AN ABSTRACT OF THE THESIS OF

Donald Duane Bills for the Ph. D. in Food Science
(Name) (Degree) (Major)

Date thesis is presented February 18, 1966

Title THE ROLE OF ETHANOL AND CERTAIN ETHYL ESTERS IN THE FRUITY FLAVOR DEFECT OF CHEDDAR CHEESE

Abstract approved ______________________
(Major professor)

During the course of ripening, Cheddar cheese frequently develops a flavor defect described as fruity. Recent work has indicated that the use of certain starter cultures ultimately results in the development of the defect as the cheese ages. The flavor compounds responsible for the defect, however, have not been elaborated. The purpose of this investigation was to isolate and identify the components responsible for the fruity flavor defect and to evaluate the role of certain cheese starter cultures in the development of the defect.

Since the fruity character of the defect is apparent in the aroma of the cheese, the compounds responsible for the defect were expected to be reasonably volatile. Volatile constituents were isolated by a distillation technique from fat expressed from a typically fruity cheese by centrifugation. The volatile constituents were then separated by gas-liquid chromatography. By monitoring the odor of the effluent stream of the column, it was possible to determine which

components had fruity odors, and these were subsequently identified by mass spectral analysis and coincidence of retention time with the authentic compounds. Ethyl butyrate, ethyl hexanoate, and ethyl octanoate were found to be the only compounds with detectable fruity odors.

The volatiles from the fat of four cheeses possessing varying degrees of the defect and their matching non-fruity controls were analyzed by a gas entrainment, on-column trapping, gas-liquid chromatographic technique. The manufacturing and curing conditions of each fruity cheese and its matching control were identical, except for the use of different starter cultures. Ethanol, ethyl butyrate, and ethyl hexanoate were more abundant in each of the fruity samples. The approximate concentration range of these compounds was as follows: In fruity cheese; ethanol 400 to 2, 040 ppm, ethyl butyrate 1. 6 to 24 ppm, ethyl hexanoate 0. 9 to 25 ppm. In non-fruity cheese; ethanol 36 to 320 ppm, ethyl butyrate 0. 7 to 4. 7 ppm, ethyl hexanoate 0. 3 to 2. 2 ppm. In ten commercial Cheddar cheeses selected at random from the market, the concentration of ethanol ranged from 5. 5 to 620 ppm.

Single-strain cultures of Streptococcus lactis, Streptococcus diacetilactis, and Streptocococcus cremoris as well as three mixed-strain commercial cultures were evaluated for ethanol and acetaldehyde production in non-fat milk medium. Among the single-strain cultures

there appeared to be no correlation between ethanol production and species, although considerable variation was noted for strains within a species. The mixed-strain cultures were designated _A_, _B_, and _C_. Cultures _B_ and _C_ had been implicated in the development of the fruity flavor defect in Cheddar cheese, while culture _A_ produced normal cheese of good quality. Cultures _B_ and _C_ produced approximately 40 times more ethanol than culture _A_ when incubated in non-fat milk medium for one month at 7° C.

Certain single-strain cultures and the three mixed-strain cultures were tested for their ability to reduce acetaldehyde and propanal, and to catalyze the formation of ethyl butyrate when ethanol and butyric acid were provided as substrates. Acetaldehyde and propanal were reduced to the corresponding alcohols by all cultures, but the formation of ethyl butyrate was not observed in any culture.

A good correlation between high levels of ethanol and high levels of ethyl butyrate and ethyl hexanoate in the fruity cheeses suggests that the quantity of ethanol present in the cheese may determine the amount of ester formed. Further, starters resulting in the defect produced considerably more ethanol than cultures resulting in normal cheese when incubated at 7° C, a normal temperature for curing Cheddar cheese. This observation adds weight to the hypothesis that certain cultures are directly responsible for the defect.

THE ROLE OF ETHANOL AND CERTAIN ETHYL ESTERS IN THE FRUITY FLAVOR DEFECT OF CHEDDAR CHEESE

by

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A THESIS

submitted to

OREGON STATE UNIVERSITY

in partial fulfillment of
the requirements for the
degree of

DOCTOR OF PHILOSOPHY

June 1966

APPROVED:

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Date thesis is presented February 18, 1966

Typed by Opal Grossnicklaus

ACKNOWLEDGMENTS

I would like to thank Dr. E. A. Day, who has served as my major professor for both graduate degrees, for his guidance during the course of the current investigation. His devotion to research has been a constant inspiration.

I would also like to express my gratitude to the members of my Program Committee: Dr. M. W. Montgomery, Dr. B. E. Christensen, and Dr. W. E. Sandine; who have contributed their time and helpful suggestions to the entire degree program. The assistance of other faculty members and graduate students of the Department of Food Science and Technology has been greatly appreciated.

Recognition is due to the Division of Environmental Engineering and Food Protection of the Public Health Service and to the National Aeronautics and Space Administration for providing the financial support for the initial and later stages of this work, respectively.

Finally, I would like to thank my wife, Marrilynn, for her patience and understanding throughout the course of my graduate career.

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THE ROLE OF ETHANOL AND CERTAIN ETHYL ESTERS IN THE FRUITY FLAVOR DEFECT OF CHEDDAR CHEESE

INTRODUCTION

The flavor of cheese varieties which are ripened before consumption is largely dependent upon the fermentation or chemical processes which occur in the cheese subsequent to the initial manufacturing step. Thus, it is possible to utilize a single lot of milk and produce from it cheeses with widely different flavor properties as exemplified by Blue, Limburger, and Cheddar cheeses. The manufacturing process, the microorganisms or enzymes incorporated into the cheese, and the curing conditions are variables which ultimately determine the type and flavor quality of the cheese. Unfortunately, in Cheddar cheese and other varieties, the variables involved in consistently producing a high-quality product are often difficult to control and incompletely understood. Consequently, a high percentage of the cheese which is eventually marketed displays defects which are objectionable to some extent. Only premium quality cheese can command a premium price; and the cheese industry is, therefore, much interested in a better understanding of the factors involved in consistently producing a good product.

The fruity flavor defect of Cheddar cheese has been described as "pear-like," "apple-like," or "pineapple-like." An accompanying defect in the body of the cheese termed slit-openness has been

associated frequently with the flavor defect. Slit-openness appears to involve the fracturing of the curd structure through the production of gases during ripening. An estimated 15 percent of the annual production of Cheddar cheese in the United States develops some degree of the fruity flavor defect.

Recent investigations have indicated that the use of certain starter cultures can result in cheese which displays the defect, but a determined effort to identify the chemical components responsible for the defect has not been made. The aroma of fruity Cheddar cheese suggests that certain esters could be responsible for the defect, but there are other compounds which also have quite fruity odors. Some acetals, aldehydes, ketones, and alcohols belong in this category and conceivably could be present in Cheddar cheese.

The purpose of this investigation was two-fold. First, to isolate and identify the components responsible for the fruity flavor defect; and second, to determine what role certain starter organisms play in the development of the compounds identified in the inital study.

REVIEW OF LITERATURE

Flavor Chemistry of Cheddar Cheese

The identification of chemical entities responsible for the flavor of Cheddar cheese has proceeded at a vigorous pace for the last decade. In spite of the knowledge accumulated in this area, a comprehensive picture of the flavor components for this variety of cheese has not yet emerged. It is not presently possible to formulate a mixture of compounds which duplicates the flavor and aroma of Cheddar cheese in all respects and even the best formulations of companies specializing in synthetic flavoring materials fall far short of imitating this elusive blend of components. The flavor of this commodity appears to be due to a large number of components of which no single compound or class of compounds is very much reminiscent of Cheddar cheese flavor, and it is probable that many important flavor compounds have not yet been identified. In addition, quantitative data is lacking for many of the compounds which have been previously identified.

Earlier workers hoped to isolate a single compound from Cheddar cheese which would have a distinct cheese-like character (71). Since such a compound has never been isolated, the balanced component theory (53, 73, 98, 138) is currently favored. The balanced component theory holds that typical Cheddar cheese flavor arises

from a combination of numerous compounds varying over a certain concentration range and present in relatively fixed ratios to one another.

Attempts to simulate Cheddar flavor with simple mixtures of compounds have met with only limited success. Silverman and Kosikowski (138) added mixtures of amino acids and free fatty acids in the proper concentrations to bland curd. The result was a Cheddar-like but incomplete Cheddar flavor. A mixture containing only acetic acid, butyric acid, methionine and butanone was reported to have an odor resembling more closely that of Cheddar cheese (73). A comment which should be made concerning the latter mixture is that methionine itself is odorless but is degraded slowly and spontaneously to yield methional (3-methylthio propanal). Methional has an odor which is somewhat cheeselike (62, 69), and this compound has been isolated from Cheddar cheese (29). Walker (147) attempted to reproduce a Cheddar-type flavor by the addition of mixtures of methyl ketones and fatty acids to bland cheese curd, but he found that the flavors produced were sharp and musty unless thioacetamide, a source of hydrogen sulfide, was added. Day et al. (29) incorporated 13 carbonyl compounds, five short-chain fatty acids, and 3-mercapto-propionic acid in a simulated Cheddar system. The aroma of the latter mixture was cheese-like but incomplete. They pointed out that numerous non-volatile proteolytic and lipolytic intermediates

occurring in cured Cheddar cheese probably have a considerable influence on the total flavor.

Free Fatty Acids

Patton (107) evaluated the contribution of various classes of compounds to Cheddar cheese aroma by adding reagents to selectively block functional groups in cheese and cheese distillates. He concluded, from the response of a panel, that acetic, butyric, caproic, and caprylic acids "constitute the backbone of Cheddar aroma".

In an earlier work, Peterson and Johnson (117) reported that the fatty acids of intermediate chain length (C_6-C_{10}) were produced during the ripening process and were characteristic of Cheddar cheese. The addition of these acids to a bland base, in concentrations found in aged Cheddar cheese, resulted in an aroma resembling that of Cheddar cheese.

Kristoffersen and Gould (76) evaluated 14 lots of Cheddar cheese for characteristic flavor, ammonia, hydrogen sulfide, free fatty acids, free amino acids, acidic and neutral carbonyl compounds, pH and total bacterial count. They reported that the characteristic flavor appeared to be related more to the ratio of free fatty acids and hydrogen sulfide concentrations than to any other compounds or combinations of compounds included in the study. The authors suggested that the measurement of free fatty acids and hydrogen sulfide might

serve as an index of Cheddar cheese flavor.

The major individual free fatty acids, acetic through linolenic, were determined in 14 samples of Cheddar cheese by Bills and Day (9). A technique utilizing two methods of column chromatography plus gas-liquid chromatography was necessary for resolution of the complete series of free fatty acids. Formic and propionic acids were not observed in any of the cheeses. Acetic acid showed the greatest variability in concentration and was usually the most abundant. Among the free fatty acids which can arise through the hydrolysis of milk fat, free butyric acid was always found in about twice the percentage reported for esterified butyric acid. The individual free fatty acids from caproic through linolenic, however, were present in nearly the same ratio as the same esterified acids in milk fat. Excluding two rancid samples, the average concentrations (expressed as mg of free fatty acid per kg of cheese) were as follows: 2:0, 865; 4:0, 115; 6:0, 38; 8:0, 41; 10:0, 49; 12:0, 81; 14:0, 218; 16:0, 503; 18:0, 172; 18:1, 467; 18:2, 69; 18:3, 40. In the two rancid samples the concentrations of acetic acid fell within the range found in the 12 other samples. Each of the individual free fatty acids from butyric through linolenic, however, was present in the rancid samples in about ten times the average concentration found in the 12 other samples.

Nitrogen Compounds

Kristoffersen (76) found that the concentration of ammonia in Cheddar cheese increased throughout the ripening period and ranged from 4.7 to 16.4 mM/g in cheese 0.5 months old and from 26.5 to 74.9 mM/g in cheese 12 months old. The average increases for raw and pasteurized milk cheeses were found to be similar. No definite relationship was found between intensity of characteristic Cheddar flavor and concentration of ammonia.

The breakdown of protein, which in Cheddar cheese is essentially casein, has long been recognized as an important part of the ripening process. Consequently, considerable effort has been directed toward establishing the pattern of amino acids present in the aged product. All of the amino acids reported to be present in casein by Gordon *et al*. (48, 49, 50) have been isolated from ripening Cheddar cheese by various workers as follows:

glycine (13, 18)
alanine (13, 18, 70)
valine (13, 18, 70)
leucine (13, 18, 70)
isoleucine (18)
proline (13, 18, 70)
phenylalanine (13, 18, 70)
tyrosine (13, 18, 70)
tryptophan (18)
serine (13, 18, 70)
threonine (13, 18, 70)
cysteine (70)
cystine (18)
methionine (13, 18, 70)
arginine (13, 18)
histidine (13)
lysine (13, 18)
aspartic acid (13, 18, 70)
glutamic acid (13, 18, 70)

In addition, the following amino acids which have not been reported

in casein or in other milk proteins (149) have been isolated from Cheddar cheese: asparagine (18, 70), glutamine (18, 70), ornithine, alpha-aminobutyric acid, and gamma-aminobutyric acid (70).

Since amino acids lack volatility, they would not be expected to contribute to the odor of Cheddar cheese. Amino acids are believed, however, to impart a brothy background taste which contributes to the total flavor of the cheese (138). Kristoffersen (76) found a wide range of concentrations of the amino acids in Cheddar cheese, but no consistent relationship between the concentration of amino acids and the quality of the cheese was noted.

Amino acids can serve as precursors for a host of volatile compounds which might contribute significantly to Cheddar flavor. An appreciable concentration of tritium was observed by Wolin and Kosikowski (150) in two carbonyl compounds isolated from Cheddar cheese prepared from labelled casein. The degradation of methionine to methional has been proposed to be an important factor contributing to the flavor of Cheddar cheese by Keeney and Day (69). Some very potently odorous amines resulting from the decarboxylation of certain amino acids have been isolated from Cheddar cheese. Tyramine, resulting from the decarboxylation of tyrosine, was reported by Bullock and Irvine (16), Kosikowski (72), and Silverman and Kosikowski (139). Cadaverine, resulting from the decarboxylation of lysine was reported by Silverman and Kosikowski (136, 137) who also found histamine

and tryptamine, the decarboxylation products of histidine and tryptophan, respectively (136). Putrescine, resulting from the hydrolysis of one molecule of urea followed by the decarboxylation of arginine, has also been isolated from Cheddar cheese by the latter authors (132). Other amino acids are also subject to strecker degradation, deamination, decarboxylation, and other degrative processes, though little is known of the importance of these reactions in the production of compounds important to the flavor of Cheddar cheese.

Carbonyl Compounds

Carbonyl compounds are usually grouped under two headings, acidic and neutral carbonyls. Under the former heading would be listed keto acids, such as pyruvic acid, which contain both the carbonyl and carboxyl function on the same molecule. Under the latter heading would be listed aldehydes and ketones which do not have the carboxyl function.

Acidic Carbonyl Compounds. Bassett and Harper (7) isolated and identified various acidic carbonyl compounds in four varieties of cheese. Alpha-ketoglutaric, oxalacetic, pyruvic, alpha-acetolactic and alpha-ketoisovaleric were found in Cheddar cheese. Comparison of relative amounts of the above compounds by observation of the paper chromatograms on which the 2, 4-dinitrophenylhydrazone

derivatives were separated revealed that alpha-ketoglutaric, pyruvic and alpha-acetolactic acids were relatively abundant; alpha-ketoisovaleric was less abundant; and oxalacetic acid occurred in trace amounts.

Kristoffersen and Gould (74) succeeded in isolating and identifying all of the above compounds with the exception of alpha-ketoisovaleric acid. These authors found in addition oxalsuccinic, glyoxylic, and alpha-ketoisocaproic acids in the extract of Cheddar cheese. The concentrations of pyruvic and alpha-ketoglutaric acids were determined quantitatively and found to average 0.9 and 20.3 micromoles per 100 g of cheese respectively in the 25 lots of cheese studied. No apparent correlation was found between the concentrations of the two keto acids and the age, grade, and flavor of the cheese.

Neutral Carbonyl Compounds. A considerable amount of effort has been expended toward elaborating the compounds in this group which are present in Cheddar cheese. While many neutral carbonyls are no doubt important to the flavor of Cheddar cheese, it should be pointed out that the large amount of work devoted to this class of compounds stems, in part at least, from the fact that stable derivatives of aldehydes and ketones can be easily formed and manipulated.

Patton (111) identified several of the more abundant neutral carbonyl compounds isolated as 2,4-dinitrophenylhydrazones from the steam distillate of a Cheddar cheese slurry. Day *et al.* (29)

succeeded in identifying several additional compounds after separation of the DNP-hydrazones by column partition chromatography.

In a recent work, Day and Libbey (32) identified a considerable number of neutral carbonyl compounds in the molecular distillate of Cheddar cheese fat obtained by centrifugation of the intact cheese. Identification was based on GLC retention time and mass spectrometry. A list of neutral carbonyls which have been isolated from Cheddar cheese is presented below.

1. acetaldehyde (29, 32, 74, 111, 147)
2. acetone (29, 32, 74, 111, 147)
3. butanone (29, 32, 111, 147)
4. 2-pentanone (29, 32, 147)
5. 2-heptanone (29, 32, 111, 147)
6. 2-nonanone (29, 32, 147)
7. n-decanal (32)
8. 2-undecanone (29, 32, 147)
9. n-dodecanal (32)
10. 2-tridecanone (29, 32, 147)
11. 2-pentadecanone (32)
12. acetoin (29, 111, 147)
13. diacetyl (29, 37)
14. formaldehyde (29, 111)
15. 3-methylbutanal (29)
16. propanal (29)
17. 3-methylthiopropanal (29)
18. butanal (23)

Sulfur Compounds

Many of the volatile sulfur compounds are substances with potent odors. Ethyl mercaptan, for example, has been reported to be detectable at a level of one part in 50 billion parts of air (62).

Since the thresholds of detection are so low for certain sulfur compounds, it is not unlikely that compounds in this class which contribute significantly to the flavor of Cheddar cheese have been overlooked due to their low concentration in the cheese. Hydrogen sulfide has been detected in Cheddar cheese by a number of investigators (75, 76, 77, 146). As previously mentioned, Kristoffersen and Gould (77) noted a distinct correlation between the level of free fatty acids, the level of hydrogen sulfide, and the flavor score of the cheese.

Methyl mercaptan was isolated from Cheddar cheese by Libbey and Day (85). Evidence for the presence of methyl mercaptan was based on retention time on both polar and non-polar GLC columns, R_f values obtained by thin-layer chromatography of 2, 4-dinitrofluorobenzene derivatives, and the U. V. spectra of the derivative. The authors estimated the concentration of methyl mercaptan in Cheddar cheese to be between three and 30 parts per billion (ppb). Day et al. (30) found the flavor threshold of methyl mercaptan to be 2 ppb in water.

Dimethyl sulfide was first isolated from milk by Patton et al. (108); and its presence in milk, cream, and butter has been confirmed by others (33, 64, 151, 153). The isolation of dimethyl sulfide from Cheddar cheese was accomplished by Patton et al. (111).

As previously mentioned, the sulfur compound methional has been isolated from Cheddar cheese (29). Jackson (62) observed that

this compound has a pumpkin-like odor at higher concentrations but has a cheese-like odor at lower concentrations. Personal observation indicates that this compound has a very strong potato-like odor at very high concentrations but does, indeed, have a cheese-like character when sufficiently dilute. A convenient method for preparing small amounts of methional for odor evaluation is to warm an aqueous solution of methionine with reagents (such as isatin) which bring about Strecker degradation of the amino acid.

Other Flavor Components

Various alcohols have been isolated from Cheddar cheese. Ethanol (11, 32, 84, 126), 1-propanol (11), 2-butanol (11, 32, 126), 1-pentanol (32), 2-pentanol (32), and 2-nonanol (32) have all been reported. Since the flavor threshold of most alcohols is relatively high, it is doubtful that many of the alcohols contribute significantly to Cheddar cheese flavor. The role of alcohols in the formation of esters, however, may be quite significant to the flavor of Cheddar cheese, since many esters have low flavor threshold values.

Esters which have been isolated from Cheddar cheese by Day and Libbey (32) are as follows: ethyl acetate, 2-butyl acetate, ethyl butyrate, 2-butyl butyrate, methyl hexanoate, ethyl hexanoate, 2-butyl hexanoate, methyl octanoate, ethyl octanoate, methyl decanoate, ethyl decanoate, methyl dodecanoate, ethyl dodecanoate, and methyl

phthalate. Some of the same esters have also been reported by Suzuki _et al._ (141), McGugan and Howsam (93), and Bills _et al._ (8) among others. The occurrence of methyl esters in Cheddar cheese is difficult to explain, since methanol does not appear to be present in significant quantities. The level of methyl esters has been found to be considerably lower than the level of ethyl esters in a given cheese sample (8, 32).

Tentative identification of delta-octalactone and delta-decalactone, intra-molecular esters of hydroxy acids, was also provided by the work of Day and Libbey (32). The _cis_ and _trans_ lactides of lactic acid were also tentatively identified in the same work. The lactide of lactic acid is an inter-molecular ester in which two molecules of lactic acid are esterified to yield a six-membered ring. Commercially prepared lactide of lactic acid was found to have a pronounced bitter taste.

Lactic acid itself probably contributes more to the acid taste of Cheddar cheese than any other single acid component. The pKa of lactic acid is 3.86 indicating that it is nearly ten times more acidic than acetic acid which has a pKa of 4.76, also the author has noted that lactic acid is usually the most abundant acid in Cheddar cheese at various stages of ripening. The latter observation resulted from a work involving the separation of the short-chain organic acids of Cheddar cheese by column chromatography (9). Undissociated salts

of lactic acid, such as calcium lactate, as well as undissociated salts of other acids may also have a bearing on flavor.

The intermediate products of proteolysis are not volatile but probably contribute to the taste of Cheddar cheese. Peptides have been implicated in the bitter defect of Cheddar cheese (36). A lesser degree of bitterness as a background taste appears to be characteristic of the typical flavor of the product.

The salt which is added to the cheese during manufacturing, the unaltered lipid and protein, and the water present in the cheese should also be considered as components in the total flavor-makeup.

It is obvious from this extensive list of compounds that the flavor chemistry of Cheddar cheese is complex. Probably many of the compounds which have been identified contribute nothing to the flavor of the cheese, because their concentrations are lower than their flavor threshold values (109). Since much of the work in this area has been purely qualitative, it is impossible at this time to conjecture on the importance of many potential flavor compounds. As Patton (106) noted, flavor thresholds can vary markedly depending on whether the flavor compound is dissolved in water or in oil, and both of these phases should be present in Cheddar cheese. Further, incompletely understood phenomena such as flavor potentiation (80) and additive effects (26) may occur between the flavor constituents to make the picture even more complex.

