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Relationship of microorganisms to the disappearance of rancidity in cheddar cheese

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NOTE TO USERS

RELATIONSHIP OF MICROORGANISMS TO THE DISAPPEARANCE OF RANCIDITY IN CHEDDAR CHEESE

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INTRODUCTION

The nature and sequence of the biological, chemical and physical changes which take place during the ripening of cheddar cheese have been considered by many investigators. These changes are believed to be the result of the combined action of a number of factors, bacteria and enzymes probably being the most important. In some instances undesirable changes occur in cheddar cheese during the ripening, and an inferior product results. Such objectionable flavors as bitter, tainted, tallowy, rancid, fruity and unclean are among those that have been encountered. Many early investigators believed that undesirable changes occurring in the cheese protein were largely responsible for these defects. Recent studies, however, have shown that the milk fat also may be involved.

Rancidity in dairy products is a defect that usually is associated with fat deterioration. The condition is very objectionable. In certain dairy products, such as butter or raw milk, rancidity sometimes increases as time goes on, and the defect may become so objectionable that the producer has difficulty in finding a market. Rancidity in certain lots of cheddar cheese also increases during holding. On the other hand rancidity in cheddar cheese often decreases, and finally disappears when it is due to the use of homogenized raw milk (29) or when it is the result of the addition to pasteurized milk of an extract containing a lipolytic enzyme (30). This cheese may even have a satisfactory flavor after the rancidity has disappeared.

In view of the relationships involved it is apparent that a detailed

study of the disappearance of rancidity in cheddar cheese would be of value, not only to the industry but as a contribution to basic information on cheese ripening.

STATEMENT OF PROBLEM

The studies reported herein were undertaken in an attempt to determine the relationship of microorganisms to the disappearance of rancidity in cheddar cheese. The investigation of this relationship included consideration of the following points:

A. Bacteria in cheddar cheese with reference to their lipolytic activity and ability to utilize sodium butyrate.

B. Disappearance of rancidity produced in cheddar cheese by the addition of butyric acid to the milk.

C. Effect of rancidity on the bacterial flora of cheddar cheese made from raw and from pasteurized milk.

D. Effect of adding the X type organism to milk used for making cheddar cheese.

E. Ability of the X type organism to utilize sodium butyrate.

F. Relationship of the X type organism to *Streptococcus lactis*.

HISTORICAL

The literature contains many reports on rancidity in dairy products, but most of the work has been concerned with rancidity in milk and butter. Relatively little information is available on rancidity in cheddar cheese. For this reason the literature reviewed herein deals largely with rancidity in dairy products other than cheddar cheese.

There is considerable confusion in the literature because the term rancidity has been used to describe various defects occurring in fatty foods. Some investigators have used the term to denote any deterioration which develops in fats or fatty foods. Such a definition, however, is much too broad, and many workers agree with Triebold (45) who listed three types of rancidity: oxidative, ketonic and hydrolytic. Oxidative rancidity is due to the addition of molecular oxygen to unsaturated glycerides with the formation of peroxides which finally break down into aldehydes, ketones and fatty acids. Ketonic rancidity is due to the formation of methyl ketones through the action of certain molds on the lower fatty acids. Hydrolytic rancidity is characterized by hydrolysis of the glycerides with the liberation of free fatty acids and glycerol. This type of rancidity is of special interest in the spoilage of dairy products due to the liberation of small amounts of butyric and caproic acids with their characteristic flavors and odors; the liberation of small amounts of most of the higher fatty acids does not appreciably affect the flavor and odor of fats.

Oxidative Rancidity

A defect of milk and milk products has long been associated with the oxidation of milk fat. In the dairy industry this condition often has been described as tallowiness. Triebold (43) believed that oxidative rancidity was due to the addition of molecular oxygen to unsaturated glycerides with the formation of peroxides which subsequently decomposed into aldehydes, ketones and fatty acids.

Anderson (2) and Guthrie and Brunker (14) produced milk which acquired an oxidized or rancid flavor by changing the feed of the cows. It was believed that a carotene deficiency in the feed was the cause of the defective flavor. Similar observations were reported by Brown, Thurston and Dustman (5). Sharp, Trout and Guthrie (42) showed that addition of ascorbic acid to the feed or to the milk greatly reduced the susceptibility of the milk to a rancid, oxidized flavor. Guthrie and Brunker (14) noted that apparently no relationship existed between the breed, lactation period or age of the cow and the development of oxidized flavor in the milk. A variation, however, was noted in the intensity of the oxidized flavor which developed in milk from different quarters of the udders of many cows. It seemed that dry feeds were not the sole cause of the development of this flavor. Brown, Thurston and Dustman (5) found that there was considerable variation among individual cows with respect to the tendency for oxidized flavor to develop in their milk. In general, dry feeding tended to increase oxidized flavor, while fresh pasture feeding tended to decrease it. They found that when cows on dry feed were given daily rations of ascorbic acid, the susceptibility of their milk to oxidized flavor was greatly reduced;

they suggested that a daily ration of ascorbic acid be given to cows.

Hammer and Cordes (18) observed that sunlight produced a distinct "off" flavor in milk. Taints of this kind developed more rapidly in pasteurized than in raw milk and were not due to the presence of enzymes or bacteria.

In studying oxidized flavor in milk, Sharp, Trout and Guthrie (42) found that enzymes, copper and oxygen all aided in its development. Of these factors, the enzymes inherent in milk were the most important. Copper catalyzed the action of the enzymes much more than it catalyzed the oxidation itself. In their efforts to prevent the development of this flavor, they employed ascorbic acid which tended to inhibit the development of oxidized flavor in the presence of copper. The ascorbic acid itself, however, was oxidized by the enzyme. The destruction of the ascorbic acid was proportional to the development of oxidized flavor. Sharp, Trout and Guthrie suggested that the effects of time, temperature, heating and addition of copper on the development of oxidized flavor and on the oxidation of ascorbic acid were proportional. Later, Krukovsky and Sharp (25) concluded that dissolved copper caused no inactivation of lipase in normal whole milk in the absence of dissolved oxygen. However, normal milk lipase was susceptible to inactivation by dissolved oxygen, and this inactivation was accelerated by heat and by dissolved copper.

Ketonic Rancidity

A peculiar type of rancidity, with a characteristic odor, found in palm oils and butter fat is attributed to the presence of ketones rather

than of aldehydes or esters during the decomposition. Anderson and Triebold (1) observed that the ketones were produced by particular groups of microorganisms. These were molds of the genus Penicillium and of the genus Aspergillus acting on fat in the presence of moisture and nitrogenous material. Lea (32) believed that the joint presence of strongly lipolytic organisms and certain ketones probably favored the production of ketonic rancidity. Similar defects developed in milk or cream owing to the activity of lipolytic organisms. He pointed out that many of the molds might attack the casein and cause a deterioration in flavor which was partially due to products of protein degradation.

Hydrolytic Rancidity

Lipolytic microorganisms bring about the break-down of fats by the secretion of lipases. The action is hydrolytic and is the cause of the rancidity. In this type of rancidity there is hydrolysis of the glycerides with the liberation of free fatty acids and glycerol. Such a change is of special interest in dairy products because of the liberation of butyric and caproic acids with their characteristic flavors and odors. Triebold (43) reported that the liberation of small amounts of the higher fatty acids did not appreciably affect the flavor and odor of fat.

Hood and White (20) found that rancid flavors developed in cheddar cheese when undesirable organisms were introduced into the milk. They concluded that bacteria which produced rancid flavors in cheese entered the milk through improperly sterilized equipment, poor starters, contaminated water and lack of suitable factory sanitation. Rancid flavors in cheese

were never noted when the quality of the milk was satisfactory and proper factory sanitation prevailed.

Orla-Jensen (38) demonstrated that microorganisms were able to hydrolyze butter fat. He showed that the characteristic odor of rancid butter was due to the action of organisms and resulted from the liberation of volatile fatty acids. He believed that light and oxygen were minor factors in the development of rancidity, except as the oxygen influenced the growth of the organisms splitting the fat. He pointed out that contamination with organisms from air, wash water and dairy equipment might bring about rancidity in such dairy products as butter.

Browne (6) concluded that rancidity in butter was the result primarily of the activity of bacteria in the entire product, with its lactose, casein and other constituents serving as bacterial food. He did not believe that pure fat alone supported the growth of organisms. The statement often made that rancidity in whole butter is produced by the action of butyric ferments on the fat with the formation of butyric acid was considered unwarranted by Browne. He thought it more likely that lactic acid was converted into butyric acid, causing the characteristic odor of rancid butter. Hammer (18) reported that microorganisms constitute the most important cause of hydrolysis of butter fat. He believed that these organisms commonly are present in raw cream. Usually they are easily destroyed by pasteurization and in this way the defect is readily controlled.

Lea (32) observed that a rancid condition in fats readily resulted from contamination with powerfully lipolytic organisms. He noted that butter, for example, became rancid through the action of Oidium lactis, Cladosporium butyri and strains of Pseudomonas, Achromobacter and yeasts.

Long and Hammer (34) noted that frequently an organism which attacked protein also hydrolysed fat. In some cases the lipase was retained within the bacterial cell, but more often it was found in the surrounding medium. A number of methods have been suggested for investigating the action of these organisms. Most of the methods involve growing the organisms on petri dishes of agar containing fat. Any diffusion of lipase from the organisms is evident by the formation of opaque spots around the colony. Various investigators have found that the addition of indicators to the medium aids in detecting lipolysis. Still another method of determining lipolysis consists of titrating the free fatty acid produced in a fatty medium.

General Considerations

An interesting type of spoilage was encountered by Hiscox and Christian (19) in commercial sterilized milk. The defective product had a characteristic "carbolic taint". It seemed apparent that both ketonic rancidity of the fat and break-down of protein were involved. They found that the causative organism was a facultative, anaerobic spore-former. Propagated by laboratory methods, the spore form was rapidly replaced by the vegetative form.

According to Davis (9), observations on the oxidation-reduction potential of ripening cheddar cheese would not only enable investigators to measure the number of viable organisms present but their reducing intensities and capacities as well. He suggested that the growth of extraneous organisms could be controlled by any mechanism capable of adjusting the poten-

tial of cheese. He pointed out that the oxidation-reduction potential of normal cheddar cheese followed a well-defined path within certain limits, and any variations from normal indicated the formation of undesired substances or the non-formation of desired substances. Lea (32) noted that the oxidation-reduction potential was one of the major factors concerned with the development of rancid flavor in cheese.

Lipase is always present in raw milk and cream. It can hydrolyze milk fat into free fatty acids and glycerol. Rice and Markley (40) found that milk could be tested for lipase by adding a high concentration of sucrose. This prevented the growth of bacteria but did not alter the action of the lipase. These investigators noted that when the raw milk-sucrose mixture was added to boiled cream a rancidity developed in 3 to 30 days at 38.0° C. to 40.0° C. They believed that much of the rancidity noted in butter and cheese was due to the lipase in milk. The rancid odor was attributed to butyric acid. Lea (32) pointed out that the action of the lipase might be obscured because the rate of hydrolysis was usually slow and was frequently overshadowed by that of the lipolytic enzymes produced by bacteria.

Mattick and Kay (35) noted that an enzyme which hydrolyzed tributyrin was present in all the samples of fresh cows' milk studied. The enzyme was present in the aqueous portion of the milk rather than in the fat. It was found in highest concentrations in the colostrum, and showed no sign of increasing toward the end of the lactation period. Palmer (39) found an abnormal amount of lipase present in the bitter milk of advanced lactation. The bitter or rancid flavor was not present when the milk was drawn and could not be transferred to normal milk. He believed that abnormal amounts of lipase were responsible for the flavor since milk fat was hydrolyzed quite

rapidly, even at low temperatures. He suggested that the liberated fatty acids contributed in large measure to the bitter flavor and rancid odor.

Dorner and Widmer (10) studied the development of rancidity in homogenized raw milk. They noted a marked difference in the flavors developing in homogenized milk as compared with untreated raw milk. The rancidity produced in the homogenized milk had a sharp, bitter flavor while that of the unhomogenized milk was of a mild, aromatic type. They believed that the rancidity produced by homogenization was probably due to the break-down of the fat and the setting free of volatile acids. Aseptically drawn milk became rancid when it was homogenized as readily as did ordinary raw milk, which shows that enzymes normally present in milk were probably responsible for the rancidity. Trout, Halloran and Gould (44) also observed that rancidity always developed in homogenized raw milk. They noted that the rancidity developed more rapidly as the pressure of homogenization was increased. Pasteurization of the milk prior to or immediately following homogenization inactivated the causative agent of rancidity.

According to Krukovsky and Sharp (22), the rate of lipolysis of milk fat was influenced by a number of activation procedures. These included (a) homogenization, (b) shaking and (c) cooling, warming and cooling, all of which brought about an increase in fat surface. When the temperature of the resurfaced fat was raised the rate of lipolysis increased, whereas with normal fat globules the rate of lipolysis increased as the temperature was lowered. The resurfaced fat globules showed no further increase in lipolysis due to cooling, warming and cooling. However, when raw milk or raw cream was cooled, warmed and cooled, the rate of lipolysis increased.

Fat is not normally utilized as readily by microorganisms as carboby-

drate and protein. Nevertheless, some molds, yeasts and bacteria are able to satisfy their carbon and energy requirements from this source. Numerous organisms have been successfully grown on artificial media containing only fat or fatty acid and mineral salts, the latter including an ammonium salt or nitrate as a source of nitrogen.

Lamm (31) observed that Penicillium glaucum and Oidium lactis grew in media containing acetic, butyric, caproic and caprylic acids. Distillations indicated that the organisms were capable of utilizing these acids. Bryant (7) also showed that some of the lower fatty acids were utilized by certain molds. Triebold (43) observed that certain molds were able to utilize some of the lower fatty acid with the formation of methyl ketones. This gave rise to ketonic rancidity when butyric, caproic, caprylic and capric acids were attacked.

