THE PRODUCTION OF VOLATILE FATTY ACIDS AND ESTERS IN CHEDDAR CHEESE AND THEIR RELATION TO THE DEVELOPMENT OF FLAVOR.

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Investigators of the chemical changes taking place in the process of cheese ripening have concerned themselves almost wholly with the nitrogenous side. The changes which the proteins undergo in the curing process, and the nitrogenous cleavage products formed have been studied by a number of investigators. Contributions to this phase of the subject have been made by Winterstein on Emmenthaler cheese, by Babcock and Russell and Van Slyke and Hart for Cheddar cheese, and Dox for the soft Camembert cheese. To these bodies, in part at least, have been attributed the cheese flavor.

Our own investigations on the failure of inherent enzymatic proteolysis alone in a curing Cheddar cheese to adequately account for the development of typical cheese flavor, led us to this study of the non-nitrogenous products formed during the normal curing process. This paper deals only with American Cheddar cheese.

According to present views, the factors involved in the curing of Cheddar cheese are the pepsin contained in the rennet; the
activating lactic acid formed from lactose fermentation; galactose, the inherent proteolytic enzyme of milk; and some biological factor other than that simply concerned in the lactose fermentation. The last, at the present state of our knowledge, is practically undefined.

There are constantly found in milk three classes of acid-forming organisms: (1) the group of bacteria represented by *B. lactis acidi*, (2) the aerogenes group, represented by *B. coli communis* and *B. lactis aerogenes*, (3) a less well defined group, the members of which are cocci, varying in color from white through the various shades of yellow to orange. These organisms may or may not liquefy gelatin, and vary greatly in their ability to produce acid. The groups of acid-forming organisms differ not only in morphology, in cultural characteristics, but especially in the products formed in their fermentation of milk.

The spontaneous souring of milk is due to the combined action of these three groups of bacteria. The relative number of bacteria belonging to these groups will vary widely from one sample of milk to another, being influenced by a large number of factors, such as cleanliness in milking, temperatures at which the milk is kept, etc. It is undoubtedly true that the majority of the bacteria in good milk ripened to a point desired for the making of Cheddar cheese, belong to the first group. If the milk is of poorer quality, the number of gas-forming organisms, the second group, will be greater. They are, however, always present in milk. The third group is likewise constantly present, as can be shown by appropriate means, but nothing is known of their relative numbers in milk.

These various organisms are enclosed in the curd and undoubtedly form the same products as when growing in milk. A number of investigators have studied in greater or less detail the by-products of the acid fermentation of milk by pure cultures of representatives of the first two groups mentioned. The organisms of the first group produce from 90 to 98 per cent of the theoretical yield of lactic acid from the sugar fermented. The remaining portion of the sugar appears in the form of other by-products, among which have been found alcohols, aldehydes and esters, whose presence indicates the formation of volatile fatty acids.

With the second group, the aerogenes-colon group, the lactic
acid may not exceed 30 per cent of the total acid formed, the remaining being largely acetic, succinic, and formic acids, with some alcohols, and in addition such gases as carbon dioxide, hydrogen and methane. The difference in taste between milk soured by a mixture of organisms, as in the spontaneous souring of milk, and milk soured by a pure culture of B. lads acidi, emphasizes the variation in the products other than lactic acid.

Nothing is known concerning the products of the third group of organisms. It is evident that during the initial fermentation of the sugar in the cheese, a considerable number of products will be formed in varying amounts, depending upon the proportion existing between the groups of acid-forming organisms present. It was thought that these various products might, through decomposition, or by combination with substances formed later in the curing of the cheese, play an essential rôle in the development of flavor.

It was also believed not improbable that the lactic acid formed in the early process of Cheddar cheese making remained only as an intermediate product and was by no means the final product, but suffered further decomposition and rearrangement. The possibility of its being the mother substance of certain volatile fatty acids, which, by further combination with alcohols and production of esters, contribute to flavor production, formed the basis of our working hypothesis.

Jensen1 has studied the occurrence of volatile fatty acids in Emmenthaler and also certain other kinds of European cheese, but his work did not involve a measure of the progressive accumulation or disappearance of such bodies during a long extended curing process. Our own work involved, at definite periods of the curing process, as complete an analysis of the cheese as available methods would allow. Quantitative estimations of lactic and volatile fatty acids, such as acetic, propionic, butyric, caproic and formic acids, were made. Qualitative separation of certain hitherto unknown bodies, such as esters, with identification of the acids and alcohols forming them, was also made at definite stages of the ripening period. In addition qualitative isolation and identification of succinic acid were made, which, we believe, has never before been reported in curing Cheddar cheese.

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EXPERIMENTAL PLAN.

A normal and a skim-milk American Cheddar cheese were made on August 1, 1908, and used for this investigation at definite intervals. Both cheeses were made from the same milk. The normal cheese was scored at the age of three months, and found to be in every way a marketable product. They were both cured at 55°F.

Description of Methods Used in the Chemical Analysis.

1. Moisture determination. About 10 grams of ground material were dried over sulphuric acid to constant weight. This required about ten days.

2. Determination of lactic and separation of succinic acids. Fifty to 100 grams of cheese were ground with pure quartz sand, strongly acidified with sulphuric acid and extracted with 75 to 100 cc. portions of ether about 24 times. The ether extract was evaporated to remove the ether, and in the case of the whole milk cheese, this ether extract residue was treated several times with hot water in a separatory funnel for the purpose of separating the fat from the extracted acids. The solution of acids was next boiled with an excess of barium hydroxide, then neutralized with dilute sulphuric acid and the precipitate removed. The filtrate and washings were evaporated to a small volume. The difficultly soluble barium succinate rapidly crystallized out. The crystals were collected and washed with 60 per cent alcohol, in which strength barium lactate is easily soluble. The mother liquor and washings from the barium succinate were evaporated and then diluted with water. Zinc sulphate was next added, care being taken to avoid an excess. Barium sulphate was removed and the filtrate evaporated until the first crystals of zinc lactate appeared. The solution was then placed in a dessicator and reduced to a small volume for further crystallization. The crystalline zinc lactate was collected and washed with a very small volume of cold water. The mother liquor and washings were treated in the same way for further crystallization. The united crystallizations were dried over sulphuric acid and quantitatively weighed. While the method is not absolutely quantitative, due to the slight solubility of the zinc salts of both inactive and active lactic acids, nevertheless by careful manipulation satisfactory results can be obtained.

Where determinations of the form of optical activity of the acids were made, definite portions were dried in an air oven at 108 to 110°C. for two hours. At a higher temperature, 115 to 120°C., there appeared to be some decomposition of zinc lactate.

3. Determination of volatile fatty acids. 250 to 300 grams of cheese were ground with sand, moistened with a dilute solution of sulphuric acid until the pasty mass gave a decided acid reaction to Congo red paper.
ass was then distilled with steam. Usually a distillate of 1000 cc. was
cient, but in certain analyses, as high as 2000 cc. were collected. The
distillate was neutralized with barium hydroxide and again redistilled,
in order to separate any neutral volatile substances, as alcohols and esters,
that may be formed during the curing process. This fraction was con-
centrated through several redistillations: as for example, the first 1000 cc.
of the steam distillate after neutralization with barium hydroxide, was
distilled to 500 cc.; 500 cc. to 250 cc.; 250 to 150 cc.; and the 150 to 75 cc.
This final and more concentrated fraction is here designated "flavor
solution" because of the strong resemblance to the cheese aroma.