Microorganisms Associated With Cheddar Cheese

The starter streptococci are normally the only organisms purposely added to Cheddar cheese during the manufacturing step. Other organisms necessary for the development of Cheddar flavor and body are introduced unintentionally into the cheese either through the milk supply or from the cheese plant environment. The cheese maker can control the former source of adventitious organisms to some extent by applying various heat treatments, up to and including full pasteurization, to the milk prior to the manufacturing process; the latter source is not so easily controlled. As a consequence, the microbiology of Cheddar cheese ripening is not well-defined, and it is difficult to determine the exact role the adventitious organisms play in ripening. It is generally agreed, however, that these organisms are necessary to the ripening process. The manufacturing of cheese aseptically to exclude organisms other than those purposely added would be extremely difficult and would require such rigorous treatment to ensure sterility of the milk that the alteration of the physical and chemical properties of the milk would make the results of the experiment rather dubious. Experiments involving the addition of selected microorganisms have, therefore, been carried out under nonaseptic conditions which explains, perhaps, some of the conflicting results reported in the literature.

The Starter Streptococci

The role of starter organisms in the development of the typical body and flavor of Cheddar cheese is still unclear. In the past, these organisms have been selected largely on the basis of their ability to produce lactic acid at a convenient rate for the initial manufacturing process.

Streptococcus cremoris, *Streptococcus lactis*, and *Streptococcus diacetilactis*, either singly or in combination, have been used conventionally as starter cultures for Cheddar cheese. A nonhemolytic strain of *Streptococcus durans* was employed experimentally as a Cheddar cheese starter by Walter *et al*. (148). The use of *S. durans* as a starter shortened the manufacturing time from renneting to pressing by three to four hours. Feagan (40) used *Streptococcus thermophilus* as the starter culture for manufacturing Cheddar cheese and reported that good-quality cheese was obtained when a higher cooking temperature of 106° F was used. Dahlberg and Kosikowski (26) concluded that the ripening process was accelerated when *Streptococcus faecalis* was used as the starter organism. It was noted that the use of *S. faecalis* plus conventional starter culture resulted in cheese with the highest score. The use of *S. durans*, *S. thermophilus*, and *S. faecalis* as starter organisms has never been adopted for commercial cheese manufacturing.

As will be discussed later, recent studies have disclosed that the species and strain of microorganisms employed in the starter culture can have a profound effect on the flavor quality of the cheese. One example of this would be the peptide bitterness which occurs in cheese if the starter has a limited proteolytic ability which allows the primary breakdown products of protein to accumulate. The bitter defect has been correlated to the inability of certain starter organisms to carry out a rapid hydrolysis of peptides by Emmons *et al.* (36, 37), by Czulak (21), and by Dawson and Feagan (28)

The Lactobacilli

Although the numbers of lactobacilli are low in newly-made Cheddar cheese, the count begins to increase after a few weeks of curing and reaches a maximum at three to six months according to Johns and Cole (66). The coincident growth of large numbers of lactobacilli with the development of typical Cheddar flavor has led many investigators to speculate on the role of these organisms in Cheddar cheese ripening. Evans *et al.* (38) were among the first to report the presence of *Lactobacillus casei* in Cheddar cheese. They noted that *L. casei* was the most prominent organism in Cheddar cheese after the decline of the starter organisms. The presence of *L. casei* in Cheddar cheese has subsequently been confirmed by Franklin and Sharpe (43), Smith and Cunningham (140), Naylor and

Sharpe (100, 101), and Perry and Sharpe (116).

Sherwood (134) reported that *Lactobacillus plantarum* was the species of lactobacillus most frequently found in New Zealand cheese. Naylor and Sharpe (99, 100), Perry and Sharpe (116), Franklin and Sharpe (43), and Smith and Cunningham (140) were also able to isolate this organism from Cheddar cheese.

Naylor and Sharpe (99, 100) isolated *Lactobacillus brevis* from ripening Cheddar cheese. Perry and Sharpe (116), in later studies, found *L. brevis* present in raw milk and then in Cheddar cheese manufactured from the same milk. The presence of this organism in Cheddar cheese has also been confirmed by Smith and Cunningham (140) and Franklin and Sharpe (43).

Other lactobacilli which have been isolated from Cheddar cheese and subsequently reported in the literature are *Lactobacillus bulgaricus* isolated by Hastings *et al.* (58) and *Lactobacillus fermenti* isolated by Perry and Sharpe (116). Judging from the literature, the latter organisms occur sporadically and probably are not truly characteristic of the microflora of Cheddar cheese. According to Tittsler *et al.* (142, 143), *Lactobacillus helveticus*, when added to cheese milk, survived in the resultant cheese for two weeks or less and probably contributed little to proteolysis or flavor development.

The Micrococci

The presence of this group of bacteria has been well documented, but little is known of the role they play in Cheddar cheese ripening. Evans *et al*. (38) reported the presence of micrococci in Cheddar cheese as early as 1914. It has been reported by Alford and Frazier (1) that two-day-old Cheddar cheese contains up to 50 million nonlactic bacteria per gram. Over 75% of the nonlactic bacteria in raw milk cheese were identified as *Micrococcus freudenreichii*, *Micrococcus caseolyticus*, or *Micrococcus conglomeratus*. Micrococci in Cheddar cheese have also been reported by Feagan and Dawson (41) and by Irvine and Beach (61).

Considering the ability of this group of bacteria to carry out proteolysis and lipolysis, it is unfortunate that so little attention has been given to their possible importance in flavor production. Perhaps this deficiency stems in part from the fact that it is difficult to classify and to estimate the numbers of these organisms in the presence of the dominating lactic flora (89).

Other Microorganisms

Dacre (24, 25) found substantial numbers of microorganisms in New Zealand Cheddar cheese which he identified as pediococci. Pediococci were also isolated from Cheddar cheese by Franklin and

Sharpe (43). The presence of bacteria of the genus *Pediococcus* has not been reported in U. S. Cheddar cheese. Dacre noted that the pediococci were about one-fourth as numerous as the lactobacilli throughout the ripening period.

Leuconostoc citrovorum was isolated from Cheddar cheese displaying the open defect, commonly associated with the fruity flavor defect, by Overcast and Albrecht (105). They suggested that heterofermentative lactic acid bacteria such as *L. citrovorum* produce large amounts of carbon dioxide which causes the fracturing of curd particles.

Chemistry of Cheddar Cheese Ripening

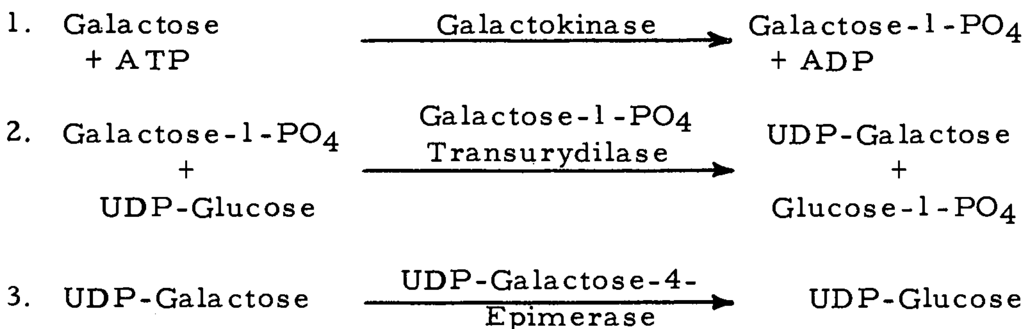
New-made Cheddar cheese has a mild, characteristic flavor but is quite bland compared to the ripened product. The body of the new-made cheese is hard and rubbery, a condition described as "corky". These observations indicate that the characteristic flavor and body of mature Cheddar cheese are evolved during the ripening period by various chemical changes involving the major milk constituents; lactose, fat, and protein; and perhaps some of the minor constituents. Reactions involving these constituents are believed to be largely catalyzed by enzymes (149, p. 651). The enzymes involved in ripening may be derived from: (a) microorganisms added to the milk as starter, (b) microorganisms present in the milk or

inadvertently incorporated into the cheese during manufacturing, (c) rennet, and (d) the milk.

Fermentation of Lactose

A considerable portion of the lactose fermentation takes place during the manufacturing process before the true ripening period begins. Nevertheless, low levels of lactose have been demonstrated in Cheddar cheese during the early stages of ripening (34, 39); and intermediates of lactose fermentation appear to be present throughout the ripening process, although the origin of intermediates such as pyruvate (7, 74) would not necessarily be lactose.

The initial step in the fermentation of lactose involves hydrolysis to yield glucose and galactose. Glucose is immediately susceptible to further catabolic processes, but galactose must first be converted to a glucose-1-phosphate as outlined by Kandler (67):

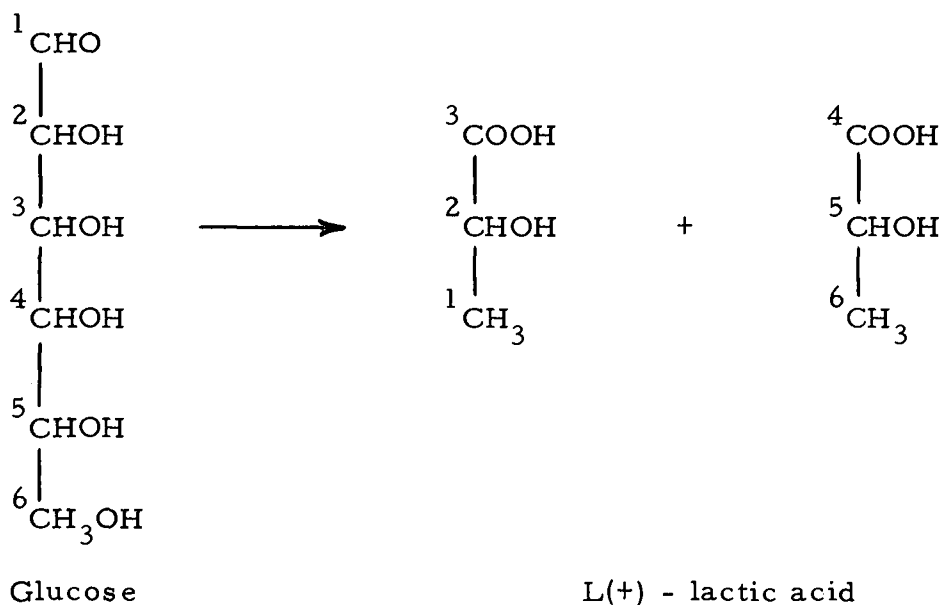


Lactose Fermentation by Homofermentative Lactic Bacteria.

Homofermentative utilization of glucose, produced from lactose as

outlined above, proceeds by the Embden-Meyerhof-Parnes (EMP) pathway. The theoretical yield of lactic acid via this route is two molecules per molecule of glucose fermented. By definition, homofermentative microorganisms are those which utilize glucose via the EMP scheme and produce lactic acid almost exclusively as a terminal product of the fermentation.

The distribution of the hexose carbon atoms in the lactic acid molecules after fermentation via the EMP scheme has been determined by means of radioactive tracer methods (67). The results of such studies are shown below:



Organisms associated with Cheddar cheese which would be classified as homofermentative are *S. lactis*, *S. diacetilactis*, *S. cremoris*, *S. faecalis*, *S. durans*, *S. thermophilus*, and *Streptococcus*

liquefaciens (121); *L. casei*, *L. plantarum*, and *L. helveticus* (42, p. 17).

It has been demonstrated that the homofermentative organisms, once believed to produce lactic acid exclusively as an end-product of glucose metabolism, produce small amounts of other compounds. Friedman (44) demonstrated the production of formic acid, acetic acid, and ethanol by *S. faecalis* in a peptone medium enriched with glucose. Gunsalus and Niven (51) showed that at alkaline pH, homofermentative organisms can produce up to 40% formic acid, acetic acid, and ethanol in the ratio of 2:1:1. In addition to lactic acid, Platt and Foster (121) reported that certain strains of *S. faecalis* produced acetic acid, formic acid, carbon dioxide, ethanol, diacetyl, acetoin, and 2, 3-butanediol. The latter authors found that a strain of *S. cremoris* produced acetic acid, formic acid, carbon dioxide, and ethanol; while a strain of *S. lactis* produced acetic acid, carbon dioxide, ethanol, and acetoin. *S. faecalis*, *S. cremoris*, and *S. lactis* produced 7. 4, 6. 4, and 2. 8 mM of ethanol per 100 mM of glucose fermented, respectively. The approximate molar equivalence of ethanol and carbon dioxide yields led Platt and Foster to speculate that carbon dioxide arises from the conversion of pyruvic acid to ethanol.

Homofermentative streptococci were reported to produce highly active alcohol dehydrogenase by Gunsalus and Wood (52). The

presence of acetaldehyde, the immediate product of the decarboxylation of pyruvic acid, has only recently been demonstrated in cultures of homofermentative lactic streptococci. Harvey (57) has reported that in milk medium strains of *S. lactis* produced from 0.4 to 4.5 mg of acetaldehyde per liter, *S. cremoris* produced from 0.5 to 9.0 mg per liter, and a strain of *S. diacetilactis* produced from 11 to 13 mg per liter. Acetaldehyde produced by *S. diacetilactis* was implicated by Badings and Galesloot (5) to be the component responsible for the green or yoghurt-like flavor defect of butter cultures. Lindsay *et al.* (88) and Lindsay (87) have shown recently that this defect is also produced by *S. lactis* when the amount of acetaldehyde produced is high compared to the amount of diacetyl produced in mixed-strain butter cultures. When *L. citrovorum*, a heterofermentative organism, was present in sufficient numbers, much of the acetaldehyde produced by the lactic streptococci was transformed into another product or products (5, 88). Galesloot (45) has suggested that under conditions of low oxygen tension where reduced pyridine nucleotides accumulate, the conversion of acetylphosphate, a form of acetaldehyde, to ethanol would afford *L. citrovorum* a means of regenerating the pyridine nucleotides. Lindsay *et al.* (88) demonstrated that added acetaldehyde was stimulatory to the growth of *L. citrovorum*, and they postulated that acetaldehyde was reduced to ethanol thereby regenerating the pyridine nucleotides.

Pyruvic acid is a key intermediate in the fermentation of lactose. Marth (91) summarized the reactions which pyruvic acid can undergo during the course of homofermentative activity. First, pyruvic acid can be reduced to lactic acid, and this is the predominant reaction which takes place. Second, pyruvic acid can be converted to alanine by means of a reductive amination. Third, malic acid can be produced by the carboxylation and reduction of pyruvic acid. Carbon dioxide is utilized in the latter reaction; and this explains, perhaps, the carbon dioxide requirement for growth of the lactic streptococci, since malic acid can be converted to oxalacetic acid, an important precursor of other amino acids. Fourth, pyruvic acid can be converted to phosphoenolpyruvic acid which can then be carboxylated to form phosphoenoloxaloacetic acid, then oxalacetic acid. Carbon dioxide is also required in the latter reaction. The formation of oxalacetic acid by these routes is necessary to the lactic acid bacteria since they are unable to utilize the citric acid cycle for the formation of this acid. Fifth, acetaldehyde, alpha-acetolactic acid and acetoin may arise from pyruvic acid.

As mentioned by Reiter and Møller-Madsen (124), there is some evidence to indicate that some homofermentative lactic streptococci are also able to utilize glucose via the Entner-Doudoroff pathway, shown in Figure 1. This route would provide another mode of synthesis of ethanol and carbon dioxide which are known to be

metabolic products of these organisms.

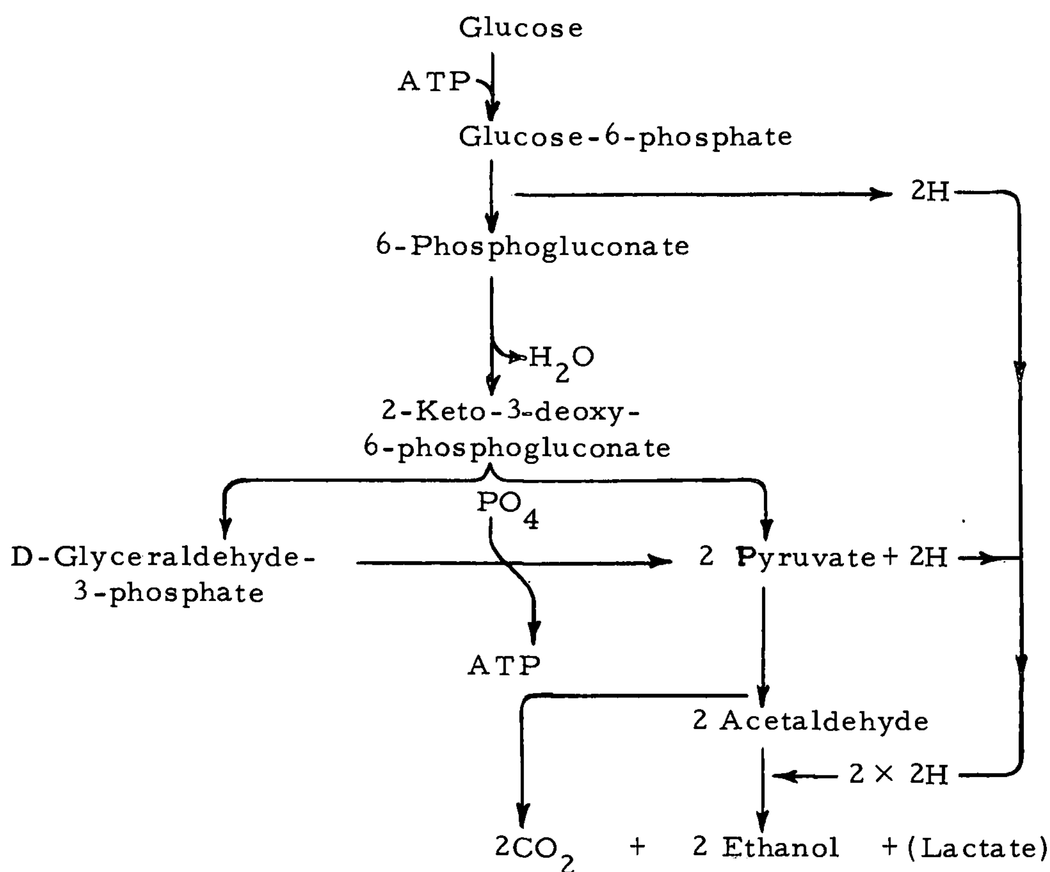


Figure 1. The Entner-Doudoroff pathway (152, p. 59).

Lactose Fermentation by Heterofermentative Lactic Bacteria. Heterofermentative utilization of lactose results in a considerable amount of end products other than lactic acid. Heterofermentative lactic bacteria produce predominately D(-)-lactic acid in contrast to the production of predominately L(+)-lactic acid by the homofermentative organisms (45). Glucose is utilized by the heterofermentative organisms by way of the hexosemonophosphate shunt (HMP)

as outlined by Kandler (67) and shown in Figure 2. Heterofermentative bacteria are forced to use this pathway for glucose metabolism because they lack the enzyme aldolase. Aldolase is required to catalyze the conversion of fructose-1, 6-diphosphate to dihydroxyacetone phosphate and 3-phosphoglyceraldehyde in the EMP scheme.

Typical heterofermentative organisms which have been associated with cheese are _L. citrovorum_ and _L. brevis_.

Reiter and Møller-Madsen (124) suggest that in addition to homofermentative and heterofermentative organisms there should be a third class of lactic acid bacteria, the facultatively homofermentative organisms. The latter class of organisms have the enzymes necessary for glucose fermentation via either the EMP or HMP scheme, but utilize the EMP pathway nearly exclusively. Some evidence has accumulated to indicate that some strains, at least, of _S. lactis_ and _S. cremoris_ have the ability to utilize the HMP pathway. Shahani and coworkers (129, 130, 131) have demonstrated key enzymes involved in both schemes for one strain of _S. lactis_. At low glucose concentrations, _L. casei_ appears to preferentially utilize the HMP pathway; and growing cells metabolize more glucose this way than by the EMP scheme (124).

Origin of Free Fatty Acids

It appears that free fatty acids play an important role in the

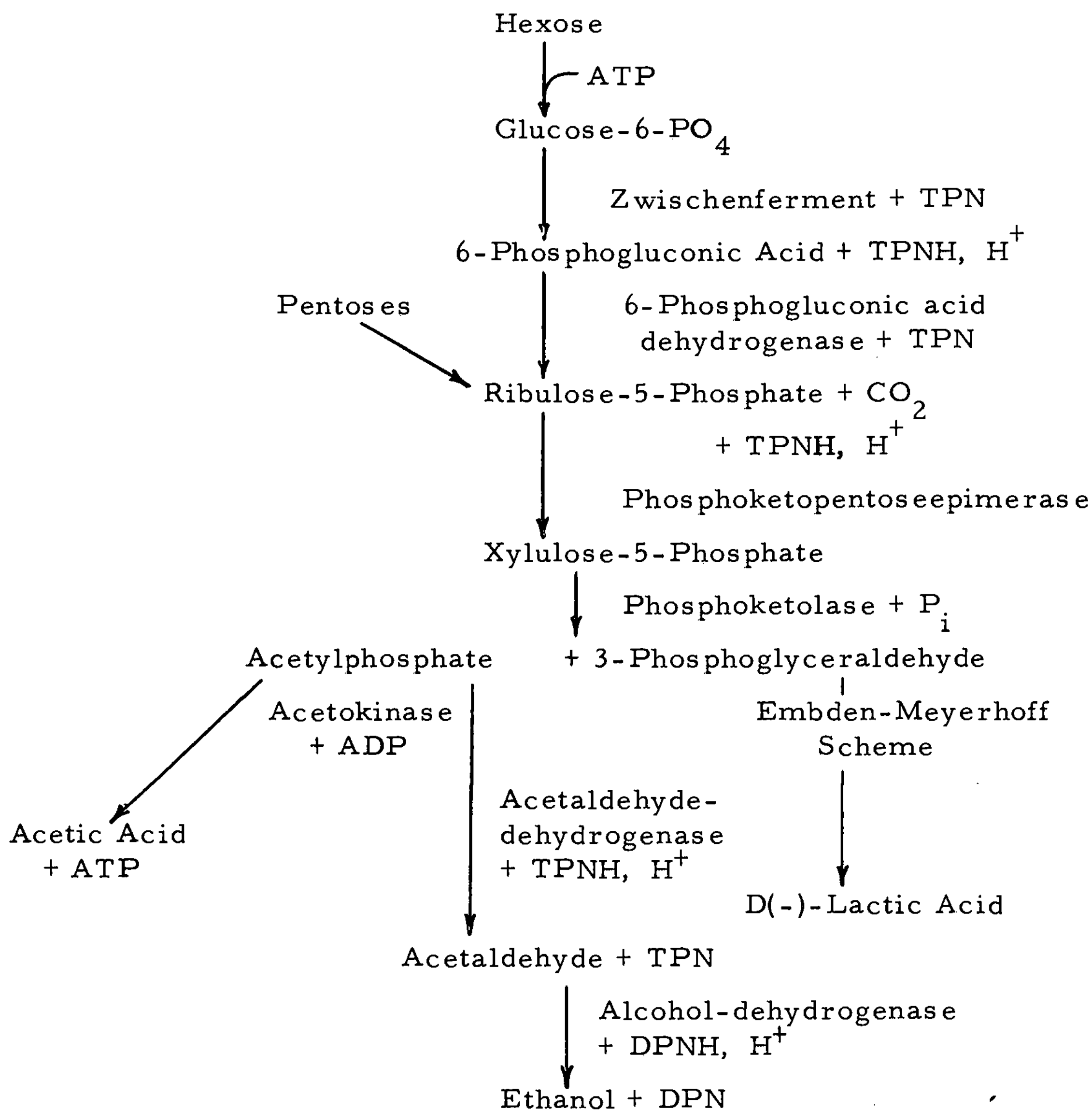


Figure 2. Carbohydrate metabolism of heretofermentative lactic acid bacteria as described by Kandler (67).

flavor of Cheddar cheese, but their origin remains uncertain. Acetic acid may be evolved through rather well known metabolic routes which have been discussed in the previous section. The precursors, metabolic route(s), enzymes, and microorganisms involved in the production of acetic acid in Cheddar cheese have not been elaborated, however. The same is true of formic and propionic acids which have been reported on occasion (77, 107). The bulk of the free fatty acids butyric through linolenic probably arise from the milk triglycerides through hydrolytic activity. The enzymes responsible for lipolysis in Cheddar cheese could be from the milk, from microorganisms associated with ripening, or from rennet preparations added to the milk. Rice and Markley (125) first suggested that milk lipase was one of the causes of rancidity in cheese, but Peterson and Johnson (118) reported that milk lipase is inactive at the pH of Cheddar cheese and is absent in the cheese after pressing. The latter authors noted the appearance of lipases in new-made cheese after five to 20 days and attributed these lipases to the growth of lipolytic bacteria in the cheese. Lipolytic organisms were isolated from Cheddar cheese by Franklin and Sharpe (43). The organisms capable of hydrolyzing milk fat consisted mainly of Gram-positive cocci, predominantly _Staphylococcus saprophyticus_. _Streptococcus faecium_, a lipolytic streptococci, was found less frequently. Lane and Hammer (82) produced typical rancid Cheddar cheese by the addition of lipase sources such

as rennet paste and pancreatin.