Long and Hammer (34) studied the action of a number of cultures of Streptococcus lactis on cottonseed oil. They found that none of the strains showed any evidence of attacking this fat, but most of them hydrolysed tripropionin. Tributyrin was less easily hydrolysed by these organisms than tripropionin. Schreiber (41) concluded that pure butter fat was not a food for microorganisms, but that a number of bacteria were capable of breaking it down when other food was present. After the fat was broken down, it was utilized by the bacteria.

Ayres, Rupp and Johnson (3) isolated a group of organisms from milk and water which used citric acid and other organic acids as sources of carbon. Some organic acids, such as g-butyric, g-valeric and caproic, were split into lower acids, which in turn were used in the formation of bicarbonates or carbonates. The author concluded that the organisms obtained

their carbon most readily from the methyl radical. Coolhaas (8) found that certain bacteria were able to use a number of salts of fatty acids and form simpler salts. Kaser (24) noted that only one of fifty strains of the colon-aerogenes group grew well in a medium in which sodium butyrate, n-valeric acid, iso-valeric acid or n-caproic acid was the sole source of carbon.

In studying the action of microorganisms on fats, Jensen and Grettie (21) noted that certain strains of bacteria produced two kinds of enzymes: lipases and oxidases. These appeared to be responsible for the rapid development of both free fatty acids and oxidation products. The lipolytic action paralleled oxidative rancidity of so-called chemical or light origin. The methods by which free fatty acids or their salts are decomposed were thought to be analogous to the process of fat-metabolism in higher animals.

Heave and Buswell (26) observed that organisms growing under anaerobic conditions on media containing fatty acids converted the latter almost quantitatively into carbon dioxide, hydrogen and methane. They noted that with the exception of acetic acid the degradation of the fatty acids yielded carbon dioxide in excess of the carboxyl group. They believed the additional oxygen came from water. The amount of water which was needed increased as the length of the carbon chain increased. In weighing the gaseous products they found a direct correlation between weight of gas produced and length of carbon chain. The weight of the gas increased as the length of the chain increased.

Keuts (12) studied the ability of certain lipolytic organisms to hydrolyze milk fat in the presence of lactic acid. He found that most of these

organisms were inhibited somewhat by the growth of butter culture organisms in cream but that some grew well in cream containing sufficient added lactic acid to give a titratable acidity of approximately 1.0 per cent. Some species caused lipolysis in cream with an acidity of more than 2.0 per cent.

Fouts (13) also studied the ability of several lipolytic organisms to utilize certain salts of the lower fatty acids as sole sources of carbon. He reported that Oidium lactis, Mycobacterium lipolyticum, Pseudomonas fluorescens, Achromobacter lipolyticum and Alcaligenes lipolyticus varied greatly in their abilities to utilize sodium butyrate, calcium butyrate, calcium caproate and calcium caprylate. Some of the organisms grew well when the fatty acid salt was the sole source of carbon, while others grew poorly or not at all. Fouts pointed out that the utilization of salts of the fatty acids in a synthetic medium did not necessarily prove that these compounds were destroyed in butter and cream. He emphasized, however, that there was a possibility that the organisms present in certain dairy products might utilize fatty acids.

Little is known, specifically, concerning rancidity in cheddar cheese. However, it seems likely that rancidity occurs in this type of cheese in much the same manner that it occurs in other dairy products.

Lane and Hammer (29) noted that a rancid flavor regularly developed very early in cheddar cheese made from homogenized raw cream added to raw skim milk. As the cheese ripened the rancid flavor diminished and finally disappeared. Following disappearance of the rancidity, a flavor characteristic of old cheese was noted. The disappearance of the rancid flavor was attributed to unknown factors.

Later, Lane and Hammer (30) observed that the addition of desiccated,

bovine mammary tissue, or a water extract of it, to pasteurized milk to be made into cheddar cheese sometimes produced a rancid flavor in the very young cheese. As the cheese aged the rancid flavor disappeared. These investigators observed that cheese made with mammary tissue usually developed "cheddar" flavor more rapidly than control cheese and also had a more desirable body and texture.

Lane and Hammer (27) did not find butyric acid prominent in the odor of cheese fat or material distilled from cheese. They suggested that it was possible that butyric acid in cheddar cheese might be changed to other compounds by microorganisms.

METHODS

Preparation of Cheddar Cheese Samples For Bacteriological Examinations

Cheese samples were taken with a sterile trier and 1 gram portions were weighed on sterile parchment paper by means of a sterile spatula. Each portion was then transferred to a sterile mortar and ground for several minutes with 9 ml. of a sterile 2.0 per cent aqueous solution of sodium citrate, using a sterile pestle. The emulsion in the mortar was considered to be a 1 to 10 dilution of the cheese. Other dilutions were made from this in 9 ml. water blanks. It was found that the best emulsion was obtained by warming the mortar, pestle and the sodium citrate solution before using; this was done by placing them in a 37.0° C. incubator for about 1 hour.

Determination of the Numbers of Total Bacteria and of Lipolytic Bacteria in Cheddar Cheese

The numbers of total bacteria and of lipolytic bacteria in samples of cheddar cheese were determined on smeared plates (26) of meat infusion agar to which had been added 0.5 per cent sterile natural fat emulsion. The fat emulsion was prepared by adding 3 grams of butter fat to 100 ml. of 0.5 per cent agar and sterilizing in a 6 ounce screw cap bottle. After sterilization the fat was well dispersed by vigorous shaking of the bottle. The fat emulsion was added to the agar just before the plates were poured, care

being taken to avoid formation of bubbles in the agar. When the plates had solidified, they were stored in the ice chest until used. Plates freshly made did not give as good results as those that had been stored for a day or two because the surface of the agar was too soft. By means of sterile pipettes, 0.1 ml. of each cheese dilution was placed on the surface of an agar plate. Sterile glass rods with bent ends were used to smear the cheese dilution over the surface of the agar. The plates were incubated at 21.0° C. for 7 days. Counts were then made and the numbers of total bacteria and of lipolytic bacteria per gram of cheese were calculated. The lipolytic colonies on a plate were detected by the opaque appearance of the fat globules around the colonies. This distinguishing characteristic was reported by Long and Hammer (34) who also observed that warming the plates to 45.0° C. before examination aided in detecting lipolytic colonies since butter fat is somewhat opaque at room temperature.

Procedure Used in Attempts to Isolate Organisms Capable of Using Sodium Butyrate as a Sole Source of Carbon

In order to determine whether cheddar cheese contained organisms capable of using butyric acid, 0.1 ml. quantities of various dilutions of cheese emulsion were inoculated into tubes of a synthetic medium in which sodium butyrate was the sole source of carbon. Sodium butyrate was used because even low concentrations of butyric acid are toxic to many organisms. The medium used was prepared according to the general formula of Ayres, Rupp and Johnson (3) and had the following composition:

Sodium ammonium phosphate	2.0 grams
Potassium chloride	0.1 gram
Salt of fatty acid	5.0 grams
Distilled water	1000.0 ml.

For the purpose of simplicity this medium will be referred to hereafter as the Ayres medium.

Six ml. portions of the medium were dispensed into cotton-plugged test tubes and sterilised in the autoclave. The tubes of medium were then inoculated with various dilutions of cheese emulsion and incubated at 21.0° C. Growth usually was visible after 4 or 5 days. Pure cultures of organisms capable of growing in the Ayres medium were isolated by streaking material from tubes showing growth on plates of tomato juice agar and picking colonies after 24 hours at 21.0° C.

The tomato juice agar was prepared according to the formula used by Lane and Hammer (26). Solution A consisted of 400 ml. of clear juice obtained from canned tomatoes by filtering through cheesecloth; it was adjusted to a pH of 7.0 by addition of sodium hydroxide. Solution B consisted of 10 grams of Beato-peptonised milk, 10 grams of Beato-peptone and 20 grams of agar dissolved by heating in 600 ml. of distilled water. Solutions A and B were mixed, filtered, dispensed in bottles and the finished medium sterilised in the autoclave at 15 lb. pressure for 20 minutes.

Procedure Used in Studying Utilisation of Sodium Butyrate by Organisms

In order to determine whether the organisms isolated from cheddar cheese were capable of utilising sodium butyrate, 50 ml. portions of the Ayres medium were placed in bottles with screw caps and sterilised in the

autoclave. Two bottles of the medium were inoculated with each of the organisms to be studied and incubated at 21.0° C. After 14 and 30 days the contents of a bottle were placed in a Kjeldahl flask. The bottle was rinsed with 15 ml. of boiled distilled water and this was added to the Kjeldahl flask. Two ml. of 5 N/1 sulfuric acid was added to each flask to free any remaining fatty acid from the salt. The flask was placed on a distilling apparatus and heated until 50 ml. of distillate was obtained. The distillate was titrated with N/10 sodium hydroxide, using phenolphthalein as an indicator. Fifty ml. bottles of medium that had not been inoculated were treated in the same manner as the inoculated bottles and served as controls. It was assumed that a decrease in yield of volatile acid was due to utilization of the butyrate by the organism that had been growing in the medium.

Procedure Used in Manufacture of Cheddar Cheese

The milk used for making experimental cheese was considered to be of good quality. It contained about 3.5 per cent fat and had a desirable flavor and odor. When pasteurized milk was used for cheese making, it was pasteurized at 145° F. (62.8° C.) for 30 minutes.

Most of the cheese was made in a vat constructed in such a way that it contained five compartments (25). Each of the compartments was independent of the others and had a capacity of 50 lb. of milk. Thus, simultaneously five lots of cheese were made under essentially identical manufacturing conditions. Additional lots of cheese were made in sterilized "shotgun" cans and in cheese vats having a capacity of 300 lb. of milk.

Except for the size of the curd knives used, the cheese was made ac-

cording to the method of Lane and Hammer (26). Each lot of milk was inoculated with 1.5 to 2.0 per cent commercial cheese culture containing 0.8 to 0.9 per cent acid, calculated as lactic acid. The milk was ripened about 30 minutes at a temperature of approximately 80° F. (26.7° C.) after which the temperature was adjusted to 86° F. (30.0° C.). Commercial cheese color and rennet were added at the rate of 1 and 3 ounces per 1,000 lb. of milk, respectively. After about 25 minutes the curd was cut into 1 inch cubes with a curd knife, and then cooked slowly to 104° F. (40.0° C.). The curd was held at this temperature until the acidity of the whey reached 0.15 to 0.16 per cent and the desired firmness of the curd was obtained. The curd was then dipped and cheddared until 0.5 to 0.6 per cent acidity in the whey was reached. After milling, the curd was forked for about 30 minutes, and 3.0 per cent salt was added. Not less than 45 minutes were required to dissolve the salt, after which the curd was rinsed with scalding water and placed in the hoops. The cheese was pressed under continuous pressure for about 18 hours, put in a drying room at 45° F. (7.2° C.) for 48 hours, paraffined, and then placed in a curing room at 50° F. (10.0° C.).

EXPERIMENTAL

Bacteria in Cheddar Cheese with Reference to Their Lipolytic Activity and Ability to Utilize Sodium Butyrate

A possible explanation for the disappearance of the rancidity that develops in cheddar cheese made with certain procedures is that butyric and other lower fatty acids responsible for the condition are destroyed by bacteria in the product. In order to determine the presence of such organisms, the microflora of a number of cheddar cheese samples was investigated. The lipolytic organisms were given special attention in this connection since it generally is believed that some organisms which hydrolyze fat also attack the products of fat hydrolysis. Fouts (13) found that various lipolytic organisms destroyed sodium butyrate in a synthetic medium in which the butyrate was the sole source of carbon.

The numbers of total bacteria and of lipolytic bacteria were determined on 27 samples of cheddar cheese from various sources. Most of the samples were considered to be good cheese. Of the samples examined, 16 were made in Iowa, 7 in Wisconsin and 4 in Canada. The age of the Iowa and Wisconsin samples varied from 2 weeks to 6 months; data on the age of the Canadian samples were not available. Smeared plates were made in duplicate on meat infusion agar containing 0.5 per cent natural fat emulsion, using dilutions of 1 to 100, 1 to 1000, 1 to 10,000, 1 to 100,000, 1 to 1,000,000 and 1 to 10,000,000. The data are presented in Table I.

The total numbers of bacteria per gram of cheese, as determined by the

TABLE I. NUMBERS OF TOTAL BACTERIA AND OF LIPOLYTIC BACTERIA IN CHEDDAR CHEESE FROM VARIOUS SOURCES

Counts made on smeared plates of meat infusion agar containing 0.5 per cent natural fat emulsion and incubated at 21.9° C. for 7 days.

Sample	Origin of cheese	Age of cheese	Quality of cheese	Bacteria per gram of cheese	
				Total	Lipolytic
1	Wisconsin	3 mo.	good	132,000,000	400
2	Wisconsin	3 mo.	good	84,000,000	300
3	Wisconsin	3 mo.	good	180,000,000	400
4	Iowa	6 mo.	sl. rancid	480,000	600
5	Iowa	6 mo.	good	142,000	100
6	Iowa	6 mo.	fair	128,000	300
7	Wisconsin	3 mo.	poor	18,000,000	< 100
8	Iowa	4 mo.	good	600,000	400
9	Iowa	5 mo.	sl. rancid	80,000	100
10	Wisconsin	4 mo.	good	15,000,000	<< 100
11	Wisconsin	4 mo.	good	35,000,000	< 100
12	Wisconsin	4 mo.	good	16,000,000	300
13	Iowa	2 wks.	good	320,000	< 100
14	Iowa	2 wks.	good	620,000	100
15	Iowa	2 wks.	good	140,000	200
16	Iowa	1 mo.	good	900,000	200
17	Iowa	1 mo.	good	13,000,000	< 100
18	Iowa	1 mo.	good	560,000	100
19	Canada	unknown	rancid	40,000,000	300
20	Canada	unknown	rancid	30,000,000	200
21	Canada	unknown	rancid	12,000,000	100
22	Canada	unknown	rancid	700,000	200
23	Iowa	3 mo.	good	400,000	400
24	Iowa	3 mo.	good	460,000	100
25	Iowa	3 mo.	good	280,000	<< 100
26	Iowa	3 mo.	good	390,000	< 100
27	Iowa	3 mo.	good	430,000	200

smeared plate method, varied from 80,000 to 180,000,000. Most of the colonies appeared to be well developed and were easily counted when the total counts were made after 7 days' incubation at 21.0° C. Only plates having between 30 and 300 colonies were used for quantitative purposes. Generally, the counts on the Iowa cheese were considerably lower than those on the cheese from other sources. No information is available that would explain these lower counts. The total numbers of lipolytic bacteria per gram of cheese, as determined by the smeared plate method, varied from less than 100 to 600. There seemed to be no relationship between the numbers of lipolytic organisms in the cheese and the presence of rancidity; several of the samples examined were rancid, and the total numbers of lipolytic organisms in these samples were no larger than in some of the samples which were not rancid. There seemed to be no relationship between the total bacterial counts and the numbers of lipolytic organisms present.

