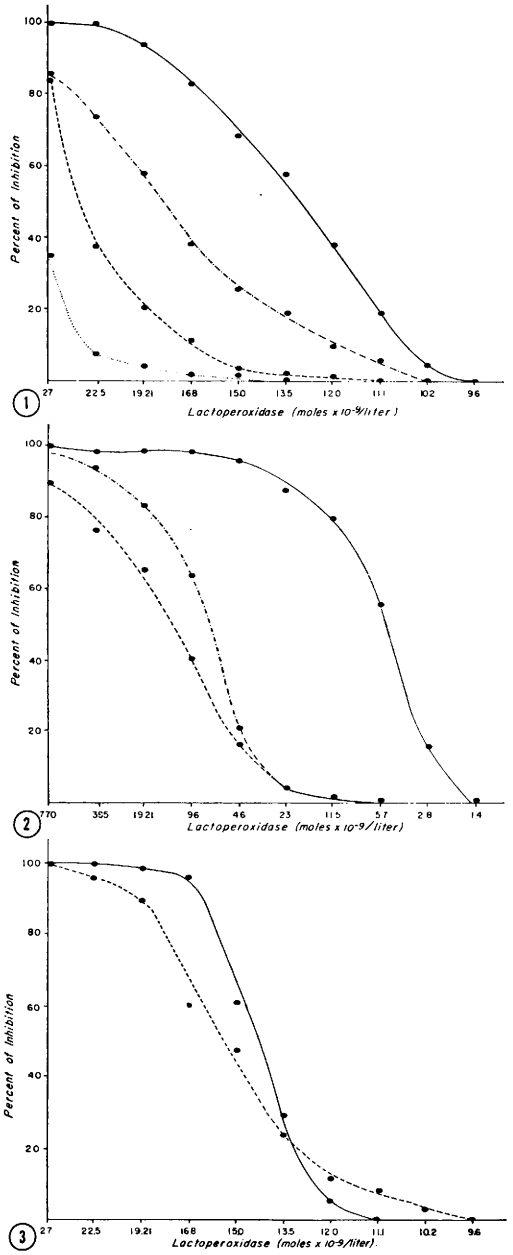

FIG. 1. Effect of lactoperoxidase on growth when number of viable cells in the inoculum was varied. —— inoculum contained 1.5 × 10^2 viable cells/ml. –•– inoculum contained 50 × 10^3 viable cells/ml. ––– inoculum contained 50 × 10^5 viable cells/ml. ••• inoculum contained 50 × 10^6 viable cells/ml. The acid produced was determined as indicated in text. Per cent inhibition equals 100 × $\frac{\text{acid produced in control} - \text{acid produced in sample}}{\text{acid produced in control}}$

FIG. 2. Effect of catalase and horse-radish peroxidase on inhibition of lactoperoxidase. —— lactoperoxidase alone. –•– lactoperoxidase plus 16 × 10^{-8} moles/l of horse-radish peroxidase. ––– lactoperoxidase plus 6 × 10^{-8} moles/l of beef liver catalase. An inoculum containing 50 × 10^3 viable cells of *Str. cremoris*, strain 972, was employed. Tubes were incubated for 16 hr at 30°. Per cent inhibition was determined as indicated in Fig. 1.

FIG. 3. Effect of cyanide on inhibition by lactoperoxidase. —— with 4 × 10^4 moles/l of cyanide in medium. ––– without cyanide in medium. An inoculum containing 50 × 10 viable cells of *Str. cremoris*, strain 972 was employed. Tubes were incubated for 16 hr at 30°. Per cent inhibition was determined as indicated in Fig. 1.

Results. Experiments were designed to establish whether lactoperoxidase functioned as (a) a, chemical which inhibits growth, (b) a catalyst to produce an inhibitor or (c) a catalyst which acts directly on the bacteria to inhibit its growth.

Fig. 1 illustrates that an increase in concentration of lactoperoxidase in the growth medium results in decreased growth of the organism. The degree of inhibition during the 16-hour incubation period was influenced by the number of viable cells in the inoculum used. When the number of cells in the inoculum was increased by a factor of 3.3 × 10^5, however, only a 2-fold increase in enzyme concentration was required to achieve a similar degree of inhibition. This suggests that lactoperoxidase does not function as a chemical to inhibit growth.

