Studies on Acetylmethylcarbinol and Diacetyl in dairy products

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Studies on Acetylmethylcarbinol and Diacetyl in Dairy Products

By M. B. Michaelian and B. W. Hammer

AGRICULTURAL EXPERIMENT STATION
IOWA STATE COLLEGE OF AGRICULTURE
AND MECHANIC ARTS

R. E. Buchanan, Director

DAIRY INDUSTRY SECTION

AMES, IOWA
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Studie s on Acety lmethylcarbinol and Diacetyl in Dairy Products

By M. B. Michaelian and B. W. Hammer

The importance of diacetyl from the standpoint of the desirable aroma of butter has been shown by various investigators. van Niel, Kluyver and Derx (6) found from 0.0002 to 0.0004 percent diacetyl in fine butter, and when these concentrations of diacetyl were added to butter neutral in odor an unmistakable aroma appeared. The important conclusion of these investigators was that diacetyl is either responsible for the aroma of butter or is the principal component of the aroma material. Schmalfuss and Barthmeyer (4) found diacetyl to be an aroma constituent of various materials, including butter. The diacetyl was considered to come from acetylmethylcarbinol. Margarine to which diacetyl had been added took on the aroma of butter. Four samples of butter, representing different conditions of feeding the producing animals, yielded from 0.0001 to 0.0006 grams of nickel salt (equivalent to diacetyl) per kilogram, and the quantity of diacetyl in the butter appeared to be correlated with the intensity of the aroma.

The investigations showing the presence of diacetyl in fine butter suggested studies on the production of diacetyl and acetylmethylcarbinol in butter cultures. Michaelian, Farmer and Hammer (2) found that: (a) Butter cultures with a satisfactory flavor and aroma contained considerable quantities of acetylmethylcarbinol + diacetyl, while cultures lacking in flavor and aroma contained small amounts or none; from a quantitative standpoint the carbinol was much more important than the diacetyl. (b) During the early stages of the ripening of satisfactory butter cultures only small amounts of acetylmethylcarbinol + diacetyl were present, while late in the ripening conspicuous increases in these materials occurred. (c) Milk cultures of Streptococcus lactis or the citric acid-fermenting streptococci normally present in butter cultures contained little or no acetylmethylcarbinol + diacetyl, but the production of these compounds by the latter type was strikingly increased by the addition of the proper amount of any one of a number of acids; it, accordingly, appears that the citric acid-fermenting streptococci produce conspicuous amounts of acetylmethylcarbinol + diacetyl only in the presence of considerable acid. (d) The source of the acetylmethylcarbinol + diacetyl produced in a butter culture is evidently citric acid.

Project No. 127 of the Iowa Agricultural Experiment Station.
Additional studies on acetylmethylcarbinol and diacetyl in dairy products have been carried out at the Iowa Agricultural Experiment Station, and the results are reported herein. The data are presented in two parts, as follows:

Part I. Comparative Amounts of Acetylmethylcarbinol Plus Diacetyl in Cream and in Buttermilk or Butter Obtained from It

Part II. General Action of Citric Acid-Fermenting Streptococci from Butter Cultures in the Production of Acetylmethylcarbinol Plus Diacetyl and of Volatile Acid.

METHODS

**ACETYLMETHYLCARBINOL + DIACETYL**

The acetylmethylcarbinol and diacetyl were commonly determined together because diacetyl is ordinarily present in a dairy product in much smaller amounts than acetylmethylcarbinol. The reagents used were: Ferric chloride solution; 40 gm. to 100 ml. with distilled water. Hydroxylamine hydrochloride solution; 20 gm. to 100 ml. with distilled water. Sodium acetate solution; 20 gm. to 100 ml. with distilled water. Nickel chloride solution; 10 gm. to 100 ml. with distilled water.

Ordinarily a 200-gm. portion of the material to be examined (cream, buttermilk, milk culture of an organism, etc.) was distilled with steam, after adding 40 ml. ferric chloride solution to oxidize the acetylmethylcarbinol to diacetyl. The distillate was collected in a mixture of hydroxylamine hydrochloride and sodium acetate solutions by means of an adapter on the end of the condenser and the nickel chloride solution added later. The quantities of reagents used depended on the amount of diacetyl expected. van Niel (5) has shown that the concentration of the reagents can fluctuate between wide limits; for about 100 mg. diacetyl he suggests 2 ml. hydroxylamine hydrochloride (20 percent solution), 3 to 5 ml. sodium acetate (20 percent solution) and 1 to 2 ml. nickel chloride (10 percent solution). The distillate with the reagents added was allowed to stand at least 2 days, and preferably longer, in order to permit complete crystallization, and the nickel salt then filtered into a weighed crucible; occasional tests showed that crystallization was complete with the periods used. The salt was washed with distilled water, dried to constant weight at 105° to 110°C. and the results recorded as the milligrams of nickel salt equivalent to acetylmethylcarbinol + diacetyl per 200 gm. of material.

In the case of butter a 400-gm. portion, rather than a 200-gm. portion, was steam distilled with 40 ml. ferric chloride solution because of the relatively small amount of acetylmethylcarbinol + diacetyl present in butter. The results obtained were calculated to a 200-gm. basis.
VOLATILE ACID

The volatile acid in a culture was determined by steam distilling a mixture of 250 gm. of the culture with 250 ml. of distilled water, after the addition of 15 ml. n/1 sulfuric acid, and titrating the first 1,000 ml. of distillate, using n/10 sodium hydroxide and phenolphthalein; the distillation was at such a rate that approximately 2 hours were required to obtain the 1,000 ml. The results were expressed as the milliliters of n/10 sodium hydroxide required. Only a portion of the volatile acid is obtained with this procedure so that the method is primarily of value for comparative purposes.

ACIDITY

The pH determinations were made electrometrically, using quinhydrone, and recorded to the nearest 0.1.

PART I. COMPARATIVE AMOUNTS OF ACETYL METHYL CARBINOL PLUS DIACETYL IN CREAM AND IN BUTTERMILK OR BUTTER OBTAINED FROM IT

The comparative amounts of acetylmethylcarbinol + diacetyl in cream at the time of churning and in the fresh buttermilk or butter obtained from it were studied in a number of trials. No attempt was made to balance the amounts of acetylmethylcarbinol + diacetyl found in the cream with the amounts found in the buttermilk and butter because of the possibility of both the production and destruction of these materials during the churning process.

COMPARATIVE AMOUNTS OF ACETYL METHYL CARBINOL + DIACETYL IN CREAM AND BUTTERMILK

The quantities of acetylmethylcarbinol + diacetyl in cream and in the fresh buttermilk obtained from it were compared on seven commercial churnings in which about 8 percent butter culture was added to pasteurized sweet cream. The mixtures of cream and butter culture were cooled to from 0° to 1° C. as soon as they were prepared and then held overnight before churning; during the holding they warmed up to from 8° to 9° C. The fat content of the cream was usually about 30 percent. The data obtained are presented in table 1.

The results show that the fresh buttermilk regularly contained more acetylmethylcarbinol + diacetyl than the cream from which it was obtained. The ratios of the acetylmethylcarbinol + diacetyl in the cream to the amounts in the butterm-
TABLE 1. COMPARATIVE AMOUNTS OF ACETYL METHYL CARBINOL PLUS DIACETYL IN CREAM AT THE TIME OF CHURNING AND IN THE FRESH BUTTERMILK OBTAINED FROM IT.

Commercial churnings of pasteurized sweet cream to which about 8 percent butter culture had been added and the mixture then held overnight at a low temperature before churning.

<table>
<thead>
<tr>
<th>Trial number</th>
<th>Mg. Ni salt equiv. to amc + aa* per 200 gm.</th>
<th>Ratio of amc + aa in cream to amount in buttermilk</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cream</td>
<td>Buttermilk</td>
</tr>
<tr>
<td>1</td>
<td>4.5</td>
<td>10.2</td>
</tr>
<tr>
<td>2</td>
<td>0.9</td>
<td>2.8</td>
</tr>
<tr>
<td>3</td>
<td>1.3</td>
<td>3.7</td>
</tr>
<tr>
<td>4</td>
<td>1.1</td>
<td>2.8</td>
</tr>
<tr>
<td>5</td>
<td>1.0</td>
<td>2.0</td>
</tr>
<tr>
<td>6</td>
<td>3.9</td>
<td>8.5</td>
</tr>
<tr>
<td>7</td>
<td>2.1</td>
<td>5.6</td>
</tr>
</tbody>
</table>

*amc + aa = acetylmethylcarbinol + diacetyl.

milk ranged from 1:2.0 to 1:3.1. The quantities of acetylmethylcarbinol + diacetyl in the cream were always relatively small, because of the method of buttermaking followed. These materials came largely from the butter culture used, although sweet cream in which there has been considerable bacterial growth may contain small quantities of them, and there may have been some increase during the holding of the mixtures of cream and butter culture.

