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Adsorption of Free Fatty Acids on Cells of Certain Microorganisms [1]

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Abstract

Information on the inhibitory effect of free fatty acids was sought by growing lactic streptococci in the presence of labeled fatty acids and collecting the cells on membrane filters. The distribution of the radioactivity indicated adsorption by the bacteria. As the number of cells in the growth medium increased, accumulation of radioactivity with the cells on the filter increased. The cells accumulated less than one-tenth as much radioactivity from sodium butyrate as from lauric acid. Washing the cells on the filter with water decreased radioactivity. More complete elution of the radioactivity was obtained by filtering the mixture after it was made alkaline. Though this implies that the association of fatty acid and cells is pH-dependent, adsorption was always evident in the pH range of normal growth. Gas-liquid chromatographic analysis indicated the fatty acids were not metabolized. All results indicate weak adsorption at the bacteria : menstruum interface. The parallels of adsorption and inhibition indicate adsorption is associated with the inhibitory process.

The presence of free fatty acids inhibits the growth of lactic streptococci (1, 2, 7, 12). Fatty acids of intermediate chain length (capric and lauric) are most inhibitory. A similar inhibitory effect, however, is not apparent with most other bacteria. The mechanism of the inhibition has not been fully explained.

Attempts to explain the process of inhibition have followed two main approaches: One understanding of the mechanism of inhibition was derived from the work on the bactericidal effect of the organic acids (3, 9, 10). The bactericidal activity was generally ascribed to the un-ionized fraction of the acids. Monocarboxylic acids were found to increase in activity with an increase in molecular weight. The work, however, was confined to caprylic and acids of shorter chain length.

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Another understanding of the mechanism of inhibition has been based on surface activity effects. As early as 1923, Ayers, Rupp, and Johnson (1) showed lactic streptococci to be inhibited in a medium to which certain materials had been added to depress the surface tension. Tarassuk and Smith (12), when working with the inhibition of lactic streptococci by fatty acids, concluded that surface-tension depression was responsible for the inhibitory effect. They further excluded the possibility of metabolic interferences by concluding that lactic streptococci metabolized fatty acids. Since surface activity arises primarily from the dipolar nature of ionized molecules of fatty acids, inhibition by surface activity might be expected to be by a totally different mechanism than with inhibition by un-ionized molecules.

Costilow and Speck (2) showed that inhibition was most pronounced in the presence of fatty acids of intermediate chain length (capric and lauric); they attributed this effect to some unknown factor other than surface tension. This path of thinking follows well the explanation of the stimulatory effect of certain fatty acids on growth of lactobacilli and the close relation to metabolism (5, 6, 8). More recent work in our laboratory (7) has indicated surface activity at the bacterium : menstruum interface to be of prime importance. Surface active agents structurally unrelated to fatty acids gave similar results to fatty acids; effectiveness was proportional to the surface-tension depression. In addition, preliminary observations showed that the fatty acids accumulated with the cells. The fate of these fatty acids, however, was not studied.

The importance of fatty acid inhibition of lactic streptococci is recognized in the dairy industry in present processes and as a limitation in developing new processes. To obtain additional information on the nature of the inhibition, with the eventual hope of eliminating the problem, observations on the accumulation and fate of fatty acids were made.

Methods

Streptococcus lactis UN-C_1, *Streptococcus faecalis* var. *liquefaciens*, and *Escherichia coli* were used. Cultures were grown at 30 C in 1% micro inoculum broth (1 g of Difco dehydrated micro inoculum broth per 100 ml), which also

served as the basal medium for study of the addition of fatty acids. This concentration has been shown to give good growth and to be a medium in which lactic streptococci are highly sensitive to added fatty acids (7).

