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Inhibitory Effect of Saliva on Glutamic Acid Accumulation by *Lactobacillus acidophilus* and the Role of the Lactoperoxidase-Thiocyanate System

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ABSTRACT

CLEM, W. H. (University of Washington, Seattle), AND S. J. KLEBANOFF. Inhibitory effect of saliva on glutamic acid accumulation by *Lactobacillus acidophilus* and the role of the lactoperoxidase-thiocyanate system. J. Bacteriol. **91**:1848–1853. 1966.—Saliva contains an antimicrobial system which inhibits the growth of *Lactobacillus acidophilus*, as well as a number of other organisms, in complete growth medium. This antimicrobial system consists of the salivary peroxidase (lactoperoxidase) and thiocyanate ions, and requires the presence of H_2O_2. Saliva inhibits the accumulation of glutamic acid and certain other amino acids by resting cells. This effect of saliva is decreased by dialysis, and thiocyanate ions restore the inhibitory effect of dialyzed saliva. The inhibitory effect of saliva is decreased by heat (100 C, 10 min), and lactoperoxidase restores the inhibitory effect of heated saliva. Thus, the inhibition of glutamic acid accumulation by saliva appears to be due in part to the lactoperoxidase-thiocyanate antimicrobial system. H_2O_2 increases the inhibitory effect of both saliva and the lactoperoxidase-thiocyanate system on glutamic acid accumulation. The inhibition of glutamic acid accumulation is not preceded by a loss in microbial viability. The glutamic acid accumulated by *L. acidophilus* under the conditions employed remains largely (over 90%) as free glutamic acid. This suggests that saliva and the lactoperoxidase-thiocyanate-H_2O_2 system inhibit the net transport of glutamic acid into the cell.

The maintenance of the delicate balance between the resident microorganisms and the host is essential for health. The mechanisms by which the body controls the microbial flora are many. Among the antimicrobial systems in saliva is one, consisting of thiocyanate ions (2, 16) and the salivary peroxidase (8), which inhibits the growth of *Lactobacillus acidophilus* as well as certain other organisms. Catalase decreases the inhibition of the growth of *L. acidophilus* by saliva (8), which suggests that H_2O_2 also is required. H_2O_2 nay be formed by the organisms or may be added. The addition of H_2O_2 or an H_2O_2 generating system greatly increases the spectrum of organisms inhibited by the peroxidase-thiocyanate antibacterial system (*7a*).

A comparable antibacterial system is present in milk. It requires the milk peroxidase, lactoperoxidase (6, 11, 13, 14), and thiocyanate ions (12). Catalase prevents the inhibition of the growth of *Streptococcus cremoris* strain 972 by milk products (6), again suggesting H_2O_2 involvement. The peroxidase-thiocyanate antimicrobial system appears also to be present in achlorhydric gastric contents (Klebanoff, *unpublished data*).

The mechanism of microbial growth inhibition by the lactoperoxidase-thiocyanate system is unknown. The present paper deals with the effect of saliva and the lactoperoxidase-thiocyanate-hydrogen peroxide system on glutamic acid accumulation by *L. acidophilus*. An inhibition of amino acid accumulation is produced under conditions in which there is little or no loss in viable-cell count. A preliminary report has appeared elsewhere (1).

MATERIALS AND METHODS

L. acidophilus ATCC 4357 was maintained on Lactobacillus-Selection Broth (LBS; BBL). An 18-hr culture in LBS was centrifuged at 3,000 × *g* for 10 min, washed twice with sterile distilled water, and diluted with water to an absorbancy at 540 mμ of 0.340 to 0.360, as measured in a Beckman B spectrophotometer. The suspension had a viable count of 2×10^8 bacteria per milliliter. Viable counts were performed by use of the pour-plate method with Lactobacillus-Selection media.

Whole paraffin-stimulated saliva was collected from 25 human subjects, pooled, centrifuged at 5,000 × *g* for 20 min, and the supernatant fraction was stored at −20 C. Prior to use, the saliva was sterilized by exposure to ultraviolet (UV) light (General Electric G8T5 lamp at a distance of 12.7 cm for 7.5 min.). No growth of organisms was observed on innoculation of Trypticase Soy Broth (BBL) with saliva treated with UV light.

Lactoperoxidase was prepared in a purified form from cow's milk by the method of Morrison and Hultquist (10). A preparation eluted from a column of CG 50 resin type 2 (and dialyzed) had a 412 to 280 mμ ratio of 0.85. Peroxidase activity of saliva and lactoperoxidase was determined by use of the orthodianisidine method (7).