The quantities of acetylmethylcarbinol + diacetyl in cream and in the fresh buttermilk obtained when the cream was churned, were also studied in several series of churnings, involving various fat percentages in the cream, that were made primarily for the purpose of investigating the influence of the acidity of the cream on the fat losses in the buttermilk. In each series of churnings a vat of pasteurized sweet cream was inoculated with about 8 percent butter culture and held at a temperature suitable for rapid growth of the added organisms (usually 21° C.). As various acidities were reached 10 gallons of the cream were removed, cooled down to 4.5° C. or lower and then churned after holding overnight at 4.5° C. or lower. Table 2 gives the results obtained in six of the series studied, together with data on the aciditie s of the cream and buttermilk.

From the data presented it is evident that the fresh buttermilk always contained more acetylmethylcarbinol + diacetyl than the cream from which it came. The ratios of the acetylmethylcarbinol + diacetyl in the cream to the amount in the buttermilk ranged from 1:1.1 to 1:2.1. In general, they were somewhat lower than for the results given in table 1, but the differences were usually rather small. In most of the series the ratios were fairly uniform with the various churnings in

---

*The studies on fat losses were carried out by Dr. E. W. Bird and Mr. D. F. Breazeale, who kindly supplied the samples of cream and buttermilk for the acetylmethylcarbinol + diacetyl determinations and also the values for the amounts of acid present.
TABLE 2. RATIOS OF TITRABLE ACID AND ACETYLMETHYLCARBINOL PLUS DIACETYL IN CREAM AT THE TIME OF CHURNING TO AMOUNTS IN FRESH BUTTERMILK, WHEN VARIOUS ACIDITIES WERE DEVELOPED IN THE CREAM BY THE USE OF BUTTER CULTURES.

Experimental churnings made primarily to study fat losses in the buttermilk.

<table>
<thead>
<tr>
<th>Series 1. Cream 21 percent fat</th>
<th>Series 2. Cream 30 percent fat</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cream at churning</strong></td>
<td><strong>Ratio of amount in cream to amount in buttermilk</strong></td>
</tr>
<tr>
<td><strong>Percent acid</strong></td>
<td><strong>Mg. Ni salt equiv. to amc +aa</strong> per 200 gm.</td>
</tr>
<tr>
<td>0.145</td>
<td>6.1</td>
</tr>
<tr>
<td>0.205</td>
<td>6.3</td>
</tr>
<tr>
<td>0.295</td>
<td>17.2</td>
</tr>
<tr>
<td>0.505</td>
<td>20.3</td>
</tr>
<tr>
<td>0.53</td>
<td>36.9</td>
</tr>
<tr>
<td>0.615</td>
<td>53.9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Series 3. Cream 30.5 percent fat</th>
<th>Series 4. Cream 38 percent fat</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cream at churning</strong></td>
<td><strong>Ratio of amount in cream to amount in buttermilk</strong></td>
</tr>
<tr>
<td><strong>Percent acid</strong></td>
<td><strong>Mg. Ni salt equiv. to amc +aa</strong> per 200 gm.</td>
</tr>
<tr>
<td>0.125</td>
<td>5.9</td>
</tr>
<tr>
<td>0.155</td>
<td>6.1</td>
</tr>
<tr>
<td>0.21</td>
<td>8.9</td>
</tr>
<tr>
<td>0.25</td>
<td>11.4</td>
</tr>
<tr>
<td>0.295</td>
<td>13.8</td>
</tr>
<tr>
<td>0.40</td>
<td>17.0</td>
</tr>
<tr>
<td>0.45</td>
<td>14.0</td>
</tr>
<tr>
<td>0.49</td>
<td>13.6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Series 5. Cream 37 percent fat</th>
<th>Series 6. Cream 38 percent fat</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cream at churning</strong></td>
<td><strong>Ratio of amount in cream to amount in buttermilk</strong></td>
</tr>
<tr>
<td><strong>Percent acid</strong></td>
<td><strong>Mg. Ni salt equiv. to amc +aa</strong> per 200 gm.</td>
</tr>
<tr>
<td>0.14</td>
<td>8.1</td>
</tr>
<tr>
<td>0.20</td>
<td>7.3</td>
</tr>
<tr>
<td>0.22</td>
<td>12.3</td>
</tr>
<tr>
<td>0.245</td>
<td>16.5</td>
</tr>
<tr>
<td>0.30</td>
<td>19.5</td>
</tr>
<tr>
<td>0.345</td>
<td>21.1</td>
</tr>
</tbody>
</table>

*amc +aa = acetylmethylcarbinol + diacetyl.

the series, but with series 3 there was considerable fluctuation. The ratios of the acid in the cream to the amount in the buttermilk ranged from 1:1.0 (with one churning of low fat cream) to 1:1.5; they were very uniform throughout the churnings in a series. In general, the ratios for the acid were lower than the ratios for the acetylmethylcarbinol + diacetyl. With both the
acid and the acetylmethylcarbinol + diacetyl the ratios were higher with a comparatively large amount of fat in the cream than with a comparatively small amount.

**COMPARATIVE AMOUNTS OF ACETYLMETHYL CARBINOL + DIACETYL IN CREAM AND IN BUTTER**

The amounts of acetylmethylcarbinol + diacetyl in cream and in the fresh butter obtained from it were studied with 56 small churnings (from 30 to 40 pounds of butter in each), involving various methods of handling sweet cream to be made into salted butter with the use of butter culture. In some cases the cream was definitely ripened while in other cases a mixture of cream and butter culture was held overnight at a relatively low temperature. The acetylmethylcarbinol + diacetyl in the butter was determined on a 400-gm. sample and the results calculated to a 200-gm. basis for comparison with the acetylmethylcarbinol + diacetyl content of the cream. The data obtained are presented in table 3.

**TABLE 3. RATIO OF ACETYLMETHYL CARBINOL PLUS DIACETYL IN CREAM AT THE TIME OF CHURNING TO THE AMOUNT IN FRESHLY CHURNED, SALTED BUTTER.**