Three predominant types of colonies were observed on most of the plates studied, regardless of the origin of the sample. These types showed the following general characteristics:

1. Smooth, raised, opaque, cream-colored colonies that under the microscope appeared to be gram-positive micrococci. Approximately 5.0 per cent of the colonies counted were of this type.

2. Small, smooth, opaque, white colonies that under the microscope appeared to be gram-positive micrococci. About 5.0 per cent of the colonies counted were of this type.

3. Small, smooth, blnish, dewdrop colonies that under the microscope appeared to be gram-positive streptococci. About 90.0 per cent of the colonies counted were of this type.

Other colonies that were noted infrequently were small, smooth, opaque, lemon-yellow colonies that under the microscope appeared to be gram-positive micrococci. Occasionally, a colony of this type exhibited lipolytic characteristics. Also observed were large, flat, irregular, white colonies that under the microscope appeared to be gram-positive rods. Some of these colonies showed lipolytic characteristics.

Several representative colonies of each of the observed types were inoculated into litmus milk and the milk incubated at 21.0° C. for several days. The micrococci produced only a slight acid reaction in the litmus milk, while the streptococci, as would be expected, produced a typical S. lactis reaction. The gram-positive rods produced an alkaline reaction and peptonized the milk.

In order to determine whether any of the organisms could utilize sodium butyrate as a sole source of carbon, a loop of each culture was transferred to tubes of the Ayres medium. No growth was visible in any of the tubes after incubating 14 days at 21.0° C., and it was assumed that the butyrate was not utilized. Similar inoculations were made into tubes of modified Ayres medium containing, in addition to the usual ingredients, 0.1 per cent glycine and also into tubes of the Ayres medium modified by the addition of 0.1 per cent peptone. No visible growth occurred during 7 days at 21.0° C. It appeared that none of the organisms isolated from cheddar cheese was capable of growing in the Ayres medium, even with the modifications used.

Organisms Obtained from the Ayres Medium Inoculated with Cheddar Cheese Emulsion

The failure of organisms isolated from cheddar cheese by the smeared plate method to utilize sodium butyrate suggested the inoculation of small quantities of cheese into the Ayres medium to determine whether uncultured species or combinations of species in the cheese might be capable of accomplishing butyrate utilization.

Samples of cheese from a number of sources were emulsified in the usual manner. After grinding each cheese, 0.1 ml. of the emulsion was inoculated directly into tubes of the Ayres medium. This quantity was the equivalent of 0.01 gram of cheese. Other tubes of the Ayres medium were inoculated with greater dilutions of cheese emulsion but the amount of material inoculated was always 0.1 ml. All tubes were incubated at 21.0° C. Growth regularly was observed when 0.01 gram of cheese had been added. Frequently, turbidity developed in the medium when 0.001 gram of cheese had been used for inoculation. After 5 days incubation, a loopful of medium from each tube showing growth was transferred to tomato juice agar plates which also were incubated at 21.0° C.

The colonies developing on the tomato juice agar were predominantly of one type. They were smooth, slightly raised, glistening and well developed. When observed with a hand lens the colonies had a rather characteristic granular appearance. It seemed logical to conclude that the organisms were capable of utilizing butyrate as a source of carbon since other organisms ordinarily present in cheese did not seem to grow. Further, a slight turbidity developed in the Ayres medium when it was inoculated with material from well-isolated colonies. Microscopically, the organisms were large

rods (0.8 to 1.3 by 4.0 to 8.0 microns) that appeared to be in pure culture. The cells were variable to the gram stain. However, when stained with carbol fuchsin an unusual structure often was noted. Each cell usually resembled two or more cocci that had merged together forming a large rod. Hereafter in this report this type of large rod will be referred to as the X type organism.

The X type organism regularly was obtained when more than 200 samples of cheddar cheese from various sources were inoculated into the Ayres medium. Most of the cheese studied was produced in Iowa and Idaho, the balance being manufactured in Wisconsin, Illinois, Oregon, New York, Washington, Nebraska and Canada; the source of three of the samples was unknown. The age of the cheese was not known in all cases but most of the samples were 3 to 6 months old.

Frequently, plates of meat infusion agar containing natural fat emulsion were smeared with 0.1 ml. of an emulsion of a cheese sample at the time the Ayres medium was inoculated. This was done to observe the types of organisms present in the samples. Very few colonies similar to those produced by the X type organism developed on these plates.

All strains of the X type organism isolated from the Ayres medium grew well on tomato juice agar and yielded abundant growth in 24 to 48 hours at 21.0° C. Plates poured with this medium were smeared with six different strains of the organism and placed in the cheese curing room, which was held at 50° F. (10.0° C.), to determine the effect of relatively low temperatures on their development. Fair growth was observed on most of the plates after 7 days and it seemed evident that the organism could grow at temperatures used for ripening cheese.

The lipolytic activities of the X type organism were tested by making transfers to plates of meat infusion agar containing 0.5 per cent natural fat emulsion, and also to plates of nile-blue sulphate agar, the latter being prepared according to the method of Kramer and Collins (17); butter fat was used in both types of media. None of the 28 strains of the organisms studied showed lipolytic activities on the media.

Cultures of the X type organism in litmus milk produced gas and became acid in reaction but did not coagulate when incubated at 21.0° C. Gas formation could be detected in the young litmus milk cultures but was not apparent in old cultures. The organism apparently is well suited to growth in milk because it was viable for several weeks in litmus milk cultures held at 21.0° C. Repeated transferring of a culture from litmus milk to litmus milk continued to produce the same cultural characteristics.

Further biochemical and cultural studies of the X type organism revealed that it produced a metallic sheen on Levine's eosin methylene blue agar, acid and gas in lactose broth and an unexplainable transition to a gram-negative rod conforming to the description of Escherichia coli recorded in Bergey's Manual of Determinative Bacteriology (4).

Disappearance of Rancidity Produced in Cheddar Cheese by the Addition of Butyric Acid to the Milk

Cheese made in stainless steel vats

As previously stated, rancidity in cheddar cheese that results from certain types of manufacturing procedures disappears as the product ripens. Presumably, the rancid flavor and odor are due to an accumulation of

butyric and other lower fatty acids which are set free by lipolytic enzymes acting on the milk fat. A study was made to determine whether rancidity produced in cheddar cheese by the addition of butyric acid would disappear during the ripening of the cheese.

Eight series of cheese were made. Each series consisted of two cheese, one being prepared from raw milk and one from pasteurized milk. Two to 5 ml. of butyric acid was added to each 50 lb. lot of milk just before the cheese culture was added. The milk was agitated vigorously while the butyric acid was being added to avoid coagulation of the milk at the point of contact with the acid and to insure even distribution of the acid. The treated milk was then made into cheese in the usual manner. A definite oder of butyric acid was noted in all lots of milk throughout the manufacturing process. During the first 14 days of the ripening, each cheese was examined several times to detect any disappearance of the rancid flavor and oder. Table II gives the results.

The first examination was made when the cheese was removed from the press (1 day), and a very rancid flavor and oder were noted in both the raw and pasteurized milk cheese. After the product had ripened 3 or 4 days there was some reduction in the rancid flavor and oder. In series 1, 5, 7 and 8 the rancidity had disappeared completely from the cheese made with pasteurized milk. After the experimental cheese had ripened 14 days, a slight flavor and oder of butyric acid could be detected in some of the raw milk cheese, but in two raw milk cheese and in all of the pasteurized milk cheese there was no evidence of rancidity. In general, the rancidity disappeared from the pasteurized milk cheese before it did from the raw milk cheese.

TABLE II. FLAVOR OF CHEDDAR CHEESE AT VARIOUS AGES WHEN MADE FROM RAW AND PASTEURIZED MILK CONTAINING ADDED BUTYRIC ACID

Two to 5 ml. of butyric acid was added to the 50 lb. of milk used in making each lot of cheese.

Series	Milk used	Degree of rancidity in cheese after		
		1 day	3 or 4 days	14 days
1	raw	very rancid	rancid	sl. rancid
	past.	very rancid	not rancid	not rancid
2	raw	very rancid	rancid	sl. rancid
	past.	very rancid	sl. rancid	not rancid
3	raw	very rancid	rancid	sl. rancid
	past.	very rancid	sl. rancid	not rancid
4	raw	very rancid	rancid	sl. rancid
	past.	very rancid	sl. rancid	not rancid
5	raw	very rancid	rancid	not rancid
	past.	very rancid	not rancid	not rancid
6	raw	very rancid	rancid	sl. rancid
	past.	very rancid	sl. rancid	not rancid
7	raw	very rancid	sl. rancid	not rancid
	past.	very rancid	not rancid	not rancid
8	raw	very rancid	rancid	sl. rancid
	past.	very rancid	not rancid	not rancid

At various times plates were also made with meat infusion agar containing natural fat emulsion to determine the numbers and types of organisms developing in each cheese. All the plates were incubated for 5 days at 21.0° C. The first bacterial counts made showed large numbers of typical S. lactis colonies. Usually no other types of organisms were observed.

Counts on the cheese after ripening 3 or 4 days showed large numbers of colonies that appeared to be of the X type. Comparatively few S. lactis colonies could be detected. Colonies of the X type were larger than those produced by S. lactis but they had the same bluish appearance. When these organisms were studied in detail they were found to be identical with the X type organisms that developed in the Ayres medium inoculated with cheddar cheese. In the case of the raw milk cheese practically pure cultures of these organisms were observed. A count of more than 400,000,000 of the bacteria per gram of cheese was noted in one case; no other types of colonies were observed on these plates. Pasteurized milk cheese also yielded a number of colonies of this type but not as many as the raw milk product. Usually other types of colonies also were present on plates made from pasteurized milk cheese. These included S. lactis and some micrococci.

When the cheese had ripened 14 days, bacterial counts showed a marked decrease in the numbers of the X type colonies developing on the plates, as compared with the numbers noted previously. In place of these, typical S. lactis colonies again were noted. This was particularly true of the raw milk cheese. The few colonies of the X type that developed in low dilutions were not typical, and seemed to be more common in cheese made from raw milk than in the cheese from pasteurized milk. With a complete disap-

pearance of rancidity in both raw and pasteurized milk cheese there was an almost complete disappearance of the X type organisms. Replacing these organisms was a more or less typical cheddar cheese flora.

Cheese made in sterilized "shotgun" cans

Four series of cheese were made in "shotgun" cans. The cans were used to avoid contamination while making the cheese. They were sterilized in the autoclave before the cheese making was begun, and the lids were kept on the cans during the manufacturing process. Twenty-five lb. lots of milk were used for the cheese. The milk was pasteurized in the cans by heating to 145° F. for 30 minutes. This was accomplished by placing the cans in a cheese vat filled with hot water. The temperature of the water was raised until the milk in each can reached 149° F.; after holding at this temperature, the milk was cooled with cold water to 86° F. Butyric acid was then added to the milk for each of the cheese in a series. The amounts of butyric acid used in series 1, 2, 3 and 4 were 1.0 ml., 1.5 ml., 2.5 ml. and 1.5 ml., respectively. The acid was well distributed in the milk by stirring with a sterile ladle. Each series consisted of three cheese; the milk for one cheese was inoculated with a cheese culture and the milk for the other two cheese with pure cultures of S. lactis. In all three cases the amount of culture used was 2.0 per cent. The cheese was then made in the usual manner. An odor of rancidity was observed throughout the manufacturing process. Care was taken during the whole procedure to avoid contamination. Table III presents the data.

Bacterial counts on the pasteurized milk were 80 per ml. for series 1, 55 per ml. for series 2, 35 per ml. for series 3 and 45 per ml. for series

TABLE XII. FLAVOR OF CHEDDAR CHEESE AFTER 1 DAY AND 7 DAYS WHEN MADE FROM PASTEURIZED MILK CONTAINING ADDED BUTYRIC ACID

Each lot of cheese was made from 25 lb. of milk pasteurized in a sterilized "shotgun" can.

Series	Bacteria per ml. of pasteurized milk	Butyric acid per 25 lb. of milk	Type of culture used	Degree of rancidity in cheese after	
				1 day	7 days
1	80	1.0 ml.	cheese culture	very rancid	not rancid
		1.0 ml.	S. lactis	very rancid	not rancid
		1.0 ml.	S. lactis	very rancid	not rancid
2	55	1.5 ml.	cheese culture	very rancid	not rancid
		1.5 ml.	S. lactis	very rancid	not rancid
		1.5 ml.	S. lactis	very rancid	not rancid
3	35	2.5 ml.	cheese culture	very rancid	not rancid
		2.5 ml.	S. lactis	very rancid	not rancid
		2.5 ml.	S. lactis	very rancid	not rancid
4	45	1.5 ml.	cheese culture	very rancid	not rancid
		1.5 ml.	S. lactis	very rancid	not rancid
		1.5 ml.	S. lactis	very rancid	not rancid

4, the counts being made on tryptone glucose extract agar with the plates incubated at 37.0° C. for 48 hours. The few colonies that developed on the medium appeared to be micrococci. When the twelve cheese were removed from the press, all of them were very rancid. Seven days later no flavor or oder of rancidity could be detected in any of them. Smeared plates were made at various times in the usual manner to determine the types of organisms in the products. The cheese made with cheese culture, as well as that made with pure cultures of S. lactis, showed a number of organisms of the X type when the cheese was 3 days old. The bacterial count of the pasteurized milk and the care taken in manufacturing the cheese make it seem unlikely that these X type organisms were present in the milk or entered as contaminants. With the disappearance of rancidity, the numbers of X type organisms decreased.