Measurement of amino acid accumulation. To an incubation mixture containing bacteria suspended in gelatin-citrate-phosphate buffer (GCP), *p*H 6.5, was added C^{14}-labeled amino acid and the additional components as indicated in the text and legends to the figures and tables. The radioactive amino acids (uniformly labeled L-glutamic acid-C^{14}, 168 mc/mmole; uniformly labeled L-phenylalanine-C^{14}, 165 mc/mmole; uniformly labeled L-valine-C^{14}, 100 mc/mmole; uniformly labeled L-lysine-C^{14}, 201 mc/mmole) were obtained from Schwarz BioResearch Inc., Orangeburg, N.Y. GCP was prepared by combining 0.1 M citric acid and 0.2 M dibasic sodium phosphate to a final *p*H of 6.5 and adding gelatin (0.1%). The incubation mixture was incubated at 37 C in 25-ml Erlenmeyer flasks with shaking for the periods indicated. At the appropriate time, a 0.5-ml sample was diluted to 5.0 ml with water and filtered through a membrane filter, with an average pore size of 0.45 μ (B-6 Bac T-Flex, 24-mm diameter; Schleicher & Scheull Co., Keene, N.H.), in an E-8B Precipitation Apparatus (Tracerlabs, Inc., Waltham, Mass.). After filtration, the membrane filter was washed with 10 ml of water, fastened to a 1-inch (2.54-cm) aluminum planchet with Elmer's Glue, dried with an infrared lamp, and counted in a Sharpe Low Beta II gas-flow counter. The results were corrected for background and for counts retained by the filter in the absence of bacteria. The latter was decreased to less than 10 counts/min in most experiments by prefiltering the C^{14}-labeled amino acid prior to use.

The trichloroacetic acid-precipitable material was prepared as follows: 0.5 ml of the incubation mixture was added to 0.5 ml of 10% trichloroacetic acid, and the mixture was allowed to stand for 15 min at room temperature. The precipitate was filtered through a membrane filter as described above and washed with 10 ml of 5% trichloroacetic acid. The samples were counted as described above.

In selected experiments, an alternate counting method was used on duplicate samples of the reaction mixtures. The membrane filter was placed in a liquid scintillation counting vial. Hyamine (0.5 ml) was added, and the filter was allowed to dissolve. A 20-ml amount of a counting mixture, containing 4 parts fluor [5 g of 2,5-diphenyloxazole and 0.3 g of 1,4-*bis*[2-(5-phenyloxazolyl)]-benzene per liter of toluene] and 1 part ethyl alcohol, was added, and the samples were counted in a Packard Tri-carb liquid scintillation counter. The results were comparable with both counting methods.

Chromatographic analysis. Extracts of *L. acidophilus* (or samples of reaction mixtures) were prepared and analyzed by paper chromatography as follows: the cells were collected by centrifugation at 4,000 × *g* for 10 min, washed twice with water, and homogenized in an all-glass Potter-Elvehjem homogenizer. The homogenate was heated for 10 min at 100 C, and the cellular debris was removed by centrifugation. The supernatant solution, approximately 5 ml, was evaporated to dryness in a rotary evaporator at 50 C (Buchler Corp., New York, N.Y.), and the residue was resuspended in 0.4 ml of water for application on Whatman no. 1 filter paper. Two-dimensional ascending paper chromatography was performed with butanol-acetic acid-water (120:30:50) and ethyl alcohol-ammonia-water (180:10:10) as the first and second solvent systems, respectively. Autoradiograms were prepared with Kodak No Screen Medical X-Ray film. The paper chromatograms which contained L-glutamic acid as a standard were stained with ninhydrin, and the spots were compared with those observed in the autoradiogram. In addition, one-dimensional descending paper chromatography was performed with butanol-acetic acid-water (120:30:50) as the solvent system, and the strips were scanned with a Packard model 7200 radiochromatogram scanner.

RESULTS

Accumulation of glutamic acid. Figure 1 demonstrates the accumulation of glutamic acid-C^{14} by *L. acidophilus*. Under the conditions employed, the uptake of radioactivity at 37 C proceeded for approximately 90 min, with no further uptake after this period. Approximately 10% of the amino acid present in the reaction mixture was accumulated by the organisms. The accumulation was temperature-dependent, with no uptake at 4 C, and required the presence of glucose. Incubation of the organisms for 4 hr in GCP containing glucose did not alter the viable-cell count.