By the above procedure, two solutions are obtained, the neutral solution
of the barium salts of the fatty acids and the so-called "flavor solution."
The former was evaporated to a volume of 150-200 cc. and after cool-
ing, 20 to 30 cc. of N/10 sulphuric acid were added. The barium sulphate
was filtered off and washed with cold water. The filtrate and washings
were distilled to separate the free fatty acids. The first distillation is
designated Fraction I. By repeating this process, a second fraction, F. II,
and third fraction, F. III, were obtained. To these fractions the Duclaux's
method for the estimation of fatty acids was applied.

O. Jensen has already used this method with interesting results in his
studies of foreign cheese.
The Duclaux method depends upon the fact that there is a constant
rate of vaporization for each volatile fatty acid. For instance, when a
definite amount of a solution of any volatile fatty acid is distilled, the
amount of acid in each successive fraction of the distillate has its own
constant proportion to the total acidity of the distillates. Duclaux dis-
tilled 110 cc. of the acid solution from a retort of 250 to 300 cc. capacity
and titrated every 10 cc. of the successive distillations with standard
lime water, until 100 cc. of the distillate were obtained. Each titre
of lime water was calculated to per cent of the total titre of the 100 cc.
of distillate. These per cents were of practically constant value for each
of the volatile fatty acids.

In a mixture of several acids, each retains its own characteristic rate of
vaporization. Because of this fact, the Duclaux method allows a quanti-
tative estimation and identification of the volatile fatty acids contained
in a solution.

Treatment of the "Flavor Solution."

The "flavor solution" was saponified with potassium hydroxide as
follows: to 50 cc. there were added 10 cc. of 20 per cent potassium hydrox-
ide and the mixture heated for two to three hours in a tightly stoppered
pressure bottle at the temperature of boiling water. After cooling, the
contents of the bottle were diluted and distilled in order that any alcohols,
either in ester combination or free, might be collected. The residue from
this distillation was acidified with dilute sulphuric acid and distilled with

1 E. Duclaux: Traité de microbiologie, iii, p. 388, 1900.
Loc. cit.
repeated additions of water. This distillate, containing volatile acids, which had existed in ester combination, was neutralized with barium hydroxide and evaporated to a small volume. Barium was separated with dilute sulphuric acid and the solution of free fatty acids treated according to Duclaux' method for their identification. The solution of alcohols from the saponification was oxidized as follows: 10 grams of potassium bichromate, and 20 grams of strong sulphuric acid were made up to 100 cc; 25 cc. of this solution were added to about 50 cc. of the alcohol solution, contained in a tightly stoppered pressure bottle, and oxidation carried on at the temperature of the water bath for 2 to 3 hours. The excess of chromic acid was removed by zinc in the presence of sulphuric acid. The volatile fatty acids were next distilled as in all previous descriptions, using Duclaux' method for identification. All reagents used in the saponification and oxidation reactions were checked for freedom from volatile acid substances. In this respect, the chemicals used were pure.

Analysis of the Cheese at Different Ages.

A consideration of the work in greater detail will be given at this place, but will suffice for reference to all of the other periods.

Three-days old. For the determination of milk sugar, 25 grams of the cheese were ground with sand and extracted with hot water. The extracts were treated according to the method proposed by Scheibe for the estimation of lactose. When the cheeses were three days old there was no reduction of the copper solution, showing that the sugar had all disappeared. In this instance, the disappearance was particularly rapid. In some previous work on the rapidity of sugar disappearance in fresh curds, lactose could be detected at the end of the third and even fifth day. It is very probable that the temperature and exposure to which the milk has been subjected prior to cheese making, would be an important factor in determining the rate of sugar disappearance in the manufactured curd.

Determination of the form of lactic acid in both whole and skim-milk cheese at the three day stage showed that it was of the inactive variety. From the whole milk cheese 0.390 gram of zinc lactate gave 0.069 gram of water of crystallization, equivalent to 17.85 per cent, while the theoretical amount for inactive zinc lactate is 18.18 per cent. From the skim-milk cheese 0.600 gram of zinc lactate gave 0.1045 gram of water of crystallization, equivalent to 17.41 per cent.

In the determination of volatile fatty acids, 250 grams of the whole milk cheese were ground, acidified and distilled to 1000 cc. The total acidity of the 1000 cc. was equivalent to 22.1 cc. of \( \frac{\text{m}}{10} \) barium hydroxide. The neutral solution of the barium salts of the volatile fatty acids was evaporated to about 100 cc. acidified and fractionally distilled.

Fraction I. 130 cc. = 6.40 cc. \( \frac{\text{m}}{10} \) Ba(OH)\(_2\)

As 10 cc. had been used in the determination of total acidity, the sum for the above fractions, corrected to 1000 cc., is equivalent to 21.60 cc. It is uncertain whether the difference of 0.5 cc. in acidity of the steam distillate and the sum of the fractions as shown above, was due to loss in manipulation or removal of barium carbonate by evaporation and filtration.

For the purpose of illustrating more in detail the method used in separating and identifying the volatile acids, data secured in the first analysis are displayed below. Tables of constants from which the calculations were made are available in Duclaux' *Traité de Microbiologie*, iii, 1900.

Fractions I and II treated according to Duclaux' method.

**Fraction I** = 130 cc. and required 6.4 cc. \( \frac{\text{m}}{10} \) Ba(OH)\(_2\),

<table>
<thead>
<tr>
<th>DISTILLATE.</th>
<th>Ba(OH)(_2).</th>
<th>FOUND.</th>
<th>CALCULATED FOR</th>
</tr>
</thead>
<tbody>
<tr>
<td>cc.</td>
<td>per cent.</td>
<td>per cent.</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>(\frac{0.46 \times 100}{1000}) = 9.91</td>
<td>8.28</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>0.91 &quot; &quot; &quot; &quot;</td>
<td>19.60</td>
<td>18.54</td>
</tr>
<tr>
<td>30</td>
<td>1.32 &quot; &quot; &quot; &quot;</td>
<td>28.45</td>
<td>27.80</td>
</tr>
<tr>
<td>40</td>
<td>1.72 &quot; &quot; &quot; &quot;</td>
<td>37.07</td>
<td>37.17</td>
</tr>
<tr>
<td>50</td>
<td>2.15 &quot; &quot; &quot; &quot;</td>
<td>46.34</td>
<td>46.35</td>
</tr>
<tr>
<td>60</td>
<td>2.58 &quot; &quot; &quot; &quot;</td>
<td>55.61</td>
<td>55.86</td>
</tr>
<tr>
<td>70</td>
<td>3.04 &quot; &quot; &quot; &quot;</td>
<td>65.52</td>
<td>65.75</td>
</tr>
<tr>
<td>80</td>
<td>3.51 &quot; &quot; &quot; &quot;</td>
<td>75.65</td>
<td>75.78</td>
</tr>
<tr>
<td>90</td>
<td>4.02 &quot; &quot; &quot; &quot;</td>
<td>86.64</td>
<td>86.81</td>
</tr>
<tr>
<td>100</td>
<td>4.64 &quot; &quot; &quot; &quot;</td>
<td>100.00</td>
<td>100.00</td>
</tr>
</tbody>
</table>

6.4 cc. Ba(OH)\(_2\) × 79.15 per cent = 5.06 cc. Ba(OH)\(_2\) for acetic acid.

6.4 " " " 5.00 " = 0.32 " " " propionic acid.

6.4 " " " 15.83 " = 1.01 " " " butyric acid.

