THE RELATION OF CARBON DIOXIDE TO PROTEOLYSIS IN THE RIPENING OF CHEDDAR CHEESE.

L. L. VAN SLYKE AND E. B. HART.

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THE RELATION OF CARBON DIOXIDE TO PROTEOLYSIS IN THE RIPENING OF CHEDDAR CHEESE.

L. L. VAN SLYKE AND E. B. HART.

SUMMARY.

1. The object of the work described in this bulletin was to ascertain the extent to which carbon dioxide is formed in American cheddar cheese during long periods of time in the process of ripening, and also to learn the nature of the chemical changes that give rise to the production of this gas.

2. Two cheeses were used for this study. One was entirely normal; the other was made from milk containing chloroform and kept under antiseptic conditions. The investigation was continued 32 weeks, when a chemical study was made of the proteolytic end-products.

3. In the normal cheese, carbon dioxide was given off continuously, though in decreasing quantities after about 20 weeks, and had not ceased at the end of 32 weeks. The total amount thus produced was 15.099 grams, equal to 0.5 per cent. of the fresh cheese. In the chloroformed cheese, the total amount of carbon dioxide produced was 0.205 gram, practically none being found after three weeks.

4. In the normal cheese, the following end-products of proteolysis were found: Tyrosine, oxyphenylethylamine,
arginine in traces, histidine, lysine, guanidine, putrescine in traces, and ammonia. In the chloroformed cheese were found the same compounds, except oxyphenylethylamine, guanidine, putrescine, and ammonia; but arginine was found in marked quantities for the first time in cheese.

5. A consideration of the possible sources of carbon dioxide in the two cheeses indicates that, in the case of the chloroformed cheese, the carbon dioxide came from that present originally in the milk and that formed in the milk from the decomposition of milk-sugar before treatment with chloroform. In the case of the normal cheese, the carbon dioxide given off in its early age came largely from the decomposition of milk-sugar by lactic acid organisms, while a small amount was probably due to the carbon dioxide present in the milk and to the respiration of living organisms present in the cheese. The carbon dioxide produced after the first few weeks came apparently from reactions taking place in some of the amido compounds, among which we were able to identify the change of tyrosine and arginine into derived products with simultaneous formation of carbon dioxide.

6. In the chloroformed cheese, the only active proteolytic agents were lactic acid, galactase and rennet-pepsin. Under the conditions of our experiment, these agents were able to form neither ammonia nor secondary amido compounds with production of carbon dioxide. The presence of chloroform could not account for this lack of action. These results suggest that, in the normal cheese, there must have been some agent at work not present in the chloroformed cheese and that this extra factor was of a biological character.
INTRODUCTION.

In 1880 Babcock\textsuperscript{1} carried on some experiments in cheese-curing, in which he attempted to measure the amount of carbon dioxide formed by cheese in ripening; but his study of each cheese was limited to short periods of time and the source of the carbon dioxide formed was not ascertained by him. The investigation described in this bulletin was undertaken primarily to learn to what extent carbon dioxide is given off by American cheddar cheese during long periods of time in the process of ripening. It was hoped that by such study we should be able also to learn the sources of the carbon dioxide thus formed and add to our knowledge in regard to some of the deep-seated chemical changes occurring in the ripening of cheddar cheese.

As material for use in carrying on the investigation, we made two cheeses. One was normal in every respect; the other was made from milk containing chloroform and was kept under antiseptic conditions, thus enabling us to suppress factors of biological activity. The study was continued for 32 weeks, at the end of which time we completed the work by making a study of the end-products in each cheese, including, more particularly, diamido compounds and their secondary cleavage products and tyrosine.

EXPERIMENTAL PART.

PREPARATION OF CHEESE.

For each cheese we used about 20 kgs. (45 pounds) of milk that had been drawn from the cows’ udders not more than three hours. One cheese was made in the usual manner, being normal in every respect. In making the other cheese, we added to the milk at the start 4 per ct. by volume of chloroform and then enough lactic acid to equal 0.2 per ct. of the milk by weight. The rest of the process of cheese-making was carried on in the usual way. In both cases salt was added at the rate of 1 part for 400 parts of milk used. The normal cheese weighed 6 pounds and 10 ounces (3000 grams); the cheese containing chloroform weighed 7 pounds and 1 ounce (3203 grams), owing to the retention of chloroform and more water.

\textsuperscript{1} Cornell Univ. Exp. Sta. Report, pp. 9-27 (1879-80).
ARRANGEMENTS FOR COLLECTING GAS EVOLVED BY CHEESE.

On April 1, 1902, each cheese was placed by itself under a bell-jar, each bell-jar being connected with its own apparatus for the absorption of carbon dioxide. During the entire period of the investigation, the cheeses were kept at a temperature of 60° F. (15.5° C.) Through the bell-jars, made tight by mercury joints, were passed daily about 8 liters of air, previously purified by passage through several wash-bottles containing potassium hydroxide. The air from the bell-jar containing the normal cheese was passed through a drying-train of strong sulphuric acid and calcium chloride and then through two Liebig bulbs, in order to absorb any carbon dioxide present. A water-bottle holding 8½ liters was used as an aspirator. The aspirator was started each morning at about 8 o’clock and stopped at 5 p. m. Over night, a stop-cock, separating the bell-jar from the wash-bottles containing potassium hydroxide and used for washing the inflowing air, was closed to prevent backward diffusion and consequent loss of carbon dioxide. The bulbs were weighed daily in the early period of the experiment, but only weekly during the later period.