The data of Bills and Day (9) and of Kristoffersen (77) suggest that butyric acid is preferentially hydrolyzed from the triglycerides or is a product of microbial synthesis. In both of these investigations, free butyric acid was found in a higher percentage, compared to other free fatty acids, than its percentage composition in milk fat triglycerides. Other investigators have demonstrated a specificity of certain lipases for the 1-position of milk triglycerides (46, 65), and it has been reported that 75% of the butyric acid is esterified at the 1- and 3-positions which are equivalent (17).

Although the development of free fatty acids during the manufacturing and ripening of Cheddar cheese has been well demonstrated, the literature provides no conclusive evidence concerning the origin or origins of individual free fatty acids.

Proteolysis

The presence of amino acids, ammonia, and other degradation products of protein in Cheddar cheese has been amply outlined in a previous section of this manuscript. Peterson et al. (119) clearly demonstrated proteinase activity in Cheddar cheese.

It is apparent from the work of Kosikowski (72) and Mabbit (90) that proteolytic activity does not proceed in a random manner and that given amino acids become detectable in the cheese at different

stages of ripening. The proteinases which act on casein could originate from the milk itself, from the rennet preparation added at the time of manufacture, or from the microorganisms which proliferate during ripening. The latter source of proteolytic enzymes has usually been considered to be the most important.

Proteolytic ability of the starter streptococci has been clearly demonstrated by several authors. Morgan (95) reported that S. lactis produced a considerable amount of free amino acids when grown in skimmilk. The intracellular proteinases of S. lactis were investigated by Baribo and Foster (6). The latter authors reported that proteinases active at pH 5.0-5.5 as well as at pH 7.0 were present in the cells and probably accounted for some of the proteolytic activity which takes place in Cheddar cheese. Czulak and Shimmin (22) compared the proteolytic ability of several strains of S. cremoris and concluded that all strains tested were able to hydrolize casein, but that some strains which produced peptide bitterness in cheese were less efficient in completing the hydrolysis of peptides to amino acids.

The proteolytic activity of some strains of L. casei in Cheddar cheese has also been demonstrated. Lane and Hammer (81) innoculated pasteurized cheese milk with eight different strains of L. casei and a lactic starter. Proteolysis occurred more rapidly in Cheddar cheese made with six of the eight strains than in the control cheeses, and this condition prevailed throughout the entire ripening period.

Cheeses innoculated with *L. casei* were found by Bullock and Irvine (16) to have a higher content of amino acids than control cheeses after eight and one-half months of ripening. Yates *et al.* (154) reported that the addition of proteolytic strains of *L. casei* to Cheddar cheese resulted in more rapid flavor development.

Proteolytic enzymes were isolated from cells of *L. casei* by Baribo and Foster (6). These authors reported maximum enzyme activity near pH 7.0. Brandsaeter and Nelson (14, 15) reported the isolation from *L. casei* of a proteinase active in the pH range 5.5 to 6.5 and a peptidase with maximum activity near pH 7.0.

As Baribo and Foster (6) observed, certain micrococci also produce proteolytic enzymes. In this study, they found that *Micrococcus freudenreichii* possessed proteolytic enzymes with pH optima of 5.0 to 5.5 and near 7.0. They concluded that micrococci probably contributed to the total proteolytic activity in Cheddar cheese.

Other Possible Mechanisms

The production of hydrogen sulfide by strains of *L. casei* has been documented by Kristoffersen and Nelson (78, 79) and by Sharpe and Franklin (132). The former authors noted that in the cheeses studied, at least one strain of hydrogen sulfide producing *L. casei* was isolated from each cheese scoring 39 to 40. Pette (120) implicated an organism resembling *S. faecalis* in the production of excess

hydrogen sulfide in Gouda cheese.

The series of methyl ketones which has been isolated from Cheddar cheese includes acetone, butanone, 2-pentanone, 2-heptanone, 2-nonanone, 2-undecanone, 2-tridecanone, and 2-pentadecanone. A possible mode of formation of the above methyl ketones, with the exception of acetone and butanone, would be the decarboxylation of the corresponding beta-ketoacids which have been identified as normal constituents of milkfat by van der Ven *et al.* (145). Beta-oxidation of even-numbered fatty acids by microorganisms could also provide the same beta-ketoacids plus beta-ketobutyric acid, the precursor of acetone. Neither of these mechanisms would provide a precursor for butanone, since neither valeric nor beta-ketovaleric acids occur to any extent in milkfat (59, 145). Scarpellino and Kosikowski (127) proposed a route for the synthesis of butanone from acetoin by way of 2, 3, butanediol. The validity of this mechanism has not been demonstrated, however.

The production of acetone by strains of *S. lactis*, *S. cremoris*, and *S. diacetilactis* has been demonstrated by Keenan (68). Harvey (57) reported that *S. cremoris* and *S. lactis* produced acetone, while *S. diacetilactis* was only able to utilize acetone. A known route of synthesis of acetone involves the condensation of acetyl phosphate and active acetate to yield beta-ketobutyric acid which is then decarboxylated to yield acetone. Pyruvic acid serves as the precursor

of both acetyl phosphate and active acetate. Clostridium butylicum is an example of an organism which utilizes this pathway (122, p. 340-344). The manner in which the lactic streptococci produce acetone has not been elaborated. Definite mechanisms and organisms associated with the formation of methyl ketones in Cheddar cheese are not presently known.

Anderson (4) has demonstrated the conversion of butanone to 2-butanol by Penicillium roqueforti and yeasts associated with the ripening of Blue cheese. A culture of S. lactis was unable to carry out the same reduction, however. Butanone is a likely precursor of 2-butanol in Cheddar cheese, but organisms and enzymes capable of this reduction have not been reported.

The synthesis of diacetyl has been thoroughly covered by Seitz (128, p. 95). It will suffice here to note that several of the precursors in the synthesis of diacetyl, as outlined by Seitz, have been isolated from Cheddar cheese. This list of precursors includes oxaloacetic acid, pyruvic acid, and alpha-acetolactic acid (7), acetaldehyde and acetoin (111).

The formation of various acidic carbonyl compounds from citric acid by lactic acid bacteria has recently been demonstrated by Harper (54). Oxalsuccinic, alpha-ketoglutaric, alpha-acetolactic, glyoxylic, and oxalacetic acids were produced by Lactobacillus bulgaricus, Lactobacillus lactis, S. thermophilus, and S. lactis from a labeled

citrate substrate.

Fruity Flavor Defect of Cheddar Cheese

Perry (115) manufactured Cheddar cheese using single strains of *S. cremoris* and *S. lactis* as starters. He reported that the three *S. lactis* strains produced an abnormal flavor in the cheese, while three strains of *S. cremoris* yielded cheese of good flavor. The abnormal flavor resulting from the use of *S. lactis* strains as starter cultures was described as dirty or fruity. The flavor defect was said to become more pronounced as the cheese aged. Perry demonstrated that the *S. lactis* strains survived in much larger numbers in the cheese than did the *S. cremoris* strains. After three weeks of ripening, the *S. cremoris* population had fallen to less than 5% of the original number, while the population of *S. lactis* remained at 42% of the original number, and reached 5% only after more than 12 weeks.

The production of the fruity flavor defect of Cheddar cheese through the use of selected starter cultures was studied by Vedamuthu (144). Two starter cultures, one designated *B* containing *S. lactis* plus *S. cremoris* strains and the other designated *C* containing *S. lactis* plus *S. cremoris* plus *S. diacetilactis* strains, were found to consistently produce the defect when used for the manufacture of Cheddar cheese. A third culture, consisting solely of *S. cremoris* strains and designated *A*, produced normal, good-quality cheese.

The starter organisms in the cheeses manufactured with cultures B and C persisted at high levels for an extended period and appeared to inhibit the normal development of the adventitious lactobacilli. The use of culture A resulted in a rapid decline of the starter organisms in the cheese and the appearance of lactobacilli at the normal time of about eight weeks. In the cheeses manufactured with cultures B and C, the appearance of the lactobacilli was often delayed until 16 to 24 weeks. Attempts to correlate differences between starters to rates of lactose utilization and protein degradation were unsuccessful. Comparison of the production of carbonyl compounds between starters A and C grown in milk cultures revealed that the latter produced higher concentrations of formaldehyde, diacetyl, and possibly pyruvic acid.

Harrison (55) isolated several yeasts from cheese samples and found that they were able to produce fruity odors when grown in milk. The production of esters by yeasts has been demonstrated more recently by Nordström (103).

A fermented flavor, often associated with the fruity flavor defect, was produced in Cheddar cheese by Sherwood (135) by incorporating lactobacilli isolated from mature cheese into the cheese milk.

Vedemuthu (144) has observed that the fruity flavor defect is generally accompanied by a defect in the body of the cheese described

as slit-openness. The production of sufficient gas during the ripening process to cause long, open fractures to develop within curd particles appears to be the cause of this defect.

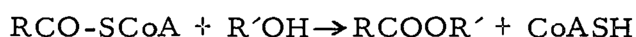
Formation of Esters in Biological Systems

Esterification reactions mediated by living cells are extremely common. The esterification of fatty acids and glycerol results in the oils and fats so universally found in the plant and animal kingdoms. Studies concerning the esterification of aliphatic monocarboxylic acids with aliphatic n-alcohols, however, have not been extensive, and mechanisms involved in such esterification reactions have only recently been investigated. The occurrence of volatile aliphatic esters in nature is not uncommon. Volatile esters are produced in many of the fruits of higher plants, but microorganisms capable of producing volatile esters are comparatively rare and have received special attention.

Some yeasts are capable of forming enormous amounts of volatile esters, especially ethyl acetate. Peel (112) measured the quantities of ethanol, acetic acid, and ethyl acetate produced by certain aerobic yeasts. The concentration of ethyl acetate was reported to exceed by 1,000 times the amount which would have resulted from the equilibrium between the acid plus alcohol and the ester. This finding suggested that a mechanism other than simple esterification

was responsible for the production of ethyl acetate in these cultures.

A comprehensive study of the mechanisms involved in the formation of volatile esters in fermentation with brewer's yeast was conducted by Nordström (103). At physiological pH values, the direct esterification involving ethanol and acetic acid was ruled out for several reasons. First, the non-enzymatic reaction would have been too slow to account for the amounts of ethyl acetate formed. At lower pH values, however, it was acknowledged that non-enzymatic, direct esterification becomes a very important factor. Second, the possibility that direct esterification was accelerated by the presence of an esterase was also ruled out, since no correlation between the amount of acetic acid produced and the amount of ethyl acetate formed was noted. Third, the equilibrium concentration was in many cases exceeded. Further experiments led to the conclusion the coenzyme A (CoA) is an essential cofactor of ester formation. Ester formation was inhibited by sodium arsenite, a substance known to inhibit CoA activity, at concentrations that did not effect growth. The manner in which CoA was believed to participate in the esterification reaction was as follows:



$$RCO\text{-}SCoA + R'OH \rightarrow RCOOR' + CoASH$$

Since this is such an important reaction in many stages of metabolism, ester formation was thought to be associated with essential

metabolic processes in the cell. The formation of esters from acids added to the medium and the formation of ethyl acetate were shown to be competitive processes. The proposed scheme showing the relationship of fatty acid activation, energy requirement, ester formation, and other metabolic pathways is shown in Figure 3. The addition of alcohols other than ethanol to the fermentation system indicated that other alcohols can serve as the substrate for ester formation. Esterification velocity decreased with increasing molecular weight of the alcohol, and iso-alcohols were found to be poorer substrates than their straight-chain isomers. Secondary and tertiary alcohols did not give rise to esters.

Newsome and Rattray (102) investigated the enzymatic esterification of ethanol with fatty acids. Pancreatin was found to catalyze the esterification of ethanol with oleic, linolenic, linoleic, arachidonic, myristic, palmitic, and stearic acids. Oleic acid was esterified most rapidly followed by the other acids in the above order. The esterification of ethanol appeared to be governed by solubility factors with the rate of esterification decreasing past the point at which definite turbidity was produced by the dispersed fatty acids. This would indicate that the reaction was favored by an esterase-like activity in the soluble state rather than a lipase-like activity at the interface between the emulsified acids and the aqueous phase. It is of interest to note, however, that the greatest degree of selectivity was

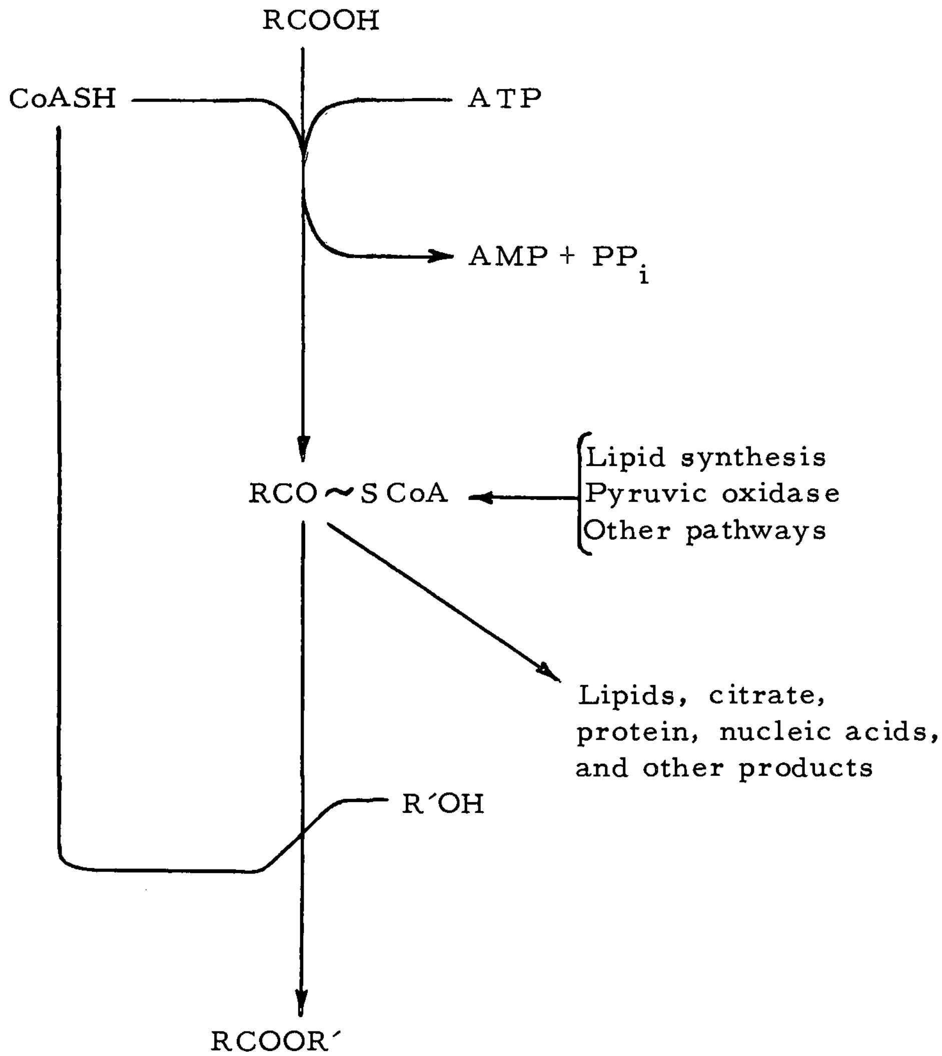


Figure 3. The relationship of fatty acid activation, energy requirement, ester formation, and other metabolic pathways in certain yeasts as outlined by Nordström (103).

evidenced when the fatty acids were present in the emulsified state. The pH optimum of the reaction varied with the fatty acid involved, with pH optima being noted for different acids as follows: myristic, 5.5; palmitic, 5.7; stearic, 5.7; oleic, 6.1; linoleic, 5.9; linolenic, 5.5; and arachidonic, 5.9.

The formation of esters by filamentous fungi has been reported by a limited number of investigators. Gordon (47) reported the formation of ethyl acetate by _Endoconidiophora moniliformis_. Ethyl acetate was also produced by _Penicillium digitatum_ according to Birkinshaw _et al._ (12). Isobutyl acetate was identified by Morgan (94) as a product of _Endoconidiophora coerulescens_. Collins and Morgan (19), using gas-liquid chromatography, identified the substances which are responsible for the ester-like aroma produced by _Chalaropsis thielavioides_ grown in potato dextrose broth; the major components were isobutyl and ethyl acetate, but lesser amounts of isoamyl acetate, ethanol and methanol were also found. In another publication Collins and Morgan (20) reported that _Ceratocystis moniliformis_, _Ceratocystis major_, _Ceratocystis coerulescens_, and _Thielaviopsis basicola_ produced appreciable quantities of ethanol and ethyl acetate. Isobutyl acetate was a major component in distillates obtained from _C. moniliformis_ and _Ceratocystis fimbriata_ var. _platani_ according to the same investigators.

A fruity aroma defect which occurs in milk, cream, and cottage

cheese is caused primarily by the psychrophile, Pseudomonas fragi. This organism was first isolated from milk having a strawberry-like odor by Eichholz (35). Pereira and Morgan (114) observed that the typical fruity aroma was produced when the organism was grown in a basal salts medium containing leucine, alanine, or glutamic acid as a carbon source; or in a glucose medium containing threonine as a nitrogen source. In a later work, Pereira and Morgan (113) identified the fruity components produced by P. fragi in milk cultures as predominately esters of isovaleric and acetic acids. Paper chromatography of the hydroxamic acid derivatives of the acids obtained from milk cultures of the organism revealed that isovaleric acid was the most abundant followed by acetic, formic, and propionic acids. Since ethanol was the only alcohol found in the cultures, they concluded that the esters detected as hydroxamates were probably ethyl esters. It was also concluded that the esterification was actually mediated by the cells of P. fragi since the addition of ethanol and isovaleric acid to sterile milk did not result in detectable ester formation over the incubation period required for ester formation in the presence of the growing cells. The production of ethanol by the organism appeared to be the limiting factor in ester formation. When one ml of ethanol was added per liter of milk prior to incubation, esters were developed sooner and in larger amounts by the organism.

Hart *et al.* (56) investigated the formation of esters in Cheddar cheese as early as 1914. They found that esters were produced in milk cultures of certain bacteria isolated from ageing cheese. One strain of Streptococcus (designated only as variety b on the basis of its ability to ferment lactose, sucrose and glycerin but not salicin or mannitol) was found to produce comparatively large quantities of alcohols and esters, and one strain of *Bacterium casei* (possibly *Lactobacillus casei*, at any rate a member of the genus now called Lactobacillus) was also shown to produce esters. They observed that esters do not appear in Cheddar cheese until it is about five weeks old. As a result of experiments in which ethanol and acetic acid were allowed to stand for a few months in dilute aqueous solutions, they came to the conclusion that esters were probably the result of biological activity and were not produced by the mere contact of alcohol and acid. Ethyl acetate was detectable after standing only when the concentration of added ethanol and acetic acid reached two percent in the initial solution. The possibility that the concentration of alcohol and acid may actually be quite high in the aqueous phase of Cheddar cheese due to the fact that there is probably little "free" water in the cheese mass was not overruled. These investigators also felt that the acids or alcohols might be associated in some manner with the constituents of the cheese so that they could be brought together in a manner favoring ester formation. They

also sought to determine whether the natural enzymes of milk could produce esters, alcohols, or volatile acids in curing cheese. When cheese was manufactured from chloroformed milk, none of the above products were isolated after five months of curing. The conclusion drawn from this experiment was that natural milk enzymes had little to do with the formation of volatile compounds.

EXPERIMENTAL

Samples of Cheddar cheese possessing varying degrees of the fruity flavor defect and their matching non-fruity controls were obtained from the Department of Microbiology and the Department of Food Science and Technology. These cheeses were the result of earlier experimental work (144) and were manufactured and ripened under identical conditions, except that starter cultures of predominately _S. lactis_ or _S. diacetilactis_ were used in the manufacture of the fruity cheeses while starters of predominately _S. cremoris_ were used in the manufacture of the control cheeses.

Identification of Compounds Responsible for the Fruity Flavor Defect

Eight kilograms of a cheese displaying a very pronounced, typical fruity flavor defect was utilized for this phase of the experimental work. The cheese was packed into 50 ml stainless steel centrifuge tubes and centrifuged 15 min at 30,000 times gravity in a Servall SS-3 Superspeed centrifuge. After centrifugation, the supernatant fat was decanted off and stored in the feed flask of the Rota-Film molecular still at 4° C. At the start of the distillation, the feed flask was fitted to the molecular still and the fat melted by means of an infrared lamp after establishing a vacuum in the system. The

fat was degassed and then passed through the still at a rate of two or three ml per min. Volatile constituents were collected during degassing and distillation in a trap packed with glass beads and cooled with liquid nitrogen. The temperature of the fat obtained from the cheese did not exceed 40° C during the centrifugation and distillation procedures, thus minimizing heat-induced artifact production.

After the distillation had been completed, a portion of the most volatile material was examined by gas-liquid chromatography. The device used for transferring volatiles from the trap cooled with liquid nitrogen to the gas chromatograph has been described in an earlier publication (84); this device consists essentially of a length of 1/8 inch OD stainless steel tubing bent into a U-shape. Volatiles are transferred from the distillation trap into the U-shaped bend, which is immersed in liquid nitrogen, by drawing a vacuum through the transfer device and allowing the distillation trap to warm slightly. The volatile components were introduced into the gas chromatograph by fitting a hypodermic needle to the transfer device, warming the U-shaped portion, and directing the carrier gas through the device. A Barber-Colman Model 20 gas chromatograph, fitted with a 1/8-inch by 10 ft column packed with 20% Apiezon M on 100-120 mesh Celite 545 and operated at 73° C, was used for the separation of this fraction. A splitter at the end of the column routed a portion of the column effluent to the beta-ionization detector and the remainder to

the atmosphere through a heated outlet at a ratio of about 1:10, thereby making it possible to monitor the odor of the separated components.

