THE ACTION OF RENNIN ON CASEIN.

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The changes produced by the action of rennin in milk and solutions of casein have been the subject of many investigations. Fremy\(^1\) was probably the first to give an explanation of this phenomenon. He believed the power to coagulate milk possessed by an extract of the mucous lining of a calf's stomach to be due to the presence of an enzyme which converted some of the lactose of the milk into lactic acid, the acid thus formed precipitating the casein.

Hammarsten\(^2\) was the first to show that this coagulation of milk was due to the presence of a soluble ferment which acted directly upon the casein, producing, as he thought, two substances, the insoluble curd, Käse, which we call paracasein and a soluble product which he called whey-protein (Molkeneiweiss). He also showed that the change of casein to paracasein was independent of coagulation, the coagulation being due to the presence of soluble calcium salts.\(^3\)

A great number of papers have been published upon this subject since the early work of Hammarsten.\(^4\) As his explanation of the action of rennin has been generally accepted as correct, most of the recent investigations have been concerned with the influence of soluble salts upon the coagulation. These investigations

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\(^1\) Fremy: Ann. de pharm. (Liebig), xxxi, p. 188, 1839.
\(^2\) Hammarsten: Maly's Jahresbericht, 1872, p. 118; 1874, p. 135; 1877, p. 158.
\(^3\) See also Arthus and Page: Arch. de physiol. (5th series), ii.
\(^4\) An excellent review of the literature with references may be found in Bulletin 56 of the Hygienic Laboratory of the Public Health and Marine Hospital Service of the United States.
have shown that the soluble salts of calcium, barium and strontium favor or hasten coagulation while salts of ammonium, sodium and potassium retard or inhibit coagulation.

Recently Van Slyke and Bosworth have shown that casein and paracasein are acids having the same percentage composition; that the molecular weight of casein is probably 8888, while the molecular weight of paracasein is one-half that of casein; that both have a combining equivalent of 1111; that combinations of casein or paracasein with one equivalent of calcium, barium or strontium are insoluble in water while the combinations with one equivalent of ammonium, sodium or potassium are soluble; and that ammonium, sodium or potassium caseinates can be changed by rennin to paracaseinates which are soluble and are precipitated by calcium chloride as calcium paracaseinates.

These facts would seem to indicate three things:

First, that rennin action consists of the hydrolytic splitting of the casein molecule into two similar molecules of paracasein; perhaps in somewhat the same manner that maltose is split into two molecules of dextrose.

Second, that, as a consequence of this cleavage it would seem to be doubtful if Hammarsten's whey-protein could be one of the products of rennin action.

Third, that rennin is not, strictly speaking, a coagulating ferment, the coagulation of paracasein being due to the fact that calcium paracaseinates are less soluble than the calcium caseinates, especially in the presence of soluble salts of calcium, barium or strontium.

This investigation was undertaken as an attempt to determine the truth of these statements. In repeating the work of Hammarsten and others a soluble substance which had not been coagulated by rennin and could not be precipitated by dilute acetic acid was always found in the filtrate. Casein solutions for such investigations have been prepared, as a general rule, by shaking pure casein with an excess of lime water or by grinding with moist calcium carbonate. The casein solutions thus obtained were made neutral to litmus and coagulated by the addition of rennin. The curds were filtered off and the filtrates examined for nitrogen.

Soluble nitrogen was always found, but the amounts were not constant and seemed to have no relation to the amounts of casein or rennin used. In the control experiments, to which no rennin had been added, similar amounts of nitrogen which could not be precipitated by dilute acetic acid were also found.

Caseinate solutions prepared in the manner described, contain basic caseinates, either neutral or alkaline to phenolphthalein. As Robertson has shown that such caseinates in solution undergo an autohydrolysis, the following experiment was carried out in order to determine if this might account for the soluble nitrogen found.

Five grams of casein were dissolved in 250 cc. of $\frac{N}{10}$ calcium hydroxide in the presence of toluol. After complete solution of the casein portions of the solution were withdrawn at intervals and the casein precipitated by means of dilute acetic acid. The casein was filtered off and the nitrogen in the filtrates determined by the microchemical method devised by Folin. The results are as follows:

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Milligrams of nitrogen in original solution</th>
<th>Milligrams of nitrogen not precipitated by rennin</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>158</td>
<td>4.0</td>
</tr>
<tr>
<td>15</td>
<td>158</td>
<td>10.0</td>
</tr>
<tr>
<td>24</td>
<td>158</td>
<td>28.8</td>
</tr>
</tbody>
</table>

Results of the same nature were obtained with solutions made by grinding casein with moist calcium carbonate. The extent of this autohydrolysis, temperature being constant, depends upon time. As dry casein goes into solution very slowly and freshly precipitated casein is quite rapidly redissolved the following procedure was adopted in order to circumvent autohydrolysis.

Ten grams of pure dry casein were dissolved in 500 cc. of $\frac{N}{10}$ calcium hydroxide. The casein was then precipitated by adding about 250 cc. of $\frac{N}{2}$ acetic acid, the liquid was siphoned off, the casein washed several times with water, placed in a linen bag and squeezed as dry as possible. It was then transferred to a mortar, ground to a paste with a little water, the paste put into

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6 Robertson: this Journal, ii, p. 344; see also Osborne: Journ. of Physiol., xxvii, p. 398.
7 Folin and Farmer: this Journal, xi, p. 493. All nitrogen determinations made in this paper were made by this method.
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a flask and 150 cc. of water, 75 cc. of lime water and some toluol were added to it. After considerable shaking the lime water became saturated with casein. By this process a solution was obtained containing a calcium caseinate neutral to litmus but acid to phenolphthalein, and containing four equivalents of base. The undissolved casein was removed by centrifuging and filtering. The amount of casein in solution was determined and the solution so diluted that each 50 cc. contained 1 gram of casein. Fifty cc. portions of this solution were withdrawn at intervals and precipitated with acetic acid. The amounts of nitrogen found in the filtrates were as follows:

<table>
<thead>
<tr>
<th>Milligrams of nitrogen in original solution</th>
<th>30 minutes</th>
<th>5 hours</th>
<th>12 hours</th>
<th>24 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milligrams of nitrogen not precipitated by acetic acid</td>
<td>0.07</td>
<td>0.92</td>
<td>1.96</td>
<td>2.00</td>
</tr>
</tbody>
</table>

Casein solutions prepared in this manner gave the following reactions. They were not coagulated by rennin. The addition of a few drops of a 10 per cent solution of calcium chloride caused them to curdle; the addition of one drop caused no change but the subsequent addition of rennin produced coagulation. If enough hydrochloric acid was added to change the caseinate to one containing two equivalents of calcium, the addition of rennin caused coagulation. That this coagulation was not due to the calcium chloride formed by the acid was shown by the fact that rennin caused coagulation after all this calcium chloride had been removed by dialysis. In both instances the coagulation removed all the nitrogen from the solution, as is shown by the following figures:

<table>
<thead>
<tr>
<th>Milligrams nitrogen in original solution</th>
<th>Milligrams nitrogen not precipitated by rennin</th>
</tr>
</thead>
<tbody>
<tr>
<td>316</td>
<td>0.8</td>
</tr>
<tr>
<td>316</td>
<td>0.6</td>
</tr>
<tr>
<td>316</td>
<td>0.2</td>
</tr>
</tbody>
</table>

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9 Robertson: ibid., ii, p. 381. Robertson believes that the addition of the common Ca ion represses the dissociation of the caseinate and thus causes precipitation.
The behavior of such caseinate solutions towards rennin can be explained by the work of Van Slyke and Bosworth as follows:

A molecule of calcium caseinate containing four equivalents of base is split by rennin into two molecules of paracaseinate, each containing two equivalents of base. Such a paracaseinate is soluble in pure water but insoluble in the presence of more than a trace of a soluble calcium salt. A molecule of calcium caseinate containing two equivalents of base is split by rennin into two molecules of paracaseinate each containing one equivalent of base. Such a paracaseinate is insoluble in pure water.

The small amounts of nitrogen recovered in the filtrates in the experiments given above may be due to autohydrolysis or to proteolysis produced by the pepsin in the rennin extract used, as is indicated by the following experiment.

Into each of several flasks were placed 50 cc. of a casein solution and a little toluol. One-half of the flasks received a few drops each of rennin solution, the others being kept as controls. The contents of the flasks were examined at intervals for autohydrolysis and proteolysis. The nitrogen in the control flasks which was not precipitated by acetic acid was considered as due to autohydrolysis; while in the case of the other flasks the nitrogen not removed by filtering was considered to be due to autohydrolysis and proteolysis. By subtracting the nitrogen found in the controls from those containing rennin a fair idea as to the extent of the proteolysis might be obtained.

<table>
<thead>
<tr>
<th>Nitrogen Analysis</th>
<th>30 minutes</th>
<th>12 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial nitrogen</td>
<td>158</td>
<td>158</td>
</tr>
<tr>
<td>Filtrate from rennin flasks</td>
<td>3.4</td>
<td>18.2</td>
</tr>
<tr>
<td>Filtrate from control autohydrolysis</td>
<td>0.1</td>
<td>2.1</td>
</tr>
<tr>
<td>Nitrogen due to proteolysis</td>
<td>3.3</td>
<td>16.1</td>
</tr>
</tbody>
</table>

Solutions of ammonium, sodium or potassium caseinates containing two or more equivalents of base could not be coagulated by rennin, but the subsequent addition of calcium chloride caused coagulation, the curd being calcium paracaseinate. That sodium caseinate in solution was changed to sodium paracaseinate was shown by the following experiment. Rennin was added to a solu-
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The action of sodium caseinate and after a short time acetic acid was added. The precipitate, after being purified and dried, was found to be paracasein.

The conclusions drawn from this investigation are as follows:

A solution of calcium caseinate neutral to litmus and free from all other salts is not curdled by rennin.

A solution of calcium caseinate acid to litmus, which contains two equivalents of base for each molecule of casein, is curdled by rennin.

Solutions of ammonium, sodium or potassium caseinates are not curdled by rennin. In such solution however the casein is changed to paracasein, the paracaseinates of these bases being soluble.

When paracasein is produced from casein by the action of rennin no other substance is formed. Two molecules of paracasein are produced from each molecule of casein as a result of this action.

Rennin is not, strictly speaking, a coagulating ferment; the coagulation being a secondary effect, the result of a change in solubilities.

Rennin action is probably a hydrolytic cleavage and may be considered the first step in the proteolysis of casein. It would follow from this that the action now attributed to rennin may be produced by any proteolytic enzyme. Work along this line is being carried out by the author.

In the light of the results reported in this paper together with those of Van Slyke and Boesworth the retarding action of soluble salts of ammonium, sodium and potassium on the coagulation of milk or casein solutions by rennin may be explained as follows. The addition of salts of these bases to milk or casein solutions results in a double decomposition whereby the calcium caseinate is changed to a caseinate of the base added. These are converted to paracaseinates by rennin, but owing to the fact that all the paracaseinates of these bases are soluble, no coagulation results.

In conclusion I wish to express my appreciation of the interest in this work shown by Dr. L. L. Van Slyke, of the Chemical Laboratory of the New York Agricultural Experiment Station, Geneva, N. Y., and Dr. Otto Folin of the Biochemical Laboratory of the Harvard Medical School, Boston, Mass.
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