CHARACTERIZATION OF RENNIN

by

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CHARACTERIZATION OF RENNIN

Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Introduction</td>
<td>1-2</td>
</tr>
<tr>
<td>II. Literature Review</td>
<td>3</td>
</tr>
<tr>
<td>A. Crude Rennin</td>
<td>3</td>
</tr>
<tr>
<td>B. Crystalline Rennin</td>
<td>4</td>
</tr>
<tr>
<td>C. Improvement of Rennet Preparations</td>
<td>7</td>
</tr>
<tr>
<td>D. The Mechanism of Milk Coagulation by Rennin</td>
<td>9</td>
</tr>
<tr>
<td>E. Amperometric Titration</td>
<td>11</td>
</tr>
<tr>
<td>III. Experimental Methods</td>
<td>12</td>
</tr>
<tr>
<td>A. Fractional Precipitation of Commercial Rennet Extract with Organic Solvents at 20°C</td>
<td>12</td>
</tr>
<tr>
<td>B. Rennin Activity Determination</td>
<td>14</td>
</tr>
<tr>
<td>C. Proteolytic Activity of Rennin in Substrate</td>
<td>15</td>
</tr>
<tr>
<td>D. Proteolytic Activity of Rennin in Cheddar Cheese</td>
<td>16</td>
</tr>
<tr>
<td>E. Kjeldahl Nitrogen Determination</td>
<td>17</td>
</tr>
<tr>
<td>F. The Effect of Different Storage Temperatures on Rennin Powder</td>
<td>17</td>
</tr>
<tr>
<td>G. Freezing Experiment on Rennin Preparations and Commercial Rennet</td>
<td>18</td>
</tr>
<tr>
<td>H. Observation of Milk Coagulation by Rennin Under the Electron Microscope</td>
<td>18</td>
</tr>
<tr>
<td>I. Paper Electrophoresis</td>
<td>19</td>
</tr>
<tr>
<td>J. Moving Boundary Electrophoresis</td>
<td>21</td>
</tr>
<tr>
<td>K. Amperometric Titration</td>
<td>21</td>
</tr>
<tr>
<td>IV. Results.</td>
<td>25</td>
</tr>
<tr>
<td>V. Discussion</td>
<td>33</td>
</tr>
<tr>
<td>VI. Summary and Conclusion</td>
<td>41</td>
</tr>
<tr>
<td>VII. Tables</td>
<td>44-57</td>
</tr>
<tr>
<td>VIII. Figures</td>
<td>58-78</td>
</tr>
<tr>
<td>IX. Bibliography</td>
<td>79-94</td>
</tr>
</tbody>
</table>
CHARACTERIZATION OF RENNIN

INTRODUCTION

Although the rennin action on milk has been investigated for more than half a century, the real mechanism of milk coagulation by rennin remains unsolved. It is generally accepted that the calcium caseinate is changed to calcium paracaseinate by rennin. Casein and paracasein are similar in many respects except that the latter has greater alkaline-binding capacity than the former.

It is a well-known phenomenon that proteolytic enzymes, such as pepsin, trypsin and papain, also can coagulate milk. It is not known, however, if they change casein to paracasein.

Commercial rennet is extracted from calves' stomachs and usually contains both rennin and pepsin. The so-called purified rennin made from commercial rennet probably also includes both enzymes. However, the optimum pH for proteolytic activity of pepsin is about 2, and at a pH less acid than 4, the proteolytic activity of pepsin rapidly approaches zero. According to recent studies the isoelectric point of pepsin is less than pH 1, but the isoelectric point of rennin is much higher than pepsin. However, recently a few investigators have been able to detect the proteolytic activity in rennin.
In the last decade, a number of investigators such as Hankinson, Berridge and Woodward, De Baun, Alais, Hostettler and Stein, Oheda and Kasai, have obtained crystalline rennin from commercial rennet, but the purification methods are rather complex and time consuming, the yield is rather poor and the crystalline preparations appear to contain more than one component when examined by electrophoretic methods.