The remainder of the molecular distillate was washed from the cold trap with 150 ml of ethyl chloride, and this extract was concentrated to a volume of about 1 ml by means of a reflux apparatus described by Bills et al. (10). This extract was chromatographed on a 1/8-inch by 10 ft column packed with 20% diethylene glycol succinate on 80-100 mesh Celite 545 at 100° C. As before, the odor of the separated components was determined in the portion of effluent split to the atmosphere.

Positive identification of the fruity components detected in the ethyl chloride concentrate was made by mass spectrometry. An Aerograph Model 600 gas chromatograph was used in conjunction with an Atlas CH-4 mass spectrometer (a 9-inch, 60-degree sector, single-focusing instrument). For the continuous analysis of the effluent from the gas chromatograph, the mass spectrometer was equipped with the EC-1 gas inlet valve. The Apiezon column previously described was used for the separation. After an initial isothermal period of 47 min at 99° C, the column was programmed to a final temperature of 170° C over a period of 12 min and held at this temperature for an additional 44 min. The column effluent was split, routing 10% to the flame ionization detector and 90% to the EC-1

inlet system. Approximately 5% of the gas stream routed to the inlet system was bled into the mass spectrometer for analysis, and the remainder was exhausted to the atmosphere for odor evaluation. Through use of appropriate lengths of capillary tubing following the effluent split, simultaneous response was obtained at the flame detector and at the mass spectrometer ion source. The mass spectrometer was operated with an accelerating potential of 3, 000 V, an ionizing current of 60 μA, and an ionizing voltage of 35 ev. Mass spectra were obtained by magnetic scanning; five sec were required to scan the mass range 25 to 250. Mass spectra were recorded with a Honeywell Visicorder Model 1508.

Comparison of the Volatile Constituents of Fruity and Control Cheeses

Cheese samples were packed into stainless steel centrifuge tubes and centrifuged as described in the previous section to obtain a clear fat-layer. The fat samples were analyzed using the gas entrainment on-column trapping technique developed by Morgan and Day (97). In essence, this technique consisted of passing a stream of nitrogen through the sample contained in a screw-capped vial (Kimble no. 60957, size no. 1) by means of a hypodermic needle. The hypodermic needle was inserted through one of two holes drilled through the cap, the original liner of the cap having been replaced by

a 1/8-inch-thick silicone rubber septum. A second shorter hypodermic needle was inserted through the second hole in the cap to provide an outlet for the nitrogen stream and the entrained volatiles. The entrained volatiles were trapped in a U-shaped bend at the head of the GLC column which was temporarily withdrawn from the oven of the F and M Model 810 instrument. The U-shaped portion of the column was immersed in a mixture of dry ice and 2-methoxyethanol. After the collection period, the head of the column was replaced in the instrument and the carrier gas flow resumed. During the collection period a waterbath was used to warm the vial containing the sample to improve the efficiency of the removal of volatile materials. The entrainment assembly is shown in Figure 4.

For the analysis of the cheese fat obtained by centrifugation, nitrogen was bubbled at a rate of ten ml per min through seven g of fat containing an excess of anhydrous sodium sulfate. The waterbath in which the vial containing the sample was immersed was held at 80° C during the collection period. A 1/8-inch by 10 ft column packed with 20% 1, 2, 3-tris (2-cyanoethoxy) propane on 80-100 mesh Celite 545 was employed. The instrument was operated at range and attenuation settings of ten and one, respectively. The entrained volatiles were separated at oven temperatures of both 50° and 80° C to provide adequate resolution of the entire spectrum of volatile constituents. Identity of the critical components separated by this system

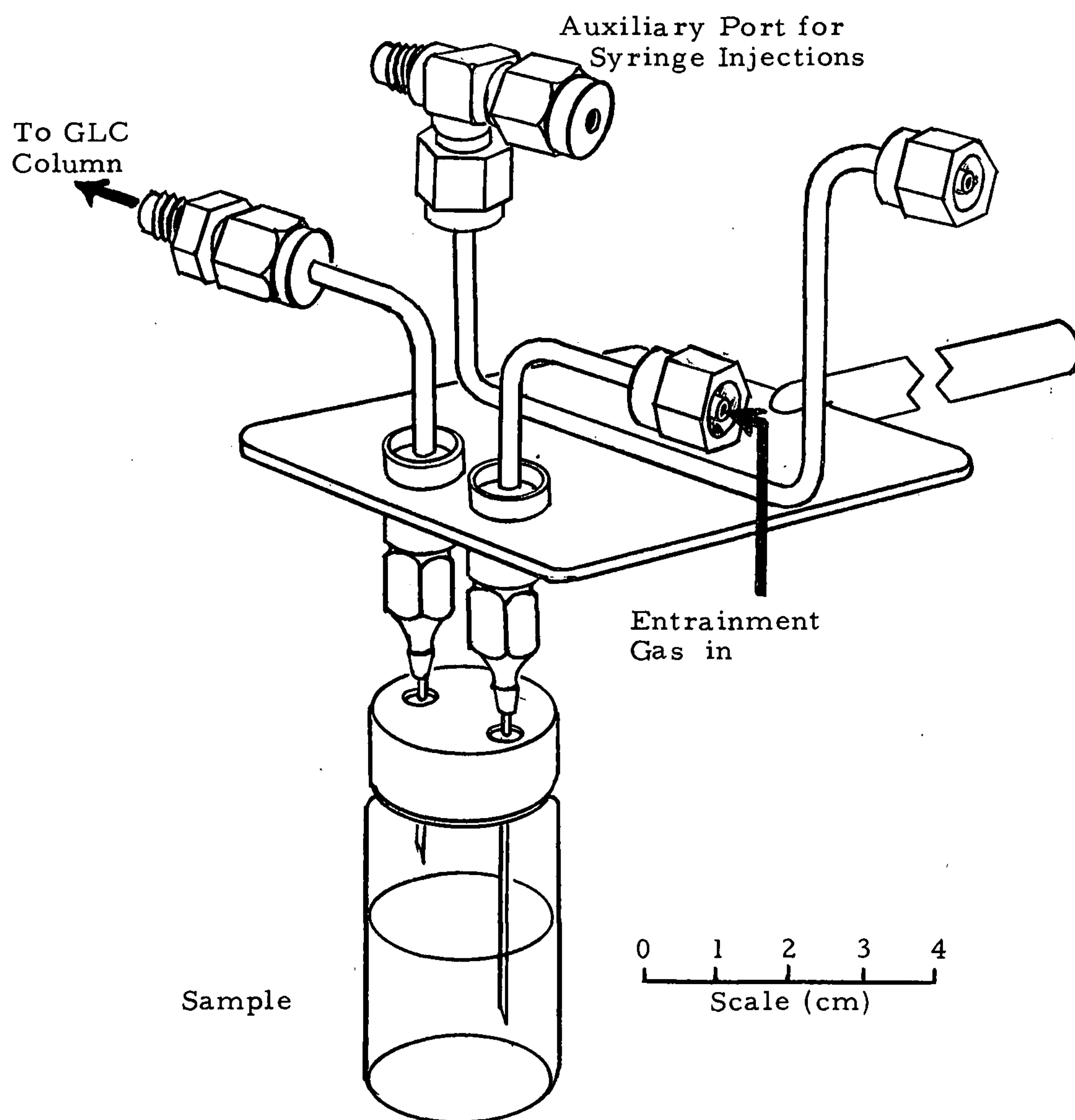


Figure 4. The entrainment assembly used in the analysis of volatiles.

was established by coincidence of retention times and mass spectral analysis.

Analysis of Cultures for Volatile Constituents

The on-column trapping technique outlined in the previous section was again utilized, except certain modifications were made which resulted in improved precision and ease of operation. The hypodermic needles were replaced with 1/16-inch OD, .04-inch ID stainless steel tempered capillary tubing. The lower tips of the capillary tubing were sharpened to resemble hypodermic needles and the tops were equipped with standard 1/16-inch Swagelok ferrules and nuts. The fabricated needles were attached to the system by means of Swagelok 1/8-inch to 1/16-inch reducing unions. This modification eliminated the leakage problems encountered occasionally with the original Luer-Lok fittings. A further modification consisted of adding a thermostatically controlled heating element to the water bath to simplify the maintenance of a given temperature. The GLC separation was carried out on a column identical in all respects to the 1, 2, 3-tris (2-cyanoethoxy) propane column previously described, except that the column length was 12 ft. Nitrogen pressure was regulated to give a flow rate of 7.65 ml/min at the exit of the column during the collection period.

The actual analysis consisted of adding 4.0 ml of culture to a

screw-cap vial containing 5.0 g of anhydrous sodium sulfate heated at least four hr at 110 °C. Four ml of water and a few milligrams (about the size of a pin head) of 1-tetradecanol were then added to the vial. A collection period of five min with a water bath temperature of 60 °C provided sufficient material for the detection of even minor components. A column temperature of 50 °C was found to give satisfactory separation of the volatile constituents.

Determination of Ethanol and Other Components in Commercial Cheeses

A 2.0 g sample of whole cheese was ground intimately with 5.0 g of anhydrous sodium sulfate in a screw-capped vial by means of a small spatula. Six ml of distilled water was added to the vial; the vial was capped and shaken vigorously to temporarily suspend the sample and the sodium sulfate. The sample was then analyzed in the manner just described for the analysis of cultures.

Methods for Relating Recorder Response to the Concentration of Volatile Constituents

Three different liquid systems were involved in the study of volatile constituents by the gas entrainment on-column trapping technique, therefore three different methods of calibrating recorder response were required.

Fat Samples

The earliest work involving fat expressed from the cheese by centrifugation was originally intended to show only relative quantitative differences in the level of certain ethyl esters between the fruity and control cheeses. It later appeared feasible to provide semi-quantitative data for the concentration of certain of these compounds in ppm. This was accomplished by dissolving such quantities of ethyl butyrate and ethyl hexanoate in high-grade mineral oil that the mixing of 1.0 g of the mineral oil solution with 100 g of cheese resulted in an increased concentration in the cheese mass of 5.0 ppm of each ester. An aqueous solution of ethanol was made up so that the addition of 1.0 ml of the aqueous solution to 100 g of cheese resulted in a concentration of 20 ppm added ethanol in the cheese mass. After adding 1.0 g of the mineral oil solution of esters and 1.0 ml of the aqueous solution of ethanol to 100 g of Cheddar cheese, the cheese was thoroughly mixed with a mortar and pestle. In order to allow the added ethanol and esters to equilibrate between the lipid and aqueous phases of the cheese mass, the sample was allowed to stand at room temperature for 4 hr. A sample of the fat was then expressed by centrifugation and analyzed by GLC as described previously. To account for the ethanol and esters indigenous to the cheese, 1.0 g of mineral oil and 1.0 ml of distilled water were added to 100 g of an identical cheese

sample which was then carried through the analytical procedure outlined above. The contribution of the added ethanol and esters to GLC peak height was taken as the difference in recorder response between the control and the sample to which the components had been added. Since the assumption must be made that the added compounds were able to equilibrate between the phases of the cheese in the same manner as the naturally occurring compounds would in ripening cheese, the data obtained by this procedure should be accepted as only semi-quantitative.

Culture Samples

A second different system involved the analysis of various cultures grown in milk. In order to simulate the liquid system involved, autoclaved milk samples were acidified with 20% phosphoric acid to a pH of 4.5. A 4.0 ml aliquot of the acidified milk was used for each calibration run. The two compounds of principal interest in this study were ethanol and acetaldehyde. Various concentrations of these components were added in aqueous solution in place of the distilled water normally added in the analysis of cultures. The GLC analysis was then carried out as previously described for the analysis of cultures. The GLC analysis of several samples of the acidified milk without added ethanol or acetaldehyde indicated that only an insignificant amount of acetaldehyde and no detectable amount of ethanol was

present. It was thus possible to refer the recorder response directly to the added amount of either component. The relationship of GLC peak height to concentration was plotted over a several-fold concentration range in order to determine the linearity of response with various concentrations.

Whole Cheese Samples

The third system involved 2.0 g of whole cheese ground with 5.0 g of sodium sulfate and then diluted with distilled water. Calibration of recorder response was carried out for acetaldehyde, acetone, ethyl acetate, butanone, 2-butanol, ethyl butyrate, ethanol, and *n*-propanol. Various concentrations of these compounds in aqueous solution were added to a sample of cheese ground with sodium sulfate in place of a portion of the 6.0 ml of distilled water normally added to each sample. The cheese sample selected for the calibration of recorder response lacked appreciable concentrations of most of the common volatile constituents, but corrections were made, when necessary, for the portion of the peak height due to components indigenous to the cheese. The concentration of added components versus GLC peak height was plotted to determine linearity of response and the slope of the line.

Maintenance of Cultures

Cultures were grown in 11% solids non-fat milk medium (Matrix Mother Culture Media, Galloway-West Co., Fond Du Lac, Wisconsin). This medium was prepared by dissolving the non-fat milk solids in distilled water and autoclaving for ten min at 121° C. Cultures were transferred every third day, using approximately two percent inoculum. Freshly inoculated cultures were incubated at 30° C until coagulation occurred and were then held at 2° C.

Experiments Involving Cultures

Production of Ethanol and Acetaldehyde

Cultures of *S. cremoris*, *S. lactis*, *S. diacetilactis*, and certain mixed-strain cultures were evaluated for ethanol and acetaldehyde production in non-fat milk medium at incubation temperatures of 21° C, 30° C, and 38° C for periods of 14 and 36 hr. Certain cultures were also incubated at 7° C for a period of one month. Incubation was carried out in screw-cap test tubes with Teflon-lined caps. Analysis at the end of the incubation period was carried out by the previously described GLC method.

Preparation of Sterile Solutions of Volatile Compounds

The problem of preparing sterile solutions of volatile compounds required for some of the subsequent experiments was solved by autoclaving the solutions in screw-capped test tubes with Teflon-lined caps. Immediately after autoclaving, the tubes were cooled in a water bath and stored at refrigerator temperature until used. Comparison of the quantity of acetaldehyde, the most volatile of the compounds, present in the solution before and after autoclaving indicated that the loss of this compound during sterilization under the conditions outlined above was negligible. This conclusion was based on the GLC peak height produced by identical quantities of the sterile and non-sterile acetaldehyde solutions added to sterile non-fat milk media and analyzed in the manner described for cultures.

Reduction of Aldehydes

The ability of single-strain cultures of *S. lactis*, *S. diacetilactis*, *S. cremoris*, *L. dextranicum*, and *L. citrovorum* to reduce acetaldehyde to ethanol was evaluated by adding 1 ml of a sterile acetaldehyde solution to 20 ml of sterile non-fat milk medium to yield a concentration of 100 ppm acetaldehyde in the resulting medium. After inoculation, the culture was incubated at 21 °C for 48 hr in screw-cap test tubes with Teflon-lined caps. Control cultures without added

acetaldehyde were concurrently incubated to account for the amount of ethanol produced from the medium through normal fermentation. An uninoculated blank consisting of non-fat milk medium acidified to pH 4.5 with sterile 20% phosphoric acid and with 100 ppm added acetaldehyde was also carried. Analysis of the acetaldehyde and ethanol concentrations in the blank and cultures was carried out by GLC after the incubation period.

The ability of certain cultures to reduce propanal to 1-propanol and butanone to 2-butanol was also evaluated in a similar manner. The reduction of propanal was later used as a measure of the ability of a culture to produce alcohols by the reduction of aldehydes.

Ester Formation by Lactic Streptococci

The ability of certain cultures to form ethyl butyrate when the ethanol and butyric acid substrates were provided was investigated. Sterile solutions of ethanol and butyric acid were added to sterile non-fat milk medium to provide a concentration of 100 and 400 ppm, respectively. After inoculation, the cultures were incubated at 7° C for a period of one month. A sterile blank containing 100 ppm ethanol and 400 ppm butyric acid and acidified to pH 4.5 with phosphoric acid was also incubated to account for any amount of ester resulting from simple acid-catalyzed esterification.

Evaluation of the Ability of Milk Enzymes to Form Ethyl Butyrate

One liter of raw milk was obtained at the time of milking and divided into two parts. One part was autoclaved to eliminate any subsequent enzyme activity and the other portion was left raw. A portion of both the raw and the autoclaved milk was acidified to pH 5.0 with 50% phosphoric acid. Ethanol and butyric acid, 100 and 400 ppm, respectively, were added to the unacidified samples and to the acidified samples (both raw and autoclaved). A drop of toluene was added to each test tube containing 20 ml of milk to inhibit microbial growth, and the tubes were incubated at 30° C for 7 and 30 hr. At the end of the incubation period, the samples were evaluated for the formation of ethyl butyrate in the manner outlined for the analysis of cultures.

RESULTS AND DISCUSSION

Compounds Responsible for the Fruity Flavor Defect

The fat obtained by centrifugation of the typically fruity cheese had the characteristic fruity odor of the intact cheese. After the molecular distillation, it became apparent that the distillation and trapping procedures had been successful when a strong fruity odor, again resembling the odor of the intact cheese, was detected around the injection device prior to injection onto the GLC column. Having previously carried out the same distillation procedure on several normal, good-quality cheeses, the fruity odor of the injection device in this case was unique because it was the only predominant detectable odor. Evaluating the odor of each component as it emerged from the GLC column, the first highly volatile fraction appeared to contain only two components with fruity odors. One component with a slightly fruity odor had the proper retention time for ethyl acetate, and the second component with a very fruity odor had the proper retention time for ethyl butyrate. It should be recalled that this first fraction was transferred directly from the trap to the GLC column and contained only the most volatile constituents, since the trap was allowed to warm only slightly during the transfer. When the concentrated ethyl chloride extract of the liquid nitrogen-cooled trap was

separated by GLC, three components with distinctly fruity odors were noted. The retention times for these three peaks agreed with the retention times for ethyl butyrate, ethyl hexanoate, and ethyl octanoate. A peak with the retention time of ethyl decanoate was also observed. The latter component did not display any appreciable fruity odor. Evaluation of the odor of the authentic compounds indicated that ethyl acetate has a sharp, solvent-like odor, ethyl butyrate and ethyl hexanoate are quite fruity, ethyl octanoate is somewhat less fruity, and ethyl decanoate has practically no fruity character. The component with the retention time of ethyl acetate, observed in the first injection of volatiles directly from the trap, apparently was lost in the concentration of the ethyl chloride extract.

The identity of ethyl butyrate, ethyl hexanoate, and ethyl octanoate was confirmed by mass spectrometry. Fragmentation peaks in the mass spectra of these components were compared to the data obtained by Sharkey _et al_. for aliphatic esters (133), the ASTM tables (3) and the API tables (2). A key rearrangement structure for ethyl esters of n-butyric and higher acids results in a strong peak representing a fragment of mass 88 arising from a cleavage between carbons two and three of the acid moiety of the molecule. All significant peaks in the proper proportions required for identification were found in the mass spectra of the three esters.

All of the esters mentioned above (ethyl acetate, ethyl butyrate,

ethyl hexanoate, ethyl octanoate, and ethyl decanoate) have been previously isolated and identified as components of good-quality Cheddar cheese by Day and Libbey (32). Since the fruity defect appeared to be due to higher levels of some of the same ethyl esters which occur in normal cheese, it was fortunate that a limited number of fruity cheeses and their matching non-fruity controls were available for comparison of the quantity of ethyl esters present in each. The GLC analysis of the volatiles entrained from the fat of these cheeses was employed to determine whether the level of ethyl esters was higher in the fruity cheeses than in the matching control cheeses. The chromatograms thus obtained from two of the fruity cheeses and their matching control cheeses are shown in Figures 5 and 6. As can be observed from the chromatograms, the levels of ethyl butyrate and ethyl hexanoate were found to be higher in the fruity cheeses than in the matching control cheeses. Ethyl octanoate was not detectably higher in any of the fruity samples compared to the matching controls.

During the course of the GLC analyses, another pertinent discovery was made; ethanol was present in each of the fruity cheeses in a considerably higher concentration than in the matching control cheeses. The identity of ethanol was confirmed by retention time and by mass spectral analysis of the compound as it emerged from the GLC column. The relative amounts of ethanol, ethyl butyrate,

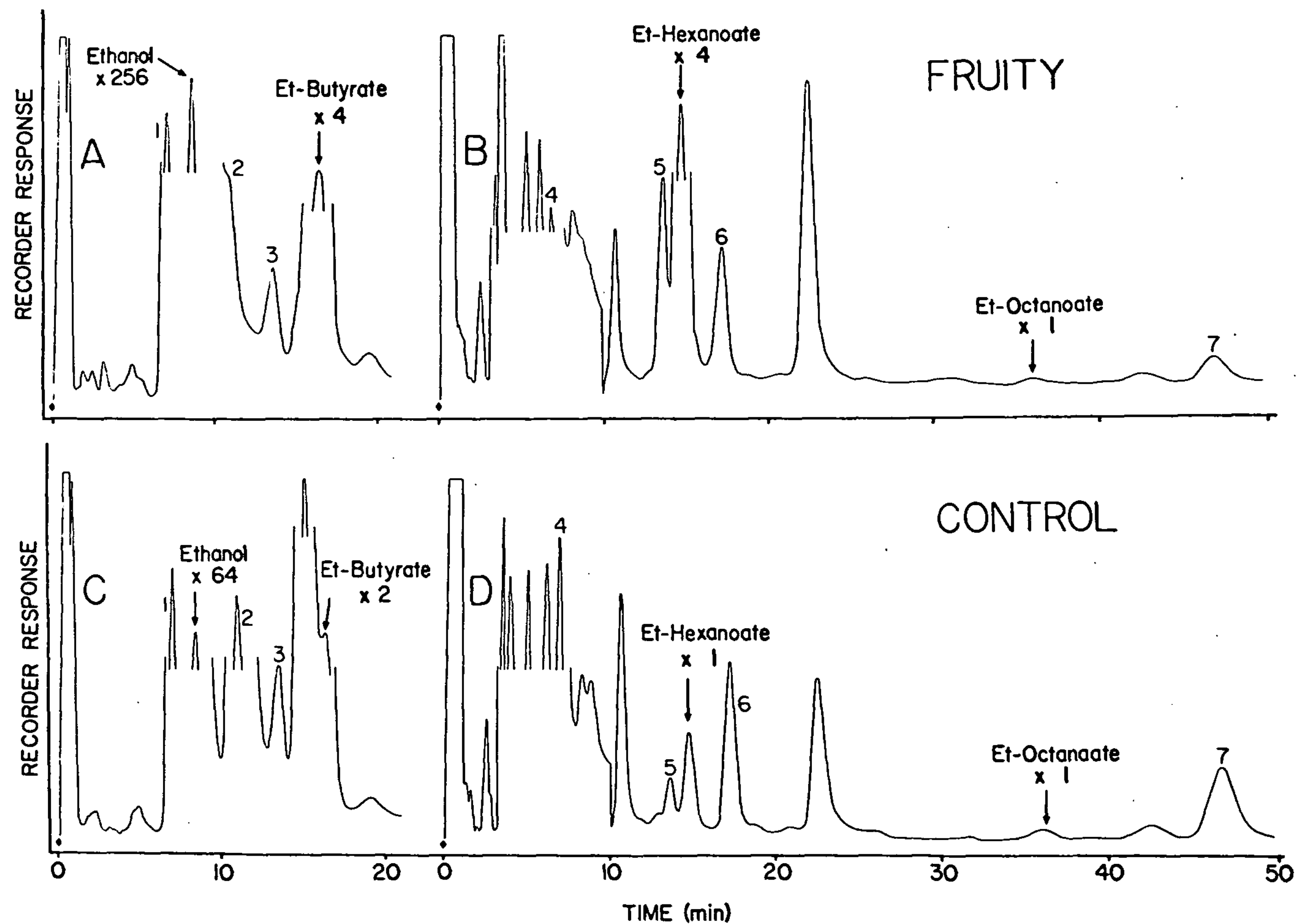


Figure 5. Chromatograms of the volatile components of a fruity cheese and its matching nonfruity control (sample pair no. 1). Chromatograms A and C obtained at 50° C. Chromatograms B and D obtained at 80° C.