*F = Formic acid; A = Acetic acid; P = Propionic acid; B = Butyric acid; V = Valerianic acid; C = Caproic acid.
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Fraction II = 150 cc. and required 15.02 cc. \( \frac{3}{10} \) \( \text{Ba(OH)}_2 \)

<table>
<thead>
<tr>
<th>DISTILLATE.</th>
<th>Ba(OH)$_2$.</th>
<th>FOUND.</th>
<th>CALULATED FOR</th>
</tr>
</thead>
<tbody>
<tr>
<td>cc.</td>
<td>per cent.</td>
<td></td>
<td>per cent.</td>
</tr>
<tr>
<td>10</td>
<td>0.63 cc. ( \times \frac{3}{8.18} ) = 7.70</td>
<td>7.67</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>1.28 &quot; &quot; &quot;</td>
<td>15.65</td>
<td>15.69</td>
</tr>
<tr>
<td>30</td>
<td>1.95 &quot; &quot; &quot;</td>
<td>23.84</td>
<td>24.00</td>
</tr>
<tr>
<td>40</td>
<td>2.65 &quot; &quot; &quot;</td>
<td>32.40</td>
<td>32.77</td>
</tr>
<tr>
<td>50</td>
<td>3.40 &quot; &quot; &quot;</td>
<td>41.56</td>
<td>41.74</td>
</tr>
<tr>
<td>60</td>
<td>4.18 &quot; &quot; &quot;</td>
<td>51.10</td>
<td>51.33</td>
</tr>
<tr>
<td>70</td>
<td>5.03 &quot; &quot; &quot;</td>
<td>61.49</td>
<td>61.37</td>
</tr>
<tr>
<td>80</td>
<td>5.96 &quot; &quot; &quot;</td>
<td>72.86</td>
<td>72.53</td>
</tr>
<tr>
<td>90</td>
<td>6.07 &quot; &quot; &quot;</td>
<td>85.20</td>
<td>84.80</td>
</tr>
<tr>
<td>100</td>
<td>8.18 &quot; &quot; &quot;</td>
<td>100.00</td>
<td>100.00</td>
</tr>
</tbody>
</table>

15.02 cc. \( \text{Ba(OH)}_2 \) \( \times \) 96.0 per cent = 14.42 cc. \( \text{Ba(OH)}_2 \) for acetic acid.
15.02 " " " 2.5 " " " = 0.38 " " " propionic acid.
15.02 " " " 1.5 " " " = 0.22 " " " butyric acid.

The total amount of volatile fatty acids in the 247.5 grams of whole milk cheese is calculated from Fractions I and II. The acids are corrected to decinormal \( \text{Ba(OH)}_2 \).

<table>
<thead>
<tr>
<th>FRACTION.</th>
<th>FRACTION.</th>
<th>TOTAL.</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td>( \text{Ba(OH)}_2 )</td>
<td>cc.</td>
<td>cc.</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>5.06</td>
<td>14.42</td>
</tr>
<tr>
<td>Propionic acid</td>
<td>0.32</td>
<td>0.38</td>
</tr>
<tr>
<td>Butyric acid</td>
<td>1.01</td>
<td>0.22</td>
</tr>
</tbody>
</table>

In a similar manner the estimation and identification of the volatile fatty acids in the skim-milk cheese were carried out. Analyses of the curd cheese were made at definite intervals and the final data on this particular phase of the subject are brought together in the two following tables. All results are expressed as decinormal acid per 100 grams of dry matter.
S. K. Suzuki, E. G. Hastings and E. B. Hart

TABLE I.

Whole Milk Cheese. In 100 grams of Dry Matter.

<table>
<thead>
<tr>
<th></th>
<th>3 DAYS OLD</th>
<th>6 WEEKS OLD</th>
<th>3 MOS. OLD</th>
<th>5½ MOS. OLD</th>
<th>10½ MOS. OLD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk sugar</td>
<td>.00</td>
<td>.00</td>
<td>.00</td>
<td>.00</td>
<td>.00</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>84.09 cc.</td>
<td>90.28 cc.</td>
<td>124.00 cc.</td>
<td>103.70 cc.</td>
<td>74.10 cc.</td>
</tr>
<tr>
<td>Total vol. acids</td>
<td>12.74 cc.</td>
<td>34.17 cc.</td>
<td>32.15 cc.</td>
<td>34.82 cc.</td>
<td>22.96 cc.</td>
</tr>
<tr>
<td>Formic acid</td>
<td>0.00 cc.</td>
<td>0.00 cc.</td>
<td>0.00 cc.</td>
<td>1.80 cc.</td>
<td>0.00 cc.</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>11.59 cc.</td>
<td>29.47 cc.</td>
<td>24.25 cc.</td>
<td>25.86 cc.</td>
<td>12.64 cc.</td>
</tr>
<tr>
<td>Propionic acid</td>
<td>0.41 cc.</td>
<td>2.15 cc.</td>
<td>3.42 cc.</td>
<td>1.07 cc.</td>
<td>2.63 cc.</td>
</tr>
<tr>
<td>Butyric acid</td>
<td>0.73 cc.</td>
<td>2.17 cc.</td>
<td>3.50 cc.</td>
<td>4.82 cc.</td>
<td>5.45 cc.</td>
</tr>
<tr>
<td>Caproic acid</td>
<td>0.00 cc.</td>
<td>0.36 cc.</td>
<td>0.96 cc.</td>
<td>1.25 cc.</td>
<td>2.23 cc.</td>
</tr>
<tr>
<td>Succinic acid</td>
<td>.00 cc.</td>
<td>.00 cc.</td>
<td>.00 cc.</td>
<td>.00 cc.</td>
<td>Trace</td>
</tr>
</tbody>
</table>

TABLE II.

Skim-milk Cheese. In 100 grams of Dry Matter.

<table>
<thead>
<tr>
<th></th>
<th>3 DAYS OLD</th>
<th>6 WEEKS OLD</th>
<th>3 MOS. OLD</th>
<th>5½ MOS. OLD</th>
<th>10½ MOS. OLD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk sugar</td>
<td>.00</td>
<td>.00</td>
<td>.00</td>
<td>.00</td>
<td>.00</td>
</tr>
<tr>
<td>Fat</td>
<td>2.12 p.c.</td>
<td>3.06 p.c.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactic acid</td>
<td>149.27 cc.</td>
<td>119.60 cc.</td>
<td>179.44 cc.</td>
<td>132.30 cc.</td>
<td>11.60 cc.</td>
</tr>
<tr>
<td>Total vol. acid</td>
<td>21.60 cc.</td>
<td>62.29 cc.</td>
<td>105.71 cc.</td>
<td>84.20 cc.</td>
<td></td>
</tr>
<tr>
<td>Formic acid</td>
<td>0.00 cc.</td>
<td>0.00 cc.</td>
<td>0.00 cc.</td>
<td>0.00 cc.</td>
<td></td>
</tr>
<tr>
<td>Acetic acid</td>
<td>19.82 cc.</td>
<td>46.13 cc.</td>
<td>74.24 cc.</td>
<td>57.10 cc.</td>
<td></td>
</tr>
<tr>
<td>Propionic acid</td>
<td>1.48 cc.</td>
<td>10.10 cc.</td>
<td>10.51 cc.</td>
<td>7.62 cc.</td>
<td></td>
</tr>
<tr>
<td>Butyric acid</td>
<td>0.29 cc.</td>
<td>4.10 cc.</td>
<td>17.42 cc.</td>
<td>15.91 cc.</td>
<td></td>
</tr>
<tr>
<td>Caproic acid</td>
<td>0.00 cc.</td>
<td>1.94 cc.</td>
<td>3.53 cc.</td>
<td>3.56 cc.</td>
<td></td>
</tr>
<tr>
<td>Succinic acid</td>
<td>.00 cc.</td>
<td>.00 cc.</td>
<td>.00 cc.</td>
<td>.00 cc.</td>
<td>Trace</td>
</tr>
</tbody>
</table>

Discussion of the Data.