With these considerations in mind it was determined: first, to devise a simplified and time-saving method of obtaining good yield of purified rennin; second, to determine the purity of purified rennin by electrophoretic methods and to investigate further the phenomenon of milk coagulation by rennin; third, to discover, if possible, the real mechanism of milk coagulation by rennin.
LITERATURE REVIEW

A. Crude Rennin

Tauber and Kleiner (86, 156, 157, 158) stated that rennin, like pepsin, is precipitated by saturated solutions of such neutral salts as NaCl, MgSO₄, and (NH₄)₂SO₄. It is for this reason that pepsin-free rennin preparations were never obtained by the salting out methods. They extracted the rennin from calves' stomachs by a 50% alcoholic solution at pH 5.4. They regarded the precipitated rennin as a thioproteinase, and found that it could be dried under vacuum over sulfuric acid, although drying inactivated about half of the enzyme. Their most active preparation had an activity of 1.4,550,000 when skim milk with added calcium chloride was used as a substrate. For example, 4,550,000 mg. of milk was clotted in 10 minutes by 1 mg. of rennin powder at 40°C. A yield of about one gram of dry substance was obtained from one kilogram of mucosa. The product contained 14.4% nitrogen, 1.19% sulfur. Tauber and Kleiner found that the isoelectric point of this product in 50% alcoholic solution was at a pH of 5.4.

In 1953, Murti and Subrahmanyan (113) purified the milk-clotting enzymes of Ficus carrica and Ram and Reddi (136) of Streblus asper by the fractional precipitation by Tauber's technique.
B. Crystalline Rennin

Hankinson's procedure for preparing crystalline rennin (61, 62) is to salt out the rennin from commercial rennet extract by adjusting its pH to 5.0, saturating with NaCl and removing the precipitated rennin by centrifuging. This precipitate, now free from the major portion of the non-rennin material of the extract, is dissolved in water of half the volume of the original extract, the pH is adjusted to 5.7-6.0, and the solution is filtered; the rennin is reprecipitated from the clear filtrate as before, by adjusting the pH to 5.0 and saturating with NaCl. This process is repeated, each time dissolving the precipitate in half the preceding volume, until the fourth precipitate is obtained. The latter is suspended in water and dialyzed against running distilled water, using a cellophane tube, until free from NaCl. The dialyzed solution of rennin is diluted to about 0.05% solids, and adjusted to pH 5.7-6.0. This is followed by slow addition of N/10 HCl at 20° to 25°C until pH 4.5 is reached. The crystals were of microscopic dimensions and were needle shaped. On a solids basis, the activity of crystalline rennin was 6 to 7 times that of a dialyzed salt-free commercial extract, and 18 to 21 times that of the commercial extract when the comparison is made on the basis of total nitrogen content. The pure rennin
contained 15.0% of nitrogen, 1.46% of sulfur and 0.041% of phosphorus and exhibited the properties of a globulin. It was 99.77% "pure" from the standpoint of peptic activity. Its isoelectric point in 16.7% NaCl solution was pH 4.5.

One part of dry rennin coagulated 72,300,000 parts of fresh raw skim milk at pH 5.75-5.80 in ten minutes at 40°C which was 4.55 times that of the dry organic matter of the original extract.

R. M. De Baun et al (42) prepared crystallized rennin by a simplified procedure, somewhat modified after Hankinson's technique. The over-all yield was approximately 5%. Electrophoretic analysis revealed the presence of three components in the crystalline rennin. The major component (25%) had both milk-clotting and proteolytic activity. The minor components did not clot milk. The ultraviolet absorption spectrum of the enzyme indicated the presence of tyrosine in the molecule.

Hostettler and Stein (74) prepared crystalline rennin from commercial rennet powder by a modification of the method of De Baun et al (42). The crystals contained 76.0 to 79.8% of protein; their milk coagulating activity was rather less than that of crystals prepared from rennet extract.

Berridge's procedure (15) for preparing crystalline rennin is to adjust the pH of commercial rennet to 5.4 and
saturate with NaCl. The supernatant is filtered for four days and the precipitated proteins are extracted exhaustively with distilled water for four times. The combined extracts are filtered and adjusted to pH 5.4 and again saturated with slow-running NaCl solution through Visking tubing for two days. The granular precipitate is then centrifuged down and redissolved in a small amount of distilled water. After standing overnight in the refrigerator, a good crop of crystals is obtained. By this procedure Berridge obtained a 12% yield of crystals. The crystals contained 13% Kjeldahl nitrogen and clotted approximately $10^7$ times their dry ash-free weight of reconstituted milk (12 grams spray-dried skim milk powder in 100 ml. N/50 CaCl$_2$ solution) in 10 minutes at 37°C. They had high proteolytic activity.