To study accumulation of fatty acids by the microorganisms, pure cultures were grown in the presence of the C^{14}-labeled fatty acid or the salt thereof. One milliliter of the culture was mixed with 5–10 ml of distilled water. Filtration was with a membrane filter 47 mm in diameter, with mean pore size of 0.45 μ (Millipore Filter Company, Bedford, Massachusetts). An aspirator for vacuum to aid filtration was allowed to operate under full force for 2 min after the visible liquid was removed from the surface. Two additional rinses each of 5 ml of distilled water were applied, since preliminary observations had shown the extent of rinsing to be critical. Deviations from this rinsing procedure are given with the specific experiment. The filters were removed to aluminum foil for drying 24 hr at 45 C, after which counts of radioactivity were made.

The radioactive materials were lauric acid 1-C^{14} and sodium butyrate 1-C^{14}. Counting was done with a Baird Atomic General Purpose Multiscaler with a thin-window Geiger flow counter. Results in this paper are corrected for a background of 30–40 counts per minute as appropriate for the time of the experiment.

To determine the fate of the fatty acids, one-liter quantities of cultures after growth for 24 hr were used. Samples were acidified to pH 3.0 and extracted three times with 100-ml portions of petroleum ether, with a 1-hr interval for a phase equilibrium to be established. The extracts were combined and concentrated to near dryness under vacuum at room temperature in a rotating evaporator. Approximately 25 ml of analytical-grade chloroform-methanol mixture (95:5 v/v) was added to the concentrate, which was then purified on a silicic acid column according to the method of Gander et al. (4). Approximately 100 ml of the chloroform-methanol mixture was used to elute the fatty acids from the column. This eluate was then concentrated to approximately 1 ml under vacuum at room temperature. The concentrate was used to prepare methyl esters according to the method of Gander et al. (4), except that the microinteresterification assembly of Stoffel et al. (11) was used for the refluxing operation.

Analyses were made with an Aerograph Model A-90-P gas chromatograph, with a 0.635-cm-od by 152.4-cm-long stainless steel column. The packing was 20% diethyleneglycol succinate on 60/80 mesh Chromosorb-P, pretreated with hexamethyldisilazane. A helium flow rate of 60 ml/minute and a column temperature of 165 C were used.

A weighed amount (approximately 50 mg) of pure stearic acid (Applied Science Laboratories, State College, Pennsylvania) was added to each sample just prior to refluxing, to provide an internal standard for quantification of the lauric acid recovery. Figure 1 represents a typical calibration curve for the quantitative determination of lauric acid by these methods. Results of several such experiments indicated that lauric acid could be detected within ±2%.

Results

Growth and accumulation of lauric acid 1-C^{14}. In a previous publication (7), lactic streptococci were shown to accumulate lauric acid, either through adsorption or transport into the cells. Knowing the site of the accumulation logically might be a significant step toward understanding the mechanism of the inhibition. To study further the nature of the accumulation process, pure cultures of lactic streptococci were grown in the presence of lauric acid

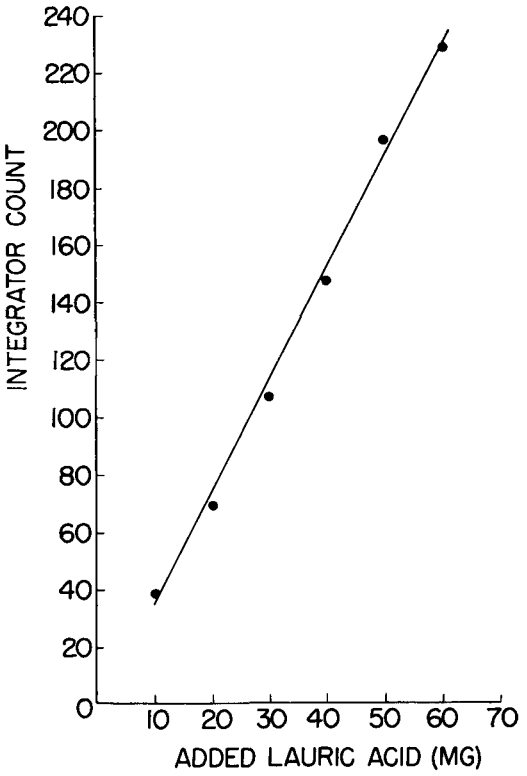

FIG. 1. Calibration curve for the quantitative determination of lauric acid by gas-liquid chromatography.