From the above tables it is apparent that the milk sugar disappeared early after manufacture. At the end of three days none could be detected.

Contrary to our working hypothesis, lactic acid did not disappear during the entire curing process. Its amount appeared to fluctuate somewhat, but in the whole milk cheese even at the end of 10 months, 88 per cent of that present at the time of the initial analysis still remained. In the case of the skim-milk cheese, during the later part of its history, lactic acid had largely disappeared.
The initial source of the lactic acid in the cheese is, of course, lactose; but the unmistakable increase in the amount of this body in both cheeses up to the end of three months and after all lactose had disappeared, raises the question of additional sources of this body during the curing process.

The supposition might be made that the lactose, during its fermentation yields, substances which later are slowly changed to lactic acid. It is a significant fact that the initial yield of lactic acid from the lactose in the curd is less than 50 per cent of the theoretical. One hundred grams of water-free cheese curd, one-day old, contain usually about 2 grams of lactose, which theoretically can yield 2 grams of lactic acid. Yet with what has been formed during the making process and up to the one-day stage, there was obtained but 0.75 gram.

In a lactose fermentation by mixed cultures of lactic acid organisms, besides the carbon dioxide, volatile fatty acids and lactic acid that are produced, such additional by-products as succinic acid and alcohol have been reported by several investigators. However, it is improbable that the latter substances are by further fermentation changed to lactic acid. It is more in agreement with the modern theory of lactic acid production, that the lactic acid itself is first formed, and by further breaking down, gives rise to simpler products. However, the occurrence of still unknown intermediary products capable of further change to lactic acid is not to be ignored.

A second possible source of lactic acid after the lactose fermentation lies in the proteolysis of the paracasein. Recht\(^1\) has already reported the formation, during casein proteolysis by gastric juice, of such products as lactic and butyric acids. Even certain amino acids are theoretically possible precursors of this acid; as for example, serin, \(\text{CH}_2\text{OH} \cdot \text{CHNH}_2 \cdot \text{COOH}\), cystein, \(\text{CH}_2\text{SH} \cdot \text{CHNH}_2 \cdot \text{COOH}\) and alanin, \(\text{CH}_2 \cdot \text{CHNH}_2 \cdot \text{COOH}\). The latter is particularly closely related to lactic acid and by simple deamidization and oxidation is converted to that body. Neuberg and Langstein\(^2\) found that after administration of alanin to rabbits, small quantities of lactic acid appeared in the urine.

\(^1\) *Compt. rend.*, lxxxvi, pp. 550–552; lxxxviii, pp. 750–751.

Experimentally we were unable to induce such a change by inoculating a sterile 1 per cent solution of alanin with a small piece of whole milk cheese, 6 months old and about the size of a pea, and incubating at 35° C. for three months. Possible modifications and extensions of this experiment are in progress to further test such a theory.

The form of lactic acid in both whole and skim-milk cheese was, as previously stated, with but one exception, of an inactive variety. The skim-milk cheese when ten months old showed the presence of active lactic acid. 0.1392 gram of the crystalline zinc salt gave 0.0176 gram of water of crystallization, equivalent to 12.64 per cent. The theoretical requirement for the active acid is 12.89 per cent. Possible selective action on the part of some biological factor operative in this cheese in its later history would explain the above phenomena. This interesting observation that lactic acid might be of the active or inactive variety in cheese, dependent upon the nature of the biological factors inducing the fermentation, warranted more extended observations. For this purpose normal Cheddar cheese made at different seasons of the year and analyzed at different ages, as well as cheese made from milk, pasteurized at 160° F., was investigated for the form of lactic acid present. The results are appended in the following table.

**TABLE III.**

<table>
<thead>
<tr>
<th>NO.</th>
<th>DATE MADE.</th>
<th>AGE.</th>
<th>REMARKS.</th>
<th>ZINC LACTATE.</th>
<th>WATER OF CRYST.</th>
<th>WATER OF CRYS T.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mos.</td>
<td></td>
<td>grams.</td>
<td>per cent.</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Mar. 1909</td>
<td>1</td>
<td>Pasteurized</td>
<td>3.967</td>
<td>0.721</td>
<td>18.18</td>
</tr>
<tr>
<td>2</td>
<td>Sept. 1908</td>
<td>9</td>
<td>&quot;</td>
<td>0.939</td>
<td>0.173</td>
<td>18.42</td>
</tr>
<tr>
<td>3</td>
<td>&quot;</td>
<td>9</td>
<td>&quot;</td>
<td>0.570</td>
<td>0.105</td>
<td>18.42</td>
</tr>
<tr>
<td>4</td>
<td>Feb. 1909</td>
<td>3</td>
<td>&quot;</td>
<td>0.819</td>
<td>0.148</td>
<td>18.14</td>
</tr>
<tr>
<td>5</td>
<td>Aug. 1908</td>
<td>10</td>
<td>Non-pasteurized</td>
<td>0.826</td>
<td>0.146</td>
<td>17.66</td>
</tr>
<tr>
<td>6</td>
<td>Feb. 1909</td>
<td>3</td>
<td>&quot;</td>
<td>0.470</td>
<td>0.085</td>
<td>18.17</td>
</tr>
<tr>
<td>7</td>
<td>Jan. 1908</td>
<td>17</td>
<td>&quot;</td>
<td>0.163</td>
<td>0.028</td>
<td>17.16</td>
</tr>
</tbody>
</table>

In all but two cases the agreement with the theoretical requirement for inactive lactic acid is close. Nos. 5 and 7 gave results lower than the theory required. Difference in the solubility of the zinc lactates made possible a separation, by fractional crystalli-
zation, of the active and inactive forms. The zinc salts of the active acids are more soluble than those of the racemic variety. This principle was applied to No. 5. From the first crystallization, 1.791 gram of the zinc salt gave 0.328 gram of water, equivalent to 18.31 per cent. From the second crystallization, 0.434 gram of zinc salt gave 0.062 gram of water, or 14.3 per cent. From the third fraction, 0.352 gram yielded 0.045 gram of water, or 12.76 per cent. The theory calls for 12.89 per cent for the active form. These results confirm the belief that there was present in cheese No. 5, active lactic acid, as well as the inactive form.

Source of the Forms of Lactic Acid.

In connection with this work on the forms of lactic acid in cheese, experiments were conducted to determine the variety of lactic acid produced in lactose solutions by various acid-producing organisms in the hope of throwing light on the source of the forms found in curing cheese.

Three hundred cubic centimeters of a lactose solution (1.8 per cent milk sugar, 0.5 per cent peptone and 5 grams of barium carbonate) were inoculated with a starter and incubated at 35° for two months. At the end of this time the lactic acid formed was examined. 0.216 gram of zinc lactate gave 0.0275 gram of water, equal to 12.73 per cent. Theory, 12.89 for the active salt. Three hundred cubic centimeters of the same solution, inoculated with a pure culture of \textit{B. lactis acidii} and incubated at 35° C. for two months gave a similar result—always the active form of lactic acid.

The lactic fermentation of Cheddar cheese, so far as our investigation has gone, usually produced the inactive variety, a result in marked contrast to the above experiments with lactose solutions.

The explanation of this result may possibly be found in that (1) there is in cheese a more varied bacterial flora than is usually supposed. The results of numerous investigations indicate that 95 to 99 per cent of the bacteria in cheese belong to the \textit{B. lactis acidii} group. It should be remembered that these results have been obtained by the use of methods that are favorable to the class of bacteria mentioned. Large numbers of other types of lactic organisms may be present. It is known that an organism similar to or identical with \textit{B. Bulgaricus} is present in cheese in
unknown numbers. This organism has been shown by Heine-
mann to produce inactive lactic acid. (2) The active acid pro-
duced by \textit{B. lactis acidi} is consumed by other organisms, while
the inactive acid remains intact. The following experiment
throws some light on the first supposition.