Alais et al (1, 2) prepared crystalline rennin successfully by a modification of the Berridge method. They found that the curdling of milk by their crystalline rennin caused an increase in nitrogen soluble in 12% trichloroacetic acid (NPN). This also was true for commercial rennet. A specific degradation reaction was involved. The NPN consists of more than one peptide from the alphacasein only.

Oheda and Kasai (123) extracted rennet powder with 13-16% of CaCl$_2$ to obtain the crystalline rennin. The crystals melted at 225°C - 227°C, were readily soluble
in water and their proteolytic activity was very slight. In December, 1955, they claimed that they obtained purified rennin from their crude rennin preparations by repeated recrystallization or by washing the first crystallized rennin crystals with 0.5 N HCl solution. It contained a single protein fraction. The mobility of the purified rennin crystals was almost the same as that of the alpha-globulin prepared from the blood serum of the calf from which rennin was prepared. The yield of rennin crystals from rennet was about 17 to 50 per cent of its milk-clotting power (124, 125, 126). The isoelectric point of their purified rennin was 4.8.

C. Improvements in and Relating to the Manufacture of Rennet Preparations.

It is reported that small quantities of milk at 35°C were coagulated by a dried rennet prepared from the stomachs of reindeer. Rennet from stomachs of 4-5 month old animals was found to possess maximum coagulating power (171).

A sterile, stable rennet powder which is practically free from pepsin was prepared by Benger's Food Ltd. (159) from the usual brine (6% NaCl) extract by adjusting the pH to 1.5 with HCl or other suitable acid, allowing to stand for 2 or 3 days, and then removing and drying the precipitate.
The moist precipitate of rennet which is obtained by salting out contains about 70% of moisture (160). It can be dried by mixing with an efflorescent salt, such as sodium sulphate or magnesium sulphate. The pH is adjusted to about 7 with di-potassium hydrogen phosphate or di-sodium hydrogen phosphate. The dry product is suitable for making into tablets.

The clarification of rennet extracts is achieved (50) by the addition of 5-10% (by weight) of finely chopped leaves or grass (or other juices) instead of the conventional precipitation of slimes and filtration. The mechanism of the clarification process, which extends over about 10-20 days is not explained.

A method for obtaining rennet extracts from calves’ stomachs was described (49). The extraction fluid is prepared by dissolving 15 kg. of pure calcium nitrate in 85 liters of water, and by the addition of acids such as boric acid in order to bring the pH to 5-6. This quantity is claimed to suffice for extraction of 200 sliced calf stomachs.

A rennet extract is divided into two or more parts, each solution containing an added substance (e.g. NaH₂PO₄, CaCl₂, etc) which will react with the substances in the other solutions. Their protein contents are adjusted to about 4 per cent, and acidified to pH 4-5. On mixing the
solutions, the active principles of the rennet are adsorbed on to the freshly-formed surfaces of the inert, readily filterable precipitate produced from the added substances. After filtration, the pH is adjusted to 7.0-7.5 so that the rennet passes into solution again, and can be separated from the adsorbent by centrifuging or filtration. It is then precipitated by saturation with salt and removed by further centrifuging or filtration (103).

D. The Mechanism of Milk Coagulation by Rennin.

The phenomenon of milk coagulation by rennin has been investigated for a long time. Bosworth stated that rennin action is probably a hydrolytic cleavage and may be considered the first step in the proteolysis of casein. He showed that the soluble salts of calcium, barium and strontium favor or hasten coagulation (109). Richardson (129, 137) summed up some factors influencing the coagulation of milk by rennin such as temperature, acidity, previous treatment and the calcium content of the milk. He stated that rennin might reduce the stability of milk by lowering the charge on the calcium caseinate micelles in suspension in milk to a critical level. Clotting is delayed or even prevented by the removal of the soluble salts by dialysis, or by the addition of oxalate or citrate. Baud et al (11) studied the coagulation of milk by rennet using the electron microscope. They observed that the spherical casein particles of 90-125 μm in diameter
agglomerated, first into fibrils one particle wide, then into bundles of fibrils and finally into a three-dimensional network. The process was compared with the clotting of other proteins, e.g., fibrin. Heinicke (65) claimed that the clotting of milk by rennin or papain is a three-step reaction. The enzyme destabilizes the casein molecule, moderate thermal energy denatures the enzyme-modified molecule, and polyvalent cations crosslink the extended polypeptide chains into a coherent clot.