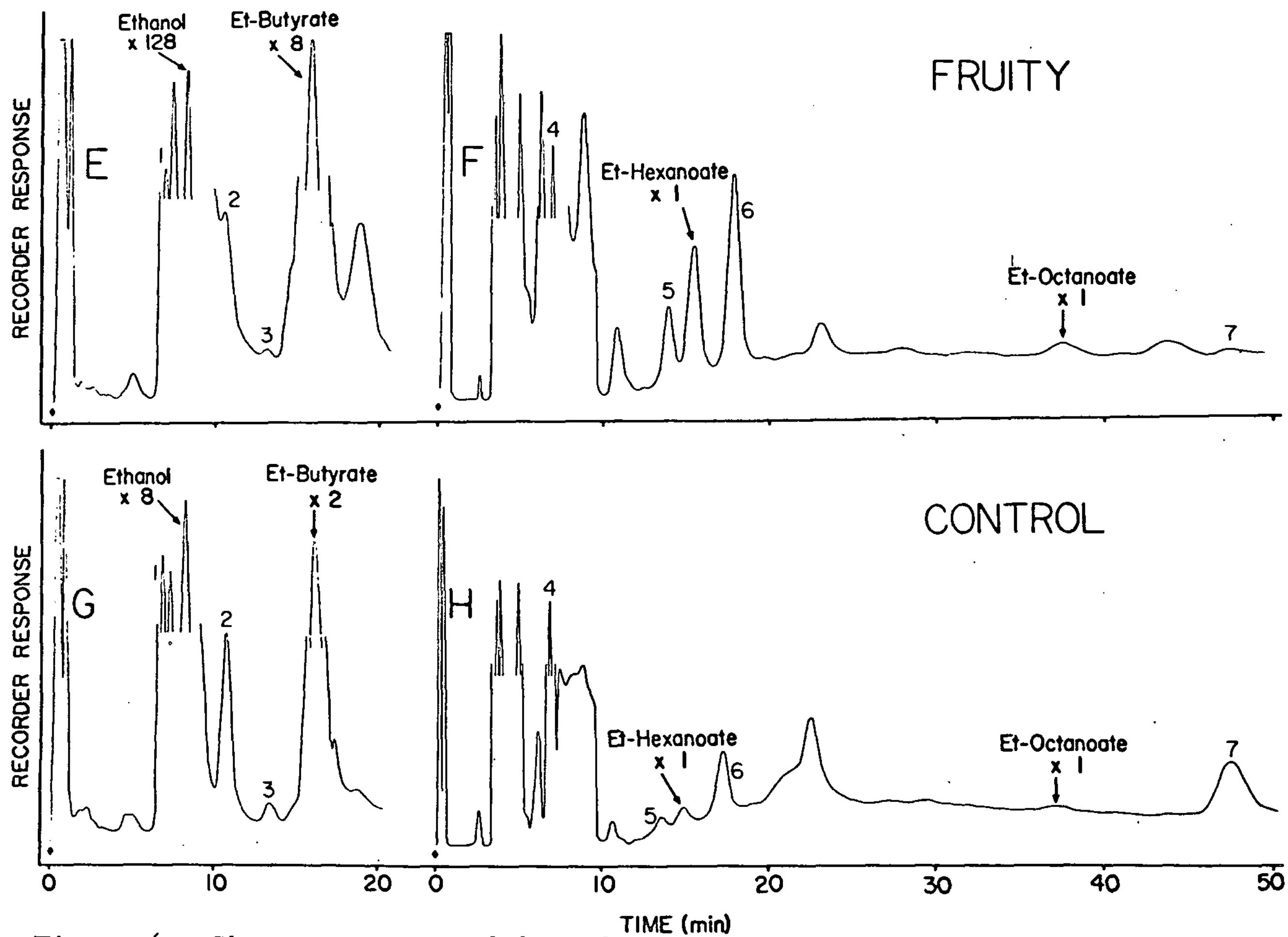


Figure 6. Chromatograms of the volatile components of a fruity cheese and its matching nonfruity control (sample pair no. 2). Chromatograms E and G obtained at 50 °C. Chromatograms F and H obtained at 80 °C.

and ethyl hexanoate found in four samples of fruity cheese and their matching non-fruity controls are reported in Table 1. On a semi-quantitative basis, the concentration of ethanol ranged from 400 to 2,040 ppm, ethyl butyrate from 1.6 to 24 ppm, and ethyl hexanoate from 0.9 to 25 ppm among the fruity samples. Among the non-fruity samples, ethanol ranged from 36 to 320 ppm, ethyl butyrate from 0.7 to 4.7 ppm, and ethyl hexanoate from 0.3 to 2.2 ppm. The semi-quantitative data were obtained in the manner outlined under experimental procedure. The peaks numbered one through seven in the chromatograms of Figures 5 and 6 were tentatively identified on the basis of retention time as follows: (1) acetone, (2) butanone, (3) 2-butanol, (4) diacetyl, (5) methyl hexanoate, (6) 2-heptanone, and (7) acetoin. The fact that these compounds have been isolated previously from Cheddar cheese and identified by rigorous analytical means adds weight to the above tentative identifications. The retention data upon which the identifications are based are presented in Table 2. In general, the level of volatile compounds other than ethanol, ethyl butyrate, and ethyl hexanoate appeared to be higher in the fruity cheeses. Individually, however, the volatile compounds other than the above three do not appear to be consistently more abundant in the fruity cheeses; for example, the component tentatively identified as 2-heptanone was more abundant in the fruity cheese than in the control cheese in one case, but less abundant in

TABLE 1. Relative amounts of ethanol, ethyl butyrate, and ethyl hexanoate found in four samples of fruity cheese and their matching non-fruity controls. Arranged in order of the intensity of the fruity defect as judged organoleptically.

Sample pair		Relative peak height[a]		
		Ethanol	Ethyl butyrate	Ethyl hexanoate
1	Fruity	230	2.44	3.08
	Control	36	1.00[b]	0.27
2	Fruity	114	7.84	0.35
	Control	7	1.52	0.05
3	Fruity	95	0.53	b
	Control	18	0.22	b
4	Fruity	45	b	0.12
	Control	4	b	0.04

[a] Full scale (1.0 mv) response equals relative peak height of 1.00 at 1 x attenuation.

[b] Incomplete or doubtful resolution of the ester peak.

TABLE 2. Compounds detected in the volatiles of Cheddar cheese fat.

Compound	t_R/t_R ethanol[a]		t_R/t_R Et-hexanoate[b]	
	Unknown	Known	Unknown	Known
Acetone	.83	.83		
Ethanol	1.00	1.00		
Butanone	1.31	1.32		
2-butanol	1.63	1.64		
Ethyl butyrate	1.94	1.94		
Diacetyl			.45	.46
Methyl hexanoate			.90	.89
Ethyl hexanoate			.99	1.00
2-heptanone			1.14	1.16
Ethyl octanoate			2.45	2.48
Acetoin			3.16	3.18

[a] Column temperature 50° C.

[b] Column temperature 80° C.

the fruity cheese of another pair. The higher overall level of volatile compounds in the fruity cheeses may have some bearing on the flavor defect, but judging from the odor of the volatile components obtained by distillation from fruity Cheddar cheese and separated by GLC, ethyl butyrate, and ethyl hexanoate appeared to be the only major components having distinctly fruity odors which resembled the aroma of the intact cheese.

From the data, it appears doubtful that there is any fixed level of ester concentration above which the flavor of the cheese becomes fruity. In the case of the samples which displayed a lesser degree of the defect, the concentration of esters was low, but the concentration of other volatiles was also low. Apparently, the ratio of esters to other components in the cheese must be taken into account, and this substantiates the balanced component theory (53, 73, 98, 138) of Cheddar cheese flavor which has been previously discussed.

The addition of 5.0 ppm of ethyl butyrate plus 5.0 ppm of ethyl hexanoate to one of the control cheeses resulted in a flavor nearly identical to that of the fruity cheese, as determined by a panel of five judges. This observation supports the conclusion that these two components are essentially responsible for the defect and adds weight to the semiquantitative data obtained for these compounds.

Quantities of Ethanol and Other Highly Volatile Constituents in Commercial Cheddar Cheese Samples

A typical chromatogram of the highly volatile constituents of a commercial Cheddar cheese sample is shown in Figure 7. The $t_R/t_{R\ ethanol}$ values of known compounds and of the components separated from cheese samples are listed in Table 3.

TABLE 3. Tentative identifications assigned to volatile components of ten Cheddar cheese samples.

		t_R/t_R ethanol	
Peak No.	Compound	Unknown	Known
1	dimethyl sulfide	0.26	0.26
2	acetaldehyde	0.31	0.31
3	unidentified	0.75	
4	acetone	0.82	0.82
5	ethyl acetate	0.88	0.87
6	unidentified	0.92	
7	ethanol	1.00	1.00
8	butanone	1.31	1.31
9	2-butanol	1.64	1.64
10	1-propanol	1.84	1.83
11	ethyl butyrate	1.99	1.97

The peak heights obtained with various concentrations of known compounds added to the sample system were plotted to yield the

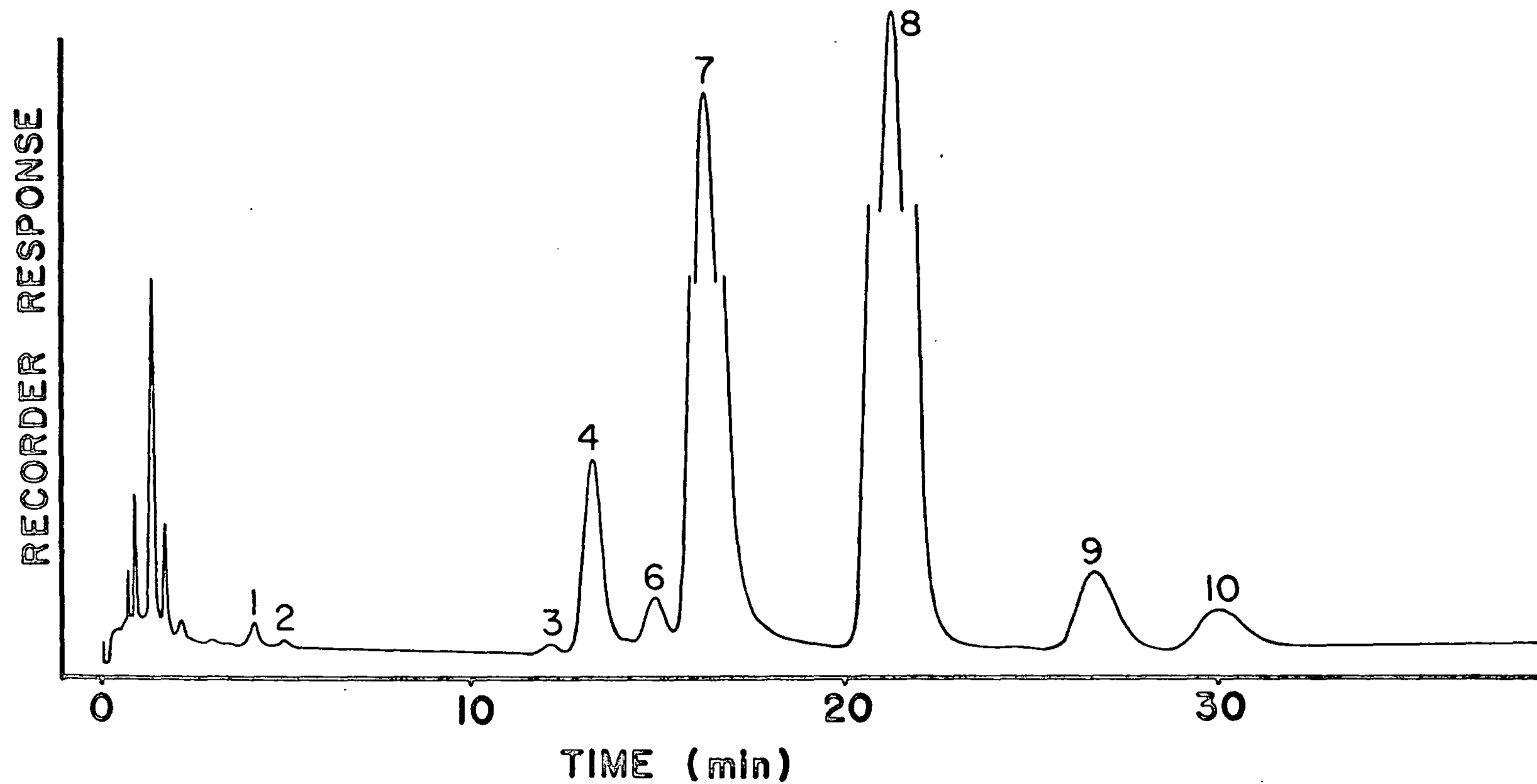


Figure 7. Typical GLC trace obtained in the analysis of the highly volatile constituents of Cheddar cheese. Peak 5 was not evident in this sample (sample no. 6). Peaks 7 and 8 were attenuated 2 and 4 times, respectively.

standard curves shown in Figure 8. Quantitative data were based on the standard curves thus obtained. The standard curve for ethanol is not shown but was linear throughout the range of occurrence in the samples, and the slope of the line yielded the equation: ppm ethanol = peak height $\times$ 0.70.

Quantitative data for compounds identified on the basis of retention time is presented in Table 4. The component identified as dimethyl sulfide was present in every sample with peak heights ranging from 0.1 to 1.1 cm. Components number three and six were not identified. Component number three was noted in four samples with peak heights ranging from 0.4 to 3.0 cm. Component number six was noted in eight samples with peak heights ranging from 0.2 to 6.8 cm. The reproducibility of $t_R/t_{R\ ethanol}$ data and of quantitative data based on peak height is demonstrated by the data presented in Table 5. The criticisms and scores assigned to each of the cheeses by a panel composed of three experienced judges are shown in Table 6.

It is apparent from the quantitative data that the concentration of some components varied widely among samples while the concentration of other components remained relatively constant in all samples. The levels of acetone and acetaldehyde remained relatively constant at low levels in all samples while the levels of ethanol, butanone, and 2-butanol varied widely between samples. The wide range of ethanol concentrations encountered in these samples is

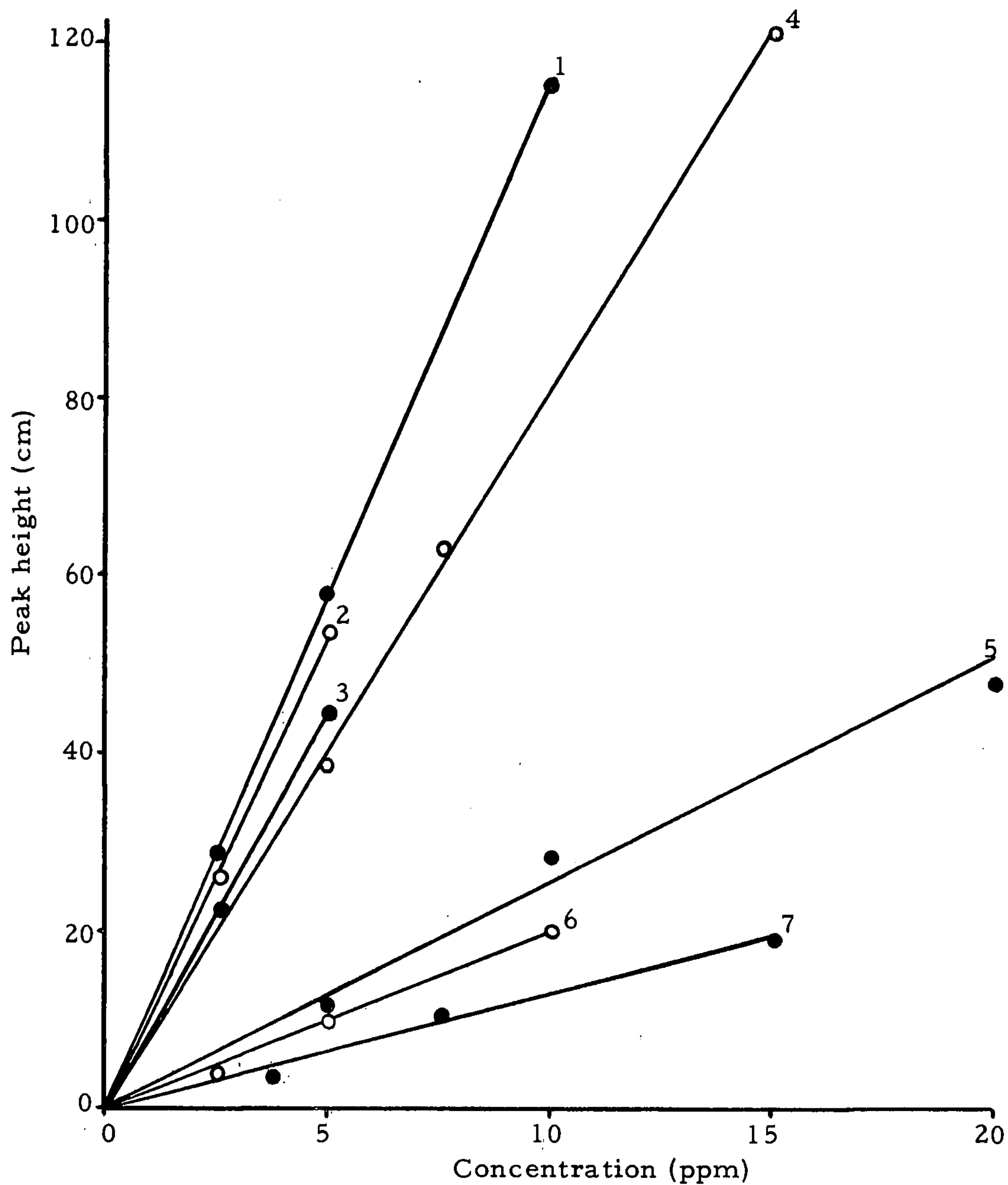


Figure 8. Recorder response obtained with various concentrations of known compounds. (1) acetaldehyde, (2) acetone, (3) ethyl acetate, (4) butanone, (5) 2-butanol, (6) ethyl butyrate, (7) 1-propanol.

TABLE 4. Concentration (ppm) of compounds identified in ten Cheddar cheese samples.

Peak No.	Compound	Cheese Sample									
		1	2	3	4	5	6	7	8	9	10
2	acetaldehyde	<0.1	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	0.2	<0.1	<0.1
4	acetone	0.3	0.1	0.2	0.4	0.5	0.7	0.1	0.1	0.5	0.3
5	ethyl acetate	0.4	0.6	0.3	0.2	a	a	a	0.8	a	a
7	ethanol	450.0	140.0	5.5	35.0	62.0	27.0	23.0	620.0	11.0	35.0
8	butanone	0.4	0.1	<0.1	0.1	<0.1	11.0	2.7	a	0.1	19.0
9	2-butanol	0.9	34.0	0.1	0.2	a	1.1	36.0	1.1	0.3	30.0
10	1-propanol	a	11.0	a	1.9	a	1.0	11.0	0.5	a	7.6
11	ethyl butyrate	a	a	a	a	1.2	a	a	0.2	a	a

[a]Indicates compound was not detected in the sample.

TABLE 5. Results of triplicate analyses of a single sample.

Trial	Peak number[a]								
	1	2	4	5	6	7	8	9	10
	t_R/t_R ethanol								
1	0.26	0.31	0.82	0.87	0.92	1.00	1.31	1.64	1.83
2	0.26	0.31	0.82	0.87	0.92	1.00	1.31	1.64	1.84
3	0.26	0.31	0.82	0.88	0.92	1.00	1.31	1.63	1.84
	Concentration found (ppm)								
1	b	0.2	0.3	0.3	b	140	0.2	35	14
2	b	0.1	0.2	0.3	b	140	0.3	32	13
3	b	0.1	0.1	0.6	b	140	0.1	34	11

[a]The components responsible for peaks three and eleven were not detected in this sample.

[b]Concentration not determined.

TABLE 6. Panel evaluation of ten Cheddar cheese samples analyzed by GLC.

Sample	Flavor	Score	Body and Texture	Score
1	Fermented, fruity, unclean	33.0	Definitely open	28.0
2	Slight acid	39.0	Slightly open	29.5
3	Flat	39.0	No criticism	30.0
4	Slight acid	39.0	Slightly pasty	29.0
5	Bitter, unclean	36.5	Slightly open	29.5
6	Acid ("sour")	37.0	Moderately open	29.0
7	Acid, bitter, unclean	36.0	Crumbly, open	28.5
8	Whey taint, unclean	36.0	No criticism	30.0
9	Definite acid	37.5	No criticism	30.0
10	Bitter, sulfide, definite Cheddar character	37.0	Slightly crumbly Slightly open	29.0

rather striking; the lowest concentration was 5.5 ppm and the highest 620.0 ppm. The cheese with the second highest ethanol concentration, 450 ppm, had a definite fermented, fruity flavor and the open body defect commonly found in fruity cheeses was quite apparent. The fact that the cheese with the highest level of ethanol was not fruity would indicate that the presence of a high level of ethanol in itself is not indicative of the defect. It is possible that this cheese might have developed the fruity defect with additional ripening, but this is only conjecture. There appeared to be no correlation between the amount of ethyl acetate and ethanol present in the samples. Ethyl butyrate was detectable in only two samples and ethyl hexanoate was not measured under the conditions of the analysis. Since the samples were selected at random from local markets, nothing can be said of the history of these cheeses, and it is therefore impossible to draw conclusions concerning the factors influencing the levels of various volatile constituents and the flavor score of the cheese. The important findings in this study are: (1) the low level of acetaldehyde in all samples, (2) the wide range of ethanol concentrations found in the samples, and (3) the lack of correlation between the concentration of ethyl acetate and ethanol. These findings will be discussed in relation to the fruity flavor defect in a later section.

