DELAYED HYDROLYSIS OF BUTTERFAT BY CERTAIN LACTOBACILLI AND MICROCOCCI ISOLATED FROM CHEESE[1]

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Early work on the ripening of hard rennet cheese established that the enzymes concerned arise from one or more of the following sources: milk, rennet extract, and the bacterial flora of the cheese. Peterson, Johnson, and Price (1948*a*,*b*) have demonstrated, however, that enzymes of milk and rennet extract constitute only a small fraction of the total hydrolytic activity present in ripening Cheddar cheese.

Many studies on the role of bacteria in the ripening of Cheddar and other hard rennet cheeses have been made. In 1889 Adametz reported that the characteristic flavor did not develop in cheese that contained added thymol to retard bacterial growth. After further work revealed that the bacterial flora of normal hard rennet cheese consists predominantly of lactic-acid-producing bacteria, Adametz proposed the "lactic acid" theory of cheese ripening which recognized these bacteria as the principal cheese-ripening agent.

The majority of the bacterial studies on the ripening of Cheddar cheese since the work of Adametz support the "lactic acid" theory from the viewpoint of predominance of organisms, increase in the rate of flavor development, or increase in the rate of nonprotein nitrogen production. There have been, however, no reports based on adequate methods on the lipolytic activity of the bacterial flora of Cheddar cheese.

In previous work Peterson, Johnson, and Price (1948*a*) suggested that the lipolytic activity at pH 5 of Cheddar cheese may represent intracellular lipases of lactic acid bacteria liberated by bacterial autolysis. Peterson, Johnson, and Price (1949) further suggested that part of the free *n*-butyric and all of the free caproic, caprylic, and capric acids present in aged Cheddar cheese are the result of the action of these liberated bacterial lipases on the milk fat of the cheese.

In an attempt to determine the validity of the above hypotheses, studies on the intracellular lipolytic activities of pure strains of bacterial species present in large numbers in Cheddar cheese during ripening have been undertaken. It has been definitely established (Hucker, 1922*a*,*b*; Sherwood, 1939) that the organisms present in Cheddar cheese in sufficiently large numbers to function in the ripening process are included in the following groups: *Streptococcus*, *Lactobacillus*, and *Micrococcus*. The purpose of the present investigation is the study

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of the intracellular lipases of a number of organisms isolated from normal Cheddar and brick cheese. These organisms fall in the *Lactobacillus* and *Micrococcus* groups. Bacterial isolates from brick cheese were also studied since the conditions of ripening and the bacterial flora of Cheddar and brick cheese are similar.

METHODS

Isolation and identification of organisms.[2] The *Lactobacillus* and *Micrococcus* organisms used in this investigation were isolated from cheese by the methods of Foster, Garey, and Frazier (1942) and Alford (1949), respectively. Group classification of all isolates and final identification of cultures shown to possess lipolytic activity were made by use of the scheme given in *Bergey's Manual* (Breed *et al.*, 1948). All stock cultures of *Lactobacillus* isolates were carried in litmus milk and as stabs in a medium consisting of 0.25 per cent Difco yeast extract, 0.25 per cent glucose, 0.25 per cent Difco peptone, 1.5 per cent agar, and 5.0 ml each of mineral salt solutions A and B (Shull, Hutchings, and Peterson, 1942) per liter. Stock cultures of *Micrococcus* isolates were carried as stabs in a medium consisting of 1.0 per cent Difco tryptone and 1.5 per cent agar.

Preparation of constituents of media. Raw milk whey was prepared by rennet curd coagulation of whole milk adjusted to pH 5.75 with lactic acid. The turbid whey was adjusted to pH 7 and clarified by filtration through a coarse, diatomaceous filter aid (celite 545; Johns-Manville). The clear yellow whey was sterilized by passage through a Mandler bacteriological filter.

Purified butterfat was prepared as follows: An ether solution of fresh butterfat was washed with dilute sodium hydroxide, then with water. After removal of ether and water under reduced pressure below 50 C, the fat was stored in the refrigerator. Small portions (100 g) were sterilized just before use by autoclaving at 121 C for 2 hours.