In the case of the cheese containing chloroform and kept in an atmosphere of chloroform, the air from the bell-jar was passed through sulphuric acid and then through silver nitrate solution, in order to absorb any hydrochloric acid formed by decomposition of chloroform; the air was then passed through three flasks containing decinormal solution of barium hydroxide, to absorb the carbon dioxide, and finally through a potassium hydroxide guard. The same precaution against backward diffusion was observed as in the case of the other cheese. Liebig absorption bulbs and direct weighing could not be employed, since the air coming from the bell-jar was constantly laden with vapor of chloroform. To replace the loss of chloroform caused by aspiration, fresh portions of chloroform were added from time to time through a separatory funnel passing through the top of the bell-jar. A small dish placed on the top of the cheese inside the bell-jar received the chloroform. Once a week the barium carbonate formed was filtered through a weighed Gooch crucible, washed with dilute ammonia, dried and weighed. From the
amount of barium carbonate thus found, the amount of carbon dioxide was calculated. On April 1 this cheese contained 12 per ct. of chloroform, and on Nov. 28th, at the close of the investigation, it contained 10.5 per ct.

The normal cheese, before being placed under the bell-jar, was completely covered on the outside by a mixture of vaseline and creosote, in order to prevent as far as possible the growth of any molds on the surface of the cheese. This was done at the suggestion of the Station bacteriologist, Mr. H. A. Harding. In a similar experiment, when no special precautions were used, Babcock\(^2\) found it impossible to prevent the growth of molds on the surface of cheese contained in a moist atmosphere under a bell-jar. He calls attention to the fact that the growth of mold was responsible for the formation of large quantities of carbon dioxide. It was absolutely essential, therefore, that in our work we should eliminate this source of carbon dioxide, if we were to learn anything definite about other sources of carbon dioxide formation within the cheese.

**PRODUCTION OF CARBON DIOXIDE IN NORMAL CHEESE.**

On the first day, we found the normal cheese had given off 0.044 gram of carbon dioxide; on the second day, 0.0978 gram; on the third day, 0.118 gram; and on the fourth day, 0.139 gram. On the eleventh day, the maximum daily record up to that time was made, 0.146 gram.

During the first week, we found 0.735 gram of carbon dioxide. This amount gradually decreased until the fourth week, when the amount was 0.364 gram. At this time the bell-jar was opened and samples taken for chemical analysis. Before opening the bell-jar, the aspiration was quickened somewhat in order to reduce the carbon dioxide in the bell-jar to the lowest amount possible. From the fourth to the ninth week, the amount of carbon produced increased gradually, reaching 0.644 gram for the ninth week. At this time a small patch of blue mold, covering about a square inch of surface, was observed. This was scraped off and more creosote applied. At the end of the eleventh week, another small patch of blue mold was noticed

and the amount of carbon dioxide formed had again risen. Again, at the end of the thirteenth week; another small patch of mold was found and the amount of carbon dioxide produced during this week was equal to that found during the first week of the experiment, 0.735 gram, the maximum weekly yield during the investigation. The whole outer surface of the cheese was then treated anew with the mixture of vaseline and creosote and afterwards no further trouble was experienced from the presence of molds. It was very noticeable that the presence of mold was quickly revealed by a sudden and marked increase in the amount of carbon dioxide formed. From the end of the thirteenth week to the close of the investigation at the end of the thirty-second week, the amount of carbon dioxide gradually decreased, being only 0.224 gram during the last week.

During the entire period of 32 weeks, the total amount of carbon dioxide produced was 15.099 grams. This is equal to 0.5 per ct. of the fresh cheese and represents a loss of solids equal to one-half pound for 100 pounds of fresh cheese. Undoubtedly other gases or volatile compounds are formed in small quantities, as shown by the blackening of the sulphuric acid in the drying-train. We hope to make later a more detailed study of the other gases formed in cheese during the ripening process.

In the table following, we present the detailed results of our work week by week:

**Table I. Amount of Carbon Dioxide formed in Normal Cheddar Cheese during Each Week of Investigation.**

<table>
<thead>
<tr>
<th>No. of week</th>
<th>Grams of C O₂ formed</th>
<th>No. of week</th>
<th>Grams of C O₂ formed</th>
<th>No. of week</th>
<th>Grams of C O₂ formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.735</td>
<td>12</td>
<td>0.406</td>
<td>23</td>
<td>0.462</td>
</tr>
<tr>
<td>2</td>
<td>0.672</td>
<td>13</td>
<td>0.735*</td>
<td>24</td>
<td>0.357</td>
</tr>
<tr>
<td>3</td>
<td>0.420</td>
<td>14</td>
<td>0.476</td>
<td>25</td>
<td>0.343</td>
</tr>
<tr>
<td>4</td>
<td>0.364</td>
<td>15</td>
<td>0.441</td>
<td>26</td>
<td>0.427</td>
</tr>
<tr>
<td>5</td>
<td>0.476</td>
<td>16</td>
<td>0.434</td>
<td>27</td>
<td>0.400</td>
</tr>
<tr>
<td>6</td>
<td>0.574</td>
<td>17</td>
<td>0.539</td>
<td>28</td>
<td>0.366</td>
</tr>
<tr>
<td>7</td>
<td>0.525</td>
<td>18</td>
<td>0.409</td>
<td>29</td>
<td>0.340</td>
</tr>
<tr>
<td>8</td>
<td>0.574</td>
<td>19</td>
<td>0.445</td>
<td>30</td>
<td>0.300</td>
</tr>
<tr>
<td>9</td>
<td>0.644*</td>
<td>20</td>
<td>0.497</td>
<td>31</td>
<td>0.260</td>
</tr>
<tr>
<td>10</td>
<td>0.539</td>
<td>21</td>
<td>0.539</td>
<td>32</td>
<td>0.224</td>
</tr>
<tr>
<td>11</td>
<td>0.651*</td>
<td>22</td>
<td>0.492</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Increase due to presence of small amount of mold.
PRODUCTION OF CARBON DIOXIDE IN CHEESE CONTAINING CHLOROFORM.

In the cheese containing chloroform, we planned to suppress all biological activity. In order to ascertain how completely we succeeded in this respect, Mr. John Nicholson, the assistant bacteriologist of the Station, made bacteriological examinations from time to time. His results showed that the cheese was practically sterile throughout the entire period of the investigation.

The quantity of carbon dioxide produced by this cheese amounted only to about 0.019 gram a day during the first week, after which it fell off rapidly, the amount during the third week being less than 0.003 gram a day. At the end of three weeks, carbon dioxide practically ceased to be formed. The total amount of carbon dioxide produced by this cheese was 0.205 gram, about three-fourths of which was given off during the first nine days.

In the accompanying diagram, we show in graphic form the amounts of carbon dioxide produced by the two cheeses during the period of investigation.

PROTEOLYTIC END-PRODUCTS IN THE NORMAL CHEESE.

At the end of 32 weeks, the normal cheese was taken from the bell-jar, the covering of vaseline and creosote removed, and also the entire outer rind of the cheese to the thickness of about one-half an inch. The remainder of the cheese was cut into small pieces and dried at 140° F. (60° C.) for several days. It was then broken into finer particles by
rubbing and again dried, after which it was extracted with ether to remove fat and then reduced to a finely powdered white mass. This mass was extracted with several portions of water at 122° F. (50° C.), until about 12 liters were collected, the water-soluble contents of the mass having been thoroughly extracted by this treatment. This extract was precipitated with tannin and filtered; the tannin in the filtrate was removed by lead acetate. The resulting precipitate of lead tannate was filtered and washed three times by suspension in water and refiltering. The excess of lead was removed by sulphuric acid and the last traces by hydrogen sulphide. The filtrate was then carefully concentrated at 55° C. to about 4 liters made acid with 5 per ct. of sulphuric acid and precipitated with phosphotungstic acid. The precipitate was washed with dilute sulphuric acid. The filtrate from the phosphotungstic acid precipitate was examined for tyrosine and the precipitate for oxyphenylethylamine and the hexon bases.

Tyrosine.—The filtrate from the precipitate by phosphotungstic acid was treated with barium oxide to remove the phosphotungstic acid and the barium hydroxide in the filtrate carefully removed by sulphuric acid. This filtrate was concentrated to a small volume. On standing, crystals separated from the solution having much the appearance of tyrosine. These were filtered, redissolved in water, recrystallized several times from water after concentration of solution and finally washed with alcohol and dried over sulphuric acid in vacuo. A nitrogen determination by the Kjeldahl process gave the following results:

Calculated for tyrosine. Found.

\[ \text{N} \quad 7.68 \text{ per ct.} \quad 7.73 \text{ per ct.} \]

The substance gave the color reactions that are characteristic of tyrosine and was undoubtedly tyrosine. The separation of other monoamido compounds was not attempted.

Oxyphenylethylamine.—About one-fourth of the phosphotungstic acid precipitate, obtained in the manner previously described, was decomposed by barium oxide and filtered. The excess of barium hydroxide was removed from the filtrate by means of carbon dioxide. The clear filtrate was concentrated at a low
temperature and then treated with benzoyl chloride in dilute alkaline solution according to the Schotten-Baumann method.  This method has been employed by Langstein in the separation of oxyphenylethylamine formed by an intense peptic digestion of egg-albumin. An abundant precipitate separated, which was filtered and washed with cold water. It was then dissolved in hot alcohol and evaporated to small bulk. On standing, an abundant crop of crystals separated, which were filtered, washed with ether and dried over sulphuric acid in vacuo.

This product had a melting-point of 169° C. (uncorrected), agreeing exactly with the oxyphenylethylamine obtained by Langstein. The following results were obtained by determining the nitrogen by the Kjeldahl method and the carbon and hydrogen by combustion.

Calculated for benzoylderivative of oxyphenylethylamine. Found.

\[
\begin{align*}
\text{C}_8\text{H}_9\text{NO} & \quad \text{(C}_6\text{H}_5\text{CO})_2 \\
76.50 & \quad 76.19 \\
5.54 & \quad 5.44 \\
4.06 & \quad 4.10
\end{align*}
\]

This product was undoubtedly oxyphenylethylamine formed, as we shall point out later, from tyrosine with the accompaniment of carbon dioxide as a by-product.