Effect of Rancidity on the Bacterial Flora of Cheddar Cheese Made From Raw and Pasteurized Milk

Bacterial flora of cheddar cheese made from raw and from pasteurized milk containing added butyric acid

Work reported earlier in this paper shows that the X type organism commonly was found in cheddar cheese made rancid by the addition of small quantities of butyric acid to the milk used in the manufacture. It was observed in one case that 1 gram of such cheese contained 400,000,000 organisms of this type.

In order to study in more detail the bacterial flora of cheddar cheese made from milk containing added butyric acid, six series of cheese were made. Each series consisted of two cheese, one made from raw milk and one

from pasteurized milk. From 3 to 5 ml. of butyric acid was added to each 50 lb. lot of milk used in the manufacture and resulted in a rancid flavor and odor in the fresh cheese.

Care was taken throughout the manufacture to eliminate as completely as possible all sources of contamination. The vats were washed carefully and treated with boiling water and live steam just before the milk was received. Only milk of the best quality available was used. The raw milk employed in making the cheese usually had a bacterial count of less than 10,000 per ml., and the pasteurized milk frequently had a count of less than 500 per ml. All counts were made on tryptone glucose extract agar with the plates incubated at 37.0° C. for 48 hours. Butyric acid was added to each lot of milk with a sterile pipette just before the addition of the cheese culture. The cheese was made in the usual manner.

At the time the cheese was removed from the press (1 day), bacterial counts were made on each lot, using smeared plates of meat infusion agar containing 0.5 per cent natural fat emulsion; the plates were incubated at 21.0° C. for 5 days. The counts showed that each cheese contained between 200 and 800 million organisms per gram. All the colonies observed in dilutions that could be counted were typical of those produced by S. lactis. Microscopically, the organisms in the colonies were gram-positive, short chain streptococci. When some of the colonies were transferred to litmus milk, a typical S. lactis reaction regularly resulted.

When the cheese was 2 days old, samples were again smeared on plates of the usual medium and the plates incubated at 21.0° C. for 5 days. The total numbers of colonies that developed were essentially the same as when the cheese was removed from the press, but the type of colonies had changed

somewhat. In most cases the colonies were much larger than those observed when the first examination was made. Frequently, the colonies observed on plates were 2 to 3 mm. in diameter. These colonies also developed much more rapidly on the agar than those produced by typical S. lactis. Often good growth was observed after 48 hours at 21.0° C. Except for size, the colonies were similar to those produced by S. lactis. Microscopic examination of smears made from a number of the colonies showed gram-positive, short chain streptococci, but their appearance was not typical of S. lactis. Chains of two or more cells seemed to have merged together to form large rods. In some cases single cocci seemed to have elongated until they appeared rod-shaped. A number of the colonies that were well isolated were transferred to litmus milk. After incubating 48 hours at 21.0° C., a typical S. lactis reaction was observed in most of the tubes, but in several cases gas was formed in the litmus milk.

The next set of samples was taken when the cheese was 4 days old. Smeared plates again were made on the usual medium and incubated at 21.0° C. for 5 days. Table IV presents data showing the numbers of the X type organism developing on the plates after incubating 48 hours. No other types of colony developed even when the plates were incubated 5 days. In some cases the total counts were not as large as when the cheese were taken from the press, but the numbers were not materially reduced. Smears made from well-isolated colonies showed large rod-shaped organisms that appeared to be the same as the X type organism. When a number of the colonies were transferred to tubes of litmus milk, a reaction similar to that produced by the X type organism was observed. All the lots of cheese were still somewhat rancid after 4 days, but those made from pasteurized milk were less

TABLE IV. NUMBERS OF THE X TYPE ORGANISM IN CHEDDAR CHEESE MADE FROM MILK CONTAINING ADDED BUTYRIC ACID

Counts made on smeared plates of meat infusion agar containing 0.5 per cent natural fat emulsion and incubated at 21.0° C. for 48 hours.

Series	Butyric acid per 50 lb. of milk	Milk used	Numbers of the X type organism per gram of cheese after	
			4 days	14 days
1	3 ml.	raw	190,000,000	<100
	3 ml.	past.	290,000	4,200
2	3 ml.	raw	126,000,000	1,200
	3 ml.	past.	160,000	22,000
3	5 ml.	raw	400,000,000	1,400
	5 ml.	past.	250,000	18,000
4	3 ml.	raw	18,000,000	4,000
	3 ml.	past.	90,000	1,500
5	5 ml.	raw	800,000,000	2,100
	5 ml.	past.	180,000	13,000
6	4 ml.	raw	48,000,000	23,000
	4 ml.	past.	14,000,000	26,000

rancid than those made from raw milk. Larger numbers of the X type organism were present in the raw milk cheese than in the pasteurized milk cheese. In the 4 day old cheese the counts on the raw milk cheese varied from 18,000,000 to 500,000,000 per gram while the counts on the pasteurized milk cheese varied from 90,000 to 14,000,000 per gram.

The next series of plates was made after the cheese was 14 days old. Table IV shows the numbers of the X type organism developing on smeared plates made with the usual medium and incubated at 21.0° C. for 48 hours. Comparatively few of the X type colonies had developed on any of the plates and no additional X type colonies were present after incubating 5 days. The X type colonies were replaced by a number of colonies that seemed to be more or less typical of the normal cheddar cheese flora. The total numbers of colonies developing were considerably less than the number observed when the cheese was first made. Very little rancidity could be detected in any of the samples. The flavor and odor of butyric acid, however, disappeared more rapidly from the pasteurized milk cheese than from the raw milk cheese. The numbers of the X type organism in the raw milk cheese and in the pasteurized milk cheese were essentially the same. In the raw milk cheese the count varied from less than 100 to 23,000 per gram, while in the pasteurized milk cheese the count varied from 1,500 to 26,000 per gram.

The additions of small amounts of butyric acid to raw milk used in making cheddar cheese brought about a definite change in the bacterial flora of the product when it was 4 or 5 days old. The change in flora was characterized by the replacement of S. lactis with the X type organism which persisted only as long as the rancid flavor and odor were noted in

the product. With the disappearance of the rancidity, the X type organism also largely disappeared. These changes made the flora of the cheese very different than that of normal cheddar cheese. Young cheddar cheese made without butyric acid always contained large numbers of S. lactis. Few organisms of the X type were ever observed on smeared plates made from young normal cheese. Since the colonies produced by the X type organism are similar to those produced by S. lactis on meat infusion agar containing natural fat emulsion, except for size, it seems likely that the X type organism is a variant of S. lactis.

Bacterial flora of cheddar cheese made from homogenized milk

Homogenization of raw milk greatly increases the surface of the fat globules and allows the natural lipase of milk a better opportunity to hydrolyze the fat. Milk treated in this manner develops a rancid flavor and odor, presumably because of the freeing of some of the lower fatty acids, such as butyric and caproic. In order to study further the effect of rancidity on the bacterial flora of cheddar cheese, milk used in the manufacture of various lots of cheddar cheese was homogenized.

Two hundred lb. of fresh, raw whole milk was homogenized using 2,000 lb. pressure at 90° F. (32.2° C.). The homogenized milk was then divided into four 50 lb. lots (lots 1, 2, 3 and 4). An additional 50 lb. lot of unhomogenized, fresh, raw whole milk from the same source (lot 5) served as a control. The five lots of milk were treated in the following manner:

Lot 1 was made into cheese without further treatment.

Lot 2 was held 2 hours before it was made into cheese; it had a definite rancid odor.

Lot 3 was at once pasteurized at 145° F. (62.8° C.) for 30 minutes and then was made into cheese.

Lot 4 was held 2 hours, pasteurized at 145° F. (62.8° C.) for 30 minutes and then made into cheese; it had a definite rancid odor.

Lot 5 was made into cheese without further treatment.

The data in Table V show the numbers of the X type organism in the different lots of cheese. After the five lots of cheese had ripened 7 days at 50° F. (10.0° C.), samples were taken and smeared plates were made on meat infusion agar containing natural fat emulsion. The plates were incubated 2 days at 21.0° C., and the numbers of the X type colonies then counted. The cheese made from homogenized milk showed only 130,000 X type organisms per gram, while the cheese made from homogenized milk that had been held 2 hours had a count of 11,340,000 X type organisms per gram. With the milk pasteurized immediately following homogenization the count of the X type organisms in the cheese was only 3,500 per gram, but with the homogenized milk held 2 hours before it was pasteurized the count of the X type organisms in the cheese was 640,000 per gram. Lot 5, which was the cheese made from unhomogenized, raw milk and used as a control, showed a count of 2,400 X type organisms per gram.

After the cheese had ripened 28 days samples were again taken and counts were made in the usual manner. A decrease in the number of X type colonies was noted in all cases. The cheese made from homogenized milk had a count of 80,000 X type organisms per gram, and the cheese made from homogenized milk that was held 2 hours had a count of 70,000 X type organisms per gram. The cheese made from pasteurized homogenized milk contained 1,500 X type organisms per gram, and the cheese made from the homogenized

TABLE V. NUMBERS OF THE X TYPE ORGANISM IN CHEDDAR CHEESE MADE FROM HOMOGENIZED MILK

Counts made on smeared plates of meat infusion agar containing 0.5 per cent natural fat emulsion and incubated at 21.0° C. for 48 hours.

Lot	Treatment of the raw milk	Numbers of the X type organism per gram of cheese after	
		7 days	28 days
1	homogenized	130,000	80,000
2	homogenized and held 2 hours	11,340,000	70,000
3	homogenized and pasteurized	3,800	1,500
4	homogenized and held 2 hours, then pasteurized	640,000	300
5	control unhomogenized, raw milk	2,400	1,500

milk that was held 2 hours before it was pasteurized had a count of 300 X type organisms per gram. The control cheese contained 1,500 X type organisms per gram. The results show that there was considerable variation in the numbers of the X type organism developing in the different lots of cheese.

After ripening 7 days the lots of cheese made from homogenized milk, from homogenized milk that was held 2 hours and from homogenized milk that was held 2 hours and then pasteurized were rancid; cheese made from homogenized milk that was pasteurized at once and from unhomogenized raw milk had no rancid flavor or odor. When the cheese had ripened 28 days only the lots made from homogenized milk and from homogenized milk held 2 hours were rancid; cheese made from milk that had been homogenized and held 2 hours before it was pasteurized were no longer rancid.

Additional lots of cheddar cheese were made from homogenized milk to study further the effect of rancidity on the bacterial flora. All the lots of cheese were made in stainless steel vats. The milk used in these experiments was of good quality and was homogenized at a pressure of 1,000 lb. per square inch and a temperature of 110° F. (43.3° C.). Two hundred lb. of milk was used for each lot of cheese. Eight lots were made from homogenized raw milk and four lots from milk that had been pasteurized at 145° F. (62.8° C.) and then homogenized.

Bacterial examinations were made of the lots of cheese when they were 2 days old. Smeared plates prepared in the usual manner showed that the lots of cheese contained between 300 and 500 million organisms per gram. All the colonies appeared to be typical *S. lactis*. When well-isolated colonies were transferred to litmus milk a typical *S. lactis* reaction resulted

after the tubes had been incubated 24 hours at 21.0° C. Microscopic examination of smears made from colonies showed gram-positive, short chain streptococci, but the cells seemed somewhat elongated.

Table VI gives additional data on the cheese. After the cheese had ripened 7 days the eight lots of cheese made from homogenized raw milk contained large numbers of the X type organism that were evident on the plates within 48 hours. The counts varied from 46 to 230 million per gram and the X type colonies seemed to be present in very nearly pure culture. The morphological and cultural characteristics of these organisms appeared to be identical with those observed previously. On the smeared plates the four lots of cheese made from homogenized pasteurized milk showed only typical S. lactis colonies. No X type colonies or types other than S. lactis were observed even when the plates were incubated 10 days at 21.0° C.

After 4 months all the lots of cheese showed comparatively few organisms on the smeared plates. However, several thousand organisms of the X type per gram of cheese were still noted on plates prepared from some of the lots of cheese made from homogenized raw milk. No organisms of the X type were noted on plates prepared from two lots of cheese made from homogenized pasteurized milk.

During the manufacturing process a rancid flavor and oder were detected in the cheese made from the homogenized raw milk. However, only a very slight rancid flavor and oder could be detected in the cheese made from the homogenized pasteurized milk. Presumably, the absence of a rancid flavor and oder in the cheese made from homogenized pasteurized milk was due to the destruction of the milk lipase by pasteurization.

Seven day old cheese made from homogenized raw milk had a definite

TABLE VI. NUMBERS OF THE X TYPE ORGANISM IN CHEDDAR CHEESE MADE FROM HOMOGENIZED RAW MILK AND HOMOGENIZED PASTEURIZED MILK

Counts made on smeared plates of meat infusion agar containing 0.5 per cent natural fat emulsion and incubated at 21.0° C. for 48 hours.

Lot	Treatment of the raw milk	Numbers of the X type organism per gram of cheese after	
		7 days	4 months
1	homogenized	46,000,000	
2	homogenized	84,000,000	20,000
3	homogenized	22,000,000	
4	homogenized	230,000,000	6,000
5	homogenized	85,000,000	24,000
6	homogenized	88,000,000	
7	homogenized	66,000,000	
8	homogenized	121,000,000	14,000
9	homogenized pasteurized	< 100	< 100
10	homogenized pasteurized	< 100	< 100
11	homogenized pasteurized	< 100	
12	homogenized pasteurized	< 100	

rancid flavor and odor; that made from homogenized pasteurized milk, however, had no rancid flavor or odor. When the cheese had ripened 4 months, that made from the homogenized raw milk was still somewhat rancid and the cheese made from homogenized pasteurized milk had become bitter.