<table>
<thead>
<tr>
<th>Trial number</th>
<th>Cream at churning</th>
<th>Ratio of amc+aa in cream to amount in butter</th>
<th>Trial number</th>
<th>Cream at churning</th>
<th>Ratio of amc+aa in cream to amount in butter</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Percent acid</td>
<td>Mg. Ni salt equiv. to amc+aa per 200 gm.</td>
<td></td>
<td>Percent acid</td>
<td>Mg. Ni salt equiv. to amc+aa per 200 gm.</td>
</tr>
<tr>
<td>1</td>
<td>0.45</td>
<td>14.1</td>
<td>29</td>
<td>0.24</td>
<td>16.8</td>
</tr>
<tr>
<td>2</td>
<td>0.49</td>
<td>13.6</td>
<td>30</td>
<td>0.21</td>
<td>6.6</td>
</tr>
<tr>
<td>3</td>
<td>0.22</td>
<td>6.8</td>
<td>31</td>
<td>0.225</td>
<td>6.7</td>
</tr>
<tr>
<td>4</td>
<td>0.25</td>
<td>9.4</td>
<td>32</td>
<td>0.225</td>
<td>8.7</td>
</tr>
<tr>
<td>5</td>
<td>0.24</td>
<td>14.6</td>
<td>33</td>
<td>0.20</td>
<td>7.7</td>
</tr>
<tr>
<td>6</td>
<td>0.22</td>
<td>11.1</td>
<td>34</td>
<td>0.225</td>
<td>10.9</td>
</tr>
<tr>
<td>7</td>
<td>0.23</td>
<td>13.3</td>
<td>35</td>
<td>0.215</td>
<td>12.0</td>
</tr>
<tr>
<td>8</td>
<td>0.24</td>
<td>20.9</td>
<td>36</td>
<td>0.20</td>
<td>3.5</td>
</tr>
<tr>
<td>9</td>
<td>0.32</td>
<td>15.9</td>
<td>37</td>
<td>0.22</td>
<td>5.0</td>
</tr>
<tr>
<td>10</td>
<td>0.325</td>
<td>17.5</td>
<td>38</td>
<td>0.205</td>
<td>4.2</td>
</tr>
<tr>
<td>11</td>
<td>0.235</td>
<td>16.1</td>
<td>39</td>
<td>0.19</td>
<td>4.4</td>
</tr>
<tr>
<td>12</td>
<td>0.195</td>
<td>6.0</td>
<td>40</td>
<td>0.195</td>
<td>6.0</td>
</tr>
<tr>
<td>13</td>
<td>0.21</td>
<td>5.9</td>
<td>41</td>
<td>0.20</td>
<td>6.7</td>
</tr>
<tr>
<td>14</td>
<td>0.20</td>
<td>7.5</td>
<td>42</td>
<td>0.19</td>
<td>1.9</td>
</tr>
<tr>
<td>15</td>
<td>0.20</td>
<td>7.6</td>
<td>43</td>
<td>0.20</td>
<td>3.3</td>
</tr>
<tr>
<td>16</td>
<td>0.225</td>
<td>9.3</td>
<td>44</td>
<td>0.19</td>
<td>2.1</td>
</tr>
<tr>
<td>17</td>
<td>0.23</td>
<td>16.2</td>
<td>45</td>
<td>0.20</td>
<td>3.4</td>
</tr>
<tr>
<td>18</td>
<td>0.22</td>
<td>10.3</td>
<td>46</td>
<td>0.205</td>
<td>3.2</td>
</tr>
<tr>
<td>19</td>
<td>0.225</td>
<td>12.4</td>
<td>47</td>
<td>0.21</td>
<td>5.1</td>
</tr>
<tr>
<td>20</td>
<td>0.21</td>
<td>12.5</td>
<td>48</td>
<td>0.21</td>
<td>0.9</td>
</tr>
<tr>
<td>21</td>
<td>0.235</td>
<td>5.8</td>
<td>49</td>
<td>0.23</td>
<td>3.5</td>
</tr>
<tr>
<td>22</td>
<td>0.25</td>
<td>7.8</td>
<td>50</td>
<td>0.21</td>
<td>2.6</td>
</tr>
<tr>
<td>23</td>
<td>0.235</td>
<td>10.2</td>
<td>51</td>
<td>0.22</td>
<td>5.8</td>
</tr>
<tr>
<td>24</td>
<td>0.20</td>
<td>3.5</td>
<td>52</td>
<td>0.29</td>
<td>12.6</td>
</tr>
<tr>
<td>25</td>
<td>0.205</td>
<td>3.1</td>
<td>53</td>
<td>0.24</td>
<td>15.3</td>
</tr>
<tr>
<td>26</td>
<td>0.21</td>
<td>7.4</td>
<td>54</td>
<td>0.19</td>
<td>3.7</td>
</tr>
<tr>
<td>27</td>
<td>0.22</td>
<td>9.7</td>
<td>55</td>
<td>0.215</td>
<td>8.7</td>
</tr>
<tr>
<td>28</td>
<td>0.23</td>
<td>12.9</td>
<td>56</td>
<td>0.22</td>
<td>10.8</td>
</tr>
</tbody>
</table>

*amc+aa = acetylmethylcarbinol + diacetyl.

**trace found in 400 gm. butter.

***not measurable in 400 gm. butter.
The results show that the butter regularly contained much less acetylmethylcarbinol + diacetyl than the cream from which it was churned. With four of the lots of butter, the acetylmethylcarbinol + diacetyl was not measurable, with the procedure used, or it was present as a trace in 400 gm. of butter. The two lots showing unmeasurable amounts of acetylmethylcarbinol + diacetyl in 400 gm. came from cream containing very little of these materials, but the two lots showing a trace came from cream that was not unusually low in acetylmethylcarbinol + diacetyl, so there may have been a destruction during the churning. For the remaining 52 lots of butter, the ratios of the acetylmethylcarbinol + diacetyl in the cream to the amounts in the butter ranged from 1:0.032 to 1:0.218. The following summary shows the general distribution of the ratios:

<table>
<thead>
<tr>
<th>Ratio of acetylmethylcarbinol + diacetyl in cream to amount in butter</th>
<th>Number of churnings</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:0.049 or less</td>
<td>3</td>
</tr>
<tr>
<td>1:0.050 to 1:0.099</td>
<td>11</td>
</tr>
<tr>
<td>1:0.100 to 1:0.149</td>
<td>21</td>
</tr>
<tr>
<td>1:0.150 to 1:0.199</td>
<td>13</td>
</tr>
<tr>
<td>1:0.200 and over</td>
<td>4</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>52</strong></td>
</tr>
</tbody>
</table>

The ratio of the acetylmethylcarbinol + diacetyl in the cream to the amount in the butter did not appear to be correlated with the quantity of acetylmethylcarbinol + diacetyl in the cream at the time of churning nor with the acidity of the cream.

**AMOUNTS OF ACETYL METHYL CARBINOL + DIACETYL IN BUTTER**

The data used in calculating the ratios (acetylmethylcarbinol + diacetyl in cream at the time of churning to the amounts in the fresh butter) presented in table 3 show the general amounts of acetylmethylcarbinol + diacetyl in fresh salted butter made with butter culture. As already pointed out, four lots of such butter showed unmeasurable amounts of acetylmethylcarbinol + diacetyl or only a trace when 400 gm. were studied with the usual procedure; this may have been due to the quality of the butter culture employed or to a destruction of the materials during the churning process. The remaining 52 samples of butter yielded from 0.1 to 3.45 mg. of nickel salt (equivalent to acetylmethylcarbinol + diacetyl) per 200 gm. of butter. The following summary shows the general distribution
of the samples on the basis of the acetylmethylcarbinol + diacetyl contained:

<table>
<thead>
<tr>
<th>Mg. Ni salt equivalent to acetylmethylcarbinol + diacetyl per 200 gm. of butter</th>
<th>Number of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.49 or less</td>
<td>11</td>
</tr>
<tr>
<td>0.50 to 0.99</td>
<td>13</td>
</tr>
<tr>
<td>1.00 to 1.49</td>
<td>10</td>
</tr>
<tr>
<td>1.50 to 1.99</td>
<td>10</td>
</tr>
<tr>
<td>2.00 to 2.99</td>
<td>7</td>
</tr>
<tr>
<td>3.00 and over</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>52</strong></td>
</tr>
</tbody>
</table>

Various other examinations of salted butter made with butter culture yielded values essentially like those given in the summary. It should be noted, however, that when relatively high acidities are developed in the cream, as in the manufacture of a great deal of the unsalted butter intended for table use, conditions are much more favorable for the production of acetylmethylcarbinol + diacetyl than when salted butter is made and comparatively large amounts are retained in the butter.

**DISTRIBUTION OF ACETYLMETHYL CARBINOL + DIACETYL IN BUTTER**

The distribution of the acetylmethylcarbinol + diacetyl in butter between the serum and the fat was studied with a series of samples of salted butter made with butter culture. The serum and fat were obtained by melting the butter, transferring it to a separatory funnel, holding it at 37° C. for a short time and then drawing off the serum. In order to reduce changes in the amounts of acetylmethylcarbinol + diacetyl to a minimum, the separation process was carried out as rapidly as possible and was usually completed within 2 hours. The fat was not filtered because of the time that would have been required. The amounts of butter used always yielded sufficient fat so that a 200-gm. portion could be employed for the determination, but frequently the amount of serum recovered was less than 200 gm., and a calculation to the usual basis was necessary.

The amount of acetylmethylcarbinol + diacetyl in the serum was always definitely larger than the amount in the fat. A 200-gm. portion of fat often showed no acetylmethylcarbinol + diacetyl, with the procedure used, while in some cases small

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*Determinations made on 400 gm. of butter and calculated to a 200-gm. basis.*
amounts were recovered. The serum regularly contained acetyl-
methylcarbinol + diacetyl, the amount per 200 gm. being de-
pendent on the character of the original cream and the method
of butter manufacture followed.
Butter made without butter culture from cream in which
there had been no bacterial growth was not studied; presum-
ably, serum from such butter would be free from acetyl-
methylcarbinol + diacetyl, because cream freshly skimmed from milk
of a low bacterial content has been found to contain no acetyl-
methylcarbinol + diacetyl.
Five lots of butter, made by working diacetyl into the product
just before it was taken from the churn, showed the usual
distribution of acetyl methylcarbinol + diacetyl between the
serum and fat.