Effect of saliva on total accumulation. The addition of saliva to *L. acidophilus* in GCP produced an inhibition of glutamic acid accumulation by the organisms (Table 1). An effect of saliva was observed at a final concentration of 2%, and approximately 65% inhibition was produced by 5% saliva. The addition of hydrogen peroxide to

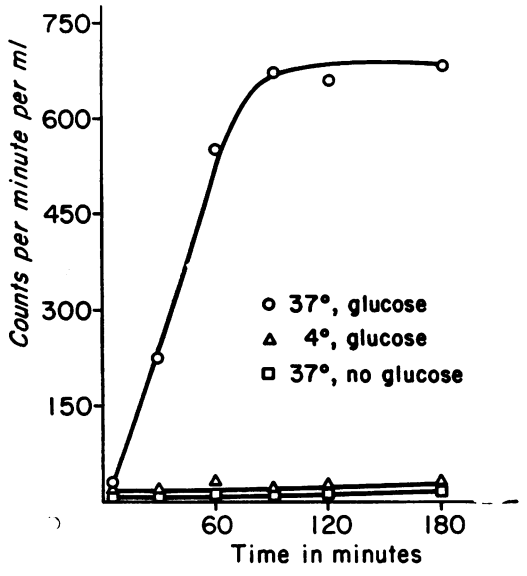

FIG. 1. *Accumulation of L-glutamic acid by Lactobacillus acidophilus. The reaction mixtures contained gelatin-citrate-phosphate buffer (GCP), 2 ml; L-glutamic acid-C^{14}, 3 × 10^{-7} M (0.05 μc/ml); L. acidophilus, 8 × 10^{7} organisms; glucose, 5 × 10^{-3} M where indicated; and water to a final volume of 4.0 ml.*

saliva considerably increased the inhibition of glutamic acid accumulation (Table 1). Hydrogen peroxide alone, at the concentration employed, was without effect. Dialysis of saliva against water overnight decreased the inhibition of glutamic acid accumulation (Table 1), although some inhibition by dialyzed saliva was consistently observed. The addition of thiocyanate to dialyzed saliva, in an amount equal to the amount present in the original whole saliva, increased the inhibition of glutamic acid accumulation. The further addition of hydrogen peroxide to the dialyzed saliva-thiocyanate system produced complete inhibition of glutamic acid accumulation. Thiocyanate and hydrogen peroxide, added separately or in combination, were without effect in the absence of dialyzed saliva.

Saliva heated to 100 C for 10 min was less effective than unheated saliva as an inhibitor of glutamic acid accumulation (Table 1). However, some inhibition was consistently observed with heated saliva. Lactoperoxidase in an amount equivalent in peroxidase activity to that present in the saliva could restore the inhibitory effect of heated saliva to that observed with unheated saliva. Lactoperoxidase when combined with thiocyanate and hydrogen peroxide produced a complete inhibition of accumulation. Lactoperoxidase alone or combined with either hydrogen peroxide or thiocyanate did not reduce glutamic acid accumulation appreciably.

TABLE 1. *Effect of saliva, hydrogen peroxide, thiocyanate, and lactoperoxidase on the accumulation of L-glutamic acid**

Supplement	Glutamic acid accumulation (counts per min per ml)	Viable-cell count × 10^{7}/ml)
None	784	1.8
Saliva (2%)	421	1.6
Saliva (5%)	270	1.4
Saliva (10%)	262	1.5
Saliva (25%)	202	1.6
Saliva + hydrogen peroxide	6	1.6
Hydrogen peroxide	750	1.5
Dialyzed saliva	524	3.1
Dialyzed saliva + thiocyanate	86	1.3
Dialyzed saliva + thiocyanate + hydrogen peroxide	8	1.6
Dialyzed saliva + hydrogen peroxide	554	1.4
Thiocyanate + hydrogen peroxide	750	1.9
Thiocyanate	740	1.6
Saliva, heated	558	2.1
Saliva, heated, + lactoperoxidase	188	1.7
Saliva, heated, + lactoperoxidase + hydrogen peroxide	20	1.6
Lactoperoxidase + hydrogen peroxide + thiocyanate	5	2.5
Lactoperoxidase + hydrogen peroxide	850	1.4
Lactoperoxidase + thiocyanate	610	1.3
Lactoperoxidase	660	2.4

* The reaction mixture contained 1.0 ml of GCP; glucose, 5 × 10^{-3} M; L-glutamic acid-C^{14}, 3 × 10^{-7} M (0.05 μc/ml); *Lactobacillus acidophilus*, 4 × 10^{7} organisms; water to a final volume of 2.0 ml; and the following supplements: saliva, 5% (v/v) unless otherwise indicated; hydrogen peroxide, 5 × 10^{-5} M; sodium thiocyanate, 5 × 10^{-5} M; lactoperoxidase, 20 *o*-dianisidine units. Viable-cell counts were performed at the end of the 2-hr incubation period.