A milk sugar solution to which a small amount of fat had been added
was inoculated with a bit of our whole milk experimental cheese. After
four weeks' incubation at 35\textdegree the kind of lactic acid produced was investi-
gated. Fractional crystallization of the zinc lactates gave the following
results: 0.762 gram of the first fraction gave 0.138 gram of water of cry-
stallization, equal to 18.12 per cent. Theory for inactive zinc lactate
18.18 per cent; 0.863 gram of the sixth fraction gave 0.110 gram of water
of crystallization, equal to 13.78 per cent. Theory for the active zinc lac-
tate 12.89 per cent. In the above experiments 1.76 gram of the zinc salt of
inactive lactic acid, and 1.42 gram of the active acid were collected.

This experiment finds its simplest explanation in the suppo-
sition that unequal amounts of the active lactic acids are formed
and that consequently several distinct lactose ferments were
present in the solution. The inactive acid generally found in
Cheddar cheese must very probably be referred to the formation
of equal quantities of the two active acids by distinct ferments
with the resultant racemic acid, or inactive form. To explain the
paradoxical condition that equimolecular quantities of the \textit{d}-acid
and \textit{l}-acid were formed by different ferments, it may be neces-
sary to assume that the excess of one suffered destruction through
the further action of other organisms. However, final conclu-
sions cannot be drawn from the limited amount of data now at
hand, and further investigations may show that small amounts
of active lactic acid are present in normal cheese, and remain in
the mother liquor after the separation of the less soluble zinc
salt of the inactive variety.

\textit{Volatile Fatty Acids.}

\textit{Whole milk cheese.} The total amount of volatile fatty acids in
100 grams of dry matter of the whole milk cheese at the end of
three days was equivalent to 12.74 cc. \% solution. This amount
rose to 34.17 cc. at six weeks; remained at about that quantity
to the end of 5\frac{1}{2} months and then fell to 22.96 cc. at the age of
ten months.
Skim-milk cheese. In the skim-milk cheese the amount of volatile acids is higher than in the whole milk cheese, probably due to the larger proportion of milk sugar in the curd, and was equivalent to 21.6 cc. $\frac{x}{10}$ acid at three days; this increased to 62.29 cc. at six weeks, 105.7 cc. at three months, and then decreased at the end of 5½ months to 84.2 cc. When we consider the individual acids, it will be seen that each had its own particular curve.

**TABLE IV.**

*Acetic Acid.*

(Results are expressed as cc. of decinormal acid.)

<table>
<thead>
<tr>
<th></th>
<th>3 Days</th>
<th>6 Weeks</th>
<th>3 Months</th>
<th>5½ Months</th>
<th>10½ Months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole milk cheese</td>
<td>11.59</td>
<td>29.44</td>
<td>24.25</td>
<td>25.86</td>
<td>12.63</td>
</tr>
<tr>
<td>Skim-milk cheese</td>
<td>19.82</td>
<td>46.13</td>
<td>74.24</td>
<td>57.10</td>
<td></td>
</tr>
</tbody>
</table>

In the whole milk cheese the maximum amount of this acid was found at the end of six weeks, and after that period it slowly decreased in amount until the end of 5½ months, after which a more rapid decrease took place.

In the skim-milk cheese there was a continual increase up to three months, after which the amount decreased.

These results in all probability only represent transition stages of the acid, and the combined result of constructive or destructive agencies. It is very probable that acetic acid is continually being formed during the cheese curing process and is also being destroyed, with the catabolic agencies more active in the later history of the cheese.

**TABLE V.**

*Propionic Acid.*

(Results are expressed as cc. of decinormal acid.)

<table>
<thead>
<tr>
<th></th>
<th>3 Days</th>
<th>6 Weeks</th>
<th>3 Months</th>
<th>5½ Months</th>
<th>10½ Months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole milk cheese</td>
<td>0.41</td>
<td>2.15</td>
<td>3.42</td>
<td>1.07</td>
<td>2.63</td>
</tr>
<tr>
<td>Skim-milk cheese</td>
<td>1.48</td>
<td>10.10</td>
<td>10.51</td>
<td>7.62</td>
<td></td>
</tr>
</tbody>
</table>
This acid behaved very similarly to acetic acid in that there was an increase during the early history of the cheese, with a gradual decrease as the cheese aged.

**TABLE VI.**

*Butyric and Caproic Acids.*

(Results are expressed as cc. of decinormal acid.)

<table>
<thead>
<tr>
<th></th>
<th>3 Days</th>
<th>6 Weeks</th>
<th>3 Months</th>
<th>5½ Months</th>
<th>10½ Months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole milk cheese:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Butyric acid</td>
<td>0.73</td>
<td>2.17</td>
<td>3.50</td>
<td>4.82</td>
<td>5.45</td>
</tr>
<tr>
<td>Caproic acid</td>
<td>0.00</td>
<td>0.36</td>
<td>0.96</td>
<td>1.25</td>
<td>2.23</td>
</tr>
<tr>
<td>Skim-milk cheese:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Butyric acid</td>
<td>0.29</td>
<td>4.10</td>
<td>17.42</td>
<td>15.91</td>
<td></td>
</tr>
<tr>
<td>Caproic acid</td>
<td>0.00</td>
<td>1.94</td>
<td>3.53</td>
<td>3.56</td>
<td></td>
</tr>
</tbody>
</table>

On the whole, butyric and caproic acids increased in amount gradually during the entire curing process, but the amount of butyric acid was much higher than that of caproic acid. Again the quantity of both acids in the skim-milk cheese is very much larger than in the cheese made from whole milk.

**Formic acid.** This acid was obtained in the whole milk cheese at the age of 5½ months, but was not detected at any of the other stages in the analysis. At no time was it found in the skim-milk cheese.

It must be understood that it is not supposed that these acids existed in the free state in the cheese, but that they were either in loose combination with the nitrogenous substances of the curing mass, or as salts of the ash elements.

**Source of the Volatile Fatty Acids.**

**Lactose as a source.** The amount of volatile fatty acids formed during the period which marks the disappearance of lactose from the ripening cheese was unquestionable to be attributed to the lactose fermentation. The source of the increasing amounts formed after that time, is not as yet clearly defined. A number of experiments planned to throw some light on the sources of these acids are given in the following pages:
One hundred cubic centimeters of sterile lactose solution (1.8 per cent milk sugar, 0.5 per cent peptone, 5 grams of barium carbonate) when inoculated with either a pure culture of *B. lactis acidi* or a starter and incubated at 35° C., gave the following results at the end of three and fifty-six days, respectively:

<table>
<thead>
<tr>
<th></th>
<th>3 Days.</th>
<th>56 Days.</th>
<th>3 Days.</th>
<th>56 Days.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>B. lactis acidi.</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Formic acid</td>
<td>0.43 cc.</td>
<td>1.68 cc.</td>
<td>0.57 cc.</td>
<td>2.20 cc.</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>3.88 cc.</td>
<td>8.06 cc.</td>
<td>16.15 cc.</td>
<td>11.25 cc.</td>
</tr>
<tr>
<td>Propionic acid</td>
<td>0.00 cc.</td>
<td>0.00 cc.</td>
<td>0.43 cc.</td>
<td>0.13 cc.</td>
</tr>
</tbody>
</table>

No propionic acid could be isolated from the *B. lactis acidi* culture; in addition no butyric or caproic acids were formed. With the starter used, propionic acid was isolated, as well as formic and acetic, but neither butyric or caproic acids. The possibility that barium carbonate may have been somewhat toxic to the *B. lactis acidi*, thereby modifying the physiological processes of the organism, suggested itself. Consequently an experiment was made, using calcium carbonate as the neutralizing agent.