ACETYLMETHYL CARBINOL + DIACETYL IN CREAM DELIVERED
TO BUTTER PLANTS

The cream delivered to butter plants often contains amounts
of acetyl methylcarbinol + diacetyl that are readily detectable
when 200-gm. portions of the cream are distilled with ferric
chloride and the distillate treated with the usual reagents. In
general, the amounts in sour cream coming to various types
of butter plants are relatively high. Acetyl methylcarbinol +
diacetyl would be expected in sour cream because of the wide
distribution of the citric acid-fermenting streptococci in dairy
products and the favorable conditions provided by the acid in
the cream for the production of these materials by such organ-
isms. It seems probable also that various other bacteria may
be involved in the production of acetyl methylcarbinol + dia-
cetyl in cream with considerable acid. Cream that would be
classed as sweet, on the basis of 0.2 percent acid calculated as
lactic, frequently contains small amounts of acetyl methyl-
carbinol + diacetyl. Such cream ordinarily has undergone
considerable bacterial action and, although the conditions are
not satisfactory for the production of acetyl methylcarbinol +
diacetyl by the citric acid-fermenting streptococci, various
organisms capable of producing these materials may have
grown in it. Streptococci that produce small amounts of
acetyl methylcarbinol + diacetyl in pure culture in milk have
repeatedly been isolated from cream. These organisms pro-
duce little or no acid in milk, and the acetyl methylcarbinol +
diacetyl production is not increased by lowering the pH with
citric or sulfuric acid. Some of the organisms appear to be
rather sensitive to acid.
In connection with the production of acetyl methylcarbinol +
diacetyl in either sour or sweet cream it should be recognized
that citric acid is only one of the sources of these materials.
Various organisms apparently break down sugar with the formation of acetylmethylcarbinol along with other products.

Several samples of cream freshly skimmed from rather low count milk showed no acetylmethylcarbinol + diacetyl when 200-gm. portions were distilled. These results, together with the larger amounts of acetylmethylcarbinol + diacetyl in sour cream than in sweet cream which has undergone considerable bacterial action, indicate that when the carbinol and its oxidation product are present in cream they are the result of the action of microorganisms.

PART II. GENERAL ACTION OF CITRIC ACID-FERMENTING STREPTOCOCCI FROM BUTTER CULTURES IN THE PRODUCTION OF ACETYL-METHYLCARBINOL PLUS DIACETYL AND OF VOLATILE ACID

In the preparation of butter cultures the most common defect is a lack of flavor and aroma. Cultures rather frequently show a good total acid development but fail to show the desirable flavor and aroma characteristic of a butter culture of satisfactory quality. Presumably, this defect is due to the failure of the citric acid-fermenting streptococci to develop in a normal manner, since in cultures showing the condition the numbers of the organisms are commonly small.

Studies were carried out on the citric acid-fermenting streptococci in an attempt to understand more fully their general action in a butter culture. The studies involved primarily cultures in milk to which considerable acid, either citric or citric and sulfuric, had been added, because in a butter culture the action of the organisms is greatly influenced by the acid formed through the activity of S. lactis. The citric acid provided additional material from which various fermentation products could be formed, while the sulfuric acid changed the pH without providing any such material.

ACTION OF KILLED ORGANISMS UNDER CONDITIONS FAVORABLE FOR THE PRODUCTION OF ACETYL-METHYL-CARBINOL + DIACETYL BY LIVING CELLS

When a rather young milk culture of a citric acid-fermenting Streptococcus is acidulated to the proper pH, the production of acetylmethylcarbinol + diacetyl is very rapid. Michaelian, Farmer and Hammer (2) found that at 21° C. considerable amounts of these materials were produced in as short a period as 15 minutes after the addition of the acid and that the production continued at a relatively rapid rate. This very active
formation suggested the possibility of the acetylmethylcarbinol + diacetyl resulting from a reaction between citric acid and some product formed by the organisms, the pH being the determining factor.

**ACTION OF ACID ON KILLED MILK CULTURES OF THE ORGANISMS**

The action of acid on killed milk cultures of the citric acid-fermenting streptococci was studied. Four flasks of sterile skim milk (1000 ml. of milk in each) were inoculated with organism 29 and incubated for 48 hours at 21° C. One flask was then heated to about 90° C. for 35 minutes, the second was thoroughly shaken with 0.5 percent formalin, the third was thoroughly shaken with 2.0 percent chloroform and the fourth was used as the control; all of the flasks were closed with rubber stoppers. After holding for 48 hours at 21° C. the milk in each flask was treated with 0.15 percent citric acid and 0.30 percent sulfuric acid. The control flask showed considerable acetylmethylcarbinol + diacetyl after 24 hours (equivalent to 51.3 mg. Ni salt) and also after 72 hours (equivalent to 63.6 mg. Ni salt), while the volatile acid after 24 hours was 32.5. The other flasks showed no acetylmethylcarbinol + diacetyl after 24 or 72 hours; each showed considerable volatile acid (from 14.2 to 15.8) after 24 hours, which was presumably produced before the various treatments for the destruction of the organisms were applied. With all the flasks the pH values established by the added acids were determined and found to be satisfactory for the production of acetylmethylcarbinol + diacetyl by living organisms.

**ACTION OF SUSPENSIONS OF THE ORGANISMS KILLED WITH CHLOROFORM**

The action of suspensions of the organisms killed with chloroform was studied in milk to which 0.50 percent citric acid had been added. In each of two trials a heavy suspension of organism 29 was secured by washing cells from growth on large whey agar plates with sterile water. The suspensions were then treated with chloroform (5 ml. to 80 ml. of suspension) and held for 7 days at 21° C. Cultures on the fifth and sixth days showed the suspensions were sterile. The suspensions were added to sterile milk containing 0.50 percent citric acid, an amount which results in a large production of acetylmethylcarbinol + diacetyl when added to an active milk culture of a citric acid-fermenting Streptococcus. Determinations made after holding 24 and 48 hours at 21° C. showed that no acetylmethylcarbinol + diacetyl had been formed in either trial.
RELATIONSHIP BETWEEN THE NUMBERS OF ORGANISMS AND THE PRODUCTION OF ACETYL METHYL CARBINOL + DIACETYL AND OF VOLATILE ACID

The relationship between the numbers of citric acid-fermenting streptococci in milk and the production of acetyl methylcarbinol + diacetyl and of volatile acid when considerable acid is present was investigated by means of suspensions of organisms. Each suspension was prepared by washing the organisms from growth on large whey agar plates with sterile water. After shaking the suspension thoroughly, various amounts were added to sterile milk and the pH adjusted at once with citric and sulfuric acids or citric acid alone. Determinations of acetyl methylcarbinol + diacetyl and of volatile acid or of acetyl methylcarbinol + diacetyl alone were made after various periods. The results obtained are presented in tables 4 and 5.

The data show a rather close relationship between the numbers of organisms added and the amounts of the usual fermentation products formed. In the results given in table 4, 1 ml. of suspension produced no acetyl methylcarbinol + diacetyl in 24 or 48 hours, and the amounts of volatile acid were negligible. With larger quantities of suspension there was a production of acetyl methylcarbinol + diacetyl, and in some cases the amounts were large; as the production of these materials in-

### Table 4. The Production of Acetyl Methylcarbinol Plus Diacetyl and of Volatile Acid in Milk Containing Considerable Acid When Inoculated with Various Numbers of Citric Acid-Fermenting Streptococci.

<table>
<thead>
<tr>
<th>Trial number</th>
<th>Ml. of susp. of organisms used per 1100 ml. milk</th>
<th>Number of organisms added per ml. of milk</th>
<th>Acid added to reduce pH</th>
<th>Period of holding after adding acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>24 hours</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>pH Mg. Ni salt equiv. to amc+aa* per 200 gm.</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>none</td>
<td></td>
<td>0.15 percent citric acid and 0.30 percent sulfuric acid to each</td>
<td>3.8</td>
</tr>
<tr>
<td>1</td>
<td>7,000,000</td>
<td></td>
<td></td>
<td>3.8</td>
</tr>
<tr>
<td>5</td>
<td>322,000,000</td>
<td></td>
<td></td>
<td>4.0</td>
</tr>
<tr>
<td>50</td>
<td></td>
<td>72.3</td>
<td></td>
<td>4.3</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td>0.65 percent citric acid to each</td>
<td>4.2</td>
</tr>
<tr>
<td>0</td>
<td>none</td>
<td></td>
<td></td>
<td>4.2</td>
</tr>
<tr>
<td>1</td>
<td>570,000</td>
<td></td>
<td></td>
<td>4.2</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td>4.3</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td>4.3</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td>4.5</td>
</tr>
<tr>
<td>50</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The numbers of organisms per ml. of milk were determined by the plate method using tomato juice agar and incubating 4 days at 21°C.