Table 1 also demonstrates the effect on the viable-cell count of the incubation of *L. acidophilus* with saliva and saliva components for 2 hr. In no instance could the decrease in glutamic acid accumulation be accounted for by a drop in the viable-cell count.

The inhibitory effect of saliva on amino acid accumulation was not limited to glutamic acid. Table 2 demonstrates the effect of 5% saliva on the accumulation of lysine, valine, and phenyl-

TABLE 2. *Effect of saliva on accumulation of amino acids other than glutamic acid**

Supplement	Amino acid accumulation (counts per min per ml)
Lysine	5,040
Lysine + saliva	970
Valine	83
Valine + saliva	10
Phenylalanine	122
Phenylalanine + saliva	81

* The reaction mixture contained 1.0 ml of GCP; glucose, 5×10^{-3} M; *Lactobacillus acidophilus*, 4×10^{7} organisms; water to a final volume of 2.0 ml; and the following supplements: saliva, 5% (v/v); L-lysine-C^{14}, 6.2×10^{-7} M (0.125 μc/ml); L-valine-C^{14}, 1.25×10^{-6} M (0.125 μc/ml); L-phenylalanine-C^{14}, 7.5×10^{-7} M (0.125 μc/ml). Incubation time was 2 hr.

alanine by *L. acidophilus*. Approximately 10% of the labeled lysine was accumulated by the organisms under the conditions employed, and this was markedly decreased by saliva. Less than 0.5% of the labeled valine or phenylalanine was taken up by the organisms under comparable conditions, and a decreased accumulation of these amino acids was consistently observed in the presence of saliva.

Effect of saliva on trichloroacetic acid-precipitable radioactivity. Less than 10% of the total radioactivity accumulated by *L. acidophilus* on incubation with glutamic acid-C^{14} was found in the material precipitable by 5% trichloroacetic acid under the conditions employed in Table 3. The amount of trichloroacetic acid-precipitable radioactivity was not decreased by the addition of chloramphenicol or puromycin, known inhibitors of protein synthesis. Thus, there is little or no incorporation of glutamic acid into protein under the conditions of incubation employed.

Table 3 demonstrates that chloramphenicol and puromycin increase the total accumulation of glutamic acid by *L. acidophilus*. A stimulatory effect of chloramphenicol on the accumulation of amino acid by *L. arabinosis* (5), *Escherichia coli* (9), and *Staphylococcus aureus* (4) has been previously reported. Saliva (5%) largely inhibits glutamic acid accumulation by *L. acidophilus* in the presence of chloramphenicol or puromycin as well as in the absence of these substances.

Extraction and chromatographic analysis revealed that the radioactivity extracted from *L. acidophilus* after incubation with glutamic acid-C^{14} was largely in the form of glutamic acid. A 10-mg (dry weight) amount of *L. acidophilus* was incubated for 2 hr at 37 C in 2.5 ml of GCP containing glucose (5×10^{-3} M) and glutamic

TABLE 3. *Effect of saliva, chloramphenicol, and puromycin on the incorporation of* L-*glutamic acid into trichloroacetic acid-precipitable material**

Supplement	Glutamic acid accumulation (counts per min per ml)	
	Whole cell	Trichloroacetic acid precipitate
None	760	60
Saliva	272	43
Chloramphenicol	1,450	80
Saliva + chloramphenicol	294	36
Puromycin	1,400	121
Saliva + puromycin	206	24

* The reaction mixture was as indicated in Table 1 except for the supplements as follows: saliva, 5% (v/v); chloramphenicol, 60 μg; puromycin, 100 μg. Incubation time was 2 hr.

acid-C^{14} (7×10^{-7} M, 0.05 μc/ml), in a final volume of 5.0 ml. Paper chromatography revealed that approximately 90% of the radioactivity was present as glutamic acid. The remaining 10% was present as three unidentified compounds. Paper chromatograms of extracts prepared from cells incubated with 5% saliva were qualitatively similar to those prepared from cells not treated with saliva.