Solid calcium carbonate was sterilized by being heated in an oven at 180 C for 6 hours.

Lipolytic activities of organisms. The lipolytic activities of isolated organisms were determined by measuring the extent of butterfat hydrolysis in whey butterfat cultures during a 60-day growth and autolysis period at 30 C. For each organism four identical sterile flasks were used. Each flask contained 100 ml of sterile whey and approximately 5 g of sterile calcium carbonate. To two of the flasks 3 g of sterile butterfat were added. One ml of inoculum from a 48-hour whey culture of the organism being studied was added to each of the four flasks. Several uninoculated control flasks, with and without butterfat, were set up for each run. In any single run only one preparation of raw milk whey and one of butterfat were used; thus the uninoculated flasks were controls for all the organisms included in that run. During the 60-day incubation period the cultures were shaken every second or third day to assure neutralization of acids formed or liberated. After growth was well under way (24 to 48 hours after inoculation),

[2] The authors are indebted to Dr. John A. Alford for the isolation and identification of the *Micrococcus* isolates used in this investigation. The *Lactobacillus* isolates were kindly furnished by Professor Edwin M. Foster.

the pH of the inoculated media for all organisms used in this investigation remained between 5 and 6 for the remainder of the 60-day period. The pH of normal Cheddar cheese during ripening lies between 5.1 and 5.3.

At 15 and 60 days, control and culture media, with and without butterfat, were analyzed for free volatile fatty acids by the following procedure: A suitable sample of each medium was adjusted to pH 2 with dilute sulfuric acid. The free fatty acids liberated were removed by extracting the acidified medium with

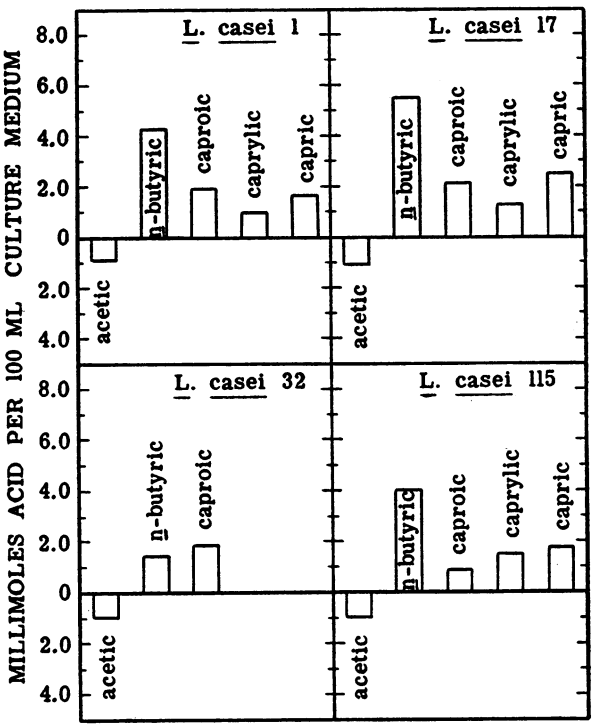

Figure 1. Butterfat hydrolysis between pH 5 and pH 6 by intracellular lipases of four *Lactobacillus casei* strains isolated from Cheddar cheese. The total block heights represent the levels of free fatty acids found in whey butterfat cultures incubated 60 days at 30 C. The block portions below the center zero line represent the fatty acid levels in similar cultures without added butterfat; hence the block heights above the center represent acids liberated from butterfat. In the case of acetic and *n*-butyric acids, the necessary corrections for small amounts of these compounds in the uninoculated media have been made.

ether. The acids were then removed from the ether extracts with dilute alkali and distilled from aqueous solution by a modification (Peterson and Johnson, 1948) of the magnesium sulfate procedure of Friedemann (1938). The distillate was titrated to the phenol red end point with 0.1 N sodium hydroxide. Although quantitative recovery of the total volatile free fatty acids is not possible by this procedure if free nonvolatile fatty acids are present (Smiley, Kosikowsky, and Dahlberg, 1946), the results obtained are sufficiently accurate for the purpose of this investigation. After suitable corrections for free volatile fatty acids pres-

ent in the uninoculated control flasks had been made, the amounts of free volatile fatty acid liberated by each organism in the presence and absence of butterfat were compared.