1-C^{14}, with a concentration of 22 μg/ml and 0.1 μc/ml of radioactivity. Initial pH was 7.0. This concentration of lauric acid was chosen to allow growth to produce visible turbidity within 16 hr by *S. lactis,* which was the most sensitive microorganism to be studied (7). Aliquots were taken to represent 0, 4, 8, and 24 hr of incubation for growth. After filtering to collect the cells, the radioactivity remaining on one filter was determined.

As growth of the cells progressed, there was accumulation (adsorption or taking into the cell for metabolic processes) of the fatty acid. Average results of three trials are shown in Table 1. There was an increase from an average of 379 counts per minute to 1,028 counts per minute for *S. lactis* after 24 hr. Even greater increases were apparent for *S. faecalis* and *E. coli.* Relatively little accumulation occurred during early phases of growth. Since accumulation was minimal during the early stages of active growth, doubt must be cast on the concept that fatty acids interfere directly wih metabolism by the microorganisms.

TABLE 1

Radioactivity in counts per minute associated with microorganisms from growth in a medium with lauric 1-C^{14} acid

Incubation time	Radioactivity associated with the microorganisms		
	Streptococcus lactis	*Streptococcus faecalis*	*Escherichia coli*
(hr)			
0	379	497	539
4	476	180	300
8	472	515	1,936
24	1,028	1,523	2,224

While results reported in a previous paper (7) indicated the accumulation was not due to collection of the micelles or aggregates of lauric acid on the filter, direct observations were made on this phenomenon. *S. lactis* was grown for 24 hr in a medium containing lauric acid 1-C^{14}. Comparable samples without bacterial inoculation were adjusted to the same pH as the media after growth. Aliquots of each were filtered, and the filters washed, dried, and counted. Results of three trials showed an average of 3,563 counts per minute for the samples with cells and 2,320 without cells. The relatively high counts, as compared to those found in Table 1, resulted from use of a higher concentration of lauric acid 1-C^{14}, so that micelle formation might be enhanced.

Growth and uptake of sodium butyrate 1-C^{14}. Another approach to the study of the accumulation process was through use of sodium butyrate 1-C^{14}, which in solution exemplified the short chain free fatty acid behavior. Neither sodium butyrate nor butyric acid produces very much surface activity and a correspondingly low inhibitory effect toward the lactic streptococci (7).

Cultures were grown in the presence of 100 μg/milliliter of sodium butyrate with 0.5 μc radioactivity per milliliter. A shorter growth time and a higher concentration of butyrate than had been used with lauric acid were possible, because of the lower inhibitory effect of butyrate (7). After 16 hr of growth, aliquots were filtered to collect the cells, and the radioactivity remaining with the filter was determined. Results of an average of three trials are given in Table 2. Less than one-tenth as much radioactivity accumulated with the cells with butyric acid as with lauric acid. The relatively low level of radioactivity remaining with the cells is even more striking when consideration is given the initital fivefold higher value of radioactivity than for those data with lauric acid.

The data also show that simple rinsing of the filter with 100 ml of distilled water removed nearly one-half the radioactivity, which indicated that the association between butyrate and the cells indeed was weak. Results are in agreement with the concept that there is little surface activity and it parallels a weak inhibitory effect (7).