*amc+aa = acetyl methylcarbinol + diacetyl.
TABLE 5. THE PRODUCTION OF ACETYLMETHYL CARBINOL PLUS DIACETYL IN MILK CONTAINING CONSIDERABLE ACID WHEN INOCULATED WITH VARIOUS NUMBERS OF CITRIC ACID-FERMENTING STREPTOCOCCI.

Organism 29. Temperature 21°C.

<table>
<thead>
<tr>
<th>Ml. of susp. of organisms used per 1100 ml. milk</th>
<th>Acid added to reduce pH</th>
<th>Period of holding after adding acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 hours</td>
<td>48 hours</td>
</tr>
<tr>
<td></td>
<td>Mg. Ni salt equiv. to ame+aa* per 200 gm.</td>
<td>pH</td>
</tr>
<tr>
<td>1</td>
<td>0.15 percent citric acid and 0.30 percent sulfuric acid to each</td>
<td>3.9</td>
</tr>
<tr>
<td>5</td>
<td>3.9</td>
<td>1.7</td>
</tr>
<tr>
<td>30</td>
<td>4.3</td>
<td>39.5</td>
</tr>
</tbody>
</table>

*ame + aa = acetylmethylcarbinol + diacetyl.

increased there was also an increase in the amount of volatile acid formed, the increases in the production of the two types of products being correlated in a general way. With the largest quantity of suspension used in trial 1 there was a conspicuous decrease from 24 to 48 hours in the amount of acetylmethylcarbinol + diacetyl present but no significant change in the amount of volatile acid. The results given in table 5 show that a small amount of acetylmethylcarbinol + diacetyl was produced with 1 ml. of suspension and that with 5 and 30 ml. quantities, large productions were obtained. With both 1 ml. and 30 ml. of suspension there was a decrease in the amount of acetylmethylcarbinol + diacetyl as the holding period was continued, but a decrease failed to occur with 5 ml. of suspension.

PRODUCTION OF RELATIVELY LARGE AMOUNTS OF ACETYLMETHYL CARBINOL + DIACETYL IN MILK

From the work of Michaelian, Farmer and Hammer (2), it appears that while the amount of acetylmethylcarbinol + diacetyl that can be produced in a butter culture varies with the milk used and certain other factors, it nevertheless is rather definitely limited. The work reported by these investigators shows that the amount of acetylmethylcarbinol + diacetyl produced in a butter culture can be materially increased by adding citric acid to the milk. It seems probable then, that the citric acid present in unmodified milk is the important limiting factor in the production of acetylmethylcarbinol + diacetyl in a butter culture.
An attempt was made to produce a large amount of acetyl-
methylcarbinol + diacetyl in milk by inoculating one of the
citric acid-fermenting streptococci, allowing it to increase in
numbers, and then adding citric acid. The data obtained are
presented in tables 6, 7 and 8.

The results given in table 6 show that 96 hours after adding
0.60 percent citric acid to a 48-hour milk culture of organism
29, the Ni salt (equivalent to the acetylcarbinol + dia-
cetyl) present in 200 gm. weighed 190.0 mg. This amount was
larger than the amounts found after 24 or 48 hours, but the in-
crease from 48 to 96 hours was considerably less than the in-
crease from 24 to 48 hours. When 0.15 percent more citric acid
was added 24 hours after the original addition, the Ni salt
equivalent to the acetylcarbinol + diacetyl present at
the end of the holding period weighed 199.9 mg.; this amount is
not significantly greater than the amount obtained without the
extra addition of citric acid. The data show the usual increases
in the pH as acetylcarbinol + diacetyl was produced.

The data presented in table 7 show that after adding 0.65
percent citric acid to a 48-hour milk culture of organism 29

**TABLE 6. ACETYL CARBINOL PLUS DIACETYL PRODUCED BY CITRIC
ACID-FERMENTING STREPTOCOCCI IN MILK WITH CONSIDERABLE
CITRIC ACID ADDED.**

Milk was inoculated with organism 29, incubated 48 hours and 0.60 percent citric acid then
added. After 24 hours the milk was divided and additional citric acid added to one-half.

<table>
<thead>
<tr>
<th></th>
<th>pH</th>
<th>Mg. Ni salt equiv. to acme + aa* per 200 gm.</th>
<th>Volatile acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk immediately after adding 0.60 percent citric acid to 48 hr. culture of org. 29</td>
<td>4.3</td>
<td>110.9</td>
<td>34.8</td>
</tr>
<tr>
<td>Milk (1) after 24 hrs. holding</td>
<td>4.7</td>
<td>172.2</td>
<td>46.5</td>
</tr>
<tr>
<td>Milk (1) after 48 hrs. holding</td>
<td>4.9</td>
<td>190.0</td>
<td>46.5</td>
</tr>
<tr>
<td>Milk (1) after 96 hrs. holding</td>
<td>5.1</td>
<td>226.0</td>
<td>50.7</td>
</tr>
<tr>
<td>Milk (2) immediately after adding 0.15 percent citric acid</td>
<td>4.3</td>
<td>176.9</td>
<td>51.7</td>
</tr>
<tr>
<td>Milk (3) after 20 hrs. holding</td>
<td>4.5</td>
<td>199.9</td>
<td>51.7</td>
</tr>
<tr>
<td>Milk (3) after 72 hrs. holding</td>
<td>4.6</td>
<td>226.0</td>
<td>50.7</td>
</tr>
</tbody>
</table>

*acme + aa = acetylcarbinol + diacetyl.

there was a production of acetylcarbinol + diacetyl for
a considerable period, although it was at a decreasing rate;
the weight of Ni salt found after 144 hours was 246.7 mg.
With the addition of small quantities of citric acid from time
to time, the amounts of acetylcarbinol + diacetyl pro-
duced after the various holding periods were slightly but not
significantly less than without the additions. During the
holding, the increases in the acetylcarbinol + diacetyl
were regularly accompanied by increases in the volatile acid.
The usual relationship between the production of acetylcarbinol-
TABLE 7. ACETYL METHYL CARBINOL PLUS DIACETYL PRODUCED BY CITRIC ACID-FERMENTING STREPTOCOCCI IN MILK WITH CONSIDERABLE CITRIC ACID ADDED.

Two lots of milk were inoculated with organism 29, incubated 48 hours and 0.65 percent citric acid added to each lot. One lot (lot A) was left untreated while additional citric acid was added to the other lot (lot B) from time to time to reduce the pH.

Temperature 21°C.

<table>
<thead>
<tr>
<th>Lot A immediately after adding 0.65 percent citric acid</th>
<th>pH</th>
<th>Mg. Ni salt equiv. to amc + aa* per 200 gm.</th>
<th>Volatile acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>A after 24 hrs. holding</td>
<td>4.1</td>
<td>113.2</td>
<td>35.9</td>
</tr>
<tr>
<td>A after 48 hrs. holding</td>
<td>4.5</td>
<td>188.0</td>
<td>45.2</td>
</tr>
<tr>
<td>A after 72 hrs. holding</td>
<td>5.0</td>
<td>218.9</td>
<td>56.9</td>
</tr>
<tr>
<td>A after 96 hrs. holding</td>
<td>5.0</td>
<td>233.1</td>
<td></td>
</tr>
<tr>
<td>A after 144 hrs. holding</td>
<td>5.2</td>
<td>246.7</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Lot B immediately after adding 0.65 percent citric acid</th>
<th>pH</th>
<th>Mg. Ni salt equiv. to amc + aa* per 200 gm.</th>
<th>Volatile acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>B immediately after adding 0.65 percent citric acid</td>
<td>4.1</td>
<td>106.9</td>
<td>35.8</td>
</tr>
<tr>
<td>B1 immediately after adding 0.15 percent citric acid</td>
<td>4.2</td>
<td>186.7</td>
<td>53.4</td>
</tr>
<tr>
<td>B2 immediately after adding 0.17 percent citric acid</td>
<td>4.3</td>
<td>216.7</td>
<td>62.0</td>
</tr>
<tr>
<td>B3 immediately after adding 0.15 percent citric acid</td>
<td>4.1</td>
<td>228.2</td>
<td>66.9</td>
</tr>
</tbody>
</table>

*amc + aa = acetylmethylcarbinol + diacetyl.

carbinol + diacetyl and an increase in the pH is also shown by the data.