If any organism was found to have liberated more than 0.5 milliequivalents of volatile fatty acid per 100 ml of culture from butterfat, levels of individual fatty acids liberated were estimated by the method of Peterson and Johnson (1948). Analyses were made on all culture and control media of that organism. The identities of the fatty acids isolated during the partition chromatographic

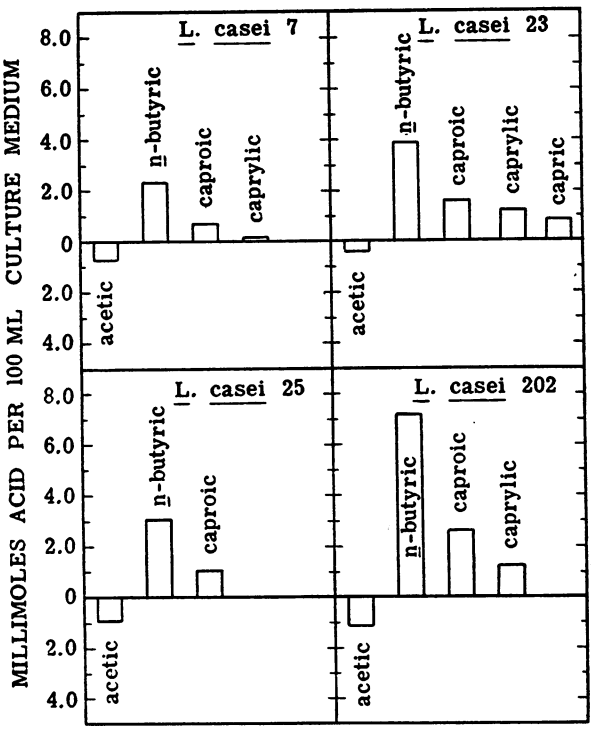

Figure 2. Butterfat hydrolysis between pH 5 and pH 6 by intracellular lipases of three *Lactobacillus casei* strains isolated from Cheddar cheese and one *L. casei* strain isolated from brick cheese (no. 202). See figure 1 for explanation.

analytical procedure were determined by their zone positions in column effluents. Fatty acids higher than capric acid, such as lauric, stearic, oleic, and so forth, were liberated only in trace amounts by the lipases of the organisms used in this investigation. Comparisons of butterfat hydrolysis at 15 and 60 days indicated whether lipolysis occurred during the normal life cycle of the organism or after autolysis.

LIPOLYTIC ACTIVITIES AND IDENTITIES OF ISOLATED CHEESE ORGANISMS

Sources and group classifications of isolated cheese organisms. Fifty-four *Lactobacillus* cultures from Cheddar and brick cheese and eight *Micrococcus* cultures

from raw milk Cheddar cheese were isolated. All *Lactobacillus* isolates are gram-positive rods with optimum temperatures between 30 C and 37 C. All strains produced lactic acid in litmus milk with subsequent curdling and litmus reduction. All *Micrococcus* isolates are gram-positive, catalase-positive cocci producing abundant growth on 1.0 per cent Difco tryptone agar.