TABLE 2

Radioactivity in counts per minute associated with microorganisms from growth in a medium with sodium butyrate 1-C^{14}

Microorganisms	Counts from unrinsed filters		Filters rinsed with 100 ml of water	
	Without bacteria	With bacteria	Without bacteria	With bacteria
Streptococcus lactis	30	49	6	36
Streptococcus faecalis	30	54	6	25
Escherichia coli	30	80	6	40

Relative binding of fatty acids by bacterial cells. Some additional observations were made on the relative binding of fatty acids by bacterial cells by altering the reaction of the bacterial culture medium before filtering. After growth for 16 hr in the presence of 22 μg/ml of lauric acid, 1-ml aliquots of the cultures were suspended in 5 ml of water or 5 ml of 0.1 N NaOH. The suspension was filtered through membrane filters and rinsed with the usual two-step 5 ml of water rinse procedure. Average results of two trials are given (Table 3).

TABLE 3

Effect of the reaction on recovery of lauric 1-C^{14} acid with bacteria from cultures on filtering through a Millipore filter

Microorganisms	Reaction of the diluent	
	Acidic	Alkaline
Streptococcus lactis	1,106[a]	80
Streptococcus faecalis	1,619	237
Escherichia coli	2,885	315

[a] Radioactivity expressed in counts per minute.

The influence of the reaction on the collection process is apparent, with much less radioactivity collecting when the medium was alkaline. There was less elution of radioactivity from cells of *S. faecalis* and *E. coli* than from those of *S. lactis*. Ease of removal of the radioactivity is striking, when compared to freeing of products that have been metabolized and have become an integral part of the cell.

Recovery of lauric acid in a growth medium with lactic streptococci. Since radioactivity from lauric acid 1-C^{14} could be removed so easily from the cells, it seemed the lauric acid was unaffected and remained outside the cell wall or had been converted to highly soluble products. Therefore, observations to differentiate between these alternatives were made. *S. lactis* was grown with and without added lauric acid, and a gas-liquid chromatographic technique was used to analyze for lauric acid. An example of the results is given in Table 4. Analyses on broth in which *S. lactis* had grown showed only a trace of lauric acid, indicating the acid was not synthesized in amounts detectable by our procedure. Broth containing lauric acid, but without the growth of microorganisms, gave a recovery of 13.5 μg, or 71.0%. Analyses on like broth after *S. lactis* had grown for 24 hr to show a highly turbid medium also gave 13.7 μg, or 72.1% recovery. The data indicated that *S. lactis* did not catabolize the lauric acid, therefore substantiating the alternative that the fatty acids remain outside the cell wall.

TABLE 4

Recovery of lauric acid from broth after growth of *Streptococcus lactis*

Treatment	Milligrams	Per cent recovery
Basal medium without added lauric acid (with growth)	Trace	.....
Basal medium plus lauric acid (without growth)	13.5	71.0
Basal medium plus lauric acid (with growth)	13.7	72.1

Discussion

The primary reason for studying the accumulation and fate of free fatty acids is their inhibitory effect on lactic streptococci. Those acids of intermediate chain length (10–12 carbons) are most inhibitory (2, 7, 12). The longer-chain fatty acids would be expected to be more inhibitory on the basis of surface activity. It is also difficult to picture metabolism in such a way that the intermediate chain lengths are most inhibitory and show progressively less inhibition with increasing or decreasing chain length. Thus, the mechanism of inhibition remains unexplained. When one considers the problem of getting long-chain fatty acids into a medium of the necessary complexity to support growth of *S. lactis,* it is apparent this failure may have clouded past observations. If long-chain fatty acids have not been so incorporated to produce the expected effect at the bacterium : menstruum interface, then the remaining observations fit well the pattern of explanation that effectiveness is at the bacterium : menstruum interface. A variety of surface active materials has been substituted for fatty acids, with similar results. Results presented in this paper indicate fatty acids are weakly adsorbed to the bacteria. The extremely short chains react in the same manner as the longer chains, but the shorter chains are less surface-active. The fatty acids can be accounted for as chemically unaltered after growth of the miroorganisms in their presence. It would seem that the evidence is strongly in favor of the concept that inhibition is a surface phenomenon at the bacterium : menstruum interface.

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