It has been suggested that imidazole or phenolic groups may be liberated from intra-molecular linkage by the action of rennin (7) and certain electrolytic balance is necessary for clotting (69).

In short, numerous theories, covering widely diversified ideas, have been proposed in explanation of the coagulation of milk by rennin. Typical examples of the ideas embodied in the various theories are: (1) Rennin causes proteolysis of casein into paracasein, (2) Rennin causes association or polymerization of casein molecules into a large paracasein molecule, (3) Rennin removes protective colloids from the surface of casein permitting its precipitation by CaCl₂, (4) Rennin opens secondary valency unions between - COOH and - NH₂ within the casein molecule, (5) Rennin carrying a positive charge combines with casein carrying a negative charge, producing mutual precipitation,
(6) Rennin releases soluble calcium from insoluble calcium phosphate, (7) Rennin reduces the zeta potential of the calcium caseinate and calcium phosphate complex to a critical degree, etc.

No one theory so far advanced is fully adequate, and no one theory is universal or even generally accepted.

The proteolytic and coagulating phenomena of milk by rennin are governed either by one single entity of enzyme or by two different enzymes: the real mechanism of milk coagulation by rennin still remains to be discovered.

E. The Determination of Protein Sulfhydryl Groups by an Amperometric Titration.

In 1946 Kolthoff and Harris (90) introduced a specific amperometric titration method for -SH groups with silver nitrate, which was adapted to the titration of -SH groups in blood serum proteins by Weissman et al (166) and Benech and Benech (114). Murayama has been able to assemble an apparatus for an automatic recording of the amperometric titration curve in determining -SH groups of serum proteins (112). Hutton and Patton (77), and Zweig and Block (170) almost at the same time used Kolthoff's technique to determine -SH groups in milk proteins.
EXPERIMENTAL METHODS

A. Fractional Precipitation of Commercial Rennet Extract With Organic Solvents at 2°C

After a number of preliminary experiments were carried out, a method modified from Tauber's fractional precipitation by ethanol to obtain a rennin concentrate and rennin powder from commercial rennet was adopted. All reagents and equipment used in the following procedure were previously cooled to $\pm 0^\circ$F. (1°C). The procedures were as follows:

1. 50 ml. of Hansen's rennet extract were dialyzed in the cellophane tube against running tap water of $60^\circ$F (15.6°C) for ten hours at a rate of half a gallon of water per minute. This was followed by dialysis against distilled water at $34^\circ$F with a running rate of about one and one-half gallons per hour for another three hours until free from NaCl. The dialyzed rennet was adjusted to pH 5.4 by adding, dropwise, 0.1 N HCl and stirring for eight minutes with a glass rod after which it was allowed to stand 30-60 minutes, or overnight, at $34^\circ$F. The precipitate formed on dialysis was removed by filtering with Whatman filter paper No. 1 or by centrifugation at $34^\circ$F at 2000 r.p.m. for fifteen minutes. The filtrate was then subjected to fractional precipitation with cold ethanol.
2. Fractionation of the filtrate, or supernatants, was carried out in a metal vessel to permit heat exchange. An equal volume (50 ml) of 95% ethanol was slowly added through capillary tubing (15-30 minutes) and permitted to flow down the cold wall of the container while the solution was kept rapidly stirred, avoiding foaming, with a motor agitator. The container was previously frozen in a water bath of one gallon tin can at 0°F (-17.8°C) before use. After all the ethanol had been added, it was allowed to stand for five minutes and then centrifuged at 2000 r.p.m. for fifteen minutes at 34°F. The supernatant (Sup. 1) was again adjusted to pH 5.4 with 0.1 NHCl and poured into the cold container and stirred for about five minutes before adding one-half volume of ethanol as before. After all the ethanol had been added and, after standing for five minutes, followed by centrifuging at 34°F for fifteen minutes, the supernatant (Sup. 2) was again adjusted to pH 5.4 with 0.1 N HCl and poured into the cold container. One-half volume of ethanol was then added. After the ethanol had been added and after standing for five minutes, the mixture was centrifuged at 34°F at 2000 r.p.m. for fifteen minutes in the same centrifuge tube as before. The supernatant (Sup. 3) was discarded, and the combined protein precipitates were dissolved in the same volume of distilled water.
as the original. A sample from this solution was removed and labelled as Fraction I. The remainder was subjected to repeated fractionation as described, yielding Fractions II to V. Fraction V was dissolved in as small an amount of distilled water as possible and dried under vacuum for 24 to 48 hours, depending on the amount of solution to be dried, for indefinite storage.