The results given in table 8 cover a longer period than those given in tables 6 and 7 and indicate that the production of relatively large amounts of acetylmethylcarbinol + diacetyl by organism 29 in milk to which considerable citric acid had been added was followed by a decrease in the amount of acetylmethylcarbinol + diacetyl just as is the production of smaller amounts of these materials.

TABLE 8. ACETYL METHYL CARBINOL PLUS DIACETYL PRODUCED BY CITRIC ACID-FERMENTING STREPTOCOCCI IN MILK WITH CONSIDERABLE CITRIC ACID ADDED.

8,700 ml. of milk were inoculated with organism 29, held 48 hours and 0.60 percent citric acid then added.

Temperature 21°C.

<table>
<thead>
<tr>
<th>Period of incubation</th>
<th>pH</th>
<th>Mg. Ni salt equiv. to amc + aa* per 200 gm.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Determination A</td>
</tr>
<tr>
<td>None</td>
<td>4.2</td>
<td>117.1</td>
</tr>
<tr>
<td>1 day</td>
<td>4.8</td>
<td>176.9</td>
</tr>
<tr>
<td>4</td>
<td>5.0</td>
<td>201.8</td>
</tr>
<tr>
<td>5</td>
<td>5.3</td>
<td>208.3</td>
</tr>
<tr>
<td>11</td>
<td>5.4</td>
<td>183.4</td>
</tr>
<tr>
<td>20</td>
<td>5.2</td>
<td>159.4</td>
</tr>
</tbody>
</table>

*amc + aa = acetylmethylcarbinol + diacetyl.
MAXIMUM PERCENTAGE OF THE CITRIC ACID ADDED TO A MILK CULTURE OF A CITRIC ACID-FERMENTING STREPTOCOCCUS THAT CAN BE RECOVERED AS ACETYLMETHYLCARBINOL + DIACETYL

The maximum percentage of the citric acid added to a milk culture of one of the citric acid-fermenting streptococci that could be recovered as acetylmethylcarbinol + diacetyl was investigated because it appeared that such information would be useful from the standpoint of adding citric acid to milk to be made into butter culture. Also it might suggest the manner in which citric acid is changed to acetylmethylcarbinol. The structure of the citric acid molecule is such that acetic acid, which is one of the important products formed by the citric acid-fermenting streptococci, could be easily split from it, but the manner in which acetylmethylcarbinol is produced is more difficult to understand. Since a citric acid-fermenting Streptococcus growing in milk at a relatively low acidity yields considerable volatile acid and little or no acetylmethylcarbinol + diacetyl while at a relatively high acidity both volatile acid and acetylmethylcarbinol + diacetyl are produced, there is, evidently, a variation in the manner in which the citric acid molecule is broken down.

The determination of the yield of acetylmethylcarbinol + diacetyl from the citric acid added to a milk culture of a citric acid-fermenting Streptococcus is greatly complicated by various factors such as (a) the influence of the pH on the nature of the fermentation, (b) the destruction of acetylmethylcarbinol by the organisms and (c) the reaction of the diacetyl, which comes from the acetylmethylcarbinol, with the milk constituents (1). The general scheme used was to add a definite quantity of citric acid to a known amount of a 24 or 48-hour milk culture of a satisfactory organism, adjust to a suitable pH with sulfuric acid and determine the amount of acetylmethylcarbinol + diacetyl present after various holding periods. Comparable cultures, to which no citric acid was added but which were acidified with sulfuric acid, provided the values representing the yields of acetylmethylcarbinol + diacetyl from the citric acid in the original milk; these values were deducted in calculating the production of acetylmethylcarbinol + diacetyl from the added citric acid. The acetylmethylcarbinol + diacetyl was regularly calculated as acetylmethylcarbinol. The trials were run in series, each series involving a number of milk cultures of a citric acid-fermenting or-

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Unpublished results obtained at the Iowa Agricultural Experiment Station suggest that with the citric acid-fermenting streptococci acetylmethylcarbinol is not formed by an acetaldehyde condensation.
ganism to which different amounts of citric acid were added. The various lots of milk in a series were adjusted to as nearly the same pH as possible. No attempt was made to determine the citric acid present in the milk at any time, because there is presumably a rather continuous production and consumption of acetylmethylcarbinol + diacetyl, and the maximum percentage of the citric acid added that could be recovered as these materials was the point of interest.

As would be expected from the procedure followed, there were great variations in the percentages of the citric acid added that were recovered as acetylmethylcarbinol + diacetyl. These were, undoubtedly, due to various factors. In some cases the incubation periods were too short for the maximum production to occur; this was evident from the fact that later determinations on the same lots of milk gave larger yields. In other cases a portion of the acetylmethylcarbinol + diacetyl that had been produced was used up; this was shown by decreases in the quantities of these materials, as compared with earlier determinations on the same lots of milk. Some of the trials gave only a very small production of acetylmethylcarbinol + diacetyl. This may have been due to any one of the various factors that are known to interfere with their production by the citric acid-fermenting organisms.

Representative results, illustrating the instances in which a comparatively large percentage of the citric acid added was recovered as acetylmethylcarbinol + diacetyl, are presented in table 9; data showing relatively small yields are not included because of the number of factors that may be responsible for such yields.

The results obtained indicate that, with the procedure employed, only a relatively small percentage of the citric acid added to a milk culture of a citric acid-fermenting Streptococcus was recovered as acetylmethylcarbinol + diacetyl. The percentages reported as representing the best yields obtained varied from 11.09 to 16.62 percent. The amounts of citric acid added to the milk ranged from 0.091 to 0.173 percent; there was no close correlation between the amount added and the percentage recovered as acetylmethylcarbinol + diacetyl but, in general, relatively high yields were more often obtained with comparatively small additions of citric acid than with comparatively large additions. The determinations made on a culture after various incubation periods showed both increases and decreases, from one determination to the next, in the percentages of citric acid recovered as acetylmethylcarbinol + diacetyl; these variations are what would be predicted from the information available on the production and destruction of acetylmethylcarbinol + diacetyl by the citric acid-fermenting streptococci.
Organism grown in milk, a known amount of citric acid added, the pH adjusted with sulfuric acid and the acetylmethylcarbinol plus diacetyl determined after various incubation periods.

**Temperature 21°C.**

<table>
<thead>
<tr>
<th>Series number</th>
<th>Organism used</th>
<th>Percent anhydrous citric acid added to milk culture</th>
<th>pH after adding acids</th>
<th>Hours incubated after adding acids</th>
<th>Final pH</th>
<th>Percent of citric acid that was recovered as <em>ame + aa</em>†</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>29</td>
<td>0.127</td>
<td>36</td>
<td>4.4</td>
<td>12.11</td>
<td>12.11</td>
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<td></td>
<td></td>
<td>0.154</td>
<td>36</td>
<td>4.4</td>
<td>12.75</td>
<td>12.75</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.127</td>
<td>72</td>
<td>4.4</td>
<td>11.58</td>
<td>11.58</td>
</tr>
<tr>
<td></td>
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<td>0.154</td>
<td>72</td>
<td>4.4</td>
<td>12.46</td>
<td>12.46</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.154</td>
<td>144</td>
<td>4.4</td>
<td>12.28</td>
<td>12.28</td>
</tr>
<tr>
<td>2</td>
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<td>48</td>
<td>3.9</td>
<td>13.19</td>
<td>13.19</td>
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<tr>
<td></td>
<td></td>
<td>0.173</td>
<td>72</td>
<td>3.9</td>
<td>13.60</td>
<td>13.60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.173</td>
<td>96</td>
<td>3.9</td>
<td>12.73</td>
<td>12.73</td>
</tr>
<tr>
<td>3</td>
<td>29</td>
<td>0.091</td>
<td>48</td>
<td>4.4</td>
<td>15.89</td>
<td>15.89</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.134</td>
<td>48</td>
<td>4.4</td>
<td>16.62</td>
<td>16.62</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.091</td>
<td>120</td>
<td>4.3</td>
<td>15.92</td>
<td>15.92</td>
</tr>
<tr>
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<td></td>
<td>0.134</td>
<td>120</td>
<td>4.3</td>
<td>12.16</td>
<td>12.16</td>
</tr>
<tr>
<td>4</td>
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<td>0.091</td>
<td>96</td>
<td>4.3</td>
<td>14.11</td>
<td>14.11</td>
</tr>
<tr>
<td>5</td>
<td>29</td>
<td>0.147</td>
<td>3.9</td>
<td>4.3</td>
<td>11.09</td>
<td>11.09</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.148</td>
<td>4.0</td>
<td>4.5</td>
<td>12.08</td>
<td>12.08</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.147</td>
<td>3.9</td>
<td>4.3</td>
<td>12.81</td>
<td>12.81</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.147</td>
<td>72</td>
<td>4.3</td>
<td>14.72</td>
<td>14.72</td>
</tr>
<tr>
<td>6</td>
<td>122</td>
<td>0.150</td>
<td>4.0</td>
<td>4.4</td>
<td>11.36</td>
<td>11.36</td>
</tr>
</tbody>
</table>

*ame + aa = acetylmethylcarbinol + diacetyl.
†Calculated as acetylmethylcarbinol from Ni salts.