(a) 200 cc. solution of

- 3.6 per cent milk sugar,
- 1 " " peptone,
- 2 " " calcium carbonate.

(b) 200 cc. solution of

- 3.6 per cent sugar,
- 1 " " peptone,
- 7 " " barium carbonate.

They were inoculated with *B. lactis acidi* and, after incubation at 35° C. for two months, the solutions were analyzed, with the following results:

<table>
<thead>
<tr>
<th></th>
<th>(a)</th>
<th>(b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formic acid</td>
<td>9.72 cc.</td>
<td>4.92 cc.</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>17.79 cc.</td>
<td>1.44 cc.</td>
</tr>
<tr>
<td>Propionic acid</td>
<td>1.51 cc.</td>
<td>2.12 cc.</td>
</tr>
</tbody>
</table>

In this instance propionic acid was formed in both solutions, and it is also clear that the barium carbonate had exerted a
depressing influence on the metabolism of the organism. The kind of products formed was not affected. Neither caproic nor butyric acids could be isolated.

Experiments on lactose solutions with two distinct acid producing organisms, one a liquefying and the other a non-liquefying coccus, and both isolated from Cheddar cheese, gave the following results. The solutions used were identical with (a) as detailed above. The period of incubation was four months at 35°C. The results are given in the following table.

<table>
<thead>
<tr>
<th></th>
<th>LIQUEFYING COCCUS</th>
<th>NON-LIQUEFYING COCCUS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid</td>
<td>28.6 cc.</td>
<td>30.35 cc.</td>
</tr>
<tr>
<td>Propionic acid</td>
<td>0.56 &quot;</td>
<td>1.07 &quot;</td>
</tr>
<tr>
<td>Butyric acid</td>
<td>0.70 &quot;</td>
<td>0.30 &quot;</td>
</tr>
<tr>
<td>Caproic acid</td>
<td>0.84 &quot;</td>
<td>0.20 &quot;</td>
</tr>
</tbody>
</table>

No formic acid was present in these solutions, but in both instances and even in the case of the non-liquefying coccus, caproic and butyric acids were formed. This is an important distinction from the products formed by the pure culture of *B. lactis acidi*, or the organisms contained in the starter used, which are largely, if not exclusively, *B. lactis acidi*. Not only was there a distinction in the character of the volatile fatty acids produced, but in addition it was impossible to separate lactic acid from the coccus fermentations.

The influence of one organism on another, whereby the character of their combined end-products is modified, finds an illustration in the following experiments. This experiment is presented at this point because of its important bearing upon interpreting results secured by fermentations with pure cultures and those in which a number of organisms have taken part.

Two hundred cubic centimeters of a lactose solution made as described above, and containing calcium carbonate, were incubated at 35°C for periods of eight and fifty-six days respectively after inoculation with a mixture of *B. lactis acidi* and the yellow coccus. The results are shown below:
In pure culture the coccus had produced both caproic and butyric acids, but in the above experiment neither of these acids could be detected. The explanation of this result may be found in the more rapid growth of *B. lactis acidii* than the yellow coccus under the conditions obtaining in the experiment. The latter organism is very slow in its growth under all conditions, and is more susceptible to acid reaction in the medium. It seems probable that the growth of the coccus was very slight, and that the acids found were the result of the activity of *B. lactis acidii*. Further experiments are necessary to fully decide this point.

The fact that butyric acid was found in but small amounts during the period of direct lactose fermentation in the cheese, and caproic acid not at all, makes it seem probable that lactose was not the mother substance of these two acids, and further, that the organisms of the *B. lactis acidii* type were not responsible for the production of these two higher acids found in the curing cheese mass.

In another experiment 200 cc. of the lactose solution of the same composition as used in previous experiments were inoculated with a bit of the whole milk cheese, five months old, about the size of a pea, and incubated at 35° C. for 30 days. It was only possible to identify acetic and propionic acids as constituting the volatile acids formed during this fermentation. From the 200 cc. of lactose solution, the following quantities were obtained:

- Acetic acid: 348.5 cc.
- Propionic acid: 174.0 cc.

In an experiment, where 200 cc. of a solution of 1 per cent peptone containing 2 per cent of calcium carbonate but no lactose, were inoculated with a similar bit of cheese, as described above, and incubated at 35° C. for 4½ months, the following results were obtained:
Putrefaction had resulted, undoubtedly occasioned by the lack of an inhibiting effect on the growth of certain organisms, which would have resulted had lactose been present with the resultant production of high acidity. This experiment, however, makes it clear that certain volatile acids can have their origin in the decomposition of the protein molecule, a fact already well-known. A peptone solution, prepared as in the previous experiment, and inoculated with \textit{B. lactis acidi} alone in pure culture, did not produce volatile fatty acids. This makes it very probable that that type of organism was not a factor in the formation of caproic and butyric acids in the cheese.

\textit{Lactates as Sources of Volatile Fatty Acids.}

The possibility that the lactates found in cheese would, by further decomposition, yield fatty acids, has already been referred to in the early part of this paper. While lactic acid did not decrease during the normal ripening period, but in both instances actually increased, it may still have been in part the mother substance of volatile fatty acids. This increase, as previously suggested, may have had its origin in protein decomposition. Two hundred cubic centimeters of a calcium lactate solution (1.0 per cent calcium lactate, 0.5 peptone, 2 per cent calcium carbonate) were inoculated with a small bit of our whole milk cheese and incubated three weeks at 35° C. The separation and identification of the volatile fatty acids were made in the usual manner.

\begin{center}
\begin{tabular}{l l l}
Acetic acid & \ldots & 19.40 cc. \\
Propionic acid & \ldots & 4.47 cc. \\
Butyric acid & \ldots & 10.85 cc. \\
Valeric acid & \ldots & 0.81 cc. \\
Caproic acid & \ldots & 3.08 cc.
\end{tabular}
\end{center}

None of the other volatile fatty acids could be detected.

In another experiment of the same character and length of time, but inoculated with a bit of skim-milk cheese, the results were:

\begin{center}
\begin{tabular}{l l l}
Acetic acid & \ldots & 8.70 cc. \\
Propionic acid & \ldots & 0.96 cc.
\end{tabular}
\end{center}
No putrefaction had occurred, as indicated by odor and the character of the acids. This makes it very probable that the lactates can act as the mother substance from which acetic and propionic acids may be formed by specific ferments\textsuperscript{1} of the cheese. It might be suggested that the above results are entirely due to enzymes contained in the cheese. The following experiment was planned to throw some light on this point. A milk sugar solution, containing peptone and calcium carbonate, as previously described, was inoculated with 2 grams of our experimental whole milk cheese, four months old, chloroform added to 2 per cent by volume, and the solution incubated 40 and 100 days respectively. No lactic acid could be isolated from the mixture while there was also a total absence of volatile fatty acids.

Even perfectly fresh milk preserved with chloroform and incubated one year at 35° C., failed to show the presence of lactic acid, indicating that there is no inherent lactose fermenting enzyme in cow's milk. A milk chloroformed and preserved at room temperature for eleven years still contained 5.06 per cent lactose and no lactic acid, confirming in every respect the above deduction.

Attempts to isolate an enzyme from curing cheese capable of attacking lactose, with production of volatile acids, also failed in every case.