Hexon bases.—The remainder of the phosphotungstic acid precipitate, obtained in the manner previously described, was decomposed by barium oxide and the excess of barium hydroxide was removed by careful addition of sulphuric acid. The resulting filtrate was worked for the hexon bases according to the Kossel-Kutscher method.

(1) Arginine.—After separating histidine from the solution, which should contain only arginine and histidine, a determination was made of the nitrogen in this solution containing only arginine. The amount of arginine, thus determined, equivalent to the nitrogen found, was only 0.364 gram, an amount too small to obtain in the form of crystals.

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3 *Ber. d. chem. Ges.*, 17: 2545 (1884) and 19: 3218 (1886).
5 *Ztschr. physiol. Chem.*, 31: 165 (1900).
(2) Histidine.—This substance was separated as the di-chloride, of which we obtained 0.850 gram. An analysis gave the following results:

Calculated for histidine hydrochloride. Found.
(C₆H₉N₃O₂ 2HCl)

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>18.42</td>
</tr>
<tr>
<td>Cl</td>
<td>31.11</td>
</tr>
</tbody>
</table>

(3) Lysine.—We separated about 2 grams of lysine in the form of picrate, which gave the following results on analysis:

Calculated for lysine picrate. Found.
(C₆H₁₄N₂O₂ C₆H₃N₃O₇)

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>18.66</td>
</tr>
<tr>
<td>C</td>
<td>38.40</td>
</tr>
<tr>
<td>H</td>
<td>4.53</td>
</tr>
</tbody>
</table>

(4) Guanidine.—The mother-liquor from the lysine precipitate was extracted by a mixture of alcohol and ether and then treated with gold chloride in very dilute hydrochloric acid solution, following the method of Winterstein and Thöny.⁶ On standing, a crystalline substance soon separated from the solution, behaving like a guanidine gold salt, yielding about 0.300 gram, which on analysis gave the following results:

Calculated for guanidine gold chloride. Found.
(CH₅N₃HCl AuCl₃)

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Au</td>
<td>49.79</td>
</tr>
</tbody>
</table>

So small an amount of this substance was obtained that we were unable to make other determinations to establish its identity with greater certainty, but it is highly probable that the substance is guanidine.

(5) Putrescine.—We expected to separate the other cleavage product of arginine, putrescine. The lysine solution had a strong odor of putrescine and it was unquestionably present but we failed in our efforts to isolate this base. It appears probable that the cleavage of arginine had only progressed as far as the formation of guanidine and ornithine, and that the latter compound had been decomposed only to a small extent, forming

merely traces of putrescine. The fact that the cheese was of
good flavor, except for a slight taste of creosote, indicates that
putrescine could not have been present in considerable
quantities.

PROTEOLYTIC END-PRODUCTS IN THE CHEESE CONTAINING
CHLOROFORM.

The cheese containing chloroform was, at the end of 32 weeks,
treated, preparatory to extraction, in the manner described
above in the case of the normal cheese. It was extracted with
several portions of water at 122° F. (50° C.) until about 12
liters of extract were obtained, the mass having been completely
extracted by this treatment. The water extract was treated with
tannin and filtered; the tannin was removed by lead acetate; the
precipitate was filtered and well washed. The excess of lead was
removed by sulphuric acid and the last traces by hydrogen sul-
phide. The filtrate was concentrated at a low temperature,
never above 131° F. (55° C.), to a small volume. It was then
precipitated by phosphotungstic acid, filtered and well washed
with dilute sulphuric acid.

Tyrosine.—After removing the phosphotungstic acid by
barium oxide and then the barium hydroxide by careful treat-
ment with sulphuric acid, the solution was concentrated to a
small volume and set aside for crystallization. After standing
several days, there separated from the solution a mixed crys-
talline and gummy mass. This precipitate was filtered, dissolved
in a small volume of water poured into cold 95 per ct. alcohol
and allowed to stand several days. A crystalline precipitate
formed at the bottom of the solution. The precipitate was
filtered, redissolved in water, decolorized with charcoal and fil-
tered. On concentration, this filtrate deposited a copious crys-
talline precipitate, greatly resembling tyrosine in appearance.
These crystals were washed with ether and dried over sulphuric
acid in vacuo. A determination gave 7.70 per ct. of nitrogen, as
compared with 7.73 calculated for tyrosine.

We were unable to find in this cheese any trace of
oxyphenylethylamine.
Hexon bases.—The phosphotungstic acid precipitate was decomposed by barium oxide, the barium hydroxide was removed from the filtrate by sulphuric acid and then arginine and histidine were precipitated by silver sulphate in barium hydrate solution in the usual way.