**INFLUENCE OF DIACETYL, ACETYLMETHYL CARBINOL AND 2.3 BUTYLENE GLYCOL ON VOLATILE ACID PRODUCTION**

The disappearance of acetylmethylcarbinol + diacetyl in milk cultures of the citric acid-fermenting streptococci (and also in butter cultures) suggests the possibility of these materials being converted into volatile acid by the organisms. Accordingly, trials were carried out to determine the effect of adding diacetyl or acetylmethylcarbinol on the volatile acid production of the organisms in milk; the effect of 2.3 butylene glycol was also investigated because of the close structural relationship of this compound to acetylmethylcarbinol.

**INFLUENCE OF DIACETYL**

The data dealing with the influence of the addition of diacetyl to milk cultures on the volatile acid production of the citric acid-fermenting streptococci are presented in tables 10 and 11. The results given in table 10 show that the addition of diacetyl had no significant effect on the volatile acid produced,
TABLE 10. EFFECT OF THE ADDITION OF DIACETYL ON THE VOLATILE ACID PRODUCTION OF CITRIC ACID-FERMENTING STREPTOCOCCI.

Organism 29 grown in milk 24 hours and reagents then added. Temperature 21°C.

<table>
<thead>
<tr>
<th>Period of holding after adding reagents</th>
<th>None</th>
<th>24 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH</td>
<td>Mg. Ni salt equiv. to amc + aa* per 200 gm.</td>
</tr>
<tr>
<td>Milk alone</td>
<td>5.9</td>
<td>none</td>
</tr>
<tr>
<td>Milk plus diacetyl (X)†</td>
<td>6.0</td>
<td>94.7</td>
</tr>
<tr>
<td>Milk plus sulfuric acid</td>
<td>4.1</td>
<td>none</td>
</tr>
<tr>
<td>Milk plus diacetyl (X) and sulfuric acid</td>
<td>4.1</td>
<td>89.9</td>
</tr>
<tr>
<td>Milk plus diacetyl (1/2X)†</td>
<td>6.0</td>
<td>44.7</td>
</tr>
<tr>
<td>Milk plus diacetyl (1/2X) and sulfuric acid</td>
<td>4.1</td>
<td>42.1</td>
</tr>
</tbody>
</table>

*amc + aa = acetylmethylcarbinol + diacetyl.
†X and 1/2X show the comparative amounts of diacetyl added.

either in the presence or absence of sulfuric acid. In the absence of sulfuric acid there was a conspicuous decrease in the diacetyl content, while in the presence of sulfuric acid there was a production of acetylmethylcarbinol + diacetyl. The amount of diacetyl that disappeared was greater with the larger addition than with the smaller, although the extent of the disappearance did not closely follow the amount originally added. The production of acetylmethylcarbinol + diacetyl, when sulfuric acid was added, was essentially the same with the two additions of diacetyl. It was relatively small in both instances, as was also the case with the culture to which sulfuric acid alone was added. With the small production of acetylmethylcarbinol + diacetyl there was no change in the pH. The volatile acid produced, either with or without added diacetyl, was regularly greater when sulfuric acid was present.

TABLE 11. EFFECT OF THE ADDITION OF DIACETYL ON THE VOLATILE ACID PRODUCTION OF CITRIC ACID-FERMENTING STREPTOCOCCI.

Organism 29 grown in milk 24 hours and reagents then added. Temperature 21°C.

<table>
<thead>
<tr>
<th>Period of holding after adding reagents</th>
<th>None</th>
<th>48 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH</td>
<td>Mg. Ni salt equiv. to amc + aa* per 200 gm.</td>
</tr>
<tr>
<td>Milk alone</td>
<td>6.0</td>
<td>none</td>
</tr>
<tr>
<td>Milk plus diacetyl</td>
<td>5.9</td>
<td>94.0</td>
</tr>
<tr>
<td>Milk plus sulfuric acid</td>
<td>4.1</td>
<td>0.5</td>
</tr>
<tr>
<td>Milk plus diacetyl and sulfuric acid</td>
<td>4.1</td>
<td>80.2</td>
</tr>
</tbody>
</table>

*amc + aa = acetylmethylcarbinol + diacetyl.
than when it was not. This relationship was reported by Michaelian and Hammer (3), who suggested the possibility of the added acid making the citrate normally present in the milk more readily available for the organisms.

The data presented in table 11 also indicate that the addition of diacetyl had no influence on the production of volatile acid by the citric acid-fermenting streptococci, either when sulfuric acid was added or when it was not. A considerable production of acetylmethylcarbinol + diacetyl was obtained when the sulfuric acid was added, either with or without diacetyl, and this is reflected in the definite increases in the pH values. The greater production of volatile acid in the presence of sulfuric acid than in its absence is again clearly evident.

**INFLUENCE OF ACETYLMETHYLCARBINOL**

The results obtained in the attempts to influence the volatile acid production of the citric acid-fermenting streptococci by adding acetylmethylcarbinol to the milk cultures are presented in table 12.

The data show that the addition of acetylmethylcarbinol had no influence on the volatile acid production, either in the presence or absence of sulfuric acid. When acetylmethyl-

### TABLE 12. EFFECT OF THE ADDITION OF ACETYLMETHYLCARBINOL ON THE VOLATILE ACID PRODUCTION OF CITRIC ACID-FERMENTING STREPTOCOCCI

Organism 29 grown in milk 24 or 48 hours before adding reagents.
Temperature 21°C.

<table>
<thead>
<tr>
<th>Period of holding after adding reagents</th>
<th>None</th>
<th>24 hours</th>
<th>48 hours</th>
<th>96 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>Mg. Ni salt equiv. to acme+aa* per 200 gm.</td>
<td>pH</td>
<td>Mg. Ni salt equiv. to acme+aa per 200 gm.</td>
<td>pH</td>
</tr>
<tr>
<td>Trial 1. Cultures grown 24 hours before adding reagents.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Milk alone</td>
<td>6.3</td>
<td>trace</td>
<td>6.9</td>
<td></td>
</tr>
<tr>
<td>Milk plus acme</td>
<td>6.3</td>
<td>27.5</td>
<td>6.3</td>
<td></td>
</tr>
<tr>
<td>Trial 2. Cultures grown 48 hours before adding reagents.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Milk alone</td>
<td>6.3</td>
<td>none</td>
<td>9.0</td>
<td>6.1</td>
</tr>
<tr>
<td>Milk plus acme</td>
<td>6.3</td>
<td>25.1</td>
<td>10.9</td>
<td>6.0</td>
</tr>
<tr>
<td>Milk plus sulfuric acid</td>
<td>4.1</td>
<td>2.4</td>
<td>16.3</td>
<td>4.3</td>
</tr>
<tr>
<td>Milk plus acme and sulfuric acid</td>
<td>4.1</td>
<td>26.3</td>
<td>14.1</td>
<td>4.3</td>
</tr>
</tbody>
</table>

*acme+aa = acetylmethylcarbinol + diacetyl.
carbinol was added without adding sulfuric acid, it decreased in amount rather rapidly. When sulfuric acid was added to a culture, either with or without the addition of acetylmethylcarbinol, there was a production of acetylmethylcarbinol + diacetyl which was followed by a partial disappearance. With a relatively short incubation period following the addition of the reagents, the volatile acid production was greater in the presence of sulfuric acid than in its absence, but with a longer incubation period this difference disappeared.