Effect of Adding the X Type Organism to Milk Used In Making Cheddar Cheese

Effect of the X type organism in cheddar cheese made from pasteurized milk containing added butyric acid

Earlier in this paper it was pointed out that the X type organism was obtained regularly when the Ayres medium was inoculated with cheddar cheese. A similar organism was observed on smeared plates prepared from cheese that was rancid because of the addition of butyric acid to the milk used. Cheese that was rancid because it was made from homogenized raw milk likewise yielded large numbers of the X type organism when smeared plates were made. As the rancidity disappeared from the experimental cheese, the X type organism also tended to disappear. In view of the observations it seemed that the X type organism might be instrumental in bringing about the disappearance of the rancidity in the cheese. Accordingly, a study was made to determine whether the inoculation of a pure culture of the X type organism into milk, containing added butyric acid that was used in making cheddar cheese might be a means of eliminating rancidity.

Table VII presents data on four series of cheese, each cheese being made from a 50 lb. lot of pasteurized milk. In series 1, 2 and 3 the amounts of butyric acid used per 50 lb. of milk were 4 ml., 3 ml. and 4 ml., respectively; series 4 contained no butyric acid and served as a control. Each

TABLE VII. EFFECT OF THE X TYPE ORGANISM ON CHEDDAR CHEESE MADE RANCID BY ADDING BUTYRIC ACID TO PASTEURIZED MILK

One day old cheese made from milk inoculated with the X type organism contained from 2 to 9 million of these organisms per gram.

Series	Treatment of 50 lb. of milk	Strain of the X type organism added; 25 ml. milk culture per 50 lb. of milk	Numbers of the X type organism and degree of rancidity after			
			1 month		2 months	
			Organisms per gram	Rancidity	Organisms per gram	Rancidity
1	4 ml. butyric acid added	none	5,000	none	3,000	none
		M	8,000	none	5,000	none
		U	88,000	none	18,000	none
2	3 ml. butyric acid added	none	38,000	none	6,000	none
		L	40,000	none	22,000	none
		K	6,000	none	2,200	none
3	4 ml. butyric acid added	none	7,000	none	5,000	none
		J	12,000	none	18,000	none
		M	16,000	none	300	none
4	none (control)	L	30,000	none	16,000	none
		M	14,000	none	3,000	none

series included lots of milk that were inoculated with 25 ml. of a milk culture of the X type organism isolated from cheddar cheese, and series 1, 2 and 3 also included one lot of milk not inoculated with the X type organism. The X type organism and the cheese culture were added to the milk at the same time. The cheese was made in the usual manner.

Bacterial counts were made on the cheese using the usual medium and incubation conditions. The 1 day old cheese made from milk inoculated with the X type organism contained from 2 to 9 million X type organisms per gram. When the cheese was 1 month old the lots made with the X type organism added contained from 6,000 to 88,000 of these organisms per gram. The lots not inoculated with the X type organism showed from 5,000 to 33,000 per gram. Without butyric acid added to the milk, the counts of the X type organism varied from 14,000 to 30,000 per gram. After the cheese had ripened 2 months the lots made with the X type organism added contained from 300 to 22,000 per gram, while those made without the X type organism contained from 3,000 to 6,000 per gram. The lots made without added butyric acid contained from 3,000 to 16,000 per gram.

After 1 day all lots of cheese made with butyric acid added to the milk were very rancid, but at the end of 1 month the rancidity had disappeared. As would be expected, after ripening 2 months and 4 months, the cheese also was free of rancidity. After ripening 4 months the cheese had the flavor of well cured cheese. The cheese made without added butyric acid was not rancid at any age.

The observations show that after 1 month ripening all the lots of cheese were free of rancidity. There appears to be little or no advantage in adding the X type organism to cheddar cheese made rancid by adding

butyric acid to the milk used in the manufacture. The disappearance of rancidity in the cheese made from milk with butyric acid added but not inoculated with the X type organism may be explained by the bacterial counts which were obtained occasionally on some of the lots of cheese during the ripening process. Counts made after the lots of cheese had ripened 3 or 4 days frequently showed large numbers of the X type organism. Probably the added butyric acid stimulated the development of this organism. With the disappearance of the rancidity in the cheese, the organism diminished in numbers.

Effect of the X type organism in cheddar cheese made from homogenized raw milk

Several investigators have noted that a rancid flavor develops in cheddar cheese made from homogenized raw milk (29). In order to study the effect of adding the X type organism to cheddar cheese made rancid by homogenizing the raw milk, four series of cheese were made.

Table VIII presents data on the cheese. The milk was homogenized at 2,000 lb. pressure and 90° F. (32.2° C.). In each series, four out of the five 50 lb. lots of milk were inoculated with 25 ml. of a milk culture of the X type organism isolated from cheddar cheese, while one lot of milk was not inoculated with the X type organism and served as a control. The X type organism and the cheese culture were added to the milk at the same time and the cheese was made in the normal manner.

Bacterial counts on the cheese were made in the usual way. The 1 day old cheese made from milk inoculated with the X type organism contained from 2 to 11 million X type organisms per gram. When the cheese was 1

TABLE VIII. EFFECT OF THE X TYPE ORGANISM ON CHEDDAR CHEESE MADE RANCID BY HOMOGENIZING THE RAW MILK

One day old cheese made from milk inoculated with the X type organism contained from 2 to 11 million of these organisms per gram.

Series	Treatment of milk	Strain of the X type organism added; 25 ml. milk culture per 50 lb. of milk	Numbers of the X type organism and degree of rancidity after 1 month		2 months		4 months	
			Organisms per gram	Rancidity	Organisms per gram	Rancidity	Organisms per gram	Rancidity
1	homogenized at 2,000 lb. pressure and 90° F. (32.2° C.)	none	5,700	rancid	3,000	sl.rancid	2,600	very sl.rancid
		B	50,000	rancid	1,000	sl.rancid	< 100	very sl.rancid
		U	15,000	rancid	200	sl.rancid	600	very sl.rancid
		X	35,000	rancid	1,100	sl.rancid	1,100	very sl.rancid
		Y	25,000	rancid	1,200	sl.rancid	1,100	very sl.rancid
2	homogenized at 2,000 lb. pressure and 90° F. (32.2° C.)	none	20,000	rancid	4,000	sl.rancid	5,000	very sl.rancid
		J	5,515,000	rancid	160,000	sl.rancid	2,000	very sl.rancid
		K	22,000	rancid	1,400	sl.rancid	1,000	very sl.rancid
		L	130,000	rancid	20,000	sl.rancid	4,000	very sl.rancid
		M	4,000,000	rancid	14,000	sl.rancid	7,000	very sl.rancid
3	homogenized at 2,000 lb. pressure and 90° F. (32.2° C.)	none	12,000	rancid	3,000	sl.rancid	3,000	very sl.rancid
		O	120,000	rancid	5,000	sl.rancid	300	very sl.rancid
		P	240,000	rancid	3,000	sl.rancid	200	very sl.rancid
		Q	16,000	rancid	14,000	sl.rancid	6,000	very sl.rancid
		Z	7,000	rancid	6,000	sl.rancid	7,000	very sl.rancid
4	homogenized at 2,000 lb. pressure and 90° F. (32.2° C.)	none	6,000	rancid	4,000	sl.rancid	3,000	very sl.rancid
		Y2	12,000	rancid	15,000	sl.rancid	12,000	very sl.rancid
		Y4	9,000	rancid	6,000	sl.rancid	300	very sl.rancid
		X3	4,000	rancid	400	sl.rancid	600	very sl.rancid
		X4	4,000	rancid	4,800	sl.rancid	300	very sl.rancid

month old the cheese with the X type organism added contained from 4,000 to 4,000,000 of these organisms per gram; the cheese without the X type organism added contained from 5,700 to 20,000 per gram. After ripening 2 months the cheese with the X type organism added contained from 200 to 214,000 of the organisms per gram, while the cheese without the X type organism added contained from 3,000 to 5,800 per gram. After ripening 4 months the cheese with the X type organism added showed from less than 100 to 12,000 per gram, while the cheese made without the X type organism added showed from 2,600 to 5,000 per gram.

After 1 day all the lots of cheese were rancid and at the end of 1 month they were still rancid. After ripening 2 months all the lots of cheese were slightly rancid, and after ripening 4 months they were very slightly rancid.

The observations indicate that rancidity in cheddar cheese due to the homogenization of the milk disappears slowly. There seems to be little or no advantage in adding the X type organism. It can be assumed that the X type organism developed in the cheese to which it was not added in much the same manner that it did in cheese made from milk containing added butyric acid. With the disappearance of the rancidity, the organism again diminished in numbers.

Effect of the X type organism in cheddar cheese made from pasteurized milk containing added pancreatin

Data already presented show that in cheddar cheese rancidity produced by adding butyric acid to the milk or by homogenizing the milk disappears during ripening. Lane and Hammer (30) observed that a rancid condition in

cheddar cheese could be produced by adding pancreatin to the milk used in the manufacture. A study was made to determine whether rancidity in cheddar cheese produced by adding pancreatin to milk inoculated with the X type organism would disappear during ripening.

Table IX presents data on four series of cheese made from 50 lb. lots of pasteurized milk. The amounts of pancreatin used per 50 lb. of milk in series 1, 2, 3 and 4 were 1.0 gram, 0.1 gram, 0.5 gram and 0.5 gram, respectively. In each series, four out of the five 50 lb. lots of milk were inoculated with 25 ml. of a milk culture of the X type organism isolated from cheddar cheese, while one lot of milk was not inoculated and served as a control. The X type organism and the cheese culture were added to the milk at the same time. The cheese was made in the usual way.

Bacterial counts on the cheese were obtained in the customary manner. The 1 day old cheese made from milk inoculated with the X type organism contained from 2 to 7 million X type organisms per gram. When the cheese was 1 month old the lots with the X type organism added contained from less than 100 to 7,700 of these organisms per gram and the lots without the X type organism added contained from less than 100 to 1,100 per gram. After the cheese had ripened 2 months the lots with the X type organism added showed from less than 100 to 22,000 X type organisms per gram; the lots without the X type organism added showed from less than 100 to 900 per gram.

After 1 day all the lots of cheese were very rancid. At the end of 1 month, 2 months and 4 months all the lots were too rancid to judge.

The observations indicate that rancidity in cheddar cheese due to the adding of pancreatin to the milk does not disappear during the ripening period. There seems to be no advantage in adding the X type organism. Ap-

TABLE IX. EFFECT OF THE X TYPE ORGANISM ON CHEDDAR CHEESE MADE RANCID BY ADDING PANCREATIN TO PASTEURIZED MILK

One day old cheese made from milk inoculated with the X type organism contained from 2 to 7 million of these organisms per gram.

Series	Treatment of 50 lb. of milk	Strain of the X type organism added; 25 ml. milk culture per 50 lb. of milk	Numbers of the X type organism and degree of rancidity after			
			1 month		2 months	
			organisms per gram	rancidity	organisms per gram	rancidity
1	pasteurized and 1.0 gram of pancreatin added	none	<100	too rancid to judge	<100	too rancid to judge
		B	<100	too rancid to judge	<100	too rancid to judge
		U	<100	too rancid to judge	<100	too rancid to judge
		X	<100	too rancid to judge	<100	too rancid to judge
		Y	<100	too rancid to judge	<100	too rancid to judge
2	pasteurized and 0.1 gram of pancreatin added	none	1,100	too rancid to judge	900	too rancid to judge
		J	1,200	too rancid to judge	1,700	too rancid to judge
		K	5,000	too rancid to judge	6,000	too rancid to judge
		L	2,200	too rancid to judge	1,000	too rancid to judge
		M	7,700	too rancid to judge	5,800	too rancid to judge
3	pasteurized and 0.5 gram of pancreatin added	none	200	too rancid to judge	200	too rancid to judge
		O	1,100	too rancid to judge	1,600	too rancid to judge
		P	2,600	too rancid to judge	2,600	too rancid to judge
		Q	2,200	too rancid to judge	1,800	too rancid to judge
		Z	200	too rancid to judge	200	too rancid to judge
4	pasteurized and 0.5 gram of pancreatin added	none	200	too rancid to judge	200	too rancid to judge
		Y2	700	too rancid to judge	2,600	too rancid to judge
		Y4	200	too rancid to judge	1,800	too rancid to judge
		X3	600	too rancid to judge	12,000	too rancid to judge
		X4	600	too rancid to judge	22,000	too rancid to judge

parently the pancreatin produced such a toxic environment that most of the X type organisms were unable to live. Total counts showed that comparatively few organisms of any type existed in the cheese after ripening 1, 2 and 4 months; those that did survive were yellow micrococci and spore-forming rods.

Ability of the X Type Organism to Utilize Sodium Butyrate

Ability of the X type organism to utilize sodium butyrate in the Ayres medium and in enriched Ayres medium

In order to determine whether the X type organism associated with rancidity in cheddar cheese was capable of destroying butyric acid, pure cultures of the organism were inoculated into bottles of media containing sodium butyrate as a carbon source. Sodium butyrate was used as the sole source of carbon rather than butyric acid because the latter is toxic to many organisms. It was assumed that a decrease in yield of volatile acid when the medium was acidified and distilled, as outlined under METHODS, was due to utilization of the butyrate by the organisms that had grown in the medium.

The X type organisms obtained from the Ayres medium inoculated with cheddar cheese emulsions did not grow well when transferred to new tubes of the medium. Growth was obtained only when rather large amounts of the cultures were transferred and even then only slight turbidity developed. Sometimes as much as 0.2 ml. of the culture in the Ayres medium had to be transferred to fresh tubes to bring about visible growth.

In an effort to obtain better growth, the Ayres medium was enriched by

adding: (a) 0.1 per cent lactose, (b) 0.1 per cent peptone and (c) 0.1 per cent lactose and 0.1 per cent peptone. Several tubes of each medium were inoculated with a loop of litmus milk cultures of the X type organisms obtained from different lots of cheddar cheese. Tubes of the Ayres medium that had not been enriched were also inoculated for comparative purposes.

After incubating at 21.0° C. for 2 days turbidity was noted in all tubes of the enriched Ayres medium. Best growth occurred in the medium containing both 0.1 per cent lactose and 0.1 per cent peptone, and next best growth in the tubes containing 0.1 per cent peptone; no visible growth occurred in any of the tubes of the plain Ayres medium.