(1) Arginine.—After the separation of histidine from arginine, a determination of nitrogen in the remaining solution indicated the presence of about 1.5 grams of arginine, which is by far the largest amount we have ever succeeded in separating from any cheddar cheese with which we have worked. The solution was evaporated, dilute nitric acid added and then set aside for crystallization. After standing about a week, the solution had partly crystallized. These crystals were removed by filtration, washed with absolute alcohol and ether and dried over sulphuric acid in vacuo. Analysis gave the following result:

Calculated for arginine nitrate. Found.

\[
\begin{align*}
(C_6H_{14}N_4O_2HNO_3\cdot\frac{1}{2}H_2O) \\
N & \quad 28.45 & 28.32
\end{align*}
\]

To the mother-liquor was added silver nitrate with 2 or 3 drops of dilute nitric acid and the solution was allowed to evaporate in vacuo. Crystals soon separated and after a few days the entire mass was crystalline. The crystals were washed with alcohol and ether and dried over sulphuric acid in vacuo. A silver determination gave the following results:

Calculated for arginine silver nitrate. Found.

\[
\begin{align*}
(C_6H_{14}N_4O_2AgNO_3HNO_3) \\
Ag & \quad 26.54 & 26.49
\end{align*}
\]

We believe we are justified in regarding this substance beyond question as arginine. So far as we are able to learn, this is the first time arginine has been separated from a ripening cheese; and, in this case, we succeeded only when all biological factors had been eliminated.

(2) Histidine.—This base was separated as a di-chloride. Analysis gave the following results:

Calculated for histidine hydrochloride. Found.

\[
\begin{align*}
(C_6H_9N_3O_2\cdot2HCl) \\
Cl & \quad 31.11 & 31.08 \\
N & \quad 18.42 & 18.59
\end{align*}
\]
(3) Lysine.—This substance was easily separated as picrate and analyzed as follows:

Calculated for lysine picrate

\[
\text{N} \quad 18.66 \\
\text{H} \quad 4.53 \\
\text{C} \quad 38.40
\]

Found.

\[
\text{N} \quad 18.78 \\
\text{H} \quad 4.38 \\
\text{C} \quad 38.51
\]

We were unable to separate guanidine from the mother-liquor of the lysine precipitate and we believe that it was not present. We were unable, also to detect any of the other possible cleavage products of arginine. The solution containing lysine had no such odor as the corresponding solution obtained from the normal cheese and was, indeed, conspicuously free from the putrescine odor that was so characteristic of the lysine solutions obtained from normal cheese ripened at about 60° F. (15.5° C.)

ANALYSIS OF CHEESES.

At intervals determinations were made of the moisture, total nitrogen, water-soluble nitrogen and nitrogen in the form of unsaturated paracasein lactate, of amido compounds and of ammonia. The results are given in the subjoined table:

**Table II.—Results of Analysis of Cheeses.**

<table>
<thead>
<tr>
<th></th>
<th>Age when analyzed.</th>
<th>Moisture in cheese.</th>
<th>Nitrogen in cheese.</th>
<th>Nitrogen expressed as percentage of total nitrogen in cheese, in form of—</th>
<th>Paracasein monolactate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal cheese.</td>
<td>Fresh</td>
<td>36.53</td>
<td>3.54</td>
<td>6.78</td>
<td>2.52</td>
</tr>
<tr>
<td></td>
<td>1 mo.</td>
<td>35.83</td>
<td>3.72</td>
<td>18.82</td>
<td>11.83</td>
</tr>
<tr>
<td></td>
<td>3 “</td>
<td>34.84</td>
<td>3.92</td>
<td>25.50</td>
<td>17.86</td>
</tr>
<tr>
<td></td>
<td>7 “</td>
<td>33.94</td>
<td>4.09</td>
<td>38.14</td>
<td>28.12</td>
</tr>
<tr>
<td>Cheese containing chloroform.</td>
<td>Fresh</td>
<td>46.23</td>
<td>2.45</td>
<td>9.80</td>
<td>3.67</td>
</tr>
<tr>
<td></td>
<td>1 mo.</td>
<td>45.77</td>
<td>2.50</td>
<td>20.80</td>
<td>11.20</td>
</tr>
<tr>
<td></td>
<td>3 “</td>
<td>44.38</td>
<td>2.51</td>
<td>27.90</td>
<td>14.74</td>
</tr>
<tr>
<td></td>
<td>8 “</td>
<td>44.37</td>
<td>2.73</td>
<td>40.30</td>
<td>23.10</td>
</tr>
</tbody>
</table>
If we compare the two cheeses in question with reference to the data contained in the preceding table, we notice:

(1st) In respect to the water-soluble compounds of nitrogen, the two cheeses did not differ greatly, the slight difference being in the favor of the chloroformed cheese. Ordinarily we should expect the normal cheese to form soluble nitrogen compounds with somewhat greater rapidity than the cheese containing chloroform. Two conditions that were present furnish an explanation of these unexpected results. In the first place, some of the creosote used in coating the normal cheese diffused into the body of the cheese and exerted some antiseptic influence, retarding enzyme and bacterial activity and giving proteolytic results lower than we commonly find in case of normal cheese. In the second place, the chloroformed cheese contained about 10 per cent. more water than the normal cheese. We have in our unpublished records numerous data which establish the fact that increase of moisture in cheese very noticeably increases the amount of water-soluble nitrogen compounds formed in a given time. The difference in results of the last analyses was made more favorable to the chloroformed cheese, since it was a month older.

(2nd) After the first month, the amount of amido compounds formed in the normal cheese was greater than in the chloroformed cheese.