Lipolytic activities of Lactobacillus isolates. After 60 days of growth and autolysis in the whey butterfat medium, only 12 of the 54 *Lactobacillus* isolates were found to have effected any appreciable butterfat hydrolysis. Seven of these 12 cultures were isolated from Cheddar cheese (numbers 1, 7, 17, 23, 25, 32, and 115)

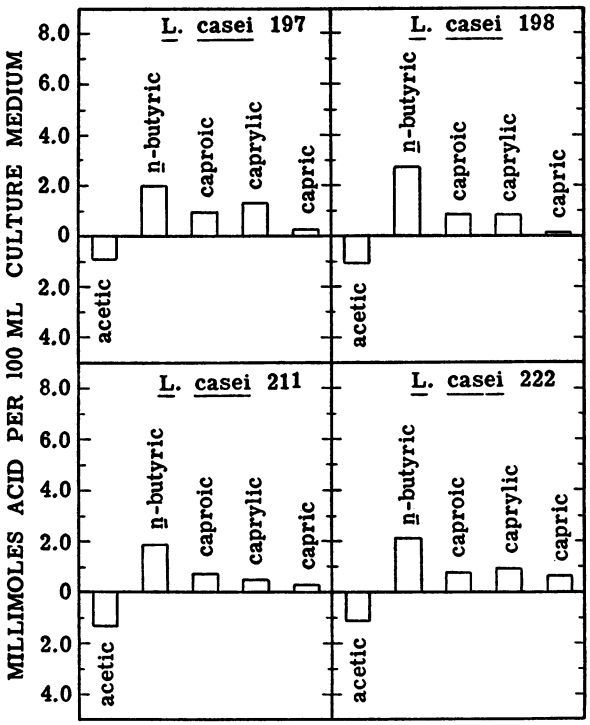

Figure 3. Butterfact hydrolysis between pH 5 and pH 6 by intracellular lipases of four *Lactobacillus casei* strains isolated from brick cheese. See figure 1 for explanation.

and the remaining 5 from brick cheese (numbers 197, 198, 202, 211, and 222). In no case was any lipolysis found to occur for any of these 12 isolates during the first 15 days of growth and autolysis at 30 C. Since these strains were all found to reach their growth peak in raw milk whey containing 0.5 molar acetate within 48 to 72 hours after inoculation, this demonstrates that these organisms are not appreciably lipolytic during their normal life cycles. Hence lipolysis occurring after 15 days and before 60 days was undoubtedly due to enzymes, active between pH 5 and pH 6, liberated by bacterial autolysis.

In figures 1, 2, and 3 the levels of individual fatty acids liberated from butterfat by the twelve *Lactobacillus* isolates having intracellular lipases active between

pH 5 and pH 6 are presented. A number of experiments were carried out, and the data presented are the results of one representative experiment for each isolate. As may be seen, there appears to be considerable lipolytic specificity among the intracellular lipases of the isolates with regard to kinds and amounts of fatty acids liberated.

Lipolytic activities of Micrococcus isolates. Although the eight *Micrococcus* isolates were not found to be lipolytic during their normal life cycles, four of them were found to possess intracellular lipases active between pH 5 and pH 6. In figure 4 the levels of individual fatty acids liberated from butterfat by these four

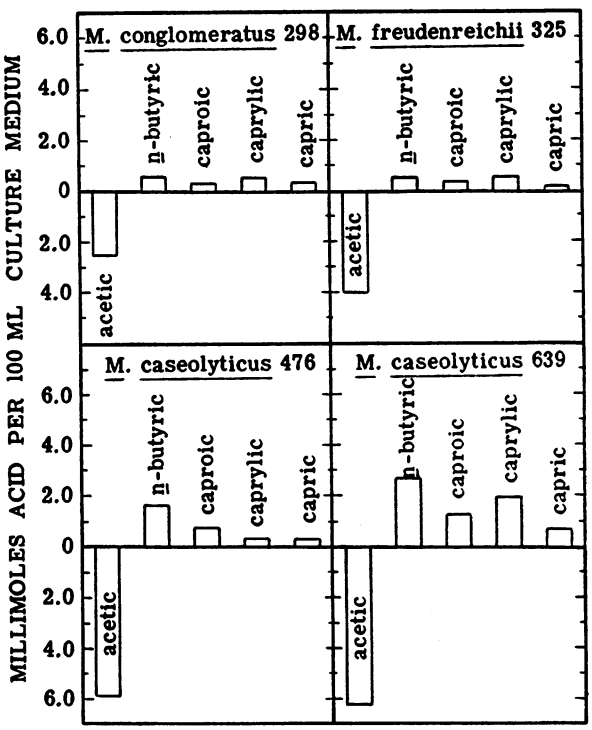

Figure 4. Butterfat hydrolysis between pH 5 and pH 6 by intracellular lipases of four *Micrococcus* cultures isolated from raw milk Cheddar cheese. See figure 1 for explanation.