INFLUENCE OF 2.3 BUTYLENE GLYCOL

In studying the effect of 2.3 butylene glycol on the production of volatile acid, organism 29 was grown in four flasks of milk (1,100 ml. each) for 48 hours at 21°C., after which sulfuric acid (to give a pH of 4.1) was added to one flask, 2.3 butylene glycol (0.5 ml.) to the second, both reagents to the third while the fourth was left as a control. Determinations after an incubation of 5 days at 21°C. indicated that the glycol had no effect on the volatile acid production, either in the presence or absence of the sulfuric acid. In the two flasks containing sulfuric acid there was considerable acetylmethylcarbinol + diacetyl (equivalent to 38.3 and 35.3 mg. Ni salt per 200 gm., with and without the glycol) present after 48 hours, while after 5 days the amounts were somewhat decreased (equivalent to 29.9 and 24.5 mg. Ni salt, with and without the glycol). The glycol seemed to have no significant effect on the production of acetylmethylcarbinol + diacetyl. In the flasks without sulfuric acid, no acetylmethylcarbinol + diacetyl was found after either 48 hours or 5 days.

DISCUSSION OF RESULTS

PART I

The results obtained on the comparative amounts of acetylmethylcarbinol + diacetyl in cream, buttermilk and butter show that during the churning process these compounds are largely concentrated in the buttermilk rather than in the butter. The presence of larger amounts of acetylmethylcarbinol + diacetyl in the serum of butter rather than in the fat is in agreement with the distribution between the buttermilk and butter. Since acetylmethylcarbinol and diacetyl are apparently of great importance from the standpoint of the aroma of butter (the diacetyl because of its odor and the acetylmethylcarbinol because it is a source of diacetyl), this general relationship is at variance with the idea that, when butter culture is used in the manufacture of butter, the butterfat largely absorbs and holds the characteristic aroma materials. There is, of course, the possibility that the comparatively small
amount of acetylmethylcarbinol + diacetyl in the fat is especially important from the standpoint of the aroma of butter because of a relatively rapid oxidation of the odorless acetylmethylcarbinol to diacetyl.

The variations in the ratios of the acetylmethylcarbinol + diacetyl in the cream to the amounts in the buttermilk or butter are presumably due to various factors, but the possibility of either the production or disappearance of these compounds during the manufacture of the butter should be recognized.

The presence of acetylmethylcarbinol + diacetyl in many lots of cream delivered to butter plants suggests that butter made without butter culture may contain these materials and the aroma of the product be due, in part, to them. With the extensive bacterial action that has taken place in much of the cream, the formation of some acetylmethylcarbinol + diacetyl would be expected because of the various types of organisms that are capable of producing these compounds. It should be noted that citric acid is not the only source of the acetylmethylcarbinol + diacetyl in cream and that sugar may be fermented by various organisms with the formation of acetylmethylcarbinol. The failure to find acetylmethylcarbinol + diacetyl in cream freshly skimmed from relatively low-count milk indicates that when these materials are present in cream they are the result of the action of microorganisms.

**PART II**

The data obtained indicate that the rapid production of acetylmethylcarbinol + diacetyl from citric acid by the citric acid-fermenting streptococci is not due to a reaction between this acid and some compound formed by the organisms. The methods used to destroy the organisms in the milk cultures, however, may have influenced the products formed by the organisms, so that trials with other procedures are advisable.

The rather close relationship between the numbers of citric acid-fermenting streptococci added to milk at a relatively low pH and the amounts of the usual fermentation products formed, is of special interest. It would be expected that, with the incubation periods employed, small differences in the numbers of organisms inoculated would be compensated for by the growth of the organisms. The results suggest that, in the presence of considerable acid, active multiplication of the organisms may not occur and that the acetylmethylcarbinol + diacetyl is primarily produced by organisms under adverse conditions. Unpublished results obtained at the Iowa Agricultural Experiment Station, which include bacterial counts on cultures of the organisms under different conditions of acidity, are in agreement with this suggestion. The failure to get a
satisfactory flavor and aroma development in certain butter cultures may be the result of a failure of the citric acid-fermenting streptococci to increase as they should during the early part of the incubation period. The maximum amount of acetylmethylcarbinol + diacetyl produced in a milk culture of one of the citric acid-fermenting streptococci to which citric acid had been added was considerably larger than the amount produced in a butter culture but was still rather sharply limited. The relatively large production was followed by a decrease, just as with a smaller production, and this emphasizes the importance of the decrease in the amount of acetylmethylcarbinol + diacetyl in milk cultures of the organisms from the standpoint of their physiology. The relatively small percentage of the citric acid added to a milk culture that could be recovered as acetylmethylcarbinol + diacetyl suggests that the manner in which the citric acid molecule is broken down is rather complex and, accordingly, difficult of determination.

The failure to increase the volatile acid production of the citric acid-fermenting streptococci by adding diacetyl or acetylmethylcarbinol to milk cultures indicates that these materials are not intermediate products from which volatile acid is eventually produced.

**SUMMARY**

**PART I**

Fresh buttermilk regularly contained larger amounts of acetylmethylcarbinol + diacetyl than the cream from which it was obtained. In seven commercial churnings the ratios of the acetylmethylcarbinol + diacetyl in the cream to the amounts in the buttermilk varied from 1:2.0 to 1:3.1, while in six series of experimental churnings, each series involving a number of lots of cream of different acidities, the ratios ranged from 1:1.1 to 1:2.1.

Butter regularly contained much smaller amounts of acetylmethylcarbinol + diacetyl than the cream from which it was churned. With 52 small churnings of butter (from 30 to 40 pounds each), in which definite amounts of acetylmethylcarbinol + diacetyl were found in 400 gm. of butter, the ratios of the acetylmethylcarbinol + diacetyl in the cream to the amounts in the butter varied from 1:0.032 to 1:0.218; the butter was salted and was made with the use of butter culture. The 52 lots of butter yielded from 0.1 to 3.45 mg. of nickel salt equivalent to acetylmethylcarbinol + diacetyl per 200 gm. of butter.

The amount of acetylmethylcarbinol + diacetyl in butter
serum was always definitely larger than the amount in the fat from the same butter.

Cream delivered to butter plants often contains acetyl-
methylcarbinol + diacetyl. In general, the amounts in sour
cream are relatively high, while sweet cream may contain small
quantities. Several samples of cream freshly skimmed from
rather low-count milk showed no acetylmehtylcarbinol +
diacetyl when 200-gm. portions were distilled.

PART II

Milk cultures of the citric acid-fermenting streptococci that
had been killed with heat, formalin, or chloroform failed to
produce acetylmehtylcarbinol + diacetyl when treated with
0.15 percent citric acid and 0.30 percent sulfuric acid. Sus-
pensions of the organisms washed from whey agar plates with
sterile water and killed with chloroform produced no acetyl-
methylcarbinol + diacetyl when added to milk containing
0.50 percent citric acid.

There was a rather close relationship between the numbers
of citric acid-fermenting streptococci added to milk having a
low pH, as a result of the addition of citric acid or citric and
sulfuric acids, and the amounts of the usual fermentation
products formed.

In milk to which considerable citric acid had been added,
relatively large amounts of acetylmehtylcarbinol + diacetyl
were produced by the citric acid-fermenting streptococci; the
maximum amount of nickel salt equivalent to acetylmehtyl-
carbinol + diacetyl that was found was 246.7 mg. per 200 gm.
The production of large amounts of acetylmehtylcarbinol +
diacetyl was followed by a decrease in these materials, just as
is the production of smaller amounts.

Only a relatively small percentage of the citric acid added to
a milk culture of a citric acid-fermenting Streptococcus could
be recovered as acetylmehtylcarbinol + diacetyl. The per-
centages representing the best yields obtained varied from 11.09
to 16.62.

The volatile acid production in milk cultures of the citric
acid-fermenting streptococci was not increased by the addition
of diacetyl, acetylmehtylcarbinol or 2.3 butylene glycol, which
indicates that when acetylmehtylcarbinol + diacetyl disap-
pars in a culture these materials are not changed to volatile
acid.
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Proctor Gull, B.S. — Research Assistant
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Robert P. Moore, B.S. — Research Fellow
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