(3rd) In the normal cheese, ammonia was formed, though somewhat less in amount than under conditions entirely normal; while in the chloroformed cheese no trace of ammonia was formed.

(4th) We have previously\(^7\) pointed out that the formation of water-soluble nitrogen compounds in cheese-ripening appears to take place at the expense of the paracasein monolactate (soluble in dilute solution of sodium chloride). The figures in the preceding table furnish confirmatory evidence of this, since the paracasein monolactate diminishes at the same time the water-soluble nitrogen increases.

(5th) The amount of water-soluble proteolytic compounds formed in cheese can not safely be used as the sole basis of comparison in respect to the extent of chemical changes taking place in ripening cheese. In the two cheeses investigated, the amounts of water-soluble nitrogen did not greatly differ, but an examination of the end-products of proteolysis showed changes much more complete in the case of the cheese containing the smaller amount of water-soluble nitrogen. The true measure of cheese-ripening must be found in the character and amount of the individual products formed rather than in the total amount of water-soluble nitrogen.

GENERAL SUMMARY OF RESULTS.

We now bring together, in a form allowing ready comparison, the results that have been presented in detail in the foregoing pages.

In Normal Cheese. In Cheese Containing Chloroform.

(1) Production of carbon dioxide.
   (a) Total in 32 weeks, 15,099 grams.
   (b) Weekly variation from 0.735 gram in first, to 0.224 gram in last, week.

(2) Proteolytic end-products formed.
   (a) Tyrosine in small amounts.
   (b) Oxyphenylethylamine.
   (c) Arginine in traces.
   (d) Histidine.
   (e) Lysine.
   (f) Guanidine.

(1) Production of carbon dioxide.
   (a) Total, 0.205 gram.
   (b) Ceased entirely after three weeks.

(2) Proteolytic end-products formed.
   (a) Tyrosine.
   (b) No oxyphenylethylamine.
   (c) Arginine in marked quantity.
   (d) Histidine.
   (e) Lysine.
   (f) No guanidine.
(g) Traces of putrescine. (g) No putrescine.
(3) Analysis of cheese. (3) Analysis of cheese.
   (a) Ammonia formed. (a) No ammonia formed.
   (b) Amido compounds more abundant. (b) Amido compounds less abundant.

DISCUSSION OF RESULTS.

We have seen above that results varying in a most marked manner were obtained from the two cheeses used in our investigation. We will now consider some of these differences with a view to finding some satisfactory explanation of the facts presented.

THE SOURCES OF CARBON DIOXIDE IN CHEESE.

What was the source of the carbon dioxide produced in each cheese? Why did the normal cheese produce relatively so large quantities of carbon dioxide over so long a period of time and why did the chloroformed cheese produce so small quantities and for so brief a period?

As possible sources of carbon dioxide in cheese, we have (1) the milk used in making cheese, (2) the decomposition of milk-sugar in the formation of lactic acid, (3) the respiration of living cells present in the cheese and (4) the chemical decomposition of compounds present in the cheese. We will consider these separately.

(1) *Milk as a source of carbon dioxide in cheese.*—According to Marshall,8 fresh milk, before exposure to air, contains on an average about 4 per ct. of free carbon dioxide by volume and this is reduced one-half by aeration. In the amount of milk used by us in making each cheese, we should have about 0.800 gram of carbon dioxide. Some of this is of necessity lost in the process of cheese-making, but we could expect to retain in the cheese 0.200 to 0.300 gram of the carbon dioxide originally present in the milk.

(2) *The decomposition of milk-sugar as a source of carbon dioxide in cheese.*—E. Kayser9 has shown that certain lactic acid bacteria produce, as the result of their action on milk-sugar, not

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only lactic acid but also certain by-products, among which is carbon dioxide. The milk-sugar is undergoing decomposition all through the normal process of cheese-making, and the carbon dioxide thus formed becomes incorporated in the cheese-curd to some extent. In our normal cheese, the milk-sugar actually present before the cheese was placed under the bell-jar amounted to 0.3 per cent. of the cheese. This was changed into lactic acid with the accompanying formation of carbon dioxide in the early period of ripening and the carbon dioxide thus formed, together with that occluded in the cheese mass, can readily account for the relatively large amount of carbon dioxide found during the first week in the normal cheese. In the case of the chloroformed cheese, a certain amount of the milk-sugar had undergone fermentation before chloroform was added, as shown by a determination of the sugar in the curd the day after the cheese was made. The amount found was low compared with the amount present in perfectly fresh curd. Such fermentation would produce small amounts of carbon dioxide, which would be absorbed by the milk and pass into the cheese mass. Carbon dioxide thus enclosed in a cheese would again be given out into an atmosphere such as was present in the bell-jar, that is, one free from carbon dioxide.