Micrococcus cultures are given. The data presented are the results of one of a number of representative experiments for each isolate.

Identities of Lactobacillus and Micrococcus isolates having intracellular lipolytic activity. All 12 *Lactobacillus* isolates having intracellular lipases active between pH 5 and pH 6 were identified as cultures of *L. casei*. These 12 cultures have an optimum temperature between 30 C and 37 C, and curdle milk in 2 to 3 days with reduction of litmus. All produce 1.1 to 1.5 per cent lactic acid in milk, are nonmotile, are catalase-negative, do not liquify gelatin, and grow better anaerobically than aerobically. Sugar fermentations are identical in all cases with those listed for *L. casei* in *Bergey's Manual* (Breed *et al.*, 1948).

The 4 *Micrococcus* isolates having intracellular lipases active between pH 5 and pH 6 were identified as 1 culture of *M. conglomeratus*, 1 culture of *M. freudenreichii*, and 2 cultures of *M. caseolyticus*. Detailed identification studies on these *Micrococcus* cultures are presented by Alford (1949).

DISCUSSION

Although the 12 *Lactobacillus casei* cultures and 4 *Micrococcus* cultures shown to possess intracellular lipases active at the pH of normal Cheddar cheese were isolated from Cheddar and brick cheese, it would be very difficult to obtain proof that they function in cheese ripening. Although it is known that *L. casei* and *Micrococcus* organisms are present in Cheddar cheese in large numbers at various stages of ripening, a practical, differential bacterial count for organisms possessing intracellular lipolytic activity would be very difficult to devise. Indirect proof that bacterial organisms, such as the 16 studied in this investigation, function

Figure 5. Free fatty acids present in Cheddar cheese. Total block heights, 360-day-old cheese; shaded block heights, 3-day-old cheese. Data from figure 1 (Peterson, Johnson, and Price, 1949).

in Cheddar cheese ripening might result from cheese-making studies in which milk cultures of these organisms are added individually and as mixtures to the cheese milk. Improved flavor development and rapid change of the individual free fatty acid picture to resemble that of aged Cheddar cheese (figure 5) would indicate activity of the added cultures. Alford (1949) found that the rate of flavor development in pasteurized milk Cheddar cheese to which milk cultures of *M. freudenreichii* 325 had been added was markedly increased. Fatty acid analyses, however, were not made.

SUMMARY

Twelve of 54 *Lactobacillus* cultures isolated from normal Cheddar and brick cheese and 4 of 8 *Micrococcus* cultures isolated from normal raw milk Cheddar cheese were found to possess intracellular lipases active between pH 5 and pH 6 and capable of considerable butterfat hydrolysis (3 to 12.8 milliequivalents of total fatty acid liberated per 100 ml of culture).

The 12 *Lactobacillus* isolates having intracellular lipolytic activity were identified as cultures of *L. casei*. The 4 *Micrococcus* isolates with similar lipolytic activity were identified as 1 culture of *M. conglomeratus*, 1 culture of *M. freudenreichii*, and 2 cultures of *M. caseolyticus*.

The intracellular lipases of 8 of the 12 *L. casei* cultures liberated *n*-butyric, caproic, caprylic, and capric acids from butterfat; the lipases of 2 of the other 4 *L. casei* cultures liberated *n*-butyric, caproic, and caprylic acids while those of the remaining 2 cultures released only *n*-butyric and caproic acids.

The intracellular lipases of all 4 *Micrococcus* cultures liberated *n*-butyric, caproic, caprylic, and capric acids from butterfat.

None of the *L. casei* or *Micrococcus* isolates found to have intracellular lipolytic activity showed lipolytic activity during their normal life cycles.

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