In order to determine whether the X type organisms actually were utilizing sodium butyrate, 50 ml. bottles of the Ayres medium and of enriched Ayres medium were inoculated, incubated at 21.0° C. for 14 days, and finally distilled. The Ayres medium was enriched by adding 0.1 per cent lactose, 0.1 per cent lactose and 0.1 per cent peptone, 0.1 per cent peptone, 0.2 per cent peptone, 0.5 per cent peptone and 1.0 per cent cheddar cheese emulsion. Five strains of the X type organism, designated as J, K, L, M and N, were tested in each medium and uninoculated bottles of the media served as controls. Little growth was observed in the plain Ayres medium until after 4 or 5 days; even then the growth was not abundant. However, turbidity developed in 2 days in all of the inoculated bottles containing lactose or peptone.

After incubating at 21.0° C. for 14 days, the contents of each bottle were acidified and distilled, and the volatile acidity was determined. Table X shows that no appreciable amounts of the butyrate were utilized by

TABLE X. ACTION OF THE X TYPE ORGANISM ON SODIUM BUTYRATE IN THE AYRES MEDIUM AND IN ENRICHED AYRES MEDIA

The values represent the ml. of N/10 sodium hydroxide required to neutralise the acid in 50 ml. of distillate obtained from 50 ml. of acidified medium.

Incubation at 21.0° C. for 14 days

Medium	Strain of the X type organism inoculated					Control
	J	K	L	M	N	
Ayres	21.2	20.3	19.6	20.7	20.8	20.1
Ayres + 0.1% lactose	21.3	20.0	21.3	20.8	21.1	21.3
Ayres + 0.1% lactose and 0.1% peptone	20.8	21.4	18.2	21.1	20.4	20.5
Ayres + 0.1% peptone	21.0	21.5	18.4	20.4	21.1	20.5
Ayres + 0.2% peptone	20.5	21.3	20.8	20.5	20.4	20.5
Ayres + 0.5% peptone	20.3	20.8	20.7	20.5	20.2	20.7
Ayres + 1.0% cheddar cheese emulsion	20.5	21.3	21.3	21.2	20.7	20.9

the organisms in any of the trials. Apparently the turbidity that developed in the enriched medium was the result of peptone or lactose utilization rather than butyrate utilization. The natural turbidity of the cheese emulsion made it difficult to observe any growth of the organisms in the bottles containing the emulsion, but the titrations indicate that very little or none of the butyrate was utilized. It is interesting to note, however, that all inoculated bottles of the Ayres medium without enrichment contained living organisms after 14 days since transfers from the bottles to tomato juice agar plates yielded good growth. This indicates that the organisms are able to live in the medium.

Since none of the organisms seemed to utilize sodium butyrate with the procedure used in the first trials, additional studies were made. A number of strains of the X type organism isolated from cheddar cheese were inoculated into bottles of the Ayres medium containing 0.125 per cent sodium butyrate as well as into bottles with the usual 0.5 per cent sodium butyrate. This was done because preliminary work had shown that smaller amounts of butyrate were very satisfactory for the isolation of the X type organism from cheddar cheese. It was believed that smaller amounts of butyrate might be less toxic to the organism. Inoculations were also made in bottles of the Ayres medium which had been acidified with lactic acid until the pH was 5.3, a reaction somewhat similar to that of cheddar cheese. Strains of the organism likewise were inoculated into the Ayres medium containing 0.5 per cent sodium butyrate and 1.0 per cent cheddar cheese emulsion, and into the Ayres medium containing 0.125 per cent sodium butyrate and 1.0 per cent cheddar cheese emulsion. All the bottles were incubated at 21.0° C. for 30 days, at which time the material was acidified and

distilled, and the distillates titrated.

The results, which are given in Table XI, show that little, if any, of the sodium butyrate was utilized by any of the strains. However, a slight turbidity developed in all of the inoculated bottles of the Ayres medium with the exception of those that had been acidified with lactic acid. Presumably, the acidified medium was so toxic that the X type organism was unable to grow. Since sodium butyrate was the sole source of carbon in the bottles showing visible growth, the organism presumably utilized the butyrate to a slight extent. All bottles containing 1.0 per cent cheese emulsion were naturally turbid so that the growth of organisms would not be evident.

Ability of 48 hour yeast extract broth cultures of the X type organism to utilize added sodium butyrate

The apparent inability of the X type organism to utilize sodium butyrate in the Ayres medium or in enriched Ayres medium does not eliminate the possibility of it destroying butyric acid in cheddar cheese. Various conditions and nutrients are present in cheddar cheese that are absent in the Ayres medium. It would seem that if large numbers of the X type organism could be grown in a suitable medium until most of the nutrients had been utilized, it might use added sodium butyrate as a source of carbon. In an effort to find more favorable conditions for butyrate utilization, yeast extract broth cultures were employed.

Large numbers of the X type organism were obtained by inoculating the organism into 2-liter flasks containing 1000 ml. of 0.1 per cent and 0.5 per cent yeast extract broth. After incubating at 21.0° C. for 48 hours,

TABLE XI. ACTION OF THE X TYPE ORGANISM ON SODIUM BUTYRATE IN THE AYRES MEDIUM AND IN MODIFIED AYRES MEDIA

The values represent the ml. of N/10 sodium hydroxide required to neutralise the acid in 50 ml. of distillate obtained from 50 ml. of acidified medium.

Incubation at 21.0° C. for 30 days

Medium	Strain of the X type organism inoculated							Control
	J	K	L	M	N	O	P	
Ayres containing 0.125% sodium butyrate	5.3	5.2	5.3	5.3	5.3	5.1	5.2	5.3
Ayres containing 0.5% sodium butyrate	20.8	19.5	19.9	20.8	20.3	20.6	21.2	20.9
Ayres + lactic acid (pH 5.3)	6.0	5.5	6.0	5.5	5.5	5.1	5.5	6.1
Ayres containing 0.5% sodium butyrate + 1.0% cheddar cheese emulsion	20.7	20.8	20.6	20.8	20.7	20.7	20.6	20.8
Ayres containing 0.125% sodium butyrate + 1.0% cheddar cheese emulsion	5.3	5.2	5.1	5.1	5.2	5.1	5.2	5.1

marked turbidity was observed in all the inoculated flasks.

When the flasks had been incubated at 21.0° C. for 48 hours, 10 ml. of sterile 10.0 per cent sodium butyrate solution was added aseptically to each of two 2-liter flasks containing 1000 ml. of 0.1 per cent yeast extract broth culture and also to an uninoculated 1000 ml. quantity of 0.1 per cent yeast extract broth which served as a control. In the case of the 2-liter flask containing 1000 ml. of a 0.5 per cent yeast extract broth culture, 20 ml. of sterile 10.0 per cent sodium butyrate solution was added, and 20 ml. of the butyrate solution also was added to an uninoculated 1000 ml. quantity of 0.5 per cent yeast extract broth. All the flasks were then incubated at 21.0° C. At intervals of 3, 7, 14 and 21 days, 100 ml. quantities of material were removed aseptically from the flasks, acidified, and distilled. When 100 ml. quantities of distillate had accumulated, they were titrated.

The data given in Table XII show that after 3 and 7 days at 21.0° C. very little of the added sodium butyrate had been utilized by the X type organism. However, at the end of 14 days considerable of the added sodium butyrate apparently had been utilized. The largest butyrate utilization occurred in the 0.1 per cent yeast extract broth cultures containing approximately 0.1 per cent sodium butyrate, but a relatively large amount of butyrate also was utilized in the case of the 0.5 per cent yeast extract broth culture containing approximately 0.2 per cent added sodium butyrate. At the end of 21 days the titration values in the case of the 0.1 per cent yeast extract broth cultures were about the same as after 14 days. Perhaps the butyrate was completely utilized after 14 days and the small titration values were caused by some compound other than liberated butyric acid. In

TABLE XII. ACTION OF THE X TYPE ORGANISM ON SODIUM BUTYRATE IN 48 HOUR YEAST EXTRACT BROTH CULTURES

The values represent the ml. of N/10 sodium hydroxide required to neutralise the acid in 100 ml. of distillate obtained from 100 ml. of acidified medium.

Medium	Inoculated with the X type organism				Control			
	After incubating at 21.0° C.							
	3 days	7 days	14 days	21 days	3 days	7 days	14 days	21 days
1000 ml. of 0.1% yeast extract broth + 10 ml. sterile 10% sodium butyrate added after 48 hours.	7.8	7.8	1.0	1.1	7.8	7.8	7.7	7.7
1000 ml. of 0.1% yeast extract broth + 10 ml. sterile 10% sodium butyrate added after 48 hours.	7.7	7.4	1.7	0.9	no control	no control	no control	no control
1000 ml. 0.5% yeast extract broth + 20 ml. sterile 10% sodium butyrate added after 48 hours.	15.0	12.0	6.5	4.0	14.8	14.6	14.7	14.4

the case of the 0.5 per cent yeast extract broth culture more utilization was evident than after 14 days.

Relationship of the X Type Organism to S. lactis

From the results of various investigators it is evident that bacterial plate counts on young cheddar cheese manufactured in the usual way commonly show large numbers of S. lactis colonies. Often the S. lactis colonies occur on the plates in nearly pure cultures. The total numbers of these colonies vary somewhat with different lots of young cheese, but frequently the numbers developing on meat infusion agar containing 0.5 per cent natural fat emulsion total 500,000,000 or more per gram of cheese. Although the numbers diminish somewhat during ripening, S. lactis colonies continue to be predominant for rather extended periods.

Various lots of cheese were made from milk containing small quantities of added butyric acid. When the cheese was 1 day old smeared plates showed that it contained approximately the same number of S. lactis colonies as cheese made without the added butyric acid. However, after the rancid cheese had ripened 3 or 4 days the bacterial flora changed very materially. The typical S. lactis colonies were no longer observed on smeared plates of meat infusion agar containing natural fat emulsion. Replacing the S. lactis colonies were the X type colonies. These observations were made many times on different lots of cheese made from milk containing added butyric acid.

Table XIII presents data on ten lots of cheddar cheese made from 50 lb. lots of milk containing from 2 to 5 ml. of added butyric acid. When

TABLE XIII. COMPARATIVE NUMBERS OF S. LACTIS AND THE X TYPE ORGANISM*, AFTER 1 DAY AND 3 OR 4 DAYS, IN CHEDDAR CHEESE MADE FROM RAW MILK CONTAINING ADDED BUTYRIC ACID

Counts made on smeared plates of meat infusion agar containing 0.5 per cent natural fat emulsion and incubated at 21.0° C. for 5 days.

Lot	Butyric acid per 50 lb. of raw milk	After ripening 1 day		After ripening 3 or 4 days	
		S. lactis per gram of cheese (dilution 1 to MM) (estimated)	Other organisms per gram of cheese (dilution 1 to MM)	S. lactis per gram of cheese (dilution 1 to MM)	The X type organisms per gram of cheese (dilution 1 to MM)
1	3 ml.	200,000,000 to 800,000,000	none	none	190,000,000
2	3 ml.	200,000,000 to 800,000,000	none	none	136,000,000
3	5 ml.	200,000,000 to 800,000,000	none	none	400,000,000
4	3 ml.	200,000,000 to 800,000,000	none	none	18,000,000
5	5 ml.	200,000,000 to 800,000,000	none	none	800,000,000
6	4 ml.	200,000,000 to 800,000,000	none	none	48,000,000
7	2 ml.	200,000,000 to 800,000,000	none	none	82,000,000
8	3 ml.	200,000,000 to 800,000,000	none	none	106,000,000
9	5 ml.	200,000,000 to 800,000,000	none	none	321,000,000
10	3 ml.	200,000,000 to 800,000,000	none	none	148,000,000

*Some of these counts have been used in a previous table.

the various lots of rancid cheese were 1 day old smeared plates were made on meat infusion agar containing natural fat emulsion, and after the plates had been held at 21.0° C. for 5 days the numbers and types of colonies were determined. Each lot of cheese had a bacterial count of 200 to 500 million typical S. lactis colonies per gram. In the dilutions used, very nearly all the colonies developing appeared to be S. lactis. After the same lots of cheese had ripened 3 or 4 days, smeared plates were made again, and the numbers and types of colonies noted. A complete change in the bacterial flora was evident. The typical S. lactis colonies could no longer be observed, and replacing them were the X type colonies. Counts of these colonies for the several lots of cheese varied from 18 to 500 million per gram.

Several lots of cheese made from milk to which butyric acid had been added were examined daily to observe, in more detail, the change in the bacterial flora. After the cheese had ripened 1 day the colonies that developed on plates of meat infusion agar containing natural fat emulsion incubated at 21.0° C. for 5 days appeared to be typical S. lactis colonies. After ripening 2 days the cheese yielded two types of colonies; one type suggested S. lactis and the other suggested the X organism. Microscopic examinations of the organisms in each type of colony showed coccus forms that seemed to be elongating and in some cases merging together. This condition was most commonly observed in the colonies suggestive of the X type. After the cheese had ripened 3 days the colonies that developed were more typical of the X type, and by the fourth day all the colonies that developed were of the X type, no typical S. lactis colonies being discernible.

It seemed very unlikely that S. lactis could be completely replaced

by a contaminant in such a short time. The acid reaction of the cheese, the low moisture content, the salt concentration and the temperature of ripening all tend to inhibit a rapid increase in most types of contaminating microorganisms. Still, when cheddar cheese was made rancid by adding a small amount of butyric acid to the milk used in the manufacturing process, the X type organisms were always observed. In normal cheese S. lactis usually constitutes a large percentage of the bacterial flora, even after several months of ripening. A possible explanation for the change in flora is that the X type organism is a variant of S. lactis.

As mentioned previously, small amounts of cheddar cheese emulsion inoculated into tubes of the Ayres medium always yielded the X type organism. Frequently, the quantity of cheese inoculated into the Ayres medium was as little as 0.001 gram. In such a quantity of cheese the only organism that ordinarily would be present in appreciable numbers is S. lactis, which also indicates that the X type organism developing from the cheese might be a variant of S. lactis. This general idea was strengthened when a number of normal cheese samples failed to show the X type colonies on smeared plates, while the same cheese inoculated into the Ayres medium produced the X type organism.