(3) Respiration of living cells present in cheese as a source of carbon dioxide.—It is well known that living cells give off carbon dioxide as the result of respiration processes. It is also known that in a fresh normal cheese of the cheddar type the number of micro-organisms, generally lactic acid formers, increases rapidly for about 10 days and then after about 25 days falls very rapidly for a period of 10 days to a relatively small number, as shown by Russell and Weinzirl. In any case, we can not look to the respiration processes of living cells in cheese as the source of the carbon dioxide formed after the first few weeks. As regards this possible source of carbon dioxide during the early age of a cheese, when the micro-organisms are present in enormous numbers, we should be justified in expecting that at this time the amount of carbon dioxide produced would be very

much greater than that formed later, when the lactic acid organisms have largely disappeared, if their respiration is the source of any appreciable amount of carbon dioxide. The results secured by us with our normal cheese do not show that there was any such comparatively large amount of carbon dioxide produced at the time the lactic acid organisms were most abundant. The somewhat larger amount of carbon dioxide produced during the first two weeks is undoubtedly due mostly to the decomposition of milk-sugar and not to the respiration process of the living cells present in the cheese. In the case of our chloroformed cheese, we inhibited the activity of living organisms and this source of carbon dioxide did not therefore exist in the cheese.

(4) Chemical decomposition of compounds present in the cheese.—Emerson\(^{11}\) has lately shown that tyrosine, through the action of the enzymes of the pancreas, can be converted into oxyphenylethylamine with simultaneous cleavage of carbon dioxide, in accordance with the following representation of the reaction:

\[
\text{HO.C}_6\text{H}_4.\text{CH}_2.\text{CH(NH}_2\text{)}\text{COOH} = \\
\text{HO. C}_6\text{H}_4.\text{CH}_2.\text{CH}_2(\text{NH}_2)+\text{CO}_2
\]

Langstein\(^{12}\) has also shown the same reaction in the case of a long-continued peptic digestion of the coagulated portion of the blood-serum of a horse.

Ellinger\(^{13}\) has shown the formation of putrescine from ornithine and of cadaverine from lysine, with the splitting off of carbon dioxide, by the action of bacterial ferments. The following equations represent these reactions:

\[
\text{CH}_2(\text{NH}_2).\text{CH}_2.\text{CH}_2(\text{NH}_2)\text{COOH} = \\
\text{CH}_2(\text{NH}_2).\text{CH}_2.\text{CH}_2(\text{NH}_2)+\text{CO}_2.
\]

\[
\text{CH}_2(\text{NH}_2).\text{(CH}_2)_3.\text{CH(NH}_2\text{)}\text{COOH} = \\
\text{CH}_2(\text{NH}_2).\text{(CH}_2)_3.\text{CH}_2(\text{NH}_2)+\text{CO}_2.
\]

Lawrow\(^{14}\) found the same reaction taking place in an intense peptic auto-digestion of the stomach.

\(^{11}\) *Beit. z. chem. Physiol. und Pathol.*, 1: 501 (1902).
\(^{12}\) Ibid. 507
\(^{13}\) *Ber. d. chem. Ges.*, 31: 3183 (1898).
\(^{14}\) *Ztschr. Physiol. Chem.*, 83: 312 (1901).
The work of Nencki and of Spiro\textsuperscript{15} has shown that phenylethylamine can be formed from phenylalanine with separation of carbon dioxide.

Of these different reactions furnishing carbon dioxide, we find in the normal cheese under investigation evidence that tyrosine has changed into oxyphenethylamine and that the decomposition of arginine has resulted in the formation of its simpler products. We cannot say whether Nencki’s reaction occurred, by which phenylalanine was changed into phenylethylamine, since we did not examine the cheese for these compounds. It is easily conceivable that such a change may take place, since E. Fischer\textsuperscript{16} has shown the presence of phenylalanine among the cleavage products of casein. There probably await discovery other similar reactions, now unknown, bearing on the formation of carbon dioxide in proteolytic changes.

It appears to us that the carbon dioxide formed after the first few weeks of ripening, in the case of the normal cheese, must have come very largely from the decomposition of such compounds as tyrosine and arginine. In the case of the different normal cheddar cheeses that we have previously investigated, the arginine and tyrosine commence to undergo proteolytic change quite early in the ripening process.

Reviewing briefly our discussion about the sources of carbon dioxide in cheese, we believe, from the evidence furnished, that the carbon dioxide given off in the early age of the normal cheese came largely from the decomposition of milk-sugar by lactic acid organisms, while a small amount was probably due to the carbon dioxide present in the milk and to the respiration of living organisms present in the cheese. The carbon dioxide produced after the first few weeks could apparently come only from the decomposition of some compounds present in the cheese, among which we were able to identify the change of tyrosine and arginine into derived products with simultaneous formation of carbon dioxide.

In the case of the chloroformed cheese, none of the carbon dioxide could have come from the respiration of living cells or

\textsuperscript{15} Beitr. z. chem. Physiol. und Pathol., 1: 347 (1901).

\textsuperscript{16} Ztsch. Physiol. Chem., 33: 151 (1901).
the decomposition of compounds like arginine and tyrosine. The amount of carbon dioxide originally present in the milk combined with that formed in the milk from the decomposition of milk-sugar before treatment with chloroform was sufficient to furnish the small amount that was given off by this cheese.

CAUSE OF DIFFERENCE IN BEHAVIOR OF NORMAL CHEESE AND CHLOROFORMED CHEESE.

We have seen that, in the chloroformed cheese, only an insignificant amount of carbon dioxide was present and after 3 weeks practically none was found. In the normal cheese, carbon dioxide was found in relatively large quantities and, even at the end of 32 weeks, more carbon dioxide was being formed in one week than the total amount produced in the other cheese. Why should there have been so marked a difference?