Glutamic acid degradation. The possibility was considered that the saliva-hydrogen peroxide or the lactoperoxidase-thiocyanate-hydrogen peroxide system might alter glutamic acid chemically, so that it is not accumulated by the organism. Saliva (5%) and hydrogen peroxide were incubated for 2 hr at 37 C with glutamic acid-C^{14}, glucose, and GCP at the same concentrations indicated in Table 1. Lactoperoxidase, thiocyanate, and hydrogen peroxide were incubated with glutamic acid-C^{14} in a similar manner. Radiochromatographs from the reaction mixtures after incubation indicated that no chemical change occurred in glutamic acid-C^{14} incubated with saliva, saliva-hydrogen peroxide, or lactoperoxidase-thiocyanate-hydrogen peroxide. In two-dimensional chromatograms, the radioactive spot which accounted for over 95% of the radioactivity corresponded exactly with the ninhydrin-positive spot of glutamic acid-C^{12} added as an internal standard.

DISCUSSION

Saliva inhibits the accumulation of glutamic acid and other amino acids by *L. acidophilus*. This effect of saliva appears to be due in part to the lactoperoxidase-thiocyanate antibacterial system. Thus, the inhibition of glutamic acid accumu-

lation is decreased by dialysis of saliva, and is restored by the addition of thiocyanate ions to the dialyzed saliva. Similarly, saliva heated to 100 C for 10 min is less inhibitory to glutamic acid accumulation, and the inhibitory effect of heated saliva is restored by lactoperoxidase. H_2O_2 increased the inhibitory effect of saliva or of lactoperoxidase-thiocyanate on glutamic acid accumulation by *L. acidophilus*. That substances in saliva other than peroxidase and thiocyanate ion contribute to the inhibition of glutamic acid accumulation is suggested by the finding that dialysis and heat did not completely abolish the inhibitory effect of saliva on glutamic acid accumulation (Table 1).

Zeldow (15) has reported that saliva has a bactericidal effect on growing cells, but does not produce any appreciable killing of resting cells. In view of the requirement for H_2O_2 for microbial growth inhibition by the antilactobacillus system of saliva (*7a*, 8), microbial metabolism in complete growth medium may be required for H_2O_2 generation. The decrease in glutamic acid accumulation produced by the saliva-H_2O_2 or the lactoperoxidase-thiocyanate-H_2O_2 system was not associated with a significant loss of viability under the incubation conditions employed in Table 1. Thus, the decrease in glutamic acid accumulation by *L. acidophilus* is not secondary to a loss in microbial viability under the conditions employed. It is possible, however, that a more prolonged absence of amino acid accumulation by the organisms may lead to cell death. Lactobacilli are nutritionally exacting organisms which require many preformed components for optimal growth. Dreizen et al. (3) reported that the growth of *L. acidophilus* was completely inhibited when glutamic acid, valine, cystine, methionine, phenylalanine, or serine was omitted from a defined synthetic medium. Less than maximal acid production occurred when any one of six other amino acids was omitted.

The glutamic acid-C^{14} accumulated by *L. acidophilus* remained largely (over 90%) as free glutamic acid, as established by extraction and chromatographic analysis. Little incorporation of glutamic acid into trichloroacetic acid-precipitable material was observed, and the level of precipitable material was unaffected by the addition of puromycin or chloramphenicol. This suggests that, under the conditions employed in this study, there was little if any incorporation of glutamic acid into protein by the intact cells, and that the accumulation of radioactivity represents the net transport of glutamic acid into the cell. It is this process which is inhibited by saliva and by the peroxidase system. The possibility was considered that a chemical alteration of glutamic acid by saliva or by the peroxidase system might prevent its uptake by the cell; however, this is unlikely, since chromatographic analysis of glutamic acid exposed to saliva or the peroxidase system revealed no chemical alteration under the conditions employed.

Amino acid transport into microbial cells is a complex process. The accumulation of glutamic acid by *L. acidophilus* requires the addition of glucose to the reaction mixture and is temperature-dependent (Fig. 1). This supports the abundance of evidence that amino acid transport in bacteria requires, at least in part, the expenditure of metabolic energy. The mechanism by which saliva or the peroxidase system inhibits amino acid accumulation by the organism is currently under study.

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