B. Rennin Coagulating Activity

Rennin activity was determined by a method modified after that of De Baun (42). A milk substrate solution was prepared by dissolving 10 g. of a low-heat skim milk powder in 90 ml. of distilled water and stored at 34°F for 5-24 hours before use. Ten ml. of substrate solution in a 7" x 1" Pyrex test tube was brought to 104-105°F (40°-41°C) in a controlled water bath, one ml. of rennin at the same temperature was blown in from a 1 ml. pipette having a wide tip. The mixture was carefully agitated with a thermometer for ten seconds. Rennin dilutions were made with 3% NaCl. A stop watch was started simultaneously with the mixing of enzyme and substrate. By slanting the test tube slightly from time to time, it was possible to observe the sudden breaking up of the milk film into discrete particles. This denotes clotting. The average of two duplicate tests was taken as a clotting time. The tests had
to agree within ten seconds. The rennin dilutions were adjusted when possible, to coagulate 10 ml. of substrate within 4-6 minutes. Milk clotting activity was estimated as follows:

\[ \text{r.u./ml} = \frac{100 \cdot d}{t} \]

Where \( r.u. \) = rennin units, \( d \) = dilution of the rennin sample, and \( t \) = clotting time in seconds.

C. Proteolytic Activity of Rennin Toward the Substrate

As early as 1913, Bosworth (28) recognized the phenomenon of autohydrolysis or autoproteolysis the extent of which, temperature being constant, was dependent on time. Several test tubes, each containing 10 ml. of substrate solution, were brought to 40-41°C in the water bath used for the rennet activity test. 1 ml. of rennin dilution was added to each tube except the controls. The contents of the test tubes were examined at intervals for autohydrolysis and proteolysis. The nitrogen in the control test tubes which was not precipitated by 10 ml. of 1 N trichloroacetic acid was considered as due to autohydrolysis. In the case of the other tubes the nitrogen in the filtrate from the clotted milk was considered to be due to autohydrolysis and proteolysis. By subtracting the nitrogen found in the controls from those containing rennin, the extent of proteolysis was obtained.
D. Proteolytic Activity by Rennin in Cheddar Cheese

The results of Zimmerman indicate that hard cheese prepared with highly purified rennin ripens essentially in the same way as cheese made with the commercial rennet. Hence the role of rennet in cheese ripening is presumably played by the agent active in the production of clot. Proteolysis by the purified enzyme is strongly inhibited by increase in pH and salt concentration. Zimmerman (169) also pointed out that although the rennet enzymes are of importance in the ripening of Cheddar cheese, and that ripening could, to some extent, be hastened by increasing the amount of rennet, other enzymes, probably of bacterial origin, were also involved in the maturation and the development of the typical flavor. However, the cheese made by Berridge with his crystalline rennin showed soft curd and lacked flavor in comparison with the control (20).

Proteolytic activity by rennin in Cheddar cheese made from Fractions I and V in powder form was measured by Dahlberg and Kosikowsky's method (39). In this method the cheese is extracted with a mixture of acetic acid, sodium acetate, sodium chloride and calcium chloride. The nitrogen in the filtrate, considered as soluble nitrogen, is converted to soluble protein using a factor of 6.38.
E. Kjeldahl Nitrogen Determination