The answer to this question involves a consideration of the causes that produce the proteolytic changes observed in the normal cheese-ripening process. The agencies sharing in this work are the following, so far as our present knowledge goes: (1) Some acid, (2) enzymes present in the milk before it is made into cheese, chief of which is galactase, (3) pepsin and pseudopepsin, added with the rennet in the process of cheese-making and (4) micro-organisms, chiefly bacteria.

In the case of our chloroformed cheese, we had present of these different agencies, acid, galactase and rennet-pepsin. These agencies, under the conditions of our experiment, were unable to split carbon dioxide from tyrosine with the formation of oxyphenylethylamine or change arginine into those of its products that we have commonly found in cheese ripening normally. As previously stated, this is the first instance in our knowledge in which arginine has been found in ripening cheese and we were able to find it only because we had inhibited the action of living organisms. These facts suggest strongly that the active cause in our normal cheese that was responsible for the deep-seated proteolysis, accompanied by production of carbon dioxide, was a biological factor.
It may be thought that our results fail to agree with those of Lawrow, cited above, in which he succeeded by an auto-digestion of a stomach in obtaining putrescine and cadaverine, probably with formation of carbon dioxide. It must be kept in mind, however, that in his work the conditions were favorable to a much more intense reaction, because he not only had a highly concentrated pepsin solution but he also kept the acid content of his digesting solution high, conditions that are not present in cheddar cheese.

It may be thought, again, that the activity of the enzymes, galactase and rennet-pepsin, in our one cheese was checked by the chloroform and that we should, under the circumstances, expect just the results we obtained. In Bulletin No. 203 of this Station, we have furnished evidence showing that chloroform does not inhibit the activity of galactase; and we shall later publish results, secured in co-operation with the bacteriological department, confirming our previous work. Lawrow’s work showed that chloroform did not inhibit the activity of a concentrated pepsin solution. In view of the evidence at hand, it appears to us quite improbable that, if chloroform has any inhibiting influence on galactase and rennet-pepsin, we should find these two enzymes, under the conditions of the experiment, able to furnish such end-products as arginine, lysine and tyrosine, but unable to produce compounds resulting from further proteolysis such as putrescine, guanidine and ammonia. If chloroform interfered with the work of these enzymes, we should expect either that there would be no proteolysis or that we should find the same compounds that are formed in the absence of chloroform but in much smaller quantities. As a matter of fact, we find these enzymes quite as active in the chloroformed cheese as in the normal cheese in forming certain compounds but they stop short in their work, appearing unable to produce the further cleavage that results in the production of carbon dioxide. This failure to furnish products beyond a certain point seems to us to depend upon other conditions than the presence of chloroform.

The only logical conclusion suggested by the results of our work appears to us to be that the enzymes, galactase and pepsin are able to furnish such end-products as arginine, lysine and
tyrosine under the conditions existing in cheddar cheese but are not able to split these compounds into simpler ones with simultaneous formation of carbon dioxide. If this is true, then we must look to some other source as the active agency in decomposing primary into secondary proteolytic cleavage products with production of carbon dioxide. The only cause that can be suggested is a biological factor. Several investigators have made a study of the gases in cheese, especially in connection with the formation of holes in emmenthaler cheese and the so-called "huffing" common to hard cheeses; and they have without exception attributed the formation of gases to micro-organisms. Thus, Baumann\textsuperscript{17} found the gas in cheese examined by him to consist of 63 per ct. of carbon dioxide. In this particular case he assigned \textit{Bacillus diatrypticus cascl} as the cause. Later von Klecki\textsuperscript{18} in a similar investigation found gas produced in an inoculated milk containing 31.76 per ct. of carbon dioxide and he attributed its formation to \textit{Bacillus saccharobutyricus}. Adametz, Freudenreich and Weigmann have assigned other organisms. Jensen\textsuperscript{19} suggests that the gas that causes holes in cheese is mostly carbon dioxide and that this comes from the action of lactic acid bacteria upon the nitrogen compounds of the cheese. While no one has probably yet solved the problem as to what specific organism or organisms are responsible for the deep seated chemical changes occurring in cheese, the general tendency has been to look to some biological source as a prominent factor in cheese-ripening.

From the consideration of quite different data, we have previously\textsuperscript{20} arrived at the conclusion that there is a biological factor at work in normal cheese-ripening. In the results presented in this paper and also in the case of a large number of results not yet published, we always find that in a chloroformed cheese, where galactase and rennet-pepsin are the only proteolytic agents present, we never have ammonia formed, while we always find

\textsuperscript{17} \textit{Landw. Versuchsta.}, \textbf{42}: 181 (1892).
it early in normal cheese. In these cases, the only difference appears to be the presence or absence of a biological agent.

What specific organism or combination of organisms may constitute this biological factor in cheese-ripening, we are not now able to say. In co-operation with the bacteriological department of this Station, we have work in progress by which we hope definitely to establish whether these deep-seated chemical changes in cheese-ripening, which can not be attributed to galactase or rennet-pepsin, are due to lactic acid organisms or to liquefying bacteria and their enzymes or to some combination of these.