\textit{Fats as a Source of Butyric and Caproic Acids.}

All our experiments point to the conclusion that in the lactose or further lactate fermentation in cheese ripening, butyric acid is formed in but traces, and caproic acid not at all; nevertheless these acids increase steadily in amounts during the curing process. A possible source of these acids is caproin and butyrin, which are known to exist in milk fat. Either enzymes or organisms could be responsible for this hydrolysis. To determine whether or not this causal factor was contained in a starter, the following experiment was made:

Three hundred grams of fresh unsalted butter were washed several times with a large volume of hot water and then subjected to steam dis-

\textsuperscript{1} The term "ferments" is here used collectively for both organisms and enzymes.
S. K. Suzuki, E. G. Hastings and E. B. Hart

tillation until the distillate was neutral. Another portion of 200 grams of butter fat was acidified with dilute sulphuric acid and distilled with steam in order to determine whether or not the fats so treated in the usual method of volatile acid separation, would yield volatile fatty acids. The distillate of 1000 cc. required but 2.2 cc. of $\frac{1}{10}$ barium hydroxide. This is a negligible factor. Ten grams of the washed fat were used in the following experiment:

(a) 400 cc. solution of

- 1.8 per cent milk sugar,
- 0.5 " " peptone,
- 1.0 " " calcium carbonate,
- 2.5 " " fat.

(b) 400 cc. solution of

- 1.8 per cent milk sugar,
- 0.5 " " peptone,
- 1.0 " " calcium carbonate,
- No fat.

Both solutions, after sterilization, were inoculated with a starter and incubated for 48 days at 35° C. The determination of the volatile fatty acids present, showed that only propionic and acetic acids had been produced in either solution, while neither butyric nor caproic acids could be identified in the distillates by the Duclaux method. This indicates that the starter contained no organisms or enzymes capable of lipolysis under the conditions of this experiment. The same experiment was repeated, but instead of inoculating with a starter, a bit of the experimental whole milk cheese, five months old, was used. At the end of a 30-day incubation, this solution was analyzed with the following results:

<table>
<thead>
<tr>
<th></th>
<th>Solution (a) plus fat</th>
<th>Solution (b) no fat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid</td>
<td>138.52 cc. $\frac{19}{10}$</td>
<td>348.59 cc. $\frac{19}{10}$</td>
</tr>
<tr>
<td>Propionic acid</td>
<td>70.87 &quot; &quot;</td>
<td>174.00 &quot; &quot;</td>
</tr>
<tr>
<td>Butyric acid</td>
<td>38.29 &quot; &quot;</td>
<td>0.00 &quot; &quot;</td>
</tr>
<tr>
<td>Caproic acid</td>
<td>6.57 &quot; &quot;</td>
<td>0.00 &quot; &quot;</td>
</tr>
</tbody>
</table>

This experiment differentiates clearly the main source of the higher fatty acids and makes it probable that there are either active enzymes or organisms contained in cheese capable of hydrolyzing glycerides in the ripening process. Whether this action is due to lipases inherent in milk, or living organisms, must be decided by further experiments.

Proteins as a Source of Volatile Fatty Acids.

The fact that volatile fatty acids have been separated from protein decomposition, suggests the factor of proteolysis as an additional source of these products. In an experiment where
perfectly fresh whole milk was preserved for one year with chloroform, 100 cc. of the milk, equivalent to from 10 to 12 grams of solids, gave volatile acids equivalent in $\frac{N}{10}$ acid to 3.28 cc. Further work along this line is being conducted, in order to obtain sufficient material for identification of the volatile acids produced. Where a milk had been preserved for eleven years with chloroform, 400 cc., equivalent to from 40 to 50 grams of dry matter, contained volatile acids in quantity equivalent to 9.0 cc. of an $\frac{N}{10}$ solution. These acids on separation gave the following results:

<table>
<thead>
<tr>
<th>Acids</th>
<th>Amount (cc)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid</td>
<td>6.64</td>
</tr>
<tr>
<td>Propionic acid</td>
<td>0.83</td>
</tr>
<tr>
<td>Butyric acid</td>
<td>0.41</td>
</tr>
<tr>
<td>Caproic acid</td>
<td>0.41</td>
</tr>
</tbody>
</table>

It is, however, necessary to note that in an equivalent quantity of whole milk cheese, three months old, four times as much butyric and an equivalent quantity of caproic acid had been produced.

From our present data it is improbable that the combined action of lipolytic and proteolytic enzymes of milk are entirely responsible for the production of the higher fatty acids occurring in ripening Cheddar cheese. Further experiments, however, must fully decide this. That fatty acids are produced from protein decomposition alone by organisms or enzymes contained in the cheese, is shown by the following experiment:

Two hundred cubic centimeters of a solution of 1 per cent peptone and 2 per cent calcium carbonate, but containing no glycerides or milk sugar, were inoculated with a small bit of our whole milk cheese. After incubation for 135 days at 35° C. it was subjected to the Duclaux method with the following results:

<table>
<thead>
<tr>
<th>Acids</th>
<th>Amount (cc)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid</td>
<td>16.40</td>
</tr>
<tr>
<td>Propionic acid</td>
<td>4.47</td>
</tr>
<tr>
<td>Butyric acid</td>
<td>10.85</td>
</tr>
<tr>
<td>Valerianic acid</td>
<td>0.81</td>
</tr>
<tr>
<td>Caproic acid</td>
<td>3.08</td>
</tr>
</tbody>
</table>

These results are in harmony with the well-known fact that extensive proteolysis gives rise to volatile fatty acid. It must
not be taken, however, as proof that this is a very important source of the volatile fatty acids produced during the cheese ripening process.

Glycerin as a Source of Certain Volatile Fatty Acids.

This body, which would be left after the hydrolysis of butyrin or caproin in the cheese mass, may by further fermentation give rise to acetic and propionic acids. This is shown in the following experiment:

Two hundred cubic centimeters of a solution of 0.9 per cent glycerin, 0.5 per cent protein and 2 per cent calcium carbonate, were treated with a small bit of the whole milk experimental cheese, and after incubation for 40 days at 35° C., gave the following results:

<table>
<thead>
<tr>
<th>Acetic acid</th>
<th>Propionic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>21.8 cc. N10</td>
<td>1.17 &quot; &quot;</td>
</tr>
</tbody>
</table>

It is apparent that under the influence of the enzymes or organisms contained in the above cheese, glycerin could yield only acetic and propionic acids.

Isolation of Succinic Acid from Curing Cheese.

Succinic acid has been isolated from Emmenthaler cheese by Winterstein1 and it has also been separated from solutions of lactose fermented by specific organisms;2 it is probable that its origin in the cheese was from the early lactose fermentation.

However, the work of Ehrlich3 on the formation of succinic acid from glutaminic acid makes it possible that protein decomposition can also be a source of this body.

In the process involving the separation of lactic acid from the three months old cheese, crystals appeared at that point in the process when the separated solution, neutralized with barium hydroxide, was evaporated to a small volume. They were

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2 Emmerling; Zersetzung stickstofffreier organische Substansen durch Bakterien, p. 59.
observed in previous analyses, but regarded as crystals of barium lactate.

**Separation.** The mother liquor from the zinc lactate was treated with hydrogen sulphide to remove the zinc, then acidified with dilute sulphuric acid and extracted with ether. The ether extract was evaporated to a small volume, taken up with water and neutralized with barium hydroxide. When this solution was evaporated to a small volume, crystals began to separate. They were difficultly soluble in water, and brownish in color, due to impurities. They contained no water of crystallization, and on analysis showed 52.4 per cent of barium. Barium succinate contains 54.1 per cent, while barium lactate, with no water of crystallization, contains but 43.5 per cent. Barium citrate, which might have been present, contains 52.1 per cent of barium.