An adequate amount of substance was transferred to a Kjeldahl flask. About seven grams of catalyst (a mixture of anhydrous Na$_2$SO$_4$ and CuSO$_4$·5H$_2$O with a ratio of 10 to 0.2) were added and 25 ml. of concentrated H$_2$SO$_4$ were then added. Digestion was conducted for at least thirty minutes after the mixture became clear. After cooling, the mixture was diluted with 200 ml. of distilled water and 4 pieces of Hengar granules or pumice stone were added. Fifty ml. concentrated sodium hydroxide solution (50%) to make the mixture alkaline, were poured down the side of the flask, so that it did not mix at once with the acid solution. The flask was then connected with distillation apparatus and mixed by rotating. Distillation was carried out until bumping started. Ammonia was received from the condensers immersed in 40 ml. 4% (saturated) boric acid; this was titrated with 0.1 N HCl or H$_2$SO$_4$, using a special indicator of methyl orange and xylene cyanate (1 g. methyl orange plus 1.4 g. xylene cyanate, made up to 500 ml. with 50% ethyl alcohol) or methyl red and bromcresol green indicator (10 ml. 0.1% solution of bromcresol green plus 2 ml. 0.1% solution of methyl red in 95% alcohol).

F. The Effect of Different Storage Temperatures on Rennin Powder

Rennin powder was stored at 8 different temperatures
(-23.3°C, -17.8°C, 1°C, 7.2°C, 10-19°C, 21°-25°C, 30°C, 36.7°C) for four months and the rennin activity was tested at various intervals in the manner described under B.

G. Freezing Experiment on Rennin Preparations and on Commercial Rennet

Fractions I, IV, V, and commercial rennet were frozen at 0°F (-17.8°C) and -10°F (-23.3°C) for a period of eight months. Three different solvents, i.e. distilled water, 0.01 N HCl solution and different levels of NaCl solution were used. Two methods of thawing were used in carrying out the rennin activity. One was to melt the frozen rennin at room temperature, the other was melted at ice water temperature.

H. Observation of Milk Coagulation by Rennin with Electron Microscope

The RCA type EMU electron microscope was used to observe the phenomenon of milk coagulation by rennin. The following technique was used: A shallow circular dish was half-filled with distilled water. Four drops of 3 per cent collodion (nitro cellulose, trade name parlodion) in amyl acetate were dropped on to the surface of distilled water with a small medicine dropper. Ten to twenty-five grids (200 per inch Lektromesh screen, C.O., Jelliff Mfg. Corp., Southport, Conn.) were placed on top of the floating film
and a glass slide was brought to contact the surfaces of grids. As the slide pushes down past the surface, the film folds back against the surface. Finally the slide was reversed, lifted clear of the water, and the excess water was blotted off by touching filter paper to the back and edges. The grids prepared this way had a film thickness of about 300 Å. The substrate was prepared by diluting reconstituted skim milk to 1% dilution with 0.01 M CaCl₂. A platinum loop of this diluted substrate and rennin mixture, taken at different intervals after the addition of rennin, were spread over the surface of grids. These grids were placed on small glass slides, which in turn were placed upon a layer of cotton wetted with 2% solution of osmic acid (in a 1% aqueous solution of chromic acid (Cr₂O₃)), in a small, wide-mouth, closed jar. After standing 5 minutes, the preparation was dried under a table lamp. The milk coagulation phenomenon by rennin of the dried samples was then examined with the electron microscope.

I. Paper Electrophoresis

The paper electrophoretic analysis was carried out according to the method of Durrum (23). The Spinco Model R paper electrophoretic apparatus (Specialized Instruments Corporation, Belmont, California) was used. Filter paper strips moistened with a buffer were placed over the three
glass rods with the ends contacting the paper wicks which were attached to separated, plastic plates and dipped into the electrode vessels. The detailed procedures were carried out according to Spinco Operating Instructions furnished with the instrument. The composition of the different phosphate buffers (55, p. 85) used in the paper electrophoresis are shown in the following table:

<table>
<thead>
<tr>
<th>Volume of Na₂HPO₄ Solution ml.</th>
<th>Volume of KH₂PO₄ Solution ml.</th>
<th>pH of Mixture</th>
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<tr>
<td>8.0</td>
<td>2.0</td>
<td>7.4</td>
</tr>
<tr>
<td>6.0</td>
<td>4.0</td>
<td>7.0</td>
</tr>
<tr>
<td>4.0</td>
<td>6.0</td>
<td>6.6</td>
</tr>
<tr>
<td>0.25</td>
<td>9.75</td>
<td>5.3</td>
</tr>
<tr>
<td>0.10</td>
<td>9.90</td>
<td>5.0</td>
</tr>
<tr>
<td>10.00</td>
<td></td>
<td>4.5</td>
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* 11.876 grams Na₂HPO₄·2H₂O in 1 liter of solution
** 9.078 grams KH₂PO₄ in 1 liter of solution.