Production of Ethanol and Acetaldehyde by Lactic Streptococci

Since a higher level of ethanol was encountered in the fruity cheeses, the production of excess quantities of ethanol which could react with free fatty acids to form esters in the ripening cheese appeared to be a likely cause of the fruity defect. Vedamuthu (144) and Perry (115) had correlated the production of fruity flavor development with the use of certain starter organisms, notably S. lactis and S. diacetilactis. S. cremoris, on the other hand, was reported by both authors to produce good-quality cheese. Consequently, it was of importance to determine the amount of ethanol produced by a few strains of each species during the fermentation of lactose. The production of acetaldehyde was also of interest since it can serve as the direct precursor of ethanol. In addition to single-strain cultures of the above species, the mixed-strain commercial starter cultures utilized by Vedamuthu (144) for manufacturing fruity and control cheeses were studied. The commercial mixed-strain starter cultures were composed of the following species according to Vedemuthu: culture A consisted of S. cremoris strains exclusively, culture B consisted of S. lactis in addition to S. cremoris, and culture C consisted of S. diacetilactis in addition to S. lactis and S. cremoris. The cultures utilized in this study are listed in Table 7.

TABLE 7. Cultures utilized in the various studies.

Number	Species	Origin[a]
1	*S. diacetilactis*	Mb culture collection, M-21-35
2	*S. diacetilactis*	Mb culture collection, DRC-1
3	*S. diacetilactis*	FST culture collection, D-3
4	*S. cremoris*	Mb culture collection, 459
5	*S. cremoris*	Mb culture collection, 799
6	*S. cremoris*	FST culture collection, C-4
7	*S. lactis*	Mb culture collection, C2-F
8	*S. lactis*	FST culture collection, LM-24
9	*S. lactis*	FST culture collection, LM-25
A	Mixed-strains[b]	Commercial culture
B	Mixed-strains[b]	Commercial culture
C	Mixed-strains[b]	Commercial culture

[a] Mb refers to the Department of Microbiology, FST refers to the Department of Food Science and Technology; Oregon State University.

[b] Refer to text for species reported to be present in these cultures.

The plot of ethanol concentration against GLC peak height for the calibration of recorder response is shown in Figure 9. The plot for acetaldehyde is shown in Figure 10. The quantities of ethanol and acetaldehyde produced at incubation temperatures of 21, 30, and 38°C for incubation periods of 14 and 36 hr are shown in Tables 8, 9, and 10.

In general, the data for ethanol and acetaldehyde production at 21, 30, and 38°C for 14 and 36 hr show two trends. First, for a given culture, the concentration of ethanol increased while the concentration of acetaldehyde usually decreased with longer incubation. Second, the concentration of ethanol and usually of acetaldehyde increased as the incubation temperature increased to the 38°C maximum. All of the _S. cremoris_ strains produced somewhat less ethanol at the higher incubation temperature, however, and this was probably due to the fact that 38°C approaches closely the 40°C temperature at which _S. cremoris_ is unable to grow. This is substantiated by the observation that the _S. cremoris_ strains also produced less lactic acid when incubated at 38°C as evidenced by the lower titratable acidity of the cultures.

Two strains of _S. lactis_, cultures eight and nine, were apparently a variety of this species designated as _S. lactis_ var. _maltigenes_ (63, 96). Although these organisms were purported to be _S. lactis_, the presence of major components with the GLC retention time of

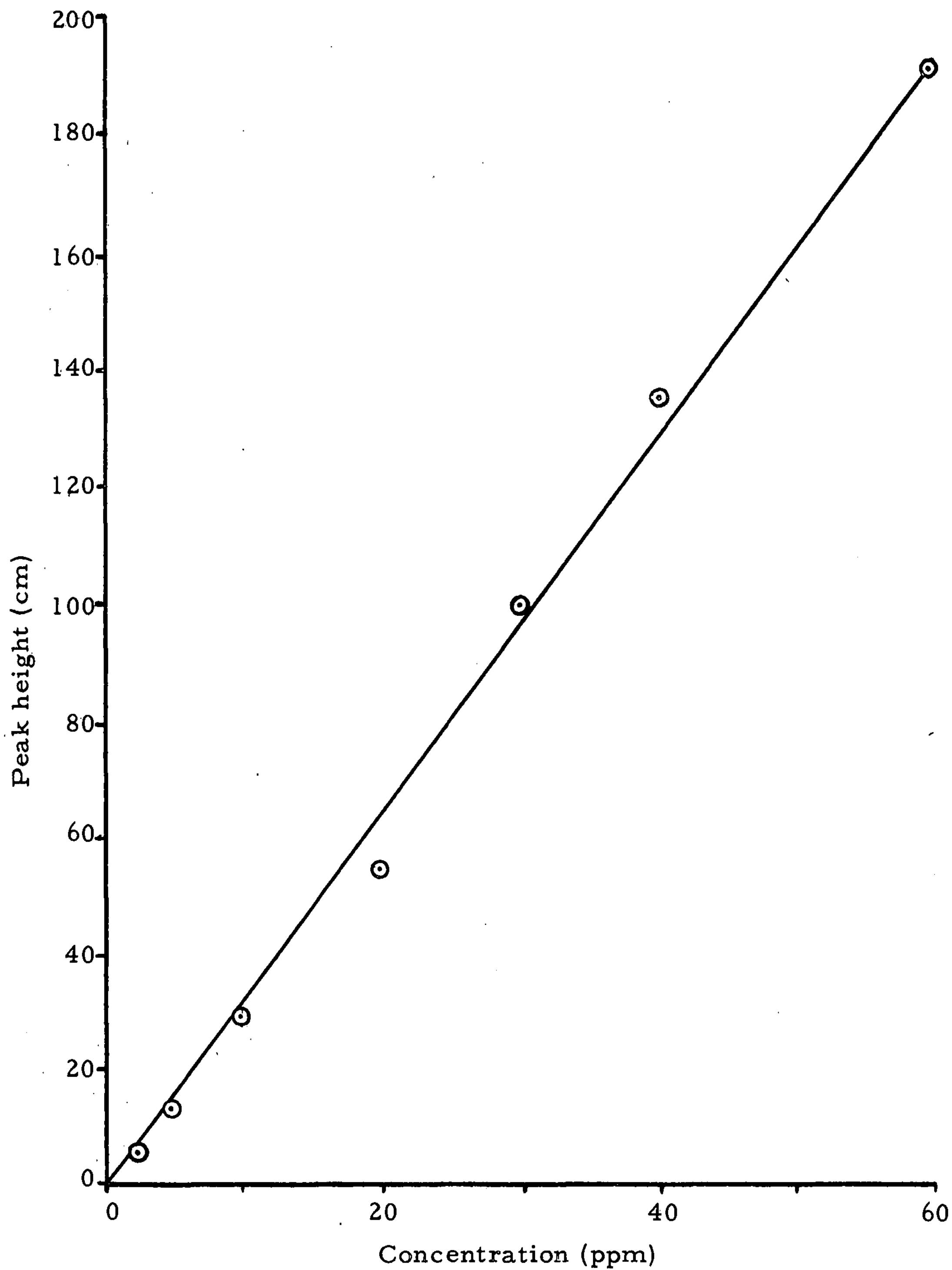


Figure 9. Recorder response obtained with various concentrations of ethanol added to a simulated culture sample.

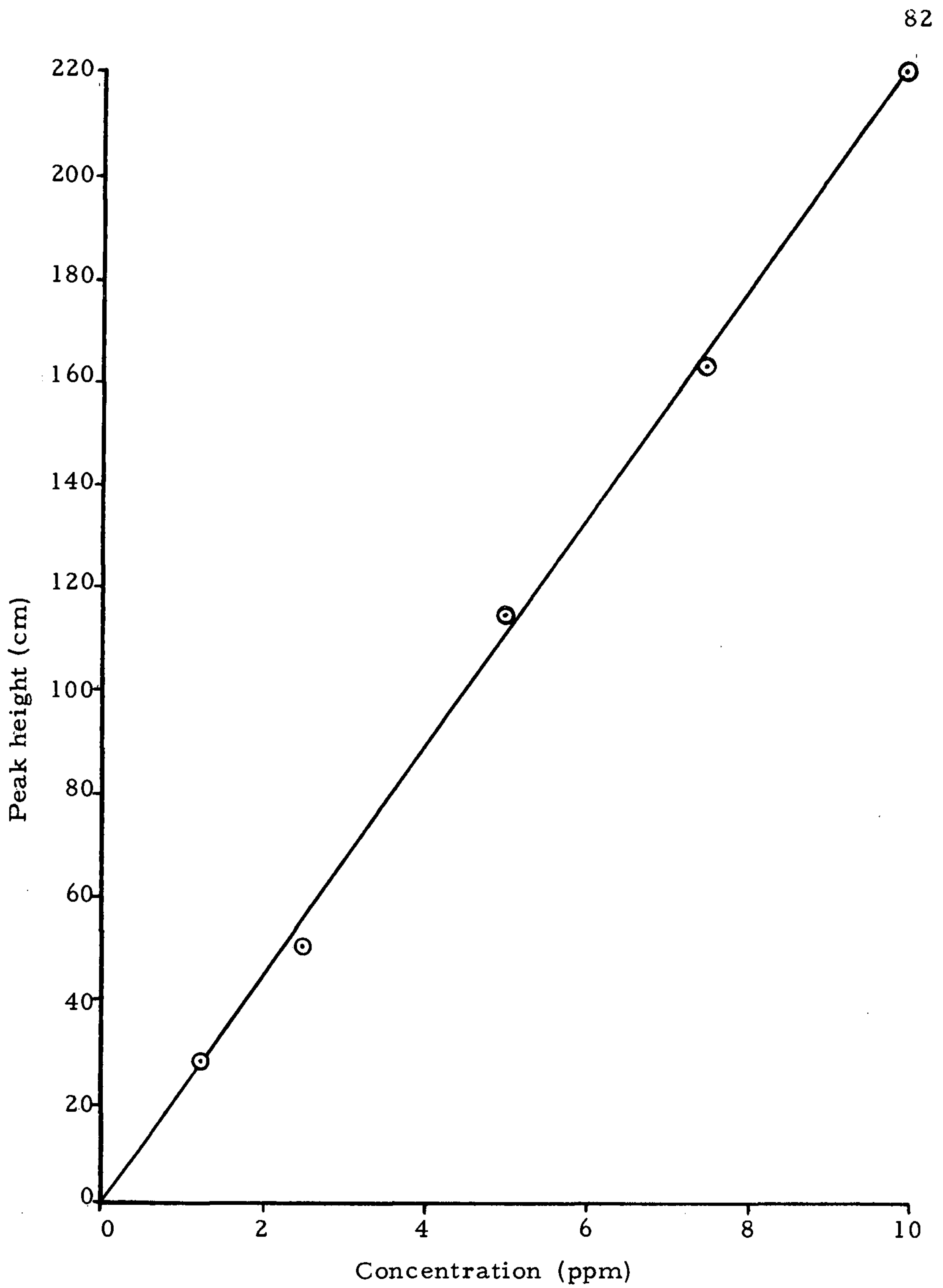


Figure 10. Recorder response obtained with various concentrations of acetaldehyde added to a simulated culture sample.

TABLE 8. Production of ethanol and acetaldehyde in milk cultures of lactic streptococci incubated at 21° C.

		Length of incubation					
		14 hr			36 hr		
No.	Species	Ethanol[a]	Acetaldehyde[a]	Titratable acidity[b]	Ethanol[a]	Acetaldehyde[a]	Titratable acidity[b]
1	S. diacetilactis	2.4	4.2	0.49	7.0	2.8	0.89
2	S. diacetilactis	0.2	3.0	0.51	0.8	4.6	0.92
3	S. diacetilactis	1.0	2.0	0.44	2.4	3.8	0.90
4	S. cremoris	1.4	7.0	0.39	3.8	4.0	0.83
5	S. cremoris	11.0	3.6	0.38	14.8	0.8	0.84
6	S. cremoris	0.6	4.6	0.44	2.6	2.2	0.84
7	S. lactis	3.4	3.0	0.35	11.8	1.4	0.84
8	S. lactis	19.8	1.8	0.44	61.6	3.2	0.72
9	S. lactis	6.2	1.4	0.55	30.4	3.4	0.83
A	mixed-strains	2.2	3.0	0.74	4.8	1.6	0.83
B	mixed-strains	2.4	2.8	0.75	5.4	0.2	0.88
C	mixed-strains	2.2	4.0	0.78	5.0	0.2	0.83

[a]Concentration expressed in ppm.

[b]Expressed as percent lactic acid.

TABLE 9. Production of ethanol and acetaldehyde in milk cultures of lactic streptococci incubated at 30° C.

		Length of incubation					
		14 hr			36 hr		
No.	Species	Ethanol[a]	Acetaldehyde[a]	Titratable acidity[b]	Ethanol[a]	Acetaldehyde[a]	Titratable acidity[b]
1	*S. diacetilactis*	6.8	2.0	0.77	18.2	0.4	0.91
2	*S. diacetilactis*	1.2	4.4	0.86	1.6	4.6	0.92
3	*S. diacetilactis*	4.0	1.0	0.76	6.8	0.4	0.88
4	*S. cremoris*	9.8	2.0	0.68	14.6	1.0	0.84
5	*S. cremoris*	16.6	1.0	0.69	25.4	0.2	0.80
6	*S. cremoris*	4.0	2.8	0.75	5.6	1.0	0.81
7	*S. lactis*	10.2	1.4	0.72	27.4	0.6	0.80
8	*S. lactis*	44.0	3.0	0.65	53.2	2.4	0.76
9	*S. lactis*	14.6	3.2	0.79	47.6	0.2	0.89
A	mixed-strains	7.4	2.0	0.81	10.2	1.4	0.84
B	mixed-strains	4.2	0.4	0.82	5.6	0.2	0.83
C	mixed-strains	6.8	0.2	0.77	6.6	0.1	0.79

[a] Concentration expressed in ppm.

[b] Expressed as percent lactic acid.

TABLE 10. Production of ethanol and acetaldehyde in milk cultures of lactic streptococci incubated at 38° C.

No.	Species	14 hr Ethanol[a]	14 hr Acetaldehyde[a]	14 hr Titratable acidity[b]	36 hr Ethanol[a]	36 hr Acetaldehyde[a]	36 hr Titratable acidity[b]
1	*S. diacetilactis*	19.0	2.0	0.67	13.6	3.6	0.68
2	*S. diacetilactis*	0.4	3.4	0.65	0.6	4.0	0.67
3	*S. diacetilactis*	2.2	0.2	0.66	4.0	0.4	0.67
4	*S. cremoris*	6.6	1.6	0.37	7.6	1.4	0.45
5	*S. cremoris*	8.6	2.2	0.35	23.0	1.8	0.44
6	*S. cremoris*	0.8	1.4	0.54	1.8	1.0	0.55
7	*S. lactis*	26.2	1.8	0.58	34.2	0.8	0.60
8	*S. lactis*	27.6	2.4	0.55	31.0	1.0	0.55
9	*S. lactis*	36.8	1.4	0.71	37.2	0.8	0.71
A	mixed-strains	2.8	1.0	0.54	2.4	0.8	0.64
B	mixed-strains	2.2	0.4	0.55	4.4	0.4	0.59
C	mixed-strains	4.0	0.2	0.51	5.2	0.4	0.57

[a] Concentration expressed in ppm.

[b] Expressed as percent lactic acid.

2-methylpropanal and 3-methylbutanal gave evidence that they should have been classified as *S. lactis* var *maltigenes*. The experiment provides interesting data on the production of ethanol and acetaldehyde by this organism, but the data should not be accepted as representative of the species *S. lactis* in general. Although ethanol production by the *S. lactis* var *maltigenes* organism was high, the presence of this variety in the commercial cheese cultures can be ruled out because of the absence of 2-methylpropanal and 3-methylbutanal in the cultures.

The results with the limited number of single-strain cultures included in this study indicate that considerable variation in ethanol and acetaldehyde production occurs within a given species. The variation in ethanol production within a species is quite apparent when the two strains of *S. cremoris* designated numbers five and six are compared. Number five consistently produced more ethanol under all conditions of incubation than number six; and under one set of conditions, number five produced over 18 times more ethanol than number six. Comparing the production of ethanol among the three species, there appears to be no correlation between species and high ethanol production, excluding the *S. lactis* var. *maltigenes* organisms.

To demonstrate that the production of certain levels of ethanol and acetaldehyde is truly characteristic for a given organism and not simply the artifact of a given inoculation, the analysis of two

single-strain cultures was repeated after making 15 consecutive transfers at three day intervals. The results of this study are presented in Table 11. The data suggest that the quantity of ethanol and acetaldehyde produced is characteristic for a given organism. After 15 transfers, the strain of S. diacetilactis was still found to produce similarly low amounts of ethanol, while the strain of S. cremoris continued to produce relatively high amounts of ethanol. The quantity of acetaldehyde produced by both cultures was also similar before and after the series of transfers.

Organisms which produced lower levels of ethanol were generally found to maintain a higher level of acetaldehyde after the prolonged incubation period, and the converse of this observation was generally true for the organisms which produced ethanol abundantly. The relationship between acetaldehyde and ethanol concentrations supports the observation that acetaldehyde is a transient product in the metabolism of these organisms as reported in earlier works (68, 88).

Certain of the single-strain cultures were incubated for one month at 7° C after a short initial incubation of 14 hr at 21° C. The quantities of ethanol and acetaldehyde found in the cultures at the end of the incubation period are reported in Table 12. The level of ethanol was considerably higher in each of the single-strain cultures at the end of the extended incubation period at 7° C than after the shorter

TABLE 11. Comparison of ethanol and acetaldehyde production initially and after 15 transfers.

No.	Species	Incubation[a]	Initially		After 15 transfers	
			Ethanol[b]	Acetaldehyde[b]	Ethanol[b]	Acetaldehyde[b]
2	*S. cremoris*	14 hr	16.6	1.0	20.6	1.2
2	*S. cremoris*	36 hr	25.4	0.2	29.2	0.4
4	*S. diacetilactis*	14 hr	1.2	4.4	1.1	5.6
4	*S. diacetilactis*	36 hr	1.6	4.6	2.2	4.4

[a]Incubation temperature 30° C.

[b]Concentrations of ethanol and acetaldehyde in ppm.

incubation periods at higher temperatures. Again, no correlation between high ethanol production and species was evident. This study did indicate, however, that ethanol production by lactic streptococci can continue at Cheddar cheese ripening temperatures.

TABLE 12. Production of ethanol and acetaldehyde by single-strain cultures of lactic streptococci incubated at 7° C for one month after an initial incubation of 14 hr at 21 ° C.

No.	Species	Ethanol (ppm)	Acetaldehyde (ppm)
1	S. diacetilactis	24.0	0.6
2	S. diacetilactis	3.2	2.6
4	S. cremoris	36.0	0.8
5	S. cremoris	58.0	0.2
7	S. lactis	43.0	0.6

The three commercial cultures were also incubated for one month at 7° C, but no initial incubation at a higher temperature was given to the newly-inoculated cultures in this case. The lyophilized culture was introduced into non-fat milk medium and incubated at 30° C until coagulation was just evident; this mother culture was then used as inoculum, and the freshly-inoculated tubes were incubated immediately at 7° C. The measurement of ethanol and acetaldehyde in the cultures at the end of the inoculation period revealed very significant differences between culture A, used in the production of normal, non-fruity cheese, and cultures B and C, used in the

production of fruity cheese. The results of this study are presented in Table 13. While cultures _B_ and _C_ produced approximately 40 times more ethanol than culture _A_, the titratable acidities of the three cultures were nearly identical. Apparently, cultures _B_ and _C_ form more ethanol per mole of lactose fermented than culture _A_ under these particular conditions. No explanation is offered for this observation, and probably no explanation is forthcoming in the literature.

TABLE 13. Production of ethanol and acetaldehyde by commercial mixed-strain cultures of lactic streptococci incubated at 7° C for one month immediately following inoculation.

Culture	Ethanol (ppm)	Acetaldehyde (ppm)	Titratable acidity[a]
_A_	4.4	0.8	0.88
_B_	176.0	0.1	0.86
_C_	191.0	0.2	0.90

[a]Expressed as percent lactic acid.

Except for cultures of _S. diacetilactis_ and _S. lactis_ var _maltigenes_, the only volatile compounds produced in quantity by the lactic streptococci included in this entire study were ethanol and acetaldehyde. Cultures of _S. diacetilactis_ produced easily detectable quantities of diacetyl, and cultures of _S. lactis_ var. _maltigenes_ produced significant quantities of the aldehydes and other components

identified by Morgan et al. (96). The concentration of dimethyl sulfide, acetone, and butanone (all tentatively identified on the basis of retention time) fluctuated little, if at all, between the sterile acidified milk medium and any of the cultures. Significant quantities of each of these three compounds were naturally present in the milk medium. A typical chromatogram of the volatile components separated from non-fat milk cultures of lactic streptococci is shown in Figure 11.

Reduction of Aldehydes by Lactic Streptococci and Leuconostoc Organisms

Acetaldehyde was reduced to ethanol by *S. lactis* (no. 7), *S. diacetilactis* (no. 1), *S. cremoris* (no. 5), one strain of *L. citrovorum*, and one strain of *L. dextranicum*. The data in Table 14 reveals that the addition of 100 ppm of acetaldehyde to newly-inoculated cultures resulted in the production of significantly higher levels of ethanol.

TABLE 14. Conversion of added acetaldehyde to ethanol by lactic streptococci and Leuconostoc organisms.

	Control		With added acetaldehyde	
Organism	Ethanol[a]	Acetaldehyde[a]	Ethanol[a]	Acetaldehyde[a]
L. citrovorum	20.2	0.2	66.0	8.0
L. dextranicum	10.0	3.4	16.0	30.0
S. lactis	11.4	0.4	36.8	5.2
S. diacetilactis	7.0	2.2	20.0	30.0
S. cremoris	7.2	1.0	17.0	13.6

[a]Concentrations reported in ppm.