These results indicated that the body was either barium citrate or an impure barium succinate. Consequently, the filtrate and washings from the determination of barium were neutralized with barium hydroxide and silver nitrate added in excess. The precipitate was collected and dried at 108° C. 0.224 gram of this compound gave 0.144 gram of silver, equivalent to 64.3 per cent. The theoretical quantity of silver in silver succinate is 64.9 per cent, while for silver citrate the per cent is 63.1. This, we believe, identifies this body as a silver-succinate.

To further establish its identity the qualitative test used by Neuberg\(^1\) was applied to the barium salt. This consists of heating in a test tube a portion of the salt with ammonia and zinc dust. When the excess of ammonia has been driven out a pine shaving, saturated with hydrochloric acid, is held at the mouth of the tube and the heating continued. A red coloration of the shaving shows the presence of succinic acid. With our preparation positive results were obtained.

The application of the test for citric acid described by Sabanin and Las- kowsky\(^2\), failed to show its presence in this preparation.

This acid was again separated from both the whole and skim-milk cheese, at the 5½ months ripening stage. 1.192 gram of barium succinate from the whole milk cheese gave 0.175 gram of barium sulphate, equivalent to 53.6 per cent of barium. Theoretical quantity, 54.1 per cent. No analysis was made of that from the skim-milk cheese. This acid could not be directed in either the whole or skim-milk cheese at the age of 10½ months.

Succinic acid was also isolated from a solution of milk sugar and peptone, fermented in the presence of calcium carbonate, and fat, by inoculation with a small bit of the whole milk experimental cheese. No attempts were made to isolate this acid.

\(^1\) Zeitschr. f. physiol. Chem., xxxi, p. 574, 1900.
from the other experimentally fermented lactose solutions, but it is very probable that it had its origin in the cheese in the initial lactose fermentation of the curing curd.

"Flavor Solution."

As previously described, this solution was obtained from the first steam distillate by neutralization and redistillation. Neutral volatile substances, as alcohols and esters, would be found here, and it is a noteworthy fact that such solutions had a very strong flavor of cheese, as indicated by the sense of smell. Saponification and separation of the acids, as well as oxidation of the alcohols to acids and their separation, was carried on in a number of instances during these studies of the progressive changes in our experimental cheese.

A partial analysis was made of the solution obtained from both whole and skim-milk cheese, when six weeks old. Qualitative reactions for alcohols and aldehydes gave positive results for the former, but negative for the latter. Saponification and application of Duclaux' method established the presence of acetic, propionic and butyric acids in the solutions obtained from both the whole and skim-milk cheese. However, the amounts secured were small, necessarily making the separations less accurate than in the later analysis of the cheese. Oxidation of the alcohols was not carried out at this time.

Further studies of these solutions were made when the cheeses were three months old. Saponification and separation of the acids showed the presence of acetic, propionic, butyric and caproic acids. These had existed in the cheese as acid radicals in ester combinations. The same kinds of acid were isolated from the "flavor solution" obtained from the skim-milk cheese.

Oxidation of the Alcohols.

The distillate obtained from the saponified alkaline solutions was oxidized with potassium bichromate and sulphuric acid, as previously described, and according to the method of Dupré.1

The solution of acids obtained by oxidation of the alcohols

1 Journ. Chem. Soc., xx, 495.
from the whole milk cheese showed the presence of acetic, propionic and butyric acids. From the skim-milk cheese the same kinds of acid were obtained, after oxidation of the alcohols contained in its "flavor solution." The predominating acid formed in the oxidation of alcohols in the whole milk cheese, was acetic acid, with but small amounts of the two other acids present. These acids have originated from the oxidation of ethyl, propyl and butyl alcohols. Further the amount of acids obtained from the oxidation of the alcohols was much greater than that obtained by direct saponification. If saponification had been complete, and we believe it must have been, then a part of the alcohols must have existed in a free state in the cheese mass.

In the examination of the "flavor solution" obtained from both cheeses at the age of 5½ months, saponification and distillation gave acetic, butyric and caproic acids, but no propionic acid could be isolated from the whole milk cheese. From the skim-milk cheese the above four acids were isolated. In the case of the whole milk cheese, acetic acid again predominated, constituting 74 per cent of the acids isolated, while in the skim-milk cheese, which at that time was rather strong and pungent, butyric and caproic acids formed 85 per cent of the total acids isolated. Oxidation of the alcohols obtained from the saponified solutions yielded in both cases acetic, propionic and butyric acids. From the whole milk cheese 90 per cent of the acids obtained by this process were acetic, while but 4 and 6 per cent of propionic and butyric acids respectively were present. From the skim-milk cheese the same general proportions prevailed. Apparently ethyl alcohol was the chief alcohol occurring in the esters of the "flavor solution" obtained from either cheese.

Practically similar results were obtained from the analyses made at the age of 10½ months. These results, while by no means final and conclusive, strongly suggest the close relation of esters to the aroma of curing cheese, and which is detected by the sense of smell. The whole milk cheese, which was of good quality, had as a predominating component of its "flavor solution," ethyl alcohol and acetic acid, in all probability as esters; while in the skim-milk cheese of poorer quality, ethyl alcohol and caproic and butyric acids predominated as esters.

It is important for clearness that we distinguish between taste
and the aroma, the latter cognizant only through the sense of
smell, and by which the quality of cheese is much more largely
judged. In how far these esters characterize the taste and typify
it for different varieties of cheese is, of course, impossible at
present to state. In this connection, it is more than probable
that the nitrogenous end-products also play an important part.

It is entirely probable that the alcohols had their origin, in
part at least, in the primary lactose fermentation and further
decomposition of lactates.

SUMMARY.

I. Lactose disappears from Cheddar cheese in from three to
six days, dependent upon the condition of the milk and the tem-
perature of curing.

II. The absolute amount of lactic acid found does not decrease
during the ripening process, but may even increase. This increase
after lactose disappears, is probably of protein origin.

III. The usual form of lactic acid found in Cheddar cheese
is racemic. However, solutions of lactose, inoculated with B.
lactis acidi or a starter, produced active acid, but when inoculated
with a bit of cheese, a mixture of active and inactive acids was
produced. Further work is necessary to explain this phenome-
non.

IV. No enzyme capable of producing lactic acid or volatile
fatty acids from lactose could be isolated from cheese. There
is present in cheese a group of acid-forming organisms which
produced no lactic acid in pure culture on a lactose solution, but
did yield volatile fatty acids.

V. Volatile fatty acids were formed in increasing amounts
during the ripening process, and after the lactose had disappeared.
Acetic and propionic acids reached a maximum at three months
and then decreased, while butyric and caproic acids continually
increased during the experimental period covered. Formic acid
was only detected in the whole milk cheese at the 5½ months
stage. Valerianic acid was never obtained.

VI. The principal sources of acetic and propionic acids were
probably lactates. Traces may have had their origin in protein
decomposition, or further fermentation of glycerin.
VII. The principal sources of butyric and caproic acids were fats and proteins.

VIII. The distillate here designated “flavor solution” and characterized by the close resemblance of its odor to the cheese aroma, contained alcohols and esters.

IX. The “flavor solution” from the mild whole milk cheese contained esters made up largely of ethyl alcohol and acetic acid, while from the more pungent skim-milk cheese the esters were largely compounds of ethyl alcohol and caproic and butyric acids.

X. The alcohol probably had its origin in the lactose fermentation and appears to be an important factor in flavor production.

XI. The agencies operative in the production of volatile acids and synthesis of esters are as yet undefined.

XII. Succinic acid was isolated from curing Cheddar cheese and identified from its silver and barium salts.
S. K. Suzuki, E. G. Hastings and E. B. Hart


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