Enzymatic peroxidation would then appear to be one of the obvious mechanisms by which lactoperoxidase could function as a catalyst to inhibit the growth of microorganisms. Such a mechanism would require the presence of peroxide in the medium from a source independent of the lactoperoxidase. The formation of hydrogen peroxide by strain 972 could be demonstrated when a whey filtrate, supplemented by addition of the mixture of 20 amino acids, was vigorously aerated and a large inoculum used. After incubating for 5 hours at 30°C with constant shaking in air, the cell-free medium was shown to contain peroxide by the oxidation of guiacol catalyzed by horse-radish peroxidase.

Any procedure which interfered with the production of hydrogen peroxide could be expected to eliminate the inhibition due to lactoperoxidase if the enzyme inhibited the growth by virtue of its peroxidase activity.

TABLE I. Effect of Lactoperoxidase Under Anaerobic Conditions.

Concentration of lactoperoxidase (Moles × 10^{-9}/liter)	O.D. at 260 mμ Aerobic (Air)	Anaerobic ($N_2 + CO_2$)
—	2.598	2.802
27	.204	2.790

Test organism *Str. cremoris*, strain 972, Inoculum: 10.5 × 10^3 cells/ml.
Incubation period 18 hr at 30°C.
The medium was inoculated with 10.5 × 10^3 cells/ml of *Str. cremoris*, strain 972, and incubated for 18 hr at 30°C.

Therefore, the effect of lactoperoxidase on the growth of strain 972 under anaerobic conditions was studied. Sterile skim milk was inoculated with strain 972 and divided into 2 parts. Lactoperoxidase was added to one part only. The tubes, with and without lactoperoxidase, were incubated in air and in an atmosphere of 95% N_2 and 5% CO_2. The nucleic acid content of each tube was determined after the cultures were incubated for 18 hours. The results (Table I) show that the enzyme does not inhibit the growth of strain 972 in the absence of oxygen, but does inhibit growth in the presence of oxygen.

An alternate method was used to demonstrate the importance of peroxide in inhibition by lactoperoxidase. This method employed catalase, which catalytically destroys peroxide. As shown in Fig. 2, catalase or horse-radish peroxidase will reverse the inhibition caused by lactoperoxidase. Catalase and horse-radish peroxidase compete with lactoperoxidase, as demonstrated by the fact that it requires increased concentrations of these enzymes to reverse the inhibition due to the increased concentration of lactoperoxidase.

From the foregoing, it is apparent hydrogen peroxide formation is an important aspect of the inhibition by lactoperoxidase. Therefore, if the inhibition is simply a function of the enzymatic activity of the peroxidase, anything which interferes with this activity should decrease the effectiveness of the enzyme in inhibiting growth. Addition of sodium cyanide to the medium, at a concentration of 20 μg per ml, was just insufficient to inhibit bacterial growth in the absence of lactoperoxidase. At this concentration of sodium cyanide, the lactoperoxidase catalyzed oxidation of guiacol was inhibited. However, the ability of lactoperoxidase to inhibit the growth of strain 972 was unimpaired (Fig. 3).

The medium was examined to determine whether the peroxidase produced an inhibitor to growth. In these experiments, a whey filtrate supplemented with amino acids was passed through a Seitz filter. The medium was then inoculated with strain 972 and divided into two parts. Lactoperoxidase was added to one portion and both samples were incubated for 2 hours at 30°C. The samples were then passed through cellophane tubing under pressure which removed both the enzyme and bacteria. Any inhibitor, other than a protein, which had been produced by the peroxidase, would be in this medium. The medium thus obtained was then sterilized by passage through a Seitz filter and both samples were reinoculated and incubated for 22 hours. Table II shows that the organism grew equally well in both samples of the medium.

In other experiments, the enzyme was added to the medium after a large inoculum had been incubated with continual shaking in air for 5 hours to insure the formation of peroxide. The enzyme and cells were removed as previously described. A filtrate of the medium was then diluted with an equal volume of fresh medium to restore the required nutrients for growth of the organism. Again it

TABLE II. Effect of Lactoperoxidase on Growth Medium.