Procedures used in attempts to produce the X type organism from S. lactis

Since small quantities of normal cheddar cheese inoculated into the Ayres medium always yielded the X type organism and since the X type organism was observed in large numbers in young cheddar cheese (3 or 4 days old) made from raw milk containing added butyric acid, it seemed probable

that the organism might be produced by inoculating S. lactis into a medium containing butyric acid or sodium butyrate.

In an attempt to produce the X type organism from S. lactis in the laboratory, pure cultures of S. lactis were inoculated into tubes of the Ayres medium and the Ayres medium containing 0.1 per cent peptone. In every case little or no growth was observed after incubating at 21.0° C. for 7 days. The failure of pure cultures of S. lactis to grow in the media suggested that perhaps certain nutrients and conditions were necessary which had not been supplied. In cheddar cheese large numbers of organisms exist under a reduced oxygen tension. It was believed that larger inoculations of litmus milk cultures of S. lactis might supply certain nutrients needed, and produce conditions somewhat similar to those in cheese.

Nine strains of S. lactis were obtained by plating nine different butter cultures on tomato juice agar, incubating the plates at 21.0° C. for 5 days and inoculating well-isolated colonies of the S. lactis type into tubes of litmus milk. After these tubes had been incubated at 21.0° C. for 48 hours, reactions typical of those produced by S. lactis were observed. These cultures again were plated on tomato juice agar, and well-isolated colonies again were transferred to tubes of litmus milk. In several instances the procedure to insure purity of the culture was repeated five times. Before using these cultures they were transferred several times in litmus milk so that they would be active. Microscopic examinations indicated that each culture was pure.

One-tenth ml. of each of the nine 48 hour litmus milk cultures of S. lactis isolated from butter cultures was inoculated into tubes of the Ayres medium containing 0.1 per cent peptone. Three stock cultures of S. lactis

grown in litmus milk 48 hours also were inoculated into the medium. After incubating at 21.0° C. for 7 days, a slight turbidity was observed in several of the tubes. Transfers from some of these tubes of the Ayres medium to tomato juice agar developed a number of typical X type colonies. Not all cultures of S. lactis, however, showed the X type organism at the same time. Sometimes transferring 0.1 ml. of the Ayres medium culture containing 0.1 per cent peptone to fresh tubes of the same medium aided in the development of the X type organism. Certain strains, however, were so stable that a number of transfers and inoculations were necessary before the X type organism could be demonstrated. Better growth always seemed to take place in the Ayres medium containing 0.1 per cent peptone than in the Ayres medium alone.

In order to produce conditions somewhat similar to these occurring in ripening cheese, several tubes of the Ayres medium containing 0.1 per cent peptone were inoculated with S. lactis strains and incubated in a Novy jar in which the air was replaced by carbon dioxide. After incubating at 21.0° C. for 7 days, no growth was observed in any of the inoculated tubes. Transfers from each tube to plates of tomato juice agar failed to grow, and it was assumed that conditions for growth had been so unfavorable that the organisms had died.

While it was possible to produce the X type organism from S. lactis by inoculating relatively large quantities of 48 hour litmus milk cultures of S. lactis into the Ayres medium containing 0.1 per cent peptone, the time required was much longer than that required for the change to take place in rancid experimental cheddar cheese. In an effort to bring about the change in a shorter time by having the conditions more comparable to

these in cheese, studies were made using the Ayres medium containing small quantities of cheddar cheese emulsion. This medium was prepared by adding 0.1 per cent cheese, emulsified in citrate solution, to the medium, and sterilizing in the autoclave. Tubes of this medium were then inoculated with 0.1 ml. quantities of 48 hour litmus milk cultures of S. lactis.

After the cultures had incubated at 21.0° C. for 7 days, transfers were made from each tube to plates of tomato juice agar to observe any change in the type of organisms developing. Several tubes containing 0.1 ml. inoculations of S. lactis showed colonies somewhat similar to the X type organism, but they were not as well developed as those obtained from the Ayres medium containing 0.1 per cent peptone. It seemed that the addition of cheese emulsion to the Ayres medium offered no advantage over the Ayres medium containing 0.1 per cent peptone. A disadvantage in using the Ayres medium containing cheese emulsion is that it is turbid and growth in the tubes cannot be detected readily.

Further efforts were made to produce the X type organism by inoculating 0.1 ml. of a 48 hour litmus milk culture of S. lactis into litmus milk containing different quantities of sodium butyrate. Varying amounts of a sterile 10.0 per cent aqueous solution of sodium butyrate were added to tubes of litmus milk so that the concentrations of butyrate in the milk were 0.1 per cent, 0.2 per cent, 0.5 per cent, 1.0 per cent, 2.0 per cent and 3.0 per cent. These tubes were then inoculated with pure cultures of S. lactis and allowed to incubate at 21.0° C. for 14 days. In every case a typical S. lactis reaction developed in the milk. It was observed, however, that the S. lactis reaction developed most readily in tubes containing only small quantities of butyrate. Daily transfers to plates of tomato

juice agar showed only typical S. lactis colonies. Transfers of each culture to fresh tubes of litmus milk without butyrate also produced typical S. lactis reactions.

Additional attempts were made to produce the X type organism by inoculating, with 48 hour litmus milk cultures of S. lactis, tubes of litmus milk to which had been added varying amounts of sterile 1.0 per cent and 10.0 per cent aqueous solutions of butyric acid; the concentrations of the acid in the milk were 0.01 per cent, 0.1 per cent, 0.5 per cent and 1.0 per cent. With 0.01 per cent and 0.1 per cent concentrations of butyric acid in litmus milk, typical S. lactis reactions resulted after incubating at 21.0° C. for 2 or 3 days. After these cultures had incubated 14 days no X type organism could be detected. Transfers of these cultures to fresh tubes of litmus milk containing similar amounts of butyric acid also failed to bring about a change in the S. lactis. S. lactis failed to grow in tubes of litmus milk containing 0.5 per cent and 1.0 per cent butyric acid.

Tubes of litmus milk containing 2.5 per cent and 5.0 per cent sodium chloride were treated with varying amounts of butyric acid and sodium butyrate; the sodium chloride was added to the milk to produce concentrations somewhat comparable to those found in cheese. The tubes were then inoculated with S. lactis and incubated at 21.0° C. for several days. Little or no growth was observed in tubes containing 5.0 per cent sodium chloride and the higher concentrations of butyric acid or sodium butyrate. With the other concentrations of sodium chloride and butyric acid or sodium butyrate, a typical S. lactis reaction was observed. Microscopic examination showed that while the cells were somewhat elongated, they were still typical S. lactis.

Additional attempts were made to produce the X type organism in another medium by inoculating S. lactis into tubes of brain heart infusion* containing added butyric acid or sodium butyrate. This medium was selected because it contains growth accessory substances and seemed capable of supporting the growth of S. lactis. Furthermore, brain heart infusion contains only small amounts of sugar which seemed desirable since young cheese contains very little sugar. Varying amounts of sterile 1.0 per cent aqueous solution of butyric acid were added to 10 ml. tubes of the medium to give concentrations of 0.005 per cent, 0.01 per cent, 0.02 per cent and 0.1 per cent of the acid. Varying amounts of sterile 10.0 per cent aqueous solution of sodium butyrate also were added to 10 ml. tubes of the medium to give concentrations of 0.01 per cent, 0.05 per cent, 0.1 per cent and 0.5 per cent of the butyrate. A series of tubes containing the various concentrations of butyric acid and sodium butyrate were inoculated with milk cultures of S. lactis and incubated at 10.0° C., 21.0° C. and 37.0° C. to determine whether the temperature of incubation was a factor in the development of the X type organism. The tubes were examined daily and transfers were made to plates of tomato juice agar to observe any change in the type of growth developing. As would be expected, the tubes incubated at 21.0° C. and 37.0° C. showed growth after 2 days, but little or no growth was observed in any of the tubes incubated at 10.0° C. Best growth occurred in the tubes containing 0.005 per cent, 0.01 per cent or 0.02 per cent butyric acid. The tubes containing sodium butyrate did not support growth of S. lactis as well as the tubes containing butyric acid. None of the cultures showed the X type organism on the plates of tomato juice agar.

*This medium was a Bacto product.

Further efforts were made to produce the X type organism by adding varying amounts of butyric acid or sodium butyrate to tubes of brain heart infusion containing 2.5 per cent and 5.0 per cent sodium chloride. All the tubes were inoculated with 0.1 ml. of a 48 hour litmus milk culture of S. lactis and incubated at 21.0 C. After 3 days microscopic examinations of material from tubes containing 2.5 per cent sodium chloride and 0.02 per cent butyric acid showed rod-shaped organisms. Similar rods were observed in brain heart infusion containing 5.0 per cent sodium chloride and 0.5 per cent sodium butyrate. When these rod-shaped organisms were first observed they appeared to be two or more cocci merged together. Inoculation of the organisms into litmus milk resulted in a typical S. lactis reaction after incubating at 21.0° C. for 3 or 4 days. Microscopic examinations of the litmus milk cultures were made daily during the incubation to observe any change in the organisms. After 24 hours the rod-shaped organisms appeared to break up into two or more cocci which seemed to be typical S. lactis organisms. This was observed in several different tubes.

After the rod-shaped organisms had grown for several days in brain heart infusion containing 5.0 per cent sodium chloride and 0.5 per cent sodium butyrate they could no longer produce an S. lactis reaction in litmus milk. Instead, an alkaline reaction was observed in the milk. The rod-shaped organisms seemed to be stable and repeated transfers to litmus milk always produced an alkaline reaction. These observations suggest that several variants of S. lactis may result when S. lactis is subjected to certain conditions.

Numerous attempts were made to repeat the changing of S. lactis into rod-shaped organisms which produced an alkaline reaction in litmus milk,

but only a few were successful. The best results were obtained when 0.1 ml. of a 48 hour litmus milk culture of S. lactis was inoculated into brain heart infusion containing 5.0 per cent sodium chloride and 0.5 per cent sodium butyrate. Attempts to produce the rod-shaped organisms by inoculating S. lactis into tubes of brain heart infusion containing butyric acid or sodium butyrate and incubating in an atmosphere of carbon dioxide were unsuccessful.

Brain heart infusion, one-tenth its usual strength and containing 2.5 per cent sodium chloride, also was used in the studies. One-tenth ml. of sterile 1.0 per cent aqueous solution of butyric acid was added to 10 ml. tubes of the medium so that a concentration of about 0.01 per cent of butyric acid was obtained. Uninoculated tubes of the medium served as a control. Tubes of this medium inoculated with 0.1 ml. of a 48 hour litmus milk culture of S. lactis occasionally yielded the rod-shaped organisms producing an alkaline reaction in litmus milk when they failed to develop in regular concentrations of brain heart infusion. The rods developing in the low concentration medium were similar to those that were observed in brain heart infusion containing 5.0 per cent sodium chloride and 0.5 per cent sodium butyrate. The rod-shaped organisms usually reverted to cocci when inoculated into tubes of litmus milk and produced a typical S. lactis reaction. When the rods were inoculated into tubes of brain heart infusion containing 0.5 per cent sodium butyrate they became permanent rods and produced an alkaline reaction whenever they were inoculated into litmus milk. These organisms differ from the typical X type organisms primarily in their action on litmus milk.

Additional attempts were made to produce the X type organism by inoculating S. lactis into a number of different culture media containing varying

amounts of butyric acid or sodium butyrate. The following media were studied:

1. Peptonized milk.
2. Lactose broth.
3. Plain broth.
4. Proteose peptone broth.
5. Yeast extract broth.
6. Nutritive caseinate broth.
7. Thioglycollate medium.

In all the types of media 2.5 per cent sodium chloride was added to some of the tubes before varying amounts of butyric acid or sodium butyrate were added. The amount of inoculation used with each medium was usually 0.1 ml. of a litmus milk culture of S. lactis. In a number of cases, however, tubes of the medium also were inoculated with a loop of the litmus milk culture. All the tubes were incubated at 21.0° C. for several days, and were examined daily to note any change in the morphology of S. lactis. None of the seven media studied were as satisfactory as brain heart infusion from the standpoint of bringing about a variation in S. lactis. The only medium giving results somewhat comparable to these obtained with brain heart infusion was peptonized milk.

Different investigators have reported that variations in organisms can be brought about by adding such compounds as lithium chloride or phenol to culture media used for growing the organisms. Various attempts were made to stimulate the formation of rod-shaped organisms from S. lactis by adding small quantities of these compounds to culture media containing butyric acid or sodium butyrate. The addition of the compounds, however, did not seem to be of value in bringing a change in the morphology of S. lactis.

It is apparent from the work reported that the formation of the X type organism from S. lactis is dependent on the proper combination of environmen-

tal factors which as yet have not been fully evaluated.

Factors that may influence the development of the X type organism from S. lactis

The characteristics and general conditions of the S. lactis cultures seemed to be important factors in the formation of the X type organism in media containing butyric acid or sodium butyrate. Some strains of S. lactis were more capable of changing than other strains. There was no uniformity in the time required for the different strains of S. lactis to change. The X type organism was obtained most regularly from an S. lactis culture by inoculating the Ayres medium containing 0.1 per cent peptone with 0.1 ml. of a 48 hour litmus milk culture, and incubating at 21.0° C. for 7 days. There were many times, however, when such inoculations failed to yield the X type organism, and repeated inoculations and transfers into fresh tubes of the Ayres medium containing 0.1 per cent peptone were necessary before a change in morphology could be noted. On the other hand, small quantities of cheese inoculated into the Ayres medium regularly yielded the X type organism after incubating at 21.0° C. for only 4 or 5 days. It appears that the organism is conditioned by growing in cheese, and then is much more capable of changing than is the organism growing in an ordinary litmus milk culture.

It is probable that one of the reasons for lack of uniformity in the attempts to change S. lactis in the laboratory was the inability to reproduce the conditions existing in cheese. Certain enzymes or similar substances may be present in cheese that are factors in the formation of the X type organism. Such enzymes or similar substances may not be present in culture media, or, if originally present, may be altered by sterilization.

Furthermore, the S. lactis organisms in cheese may be conditioned by their environment. Very little carbohydrate is available as a source of carbon and energy. Other food materials may be in such forms that the organisms cannot utilize them readily. These and probably other factors may be of importance in the development of the X type organism.