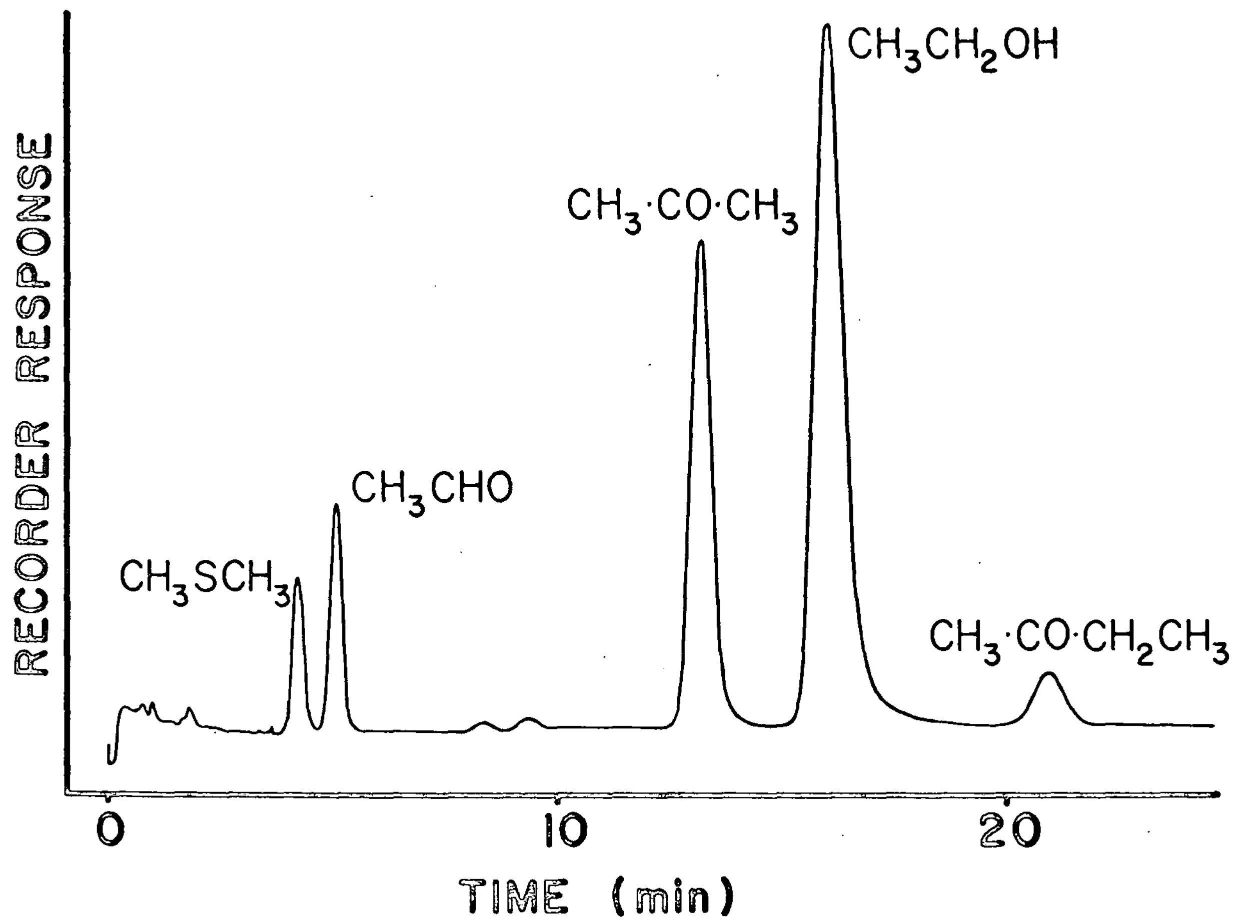


Figure 11. Typical chromatogram of the volatile components separated from non-fat milk cultures of lactic streptococci.

The three species of lactic streptococci were subsequently shown to be capable of reducing added propanal to 1-propanol. Since neither propanal nor 1-propanol were detected by GLC in any of the cultures of lactic streptococci, it appeared that neither of these compounds was produced to any extent during the fermentation process and that the reduction of added propanal might serve as an index of the aldehyde-reducing ability of a given culture. The relative amounts (as indicated by GLC peak heights) of propanal and 1-propanol after the addition of 100 ppm propanal to the freshly-inoculated culture followed by incubation for 48 hr at 21° C are shown in Table 15.

TABLE 15. Conversion of added propanal to 1-propanol by single-strain cultures of lactic streptococci.

Organism	Propanal, GLC Peak height (cm)	1-propanol, GLC Peak height (cm)
S. lactis (no. 7)	34	80
S. diacetilactis (no. 1)	219	58
S. diacetilactis (no. 2)	576	13
S. cremoris (no. 5)	8	89
Control[a]	1203	0

[a]Control consisted of sterile milk medium acidified to pH 4.5 and with 100 ppm added propanal.

The results of this study indicated that organisms which produce lower amounts of ethanol while retaining higher levels of acetaldehyde; such as *S. diacetilactis*, culture number two; also have a lesser capability for the reduction of propanal to 1-propanol. On the

basis of this observation, it seemed worthwhile to compare the aldehyde-reducing ability of the commercial cultures used earlier for the production of normal and fruity-flavored cheeses. The results of this study are shown in Table 16. Under the conditions of the experiment, which were identical to those employed for the single-strain cultures, there appeared to be little difference in the ability of the three commercial cultures to reduce propanal. Culture A, which was reported to produce consistently good cheese, produced slightly more 1-propanol. The propanal-reducing ability of the three cultures fell about midway between the extremes found for the single-strain organisms.

TABLE 16. Conversion of added propanal to 1-propanol by mixed-strain commercial cultures.

Culture	Propanal, GLC peak height (cm)	1-propanol, GLC peak height (cm)
A	515	38
B	713	22
C	560	23

The reduction of aldehydes other than acetaldehyde by the lactic streptococci is not a new concept. Gunsalus and Wood (52) reported the production of highly active alcohol dehydrogenase by homofermentative lactic streptococci. Resting cells of Streptococcus mastiditis, Lancefield group B, were able to oxidize a number of

alcohols, as indicated by methylene blue reduction. Several of the alcohols including the normal alcohols C_2 to C_5, secondary alcohols from C_3 to C_5, tertiary amyl alcohol and 2, 3-butylene glycol were more active donors of hydrogen to methylene blue than glucose. Methyl alcohol was not activated by these organisms. In a recent publication, Morgan _et al_. (96) reported that _S. lactis_ var. _maltigenes_ cultures produce in addition to 2-methylpropanal and 3-methylbutanal the corresponding alcohols, 2-methylpropanol and 3-methylbutanol. The latter authors suggested that the presence of the alcohols in the cultures might indicate that _S. lactis_ var. _maltigenes_ possesses a yeast-like alcohol dehydrogenase.

No evidence was obtained for the reduction of butanone to 2-butanol by _S. lactis_ (no. 7), _S. diacetilactis_ (no. 1), and _S. cremoris_ (no. 5). Although the mode of synthesis of butanone in Cheddar cheese is not definitely known (127), a relationship between butanone and 2-butanol seems probable. Anderson (4) demonstrated the interconversion of 2-pentanone and acetone with their corresponding secondary alcohols by _Mycoderma_, _Torulopsis_, _Geotrichum_, and _Penicillium_ cultures. A strain of _S. lactis_ was reported to be unable to bring about the same conversions. The reduction of ketones to secondary alcohols does not appear, therefore, to be a general attribute of the lactic streptococci.

Formation of Ethyl Butyrate in Cultures

Single-strain cultures of *S. lactis* (number seven), *S. diacetilactis* (numbers one and two), and *S. cremoris* (numbers four and five), as well as the mixed-strain commercial cultures *A*, *B*, and *C* were evaluated for their ability to catalyze the formation of ethyl butyrate when ethanol and butyric acid were provided as substrates. In no case was the concentration of ethyl butyrate found to be significantly higher at the end of the incubation period in the cultures than in the control acidified to pH 4. 5 with phosphoric acid. The amount of ethyl butyrate present in both cultures and control amounted to about 0. 1 ppm on the basis of GLC peak height. The results of this experiment indicate that the cultures in question have no detectable ability to bring about the formation of ethyl butyrate even when the alcohol and acid substrates are provided. The small amount of the ester produced in the cultures and control can be attributed simply to the acid catalyzed reaction of ethanol and butyric acid. Even the presence of intracellular esterases capable of catalyzing the esterification reaction appears doubtful since the long incubation period (one month) should have resulted in the release of substantial amounts of intracellular enzymes. The experiment does, however, demonstrate the formation of esters by non-biological means in very dilute solutions of the alcohol and acid.

Formation of Ethyl Butyrate in Raw and Heated Milk

Recent work by Patton, McCarthy and co-workers has indicated that triglyceride synthesis takes place in freshly secreted milk (92, 110). The formation of labelled ethyl esters in milk when ethanol was used as the solvent for incorporating labelled fatty acids is mentioned briefly in the latter publication (110). Microsome-like particles have been implicated as the site of glyceride formation.

The possible formation of ethyl esters by mechanisms inherent in milk appeared to be worthy of investigation with regard to the explanation of the formation of esters in Cheddar cheese.

A barely-detectable GLC peak with the proper retention time for ethyl butyrate was observed for the samples of both raw and autoclaved milk to which ethanol and butyric acid had been added. Since the peak was so small, it was difficult to determine whether it was actually larger in the raw and autoclaved samples acidified to pH 5.0. It was apparent, however, that no appreciable amount of ethyl butyrate was formed under the experimental conditions. It is, therefore, difficult to visualize the presence in milk of a mechanism capable of forming ethyl esters in high yields. The experiment, at any rate, would seem to rule out any significant enzymatic activity in the formation of ethyl butyrate.

Possible Mechanisms Involved in the Development of the Fruity Flavor Defect of Cheddar Cheese

The portion of this work devoted to the identification and quantitative comparison of the volatile compounds associated with Cheddar cheese displaying both excellent flavor and the flavor defect described as fruity yielded evidence that ethyl butyrate and ethyl hexanoate are responsible for the defect. The results indicated further that the defect was due to a high proportion of these two esters in relation to other flavor components in the cheese. Ethanol was also found to be much more abundant in the fruity cheeses. The latter compound probably contributes little to the flavor of Cheddar cheese even at very high concentrations, since informal taste trials with ethanol solutions indicated that the flavor of 1,000 ppm of ethanol in distilled water is just perceptible.

The formation of ethyl esters in Cheddar cheese, however, could be quite dependent upon the concentration of ethanol. The usual concentration of free fatty acids in Cheddar cheese has been shown to vary over a fairly narrow range, and the concentration of butyric and hexanoic acids in two samples of fruity cheese was about average for the 12 samples analyzed (9).

Since certain lactic streptococci have been implicated in the production of the fruity defect, it seemed appropriate to determine

whether there was a significant difference in ethanol production by different species, strains within a species, and the commercial mixed-strain cultures used in the manufacture of fruity and control cheeses. Significantly different amounts of ethanol were produced by strains within a species; but no real difference was demonstrated among the three species; _S. lactis_, _S. diacetilactis_, and _S. cremoris_. The three commercial cultures: _A_ which produced normal cheese, and _B_ and _C_ which produced fruity cheese; were shown to produce roughly equivalent amounts of ethanol when incubated at 21, 30 and 38° C for 14 or 36 hr. When incubated at 7° C, however, _B_ and _C_ produced approximately 40 times more ethanol than _A_. This evidence suggests that organisms within the mixed-strain cultures _B_ and _C_ may be directly responsible for the production of high levels of ethanol in ageing cheese. The incubation of mixed-strain cultures in milk medium for one month at 7° C would appear to be a potential screening test for the detection of cultures which produce the fruity flavor defect in Cheddar cheese. Determination of tolerable levels of ethanol production in cultures thus incubated would be necessary. Isolation of the species and strains involved in cultures _B_ and _C_ and the subsequent incubation of these isolates under the above conditions should also provide interesting data concerning species and strain involvement in the defect.

A ready explanation of the body defect termed slit-openness

which is associated with the fruity flavor defect can be based upon the manner in which ethanol arises. In all known routes of synthesis, the immediate precursor of ethanol is acetaldehyde. Acetaldehyde in turn arises through the decarboxylation of pyruvic acid. The theoretical yield of ethanol would then be one mole of ethanol per mole of carbon dioxide produced in the decarboxylation reaction. On this basis, the production of 1,000 ppm ethanol within the cheese mass would result in the production of 485 ml of carbon dioxide per kilogram of cheese at standard conditions of pressure and temperature. It is not unreasonable that this amount of gas would be capable of fracturing the body of the cheese and producing the defect.

Another important aspect worthy of discussion at this point is the extended survival of culture organisms responsible for the defect and the suppression of the growth of adventitious organisms. After having established that *S. lactis* survived in higher numbers for longer periods of time than *S. cremoris*, Perry (115) suggested two alternate hypotheses to explain the development of the flavor defect of Cheddar cheese resulting from the use of *S. lactis* starter cultures. The defect, described as fruity or dirty, was proposed to be due to either a direct effect, the production of flavor compounds by *S. lactis*, or to an indirect effect, the suppression of the growth of the normal adventitious microflora which are required to yield a normal Cheddar cheese flavor. Perry favored the first hypothesis and suggested

further that the resting cells of *S. lactis*, although unable to multiply in the cheese, are able to impart the characteristic flavor which distinguishes cheeses made with *S. lactis* from those made with *S. cremoris*. It was ruled unlikely that the *S. lactis* organisms had any stimulatory effect on the production of flavor compounds by the adventitious bacteria.

A study of the survival of various cheese starter organisms was conducted by Dawson and Feagan (27). These authors noted that *S. lactis* maintained a high but declining population during maturing of the cheese, the organisms beginning to die out gradually after eight weeks. Similar trends were found for *S. diacetilactis*, but the population for this organism was somewhat lower. Much lower initial populations and a rapid decrease in numbers after two weeks was observed for *S. cremoris*. A correlation between persistence in the cheese and salt tolerance of the organisms was noted. No mention is made in this publication of the relationship between starter organisms and Cheddar cheese flavor.

The suppression of the growth of adventitious organisms and the survival of the starter organisms for a prolonged period has also been reported by Vedamuthu (144) for the starter cultures which produce the fruity flavor defect.

The results of the current investigation indicate that both the production of excess amounts of ethanol by the starter organisms

and their repression of the growth of adventitious organisms may have a bearing on the development of the defect. The formation of high levels of ethanol appears to be linked to the formation of high levels of ethyl esters, while the suppression of the growth of adventitious organisms may prevent the formation of other flavor compounds necessary to balance out the esters which appear when certain starter streptococci predominate.

The mechanism of ester formation within the cheese remains obscure. Resorting to the proposals originally set forth by Hart et al in 1914 (56), there are three basic mechanisms to consider. First, the esterification reaction may be catalyzed by enzymes produced by microorganisms or inherent in the milk. Second, the adsorbtion of the acid or alcohol at surfaces within the cheese mass may favor the esterification reaction. Third, the formation of esters may come about in a simple acid-catalyzed esterification reaction. Regardless of which of these mechanisms is responsible for ester formation, it should be pointed out that the amount of ester formed would reflect the amount of alcohol and acid available for esterification.

The concentration of ethyl octanoate did not appear to be greater in the fruity samples, while ethyl butyrate and ethyl hexanoate were found at much higher levels. This observation suggests that some selectivity may be involved in the esterification reaction. Biological mechanisms usually exhibit selectivity to some degree in the

catalysis of reactions, but purely mechanical factors could also be involved. Rammell (123) has demonstrated that the fat in Cheddar cheese is distributed throughout the cheese, partially in the form of droplets. Thus, a considerable interface between lipid and non-lipid phase is present in the cheese mass. Lipolytic activity would be expected to occur at such an interface and the resulting free fatty acids would be able to partition between the two phases. Butyric and hexanoic acids are considerably more water-soluble than octanoic acid, and ethanol is much more soluble in water than in lipid. Esterification could thus be dependent on the solubility of the acids in the aqueous phase where the concentration of ethanol is greatest. The fact that the concentration of ethyl acetate in the commercial cheese samples did not appear to be related to the concentration of ethanol could suggest that factors other than solubility influence the selectivity of the esterification reaction, since acetic acid would be expected to be associated almost exclusively with the aqueous phase.

As pointed out by Hart (56), the concentration of ethanol in the free water of the cheese may be quite high. Although Cheddar cheese may contain up to 39% water, a considerable portion of the total water is associated with the protein of the cheese. The binding of much of the water is apparent when Cheddar cheese is centrifuged at high speeds; usually it is impossible to obtain an aqueous layer even with prolonged centrifugation.

Until the physical and biochemical environment in which esterification takes place in Cheddar cheese has been more thoroughly investigated, it will not be possible to conclude the mechanism underlying the reaction. The micro-environment in which the reaction occurs may actually be quite different than would be anticipated from the gross composition of the cheese.

SUMMARY AND CONCLUSIONS

The volatile flavor components of Cheddar cheese displaying a pronounced fruity flavor defect were separated by GLC. By monitoring the odor of the effluent stream of the column, it was possible to determine which components had fruity odors, and these were subsequently identified by mass spectral analysis and coincidence of retention time with the authentic compounds. Comparison of the quantity of these compounds in fruity cheeses and non-fruity control cheeses both manufactured under experimental conditions was accomplished by means of a gas entrainment, on-column trapping, GLC technique. In addition, ten samples of commercial Cheddar cheese selected at random were analyzed in a similar manner for the concentration of ethanol and other highly volatile constituents.

Certain cheese-starter cultures of lactic streptococci had been implicated earlier in the development of the fruity flavor defect. Using GLC methods, the volatile metabolic products of these mixed-strain cultures and other selected single-strain cultures were investigated. The ability of these lactic streptococci to produce high levels of ethanol and acetaldehyde, and to reduce acetaldehyde to ethanol in milk cultures was of special interest. The reduction of propanal to 1-propanol was used as an index of the reducing ability of a given culture. Catalysis of the esterification reaction of ethanol and

butyric acid by cultures of lactic streptococci or the enzymes inherent in milk was also investigated.

The following results and conclusions were obtained from the investigation:

1. Ethanol, ethyl butyrate, and ethyl hexanoate were more abundant in proportion to other volatile compounds in Cheddar cheese displaying the fruity flavor defect.
2. The addition of 5.0 ppm of ethyl butyrate and 5.0 ppm of ethyl hexanoate to a non-fruity cheese sample resulted in a flavor which closely duplicated the typical fruity flavor defect. Since these esters were the only abundant components with fruity odors isolated from fruity Cheddar cheese, it was concluded that they are mainly responsible for the defect.
3. The concentration of these esters in relation to the concentration of other volatile constituents appears to be important. A relatively high concentration of the esters did not result in the fruity defect when other volatile constituents were also present in relatively high concentrations.
4. A good correlation between high levels of ethanol and high levels of the ethyl esters of butyric and hexanoic acids suggests that the quantity of ethanol present in the cheese may determine the amount of these esters formed.
5. All strains of _S. lactis_, _S. diacetilactis_, and _S. cremoris_

investigated produced ethanol when grown in milk medium. No correlation was found between species and ethanol production. Considerable variation was noted between strains within a species.

6. All organisms of the above species were capable of reducing added acetaldehyde or propanal to the corresponding alcohol when grown in milk medium. The organisms which produced ethanol most abundantly when incubated at 21, 30, or 38° C for 14 or 36 hr were found to reduce propanal most rapidly.

7. The commercial cultures used in the manufacture of fruity and control cheeses produced nearly equivalent amounts of ethanol and acetaldehyde when incubated at 21, 30, and 38° C for 14 or 36 hr. When incubated at 7° C for one month, however, the two commercial cultures reported to result in the fruity flavor defect produced approximately 40 times more ethanol than the culture reported to produce normal, good-quality cheese. This observation further substantiates the hypothesis that certain cultures are directly responsible for the development of the defect.

8. Ethyl butyrate was not detected in significant quantities after the incubation of certain of the single-strain cultures and the mixed-strain commercial cultures with ethanol and butyric acid provided as substrates. From this it was concluded that these organisms have little or no ability to catalyze the esterification reaction.

9. The addition of ethanol and butyric acid to freshly-drawn raw

milk did not result in the formation of detectable amounts of ethyl butyrate. This indicates that the enzymes or biological systems inherent in milk have little ability to catalyze the esterification reaction.

10. The body defect which has been termed slit-openness could be due to the production of carbon dioxide through the decarboxylation of pyruvic acid to yield acetaldehyde. The reduction of acetaldehyde ultimately yields ethanol. On the basis of the ethanol concentration in cheeses displaying both the flavor defect and the body defect, the production of carbon dioxide could be as high as one liter, at standard conditions, per kilogram of cheese.

BIBLIOGRAPHY

1. Alford, J. A. and W. C. Frazier. Occurrence of micrococci in Cheddar cheese made from raw and from pasteurized milk. Journal of Dairy Science 33:107-114. 1950.

2. American Petroleum Institute. Research Project 44 Catalog of mass spectral data. College Station, Texas, Agricultural and Mechanical College of Texas, Chemical Thermodynamics Properties Center. 1948-to date.

3. American Society for Testing and Materials Committee E-14 on Mass Spectrometry. Index of mass spectral data. Philadelphia, 1963. Various paging, loose-leaf numbers.

4. Anderson, D. F. Flavor chemistry of Blue cheese. Ph. D. thesis. Corvallis, Oregon State University, 1966. 95 numb. leaves.

5. Badings, H. T. and T. E. Galesloot. Studies on the flavour of different types of butter starters with reference to the defect "yoghurt flavor" in butter. In: Proceedings of the 16th International Dairy Congress, København, 1962. Vol. B. Odense, Andelsbogtrykkeriet. p. 199-208.

6. Baribo, L. E. and E. M. Foster. The intracellular proteinases of certain organisms from cheese and their relationship to the proteinases in cheese. Journal of Dairy Science 35: 149-160. 1952.

7. Bassett, E. W. and W. J. Harper. Isolation and identification of acidic and neutral carbonyl compounds in different varieties of cheese. Journal of Dairy Science 41:1206-1217. 1958.

8. Bills, D. D. et al. Identification of compounds responsible for fruity flavor defect of experimental Cheddar cheeses. Journal of Dairy Science 48:1168-1173. 1965.

9. Bills, D. D. and E. A. Day. Determination of the major free fatty acids of Cheddar cheese. Journal of Dairy Science 47:733-738. 1964.

10. Bills, D. D., L. L. Khatri and E. A. Day. Method for the determination of the free fatty acids of milk fat. Journal of Dairy Science 46:1342-1347. 1963.

11. Bills, D. D., R. E. Willits and E. A. Day. Determination of some neutral volatile constituents in ten commercial Cheddar cheeses. Submitted to Journal of Dairy Science, 1965.

12. Birkinshaw, J. H., V. Charles and H. Rainstrick. Studies in the biochemistry of micro-organisms. XVIII. Biochemical characteristics of species of Penicillium responsible for the rot of citrus fruits. Philosophical Transactions of the Royal Society of London, series B, 220:355-367. 1931.

13. Brand, E. et al. The empirical formula of beta-lactoglobulin. Journal of the American Chemical Society 67:1524-1532. 1945.

14. Brandsaeter, E. and F. E. Nelson. Proteolysis by Lactobacillus casei. I. Proteinase activity. Journal of Bacteriology 72:68-72. 1956.

15. ______________. Proteolysis by Lactobacillus casei. II. Peptidase activity. Journal of Bacteriology 72:73-78. 1956.

16. Bullock, D. H. and O. R. Irvine. A chromatographic study of Cheddar cheese ripening. Journal of Dairy Science 39: 1229-1235. 1956.

17. Clément, G. et al. Hydrolysis of butter triglycerides by pancreatic lipase: localization of butyric acid. Archives des Sciences Physiologiques 16:237-243. 1962. (Abstracted in Dairy Science Abstracts 25:no. 1806. 1963.)

18. Cohn, E. J. and J. L. Hendry. Studies in the physical chemistry of the proteins. II. The relation between the solubility of casein and its capacity to combine with base. The solubility of casein in systems containing the protein and sodium hydroxide. Journal of General Physiology 5:521-554. 1923.