Concentration of lactoperoxidase (Moles × 10^{-7}/liter)	Inoculum (viable cells/ml)	Log I_o/I at 500 mμ
Before removal of enzyme		
None	22.5 × 10^5	.570
4.2	22.5 × 10^5	.040
After removal of enzyme		
None	52 × 10^4	.600
	12.5 × 10^4	.510
	2.5 × 10^4	.355
4.2	21 × 10^4	.590
	4.5 × 10^4	.570
	9 × 10^3	.530

The *Str. cremoris*, strain 972, was incubated for 22 hr at 30° as described in text.

was found that the organism grew equally well in both samples of the medium.

Discussion. The present study is in agreement with the previous work of Portmann and Auclair(3) and shows that a highly purified preparation of lactoperoxidase can inhibit growth of *Streptococcus cremoris,* strain 972.

The results indicate that *Streptococcus cremoris,* strain 972, can produce peroxide when grown under aerobic conditions, and that it is only under those conditions where peroxide is formed that lactoperoxidase functions as an inhibitor of growth. Under anaerobic conditions, lactoperoxidase does not function as an inhibitor of growth for this organism.

Catalase, which destroys peroxide, can also reverse the inhibitory activity of lactoperoxidase, and would appear to do so by competing with lactoperoxidase for the available peroxide. However, in light of its high turnover number, the amount of catalase required to reverse the inhibitory action of lactoperoxidase was surprising.

There are two possible reasons for the failure of Wilson and Rosenblum(4) to show that catalase reversed the effect of the inhibitor in raw milk. Since raw milk is now known to contain a number of inhibitory substances (5) they may have been concerned with something other than lactoperoxidase. More probably it was due to the large excess of catalase they indicated was employed in their study. In the present investigation, it was observed that catalase will itself inhibit growth when added in excess.

The results suggest that lactoperoxidase functions to inhibit growth under conditions where peroxide is produced and not destroyed. Under these conditions, an oxidized product of a substrate present in the medium, or elaborated into the medium, could possibly be the direct inhibitor of growth. Our efforts to detect such an inhibitor, were, however, negative.

Cyanide does not appear to reverse the inhibition caused by lactoperoxidase even at sufficient concentrations to decrease the oxidation of guiacol by the enzyme. It is difficult to evaluate the role of cyanide, since in higher concentrations it will directly inhibit the organism.

It is interesting to note that the inhibiting properties of lactoperoxidase are not general to other peroxidases. The activity of lactoperoxidase would appear to be specific, for horse-radish peroxidase does not inhibit bacterial growth, and will, in fact, actually reverse the inhibition caused by lactoperoxidase. This could readily be explained on the basis of substrate specificity. The role of lactoperoxidase in inhibition of growth is probably to react with a specific hydrogen donor group on the bacterial membrane. The oxidation of the membrane results in the inhibition of growth which has been observed. Horse-radish peroxidase, in contrast, may either react with this group on the membrane very slowly or not at all, while reacting with other hydrogen donors more readily than does lactoperoxidase. Thus, horse-radish peroxidase would reduce the available hydrogen peroxide and reverse the inhibition due to lactoperoxidase.

Summary. The growth of *Str. cremoris,* strain 972, is inhibited by a purified preparation of the enzyme lactoperoxidase under aerobic conditions. Lactoperoxidase does not inhibit the organism anaerobically. The inhibitory activity of lactoperoxidase can be reversed by catalase or horse-radish peroxidase. The inhibitory activity is not the result of oxidation of low molecular weight substances. The mechanism of the inhibition of growth by lactoperoxidase is discussed.

1. Jones, F. S., Simms, H. S., *J. Exp. Med.,* 1930, v51, 327.

2. Wright, A. T., Tramer, J., *J. Dairy Res.,* 1958, v25, 104.

3. Portmann, A., Auclair, J. E., *Le Lait,* 1959, v39, 147.

4. Wilson, A. T., Rosenblum, H., *J. Exp. Med.,* 1952, v95, 39.

5. Auclair, J. E., Hirsch, A., *J. Dairy Res.,* 1953, v20, 45.

6. Morrison, M., Hamilton, H. B., Stotz, E., *J. Biol. Chem.,* 1957, v228, 767.

7. Maehly, A. C., Chance, B., in *Methods of Biochemical Analysis,* v1, ed. David Glick, Interscience Publishers, Inc., New York, 1954.

Received August 6, 1962. P.S.E.B.M., 1962, v111.