Observations of several investigators on bacterial variation

As pointed out earlier, the X type organism seems to be a variant of S. lactis. It is not the purpose of this paper to give a detailed discussion of bacterial life cycles and mechanisms of variability. Hadley (15) and other investigators have made extensive reviews of the literature on microbic dissociation. However, certain references seem to be of special importance from the standpoint of the results obtained.

Löhnis and Smith (33) studied the life cycles of a number of bacteria. They believed that each life cycle is composed of several subcycles showing wide morphological and physiological differences.

Evans (11) reported that "a Streptobacterium, a filterable form, and an aerobic spore-bearing rod are phases in the life cycle of an organism cultivated from cases of epidemic encephalitis and from the so-called herpetic and encephalitic viruses." The metamorphosis was observed many times. The transformation from the Streptobacterium to a rod appeared to take place by elongation of cells. At least two ways by which cocci might be derived from rods were noted: (a) The rods swell and disintegrate, freeing granules which enlarge and multiply as cocci; (b) cocci may be derived from rods without disintegration by the development of coccoid bodies on the walls.

The coccoid bodies become detached when very small, and increase in size to ordinary cocci independent of the rod. Evans believed that complex life cycles, with metamorphosis accompanied by changes in habitat and biological behavior, should be expected in bacteria, rather than monomorphism.

Tunnicliff (45) observed bacillary forms in cultures from convalescing scarlet fever patients. These forms reverted to typical streptococci in sub-cultures.

Noguchi (37) concluded that the Bacillus bifidus communis has an aerobic phase in which it resembles Bacillus mesentericus. Numerous intermediate phases also were observed. He concluded that these "morphological and biological variabilities demand the utmost attention in order to interpret more intelligently the various phases of a given organism, constantly found in the stools of sucklings, and to avoid the artificial creation of two or more organisms from a single microbic type."

Hadley (18) believed that microorganisms are capable of responding to a changing environment by alterations in body state, both morphological and biochemical, in order to generate another and more stable type.

The observations cited suggest that bacterial variation is rather common, and should be expected under certain conditions. It seems likely that the X type organism develops from S. lactis in cheddar cheese made rancid by various manufacturing procedures because of the unfavorable environment. With the disappearance of the butyric and other lower fatty acids in the cheese, S. lactis presumably returns to its original form.

DISCUSSION

In considering the relationship of microorganisms to the disappearance of rancidity in cheddar cheese, it seemed logical to assume that lipolytic organisms might be of importance since a number of these organisms are capable of utilizing the products of fat hydrolysis. The hydrolysis of fat and the destruction of at least certain products of the hydrolysis might be the sequence of changes in rancid cheese from which the rancidity disappears. In attempting to isolate the lipolytic organisms from cheddar cheese the flora of normal cheese was studied. Only a few lipolytic organisms were ever found. The organisms that were most prominent were S. lactis and certain micrococci. None of these organisms, non-lipolytic or lipolytic, appeared to utilize sodium butyrate when loops of litmus milk cultures were inoculated into the Ayres medium.

When inoculated with small quantities of cheddar cheese emulsified in 2.0 per cent sodium citrate solution, the Ayres medium regularly yielded large rod-shaped organisms. These organisms were obtained in practically pure culture from more than 200 samples of cheddar cheese from various sources. The organisms grew readily on tomato juice agar, and were arbitrarily designated the X type organism. They were found to be non-lipolytic on natural fat agar and on nile-blue sulphate agar. When inoculated into litmus milk, acid and gas were formed. After these organisms had grown in lactose broth, they were found to be small gram-negative rods which conformed to Bergey's (4) description of Bu. coli.

Since the X type organism regularly developed in the Ayres medium in-

oculated with cheddar cheese, the question arose as to how the addition of small quantities of butyric acid would affect the flora of cheese. In 1 day old cheese made from raw milk containing added butyric acid, enormous numbers of S. lactis organisms were present. The numbers of these organisms in the cheese were essentially the same as the numbers in normal cheddar cheese of the same age. Few organisms of any other type were observed. After the cheese had ripened 3 or 4 days S. lactis was no longer detected, and was replaced by the X type organisms. After the cheese had ripened 14 days the X type organisms had diminished in numbers. With their disappearance a more or less typical cheddar cheese flora was observed. Similar observations were made when cheese was made from pasteurized milk containing added butyric acid, but the numbers of the X type organisms that developed were usually not as large as with the raw milk cheese. These experiments were repeated many times, great care being exercised to eliminate the possibility of contamination. In some cases the cheese was made with pure cultures of S. lactis instead of cheese culture, and still large numbers of the X type organisms were observed. The presence of millions of the X type organisms per gram in cheese 3 or 4 days old probably can not be ascribed to contamination. It appears that the X type organisms developing in cheese containing butyric acid are variants of S. lactis. The change in morphology may be due to the environment produced in cheese by the added butyric acid. This hypothesis is in conformity with Hadley's (15) statements. As the flavor and oder of butyric acid disappeared from the cheese, many of the organisms seemed to return to their original form.

All lots of cheese made from milk containing added butyric acid were very rancid when 1 day old. However, cheese made from pasteurized milk lost

much of its rancidity after 3 or 4 days, and raw milk cheese was definitely less rancid at the same age. After the cheese had ripened 14 days most of the rancidity had disappeared in both the pasteurized and raw milk cheese.

The bacterial flora of 7 day old cheese made from homogenized raw milk usually consisted of rather large numbers of the X type organisms. When the homogenized milk was held 2 hours before it was made into cheese even larger numbers of the X type organisms developed. However, cheese made from milk that was homogenized and immediately pasteurized showed very few of these organisms. The X type organisms seemed to develop less rapidly in cheese from homogenized milk than in cheese from raw milk containing added butyric acid; however, they appeared to persist in the former cheese for a longer period of time.

Cheese made from homogenized raw milk always became rancid. However, cheese made from milk that was homogenized and immediately pasteurized did not become rancid. Rancidity developing in cheese from homogenized raw milk persisted for a longer period than rancidity in cheese from raw milk containing added butyric acid. Presumably, in homogenized raw milk cheese the lipase continued to act on the fat and set free butyric acid during the ripening process. This would explain why the rancidity persists over a longer period of time.

Inoculation of pure cultures of the X type organism into cheese made from milk containing added butyric acid did not accelerate the rate at which this type of rancidity disappeared, the rancidity disappearing as rapidly without the inoculation as with it. Similar inoculations were made into homogenized raw milk used in cheese manufacture. Here, as in the case of cheese made from milk containing added butyric acid, rancidity disappeared

rapidly either with or without inoculation. When milk containing pancreatin was made into cheese, both the inoculated and uninoculated lots became more rancid as the product aged. Lots of cheese 4 months old were so rancid that they could not be judged. Presumably, the environment produced by the pancreatin was so unfavorable that few organisms were able to survive. It appears that the organisms normally present in young cheese are capable of destroying small amounts of butyric acid, and perhaps other fatty acids. It may be that L. lactis changes its morphological and biochemical properties in order to better utilize butyric acid. When, however, large amounts of free fatty acids are present many of the organisms are unable to survive.

The X type organism did not grow well when inoculated into the Ayres medium. Growth was obtained by transferring rather large amounts of inoculating material, but even then only a slight turbidity developed. Since sodium butyrate was the sole source of carbon in this medium and some growth did take place, the organisms may have utilized butyrate to a slight extent. Better growth was obtained when the Ayres medium was enriched with small quantities of peptone or lactose or both. However, acidifying and distilling the medium and titrating the distillates showed that little or none of the butyrate had been utilized. The ability of the X type organism to utilize butyrate was further studied by adding sterile sodium butyrate solution to active cultures growing in dilute yeast extract broth. Under these conditions the organism utilized the butyrate. Disappearance of butyrate was apparent after 14 days at 21.0° C., and was more complete after 21 days. These observations indicate that the X type organism has the ability to bring about the disappearance of rancidity in cheddar cheese.

In attempts to produce the X type organism from pure cultures of

S. lactis in the laboratory, a number of media containing varying amounts of sodium butyrate or butyric acid were studied. The attempts were not always successful. Best results were obtained when tubes of the Ayres medium containing 0.1 per cent peptone were inoculated with 0.1 ml. of a vigorous 48 hour litmus milk culture of S. lactis. Frequently, however, it was necessary to incubate the enriched Ayres medium cultures for 1 week or more at 21.0° C. before the X type organism developed. Sometimes a culture of S. lactis had to be transferred in the medium several times before a change could be noted. There seemed to be no uniformity in the time required for the change to take place in the cultures studied.

Tubes of brain heart infusion containing sodium chloride and butyric acid or sodium butyrate occasionally yielded rod-shaped organisms when inoculated with 0.1 ml. of a litmus milk culture of S. lactis. These organisms, however, were not the X type; they produced an alkaline reaction in litmus milk rather than the acid reaction usually noted with the X type organism.

Inoculation of litmus milk containing butyric acid or sodium butyrate with 0.1 ml. quantities of S. lactis failed to produce any change in the S. lactis. It seems likely that the lactose in milk is such a readily available source of carbon for the organism that it does not utilize the added sodium butyrate or butyric acid. On the other hand the Ayres medium contains sodium butyrate as the only source of carbon, and in order to utilize it the organism may be forced to change its general characteristics.

It seems that the formation of the rod-shaped organism from pure cultures of S. lactis depends on a rather delicate balance of a number of factors. Some cultures of S. lactis changed into rod-shaped organisms rather

readily, while other cultures did not change until they had been transferred a number of times. Furthermore, the amount of culture transferred appeared to be an important factor in the development of the X type organism. When only a small loop of an S. lactis culture was transferred to the medium containing butyrate it was difficult to obtain the large rods. The difficulty experienced in regularly producing the X type organism in the Ayres medium inoculated with S. lactis perhaps can be ascribed to the inability to reproduce the conditions occurring in cheddar cheese.

The data presented indicate that the disappearance of rancidity produced in cheddar cheese in various ways is due to the activity of microorganisms. In young cheddar cheese large numbers of S. lactis organisms are normally present, but the readily available food supply is limited. It seems probable that these organisms utilize butyric and other lower fatty acids as a source of carbon. In order to utilize these acids under the environmental conditions present in cheddar cheese, S. lactis is forced to change its morphological and physiological properties to those of the X type organism. With the disappearance of rancidity in cheddar cheese the X type organism decreases in numbers and is replaced with a more typical cheddar cheese flora. Presumably, the same general mechanism is operative in cheese made in the usual way, and may explain why raw milk cheese commonly does not develop rancidity, even when the acidity in the fat increases considerably.

SUMMARY AND CONCLUSIONS

The work reported involved a study of the relationship of microorganisms to the disappearance of rancidity produced in cheddar cheese with various procedures. The following observations were made:

1. There was no relationship between the total bacterial counts and the numbers of lipolytic organisms in cheddar cheese, the numbers of lipolytic organisms regularly being very low.

2. When a loop of litmus milk culture of various organisms isolated from cheddar cheese was inoculated into the Ayres medium, no growth was visible after 14 days at 21.0° C.

3. The Ayres medium, inoculated with emulsified cheddar cheese and incubated 4 or 5 days at 21.0° C., regularly yielded a large rod-shaped organism which was arbitrarily designated the X type organism. When grown in lactose broth this organism had characteristics conforming to the accepted description of *Es. coli*.

4. The X type organism was obtained regularly when more than 200 samples of cheddar cheese from various sources were inoculated into the Ayres medium.

5. The X type organism developed regularly after 3 or 4 days in cheddar cheese made rancid by adding butyric acid to the milk used in the manufacture. As the cheese ripened the rancidity disappeared, and the X type organism diminished in numbers. The disappearance of rancidity was more rapid in pasteurised milk cheese than in raw milk cheese.

6. The X type organism developed regularly after 6 or 7 days in cheddar cheese made rancid by homogenising the raw milk used in the manufacture.

As the cheese ripened the rancidity slowly disappeared and the X type organism diminished in numbers.

7. Cheddar cheese made from pasteurized milk that was homogenized and from milk pasteurized immediately following homogenization failed to become rancid, and the X type organism was not obtained from it. However, cheddar cheese made from raw homogenized milk held for 2 hours and then pasteurized became rancid, and the X type organism was recovered from it. The rancidity disappeared as the cheese ripened, and the numbers of the X type organism diminished.

8. Frequently, the X type organism completely replaced S. lactis in rancid cheese made from milk containing added butyric acid and in rancid cheese made from raw homogenized milk. The X type organism appears to be a variant of S. lactis.

9. The rate at which rancidity disappeared in cheddar cheese made from milk containing added butyric acid or from raw homogenized milk was not accelerated by adding pure cultures of the X type organism to the milk.

10. Rancidity did not disappear in cheese made from milk containing pancreatin, and inoculation of pure cultures of the X type organism into the milk did not result in a disappearance of the rancidity in the cheese.

11. Inoculation of pure cultures of the X type organism into the Ayres medium and into enriched Ayres medium failed to show utilization of the sodium butyrate after 14 days or 30 days at 21.0° C.

12. Sodium butyrate was utilized when added to 48 hour yeast extract broth cultures of the X type organism. Utilization of the butyrate was evident after 14 days at 21.0° C. and was more complete after 21 days.

13. Tubes of the Ayres medium, containing 0.1 per cent peptone, that were inoculated with 0.1 ml. of a 48 hour litmus milk culture of S. lactis occasionally yielded the X type organism after 7 days at 21.0° C.

14. Tubes of litmus milk, containing butyric acid or sodium butyrate, that were inoculated with 0.1 ml. of a 48 hour litmus milk culture of S. lactis failed to yield the X type organism after 14 days at 21.0° C.

15. Tubes of brain heart infusion, containing sodium chloride and butyric acid or sodium butyrate, that were inoculated with 0.1 ml. of a 48 hour litmus milk culture of S. lactis occasionally yielded a large rod-shaped organism. However, this organism was not a typical X type organism.

16. The disappearance of rancidity produced in cheddar cheese with various procedures appears to be due to the activity of microorganisms normally present in the cheese.

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