19. Collins, R. P. and M. E. Morgan. Esters produced by Chalaropsis thielaviodes. Science 131:933-934. 1960.

20. ______________. Identity of fruit-like aroma substances synthesized by endoconidial-forming fungi. Phytopathology 52:407-409. 1962.

21. Czulak, J. Bitter flavour in cheese. Australian Journal of Dairy Technology 14:177-179. 1959.

22. Czulak, J. and P. D. Shimmin. Further notes on bitter flavour in cheese. Australian Journal of Dairy Technology 16:96-98. 1961.

23. Dacre, J. C. A chemical investigation of the volatile flavor principle of Cheddar cheese. Journal of Dairy Research 20:217-223. 1955.

24. ________________. A note on the pediococci in New Zealand Cheddar cheese. Journal of Dairy Research 25:414-417. 1958.

25. ________________. Characteristics of a presumptive Pediococcus occurring in New Zealand Cheddar cheese. Journal of Dairy Research 25:409-413. 1958.

26. Dahlberg, A. C. and F. V. Kosikowski. The development of flavor in American Cheddar cheese made from pasteurized milk with Streptococcus faecalis starter. Journal of Dairy Science 31:285-292. 1948.

27. Dawson, D. J. and J. T. Feagan. Bacteriology of Cheddar cheese: A study of starter organisms in manufacture and maturing. Journal of Dairy Research 24:210-224. 1957.

28. ________________. Bitter flavours in cheese. Australian Journal of Dairy Technology 15:65-66. 1960.

29. Day, E. A., Richard Bassette and Mark Keeney. Identification of volatile carbonyl compounds from Cheddar cheese. Journal of Dairy Science 43:463-474. 1960.

30. Day, E. A., D. A. Forss and Stuart Patton. Flavor and odor defects of gamma-irradiated skimmilk. I. Preliminary observations on the role of volatile carbonyl compounds. Journal of Dairy Science 40:922-931. 1957.

31. Day, E. A. and M. Keeney. Identification of volatile carbonyls from Cheddar cheese. (Abstract) Journal of Dairy Science 41:718. 1958.

32. Day, E. A. and L. M. Libbey. Cheddar cheese flavor: Gas chromatographic and mass spectral analyses of the neutral components of the aroma fraction. Journal of Food Science 29:583-589. 1964.

33. Day, E. A., R. C. Lindsay and D. A. Forss. Dimethyl sulfide and the flavor of butter. Journal of Dairy Science 47:197-199. 1964.

34. Dolby, R. M., F. H. McDowall and A. K. R. McDowall. Studies on the chemistry of Cheddar cheese making. VI. Factors influencing the acidity and mineral content of cheese. Journal of Dairy Research 8:74-85. 1937.

35. Eichholz, W. Erdbeerbacillus (Bacterium fragi). Centralblatt für Bakteriologie, Abteilung 2, 9:425-428. 1902.

36. Emmons, D. B., W. A. McGugan and J. A. Elliot. Effect of strain of starter culture and manufacturing procedure on bitterness in Cheddar cheese. (Abstract) Journal of Dairy Science 43:861. 1960.

37. ______________. Nitrogen distribution in Cheddar cheese as related to bitterness and strain of starter culture. (Abstract) Journal of Dairy Science 43:862. 1960.

38. Evans, A. C., E. G. Hastings and E. B. Hart. Bacteria concerned in the production of the characteristic flavor in cheese of the Cheddar type. Journal of Agricultural Research 2:167-192. 1914.

39. Fagan, H. J., J. B. Stine and R. V. Hussong. The identification of reducing sugars in Cheddar cheese during the early stages of ripening. Journal of Dairy Science 35:779-782. 1952.

40. Feagan, J. T. A modification to the short method for Cheddar cheese manufacture. Australian Journal of Dairy Technology 11:149-152. 1956.

41. Feagan, J. T. and D. J. Dawson. Bacteriology of Cheddar cheese. Some observations on the microflora during maturing. Australian Journal of Dairy Technology 14:59-66. 1959.

42. Foster, Edwin M. et al. Dairy microbiology. Englewood Cliffs, Prentice-Hall, 1957. 492 p.

43. Franklin, J. G. and M. Elisabeth Sharpe. The incidence of bacteria in cheese milk and Cheddar cheese and their association with flavour. Journal of Dairy Research 30:87-99. 1963.

44. Friedman, T. E. The carbohydrate metabolism of streptococci. Journal of Biological Chemistry 130:757-761. 1939.

45. Galesloot, T. E. The bacteriology and biochemistry of starters and ripened cream. In: Proceedings of the 16th International Dairy Congress, København, 1962. Vol. D. Odense, Andelsbogtrykkeriet. p. 143-167.

46. Gander, G. W. and R. G. Jensen. Specificity of milk lipase toward the primary ester groups of some synthetic triglycerides. Journal of Dairy Science 43:1762-1765. 1960.

47. Gordon, M. A. The physiology of a blue stain mold with special reference to production of ethyl acetate. Mycologia 42:167-185. 1950.

48. Gordon, W. G. et al. Amino acid composition of alpha-casein and beta-casein. Journal of the American Chemical Society 71:3293-3297. 1949.

49. Gordon, W. G., W. F. Semmett and M. Bender. Alanine, glycine and proline contents of casein and its components. Journal of the American Chemical Society 72:4282. 1950.

50. ________________. Amino acid composition of gamma-casein. Journal of the American Chemical Society 75:1678-1679. 1953.

51. Gunsalus, I. C. and C. F. Niven. The effect of pH on the lactic acid fermentaion. Journal of Biological Chemistry 145: 131-136. 1942.

52. Gunsalus, I. C. and A. J. Wood. The dehydrogenation of alcohols by streptococci of group B. Journal of Bacteriology 44:523-528. 1942.

53. Harper, W. J. Chemistry of cheese flavors. Journal of Dairy Science 42:207-213. 1959.

54. ________________. The metabolism of some carbon-14 labeled substrates in certain cheeses and in cultures of lactic acid bacteria. Milchwissenschaft 20:354-356. 1965.

55. Harrison, F. C. Cheese torulae. Transactions of the Royal Society of Canada, Ser. 3, 21 (sec. 5):341-380. 1927.

56. Hart, E. B. et al. Relation of the action of certain bacteria to the ripening of cheese of the Cheddar type. Journal of Agricultural Research 2:193-216. 1914.

57. Harvey, R. J. Production of acetone and acetaldehyde by lactic streptococci. Journal of Dairy Research 27:41-45. 1960.

58. Hastings, E. G., Alice C. Evans and E. B. Hart. The bacteriology of Cheddar cheese (U. S. Dept. of Agriculture Bureau of Animal Industry, Bulletin 150) 1912. 52 p.

59. Herb, S. F. et al. Fatty acids of cow's milk. B. Composition by gas-liquid chromatography aided by other methods of fractionation. Journal of the American Oil Chemists' Society 39:142-146. 1962.

60. Hussong, R. V., H. F. Long and B. W. Hammer. Classification of the organisms important in dairy products. II. Pseudomonas fragi. Ames, 1937. p. 119-136. (Iowa Agricultural Experiment Station. Research Bulletin 225)

61. Irvine, O. R. and M. E. Beach. Method for enumerating nonlactic organisms in fresh Cheddar cheese. (Abstract) Journal of Dairy Science 45:648. 1962.

62. Jackson, H. W. Flavor research in cheese. Perfumery and Essential Oil Record 49:256-258. 1958.

63. Jackson, H. W. and M. E. Morgan. Identity of the malty aroma substance from milk cultures of Streptococcus lactis var. maltigenes. Journal of Dairy Science 37:1316-1324. 1954.

64. Jennings, W. G., S. Viljhalmsson and W. L. Dunkley. Direct gas chromatography of milk vapors. Journal of Food Science 27:306-308. 1962.

65. Jensen, R. G. et al. Some evidence supporting the specificity of milk lipases for the primary hydroxyl positions of milk glycerides. Journal of Dairy Science 43:96-97. 1960.

66. Johns, C. K., and S. E. Cole. Lactobacilli in Cheddar cheese. Journal of Dairy Research 26:157-161. 1959.

67. Kandler, O. Stoffwechsel der Saüreweckerorganismen. Milchwissenschaft 16:523-581. 1961.

68. Keenan, T. W. Acetaldehyde production and utilization by lactic cultures. Master's thesis. Corvallis, Oregon State University, 1966. 71 numb. leaves.

69. Keeney, M. and E. A. Day. Probable role of the strecker degradation of amino acids in development of cheese flavor. Journal of Dairy Science 40:874-876. 1957.

70. Klostergaard, H. and R. A. Pasternak. Electrophoresis and ultracentrifuge studies of milk proteins. II. Alpha-lactalbumin. Journal of the American Chemical Society 79: 5674-5676. 1957.

71. Kosikowski, F. V. Milk and cheese flavor. In: Proceedings Flavor Chemistry Symposium, Camden, 1961. Campbell Soup Company, 1961. p. 179-181.

72. ________________. The liberation of free amino acids in raw and pasteurized milk Cheddar cheese during ripening. Journal of Dairy Science 34:235-241. 1951.

73. Kristoffersen, T. Flavor chemistry of cheese--with emphasis on Cheddar. In: Proceedings Flavor Chemistry Symposium, Camden, 1961. Campbell Soup Company, 1961. p. 201-219.

74. Kristoffersen, T. and I. A. Gould. Carbonyl compounds in Cheddar cheese and their possible relationship to flavor. In: Proceedings of the 15th International Dairy Congress, London, 1959. Vol. 2. Suffolk, Richard Clay. p. 720-728.

75. ________________. Characteristic Cheddar cheese flavor in relation to hydrogen sulfide and free fatty acids. (Abstract) Journal of Dairy Science 41:717. 1958.

76. ________________. Cheddar cheese flavor. II. Changes in flavor quality and ripening products of commercial Cheddar cheese during controlled curing. Journal of Dairy Science 43:1202-1215. 1960.

77. Kristoffersen, T., I. A. Gould and W. J. Harper. Cheddar cheese flavor. I. Flavor and biochemical relationships of commercial Cheddar cheese. Milk Products Journal 50:14-17. 1959.

78. Kristoffersen, T. and F. E. Nelson. Metabolism of amino acids by _Lactobacillus casei_. (Abstract) Journal of Dairy Science 37:635. 1954.

79. ________________. The relationship of serine deamination and hydrogen sulfide production by _Lactobacillus casei_ to Cheddar cheese flavor. Journal of Dairy Science 38:1319-1325. 1955.

80. Kuninaka, Akira. Flavor Potentiators. In: Symposium on Foods: The Chemistry and Physiology of Flavors, ed. by H. W. Schultz and E. A. Day. Corvallis, 1965. Westport, Avi Publishing Co., In press.

81. Lane, C. B. and B. W. Hammer. Bacteriology of cheese. II. Effect of _Lactobacillus casei_ on the nitrogenous decomposition and flavor development in Cheddar cheese made from pasteurized milk. Ames, 1935. (Iowa. Agricultural Experiment Station. Bulletin 190)

82. ________________. Effect of lipolytic enzymes on the ripening of Cheddar cheese. (Abstract) Journal of Dairy Science 23:519. 1940.

83. ________________. Relation of fat hydrolysis to flavor development in Cheddar cheese. Journal of Dairy Science 22:432-433. 1939.

84. Libbey, L. M., D. D. Bills and E. A. Day. A technique for the study of lipid-soluble food flavor volatiles. Journal of Food Science 28:329-333. 1963.

85. Libbey, L. M. and E. A. Day. Methyl mercaptan as a component of Cheddar cheese. Journal of Dairy Science 46:859-861. 1963.

86. Lillard, D. A. and E. A. Day. Autoxidation of milk lipids. II. The relationship of sensory to chemical methods for measuring the oxidized flavor of milk fats. Journal of Dairy Science 44:623-632. 1961.

87. Lindsay, R. C. Flavor chemistry of butter culture. Ph. D. thesis. Corvallis, Oregon State University, 1965. 228 numb. leaves.

88. Lindsay, R. C., E. A. Day and W. E. Sandine. Studies on the green flavor defect in lactic starter cultures. Journal of Dairy Science 48:863-869. 1965.

89. Mabbitt, L. A. The flavor of Cheddar cheese. Journal of Dairy Research 28:303-318. 1961.

90. ________________. Quantitative estimation of the amino acids in Cheddar cheese and their importance in flavor. Journal of Dairy Research 22:224-231. 1955.

91. Marth, E. H. Microbiological and chemical aspects of Cheddar cheese ripening. A review. Journal of Dairy Science 46:869-890. 1963.

92. McCarthy, R. D. and S. Patton. Biosynthesis of glycerides in freshly secreted milk. Nature 202:347-349. 1964.

93. McGugan, W. A. and S. G. Howsam. Analysis of neutral volatiles in Cheddar cheese. Journal of Dairy Science 45:495-500. 1962.

94. Morgan, E. N. Volatile metabolic products of _Endoconidiophora coerulescens_ Munch. Biochemical Journal (Proceedings of the Biochemical Society) 43:xxxvii. 1948.

95. Morgan, M. E. The distribution of certain amino acids in the soluble nitrogen fraction of milk cultures of _Streptococcus lactis_. Iowa State College Journal of Science 24:87-89. 1949.

96. Morgan, M. E. _et al_. Identity of additional aroma constituents in milk cultures of _Streptococcus lactis_ var. _maltigenes_. Journal of Dairy Science: In press.

97. Morgan, M. E. and E. A. Day. Simple on-column trapping procedure for gas chromatographic analysis of flavor volatiles. Journal of Dairy Science 48:1382-1384. 1965.

98. Mulder, H. Taste and flavor forming substances in cheese. Netherlands Milk and Dairy Journal 6:157-168. 1952.

99. Naylor, Jill and M. Elisabeth Sharpe. Lactobacilli in Cheddar cheese. I. The use of selective medium for isolation and of serological typing for identification. Journal of Dairy Research 25:92-103. 1958.

100. ______________. Lactobacilli in Cheddar cheese. II. Duplicate cheeses. Journal of Dairy Research 25:421-430. 1958.

101. ______________. Lactobacilli in Cheddar cheese. III. The source of lactobacilli in Cheddar cheese. Journal of Dairy Research 25:431-438. 1958.

102. Newsome, W. H. and J. B. M. Rattray. The enzymatic esterification of ethanol with fatty acids. Canadian Journal of Biochemistry 43:1223-1233. 1965.

103. Nordström, Kurt. Studies on the formation of volatile esters in fermentation with brewer's yeast. Svensk Kemisk Tidskrift 76:510-543. 1964.

104. Omelianski, V. L. Aroma-producing microorganisms. Journal of Bacteriology 8:393-419. 1923.

105. Overcast, W. W. and T. W. Albrecht. Gas production in Cheddar cheese caused by Leuconostoc citrovorum. Journal of Dairy Science 35:554-558. 1952.

106. Patton, Stuart. Flavor thresholds of volatile fatty acids. Journal of Food Science 29:679-680. 1964.

107. ______________. Volatile acids and the aroma of Cheddar cheese. Journal of Dairy Science 46:856-858. 1963.

108. Patton, S., D. A. Forss and E. A. Day. Methyl sulfide and the flavor of milk. Journal of Dairy Science 39:1469-1470. 1956.

109. Patton, S. and D. V. Josephson. A method for determining significance of volatile flavor compounds in foods. Food Research 22:316-318. 1957.

110. Patton, S., R. D. McCarthy and P. S. Dimick. Structure and synthesis of milk fat. IX. Site of lipid synthesis in freshly secreted milk. Journal of Dairy Science 48:1389-1391. 1965.

111. Patton, Stuart, N. P. Wong and D. A. Forss. Some volatile components of Cheddar cheese. Journal of Dairy Science 41:857-858. 1958.

112. Peel, J. L. Ester formation by yeasts. 2. Formation of ethyl acetate by washed suspensions of Hansenula anomala. Biochemical Journal 49:62-67. 1951.

113. Pereira, J. N. and M. E. Morgan. Identity of esters produced in milk cultures of Pseudomas fragi. Journal of Dairy Science 41:1201-1205. 1958.

114. ________________. Nutrition and physiology of Pseudomonas fragi. Journal of Bacteriology 74:710-713. 1957.

115. Perry, K. D. A comparison of the influence of Streptococcus lactis and Str. cremoris starters on the flavour of Cheddar cheese. Journal of Dairy Research 28:221-229. 1961.

116. Perry, K. D. and M. Elisabeth Sharpe. Lactobacilli in raw milk and in Cheddar cheese. Journal of Dairy Research 27:267-275. 1960.

117. Peterson, M. H. and M. J. Johnson. Liberation of fatty acids during making and ripening of Cheddar cheese. Journal of Dairy Science 32:862-869. 1949.

118. Peterson, M. H., M. J. Johnson and W. V. Price. Lipase activity during making and ripening of Cheddar cheese. Journal of Dairy Science 31:39-46. 1948.

119. ________________. Proteinase content of Cheddar cheese during making and ripening. Journal of Dairy Science 31:55-61. 1948.

120. Pette, J. W. Lactic acid bacteria producing hydrogen sulfide

in Gouda cheese. Netherlands Milk and Dairy Journal 9:291-302. 1955.

121. Platt, T. B. and E. M. Foster. Products of glucose metabolism by homofermentative streptococci under anaerobic conditions. Journal of Bacteriology 75:453-459. 1958.

122. Prescott, Samuel C. and Cecil G. Dunn. Industrial microbiology. New York, McGraw-Hill, 1949. 923 p.

123. Rammell, C. G. The distribution of bacteria in New Zealand Cheddar cheese. Journal of Dairy Research 27:341-351. 1960.

124. Reiter, B. and A. Møller-Madsen. Reviews of the progress of Dairy Science. Section B. Cheese and butter starters. Journal of Dairy Research 30:419-456. 1963.

125. Rice, F. E. and A. L. Markley. Proof of the presence of lipase in milk and a new method for the detection of the enzyme. Journal of Dairy Science 5:64-82. 1922.

126. Scarpellino, R. and F. V. Kosikowski. A method of concentrating ripened cheese volatiles for gas chromatography. Journal of Dairy Science 44:10-15. 1961.

127. ________________. Evolution of volatile compounds in ripening raw and pasteurized milk Cheddar cheese observed by gas chromatography. Journal of Dairy Science 45:343-348. 1962.

128. Seitz, Eugene Walter. Studies on diacetyl production by Streptococcus diacetilactis Matuszewski et al. Ph. D. thesis. Corvallis, Oregon State University, 1962. 113 numb. leaves.

129. Shahani, K. M. Galactokinase and Galactowaldenase enzyme activity of Streptococcus lactis. (Abstract) Journal of Dairy Science 43:852. 1960.

130. Shahani, K. M. and W. J. Harper. Differences in the acid production and phosphorus metabolism of oxytetracycline sensitive and resistant organisms. Applied Microbiology 6:9-14. 1958.

131. Shahani, K. M. and J. R. Vakil. Certain enzymes of glycolytic and hexosemonophosphate shunt pathways of _Streptococcus lactis_. (Abstract) Journal of Dairy Science 45: 655. 1962.

132. Sharpe, M. Elisabeth and J. G. Franklin. Production of hydrogen sulfide by lactobacilli with special reference to strains isolated from Cheddar cheese. (Abstract) In: Abstracts of the VIII International Congress for Microbiology, Montreal, 1962. p. 46.

133. Sharkey, A. G., Jr., Janet L. Shultz and R. A. Friedel. Mass spectra of esters: Formation of rearrangement ions. Analytical Chemistry 31:87-94. 1959.

134. Sherwood, I. R. The bacterial flora of New Zealand Cheddar cheese. Journal of Dairy Research 10:426-448. 1939.

135. ______________. The relation of certain lactic acid bacteria to open texture in cheese. Journal of Dairy Research 10: 326-335. 1939.

136. Silverman, G. J. and F. V. Kosikowski. Amines in Cheddar cheese. Journal of Dairy Science 39:1134-1141. 1956.

137. ______________. Decarboxylation products of amino acids in Cheddar cheese. (Abstract) Journal of Dairy Science 37: 645. 1954.

138. ______________. Observations on cheese flavor production by pure chemical compounds. (Abstract) Journal of Dairy Science 36:574. 1953.

139. ______________. Tyrosine in Cheddar cheese. Journal of Dairy Science 39:950-958. 1955.

140. Smith, R. E. and J. D. Cunningham. The characterization of lactobacilli from Cheddar cheese. I. An evaluation of physiological and biochemical tests. Canadian Journal of Microbiology 8:727-735. 1962.

141. Suzuki, S. K., E. G. Hastings and E. B. Hart. The production of volatile fatty acids and esters in Cheddar cheese and their relation to the development of flavor. Madison, 1910. p. 127-154. (Wisconsin. Agricultural Experiment Station. Research Bulletin No. 11)

142. Tittsler, R. P. et al. The effects of lactobacilli on the quality of Cheddar cheese made from pasteurized milk. (Abstract) Journal of Bacteriology 51:276. 1946.

143. ________________. The influence of various lactobacilli and certain streptococci on the chemical changes, flavor development, and quality of Cheddar cheese. (Abstract) Journal of Dairy Science 31:716. 1948.

144. Vedamuthu, E. R. Production of carbonyl compounds by lactic streptococci: Relation to Cheddar cheese flavor. Ph. D. thesis. Corvallis, Oregon State University, 1965. 152 numb. leaves.

145. Ven, B. van der, P. Haverkamp Bergemann and J. C. M. Schogt. Precursors of methyl ketones in butter, Journal of Lipid Research 4:91-95. 1963.

146. Walker, J. R. L. Some volatile compounds in New Zealand Cheddar cheese and their possible significance in flavor formation. II. Volatile compounds of sulfur. Journal of Dairy Research 26:273-276. 1959.

147. Walker, J. R. L. and R. J. Harvey. Some volatile compounds in New Zealand Cheddar cheese and their possible significance in flavor formation. I. Identification of the volatile carbonyl fraction. Journal of Dairy Research 26:265-272. 1959.

148. Walter, H. E. et al. A simplified short time method for making Cheddar cheese from pasteurized milk. (Abstract) Journal of Dairy Science 39:917. 1956.

149. Webb, Byron H. and Arnold H. Johnson. Fundamentals of dairy chemistry. Westport, Connecticut, Avi Publishing Company, 1965. 827 p.

150. Wolin, A. G. and F. V. Kosikowski. Tritium radioactive-casein and cheese ripening. Journal of Dairy Science 42: 998-1007. 1959.

151. Wong, N. P. and S. Patton. Identification of some volatile compounds related to the flavor of milk and cream. Journal of Dairy Science 45:724-728. 1962.

152. Wood, W. A. Fermentation of carbohydrates and related compounds. In: The Bacteria, ed. I. G. Gunsalus and R. Y. Stanier. vol. 2, New York and London, Academic Press, Inc. 1961. p. 59-149.

153. Wynn, J. D., J. R. Brunner and G. M. Trout. Gas chromatography as a means of detecting odors in milk. Journal of Food Technology 14:248-250. 1960.

154. Yates, A. R., O. R. Irvine and J. D. Cunningham. Chromatographic studies on proteolytic bacteria in their relationship to flavor development in Cheddar cheese. Canadian Journal of Agricultural Science 35:337-341. 1955. (Abstracted in Chemical Abstracts 50:1652 a. 1955.)