The Spinco buffers (barbiturate buffer) were prepared by wetting a mixture of barbital and sodium barbital with about 25 ml. of 95% ethanol in a liter volumetric flask and then diluting to 1 liter with 5% acetic acid. The composition of barbital and sodium barbital in Spinco buffers B-1 and B-2 were as follows:
Spinco buffer B-1, pH 8.6, \( u = 0.05 \)
1.84 g. barbital
10.30 g. sodium barbital

Spinco buffer B-2, pH 8.6, \( u = 0.075 \)
2.76 g. barbital
15.45 g. sodium barbital

J. Moving Boundary Electrophoresis

The moving boundary electrophoretic experiments were carried out with electrophoresis apparatus Model 38 (The Perkin-Elmer Corporation, Norwalk, Connecticut, U.S.A.). A 2-ml. Tiselius cell was used in the usual manner. The specific conductance was determined with the slide wire potentiometer (Leeds & Northrup Co., Philadelphia, Pennsylvania, U.S.A.) using 0.02 M KCl in determining the cell constant. A phosphate buffer pH 6.6 (3.7908 g. of NaH₂P₀₄ and 5.4468 g. of KH₂P₀₄ diluted to 1 liter) of an ionic strength of 0.12 was used. The rennin sample was adjusted to about 1% of protein after dialysis at 10°C for 48 hours against the corresponding buffer solution for electrophoretic analysis. The instructions supplied with the instrument were followed.

K. Amperometric Titration

The method and apparatus employed for the titration of \(-SH\) groups were essentially those of Kolthoff and Harris (90) with minor modifications.

Apparatus. The apparatus is shown in Figure 17. The
rotating indicator electrode, A, consists of a platinum wire about 6 to 8 mm. long and 0.5 mm. in diameter, sealed in 6 mm. soft glass tubing. A simple device for rotating the electrode was arranged using a laboratory motor stirrer and accessory pulleys and chuck. The 6 mm. glass tubing of the electrode was fastened inside a short length of brass tubing which was made to rotate. Electrical contact was made by dipping a No. 18 copper wire into the mercury inside the glass tubing. A reference electrode, B, was used which had a potential of -0.23 volt against a saturated calomel electrode. The electrode solution for the reference half-cell was prepared by dissolving 4.2 grams of potassium iodide and 1.3 grams of mercuric iodide in 100 ml. of saturated potassium chloride solution. The electrode solution was poured into a wide-mouth glass jar. A layer of mercury at the bottom of the jar served as an electrode. Glass tubing with a platinum wire sealed at the tip and containing mercury was inserted into the jar with the tip of platinum contacting the mercury. Electrical connection with the microammeter was made by dipping No. 18 copper wire into the mercury inside the glass tubing. Electrical connection between the reference electrode and the sample was made by connecting a salt bridge, C, to a jar, I, which was filled with saturated potassium chloride and was connected to the reference electrode by
an U-shaped, soft glass tubing. The salt bridge, consisting of about 1-foot of 6 mm. (inside diameter), U-shaped, soft glass tubing, was filled with a gel of 3% agar in saturated potassium chloride. It was changed daily or every other day. To complete the circuit, the two half-cells were short-circuited through a microammeter, D. The No. 18 copper wire in the rotating electrode was connected to the positive pole of the microammeter and that of reference electrode to the negative pole. A Simpson microammeter (Model 29) with a range of 0-25 microamperes was used.

**Procedure.** In preparing samples for titration, the following procedure was adopted: Into a 100-ml. graduate cylinder were placed 1.25 g. of NH₄NO₃, 0.6 ml. of 28% NH₄OH and sufficient distilled water to bring the volume to 15 ml. Ten ml. of milk to be titrated was added. The addition of 25 ml. of 95 per cent ethanol brought the final volume to 47.5% alcoholic solution. This mixture was carefully transferred to a 250-ml. beaker, avoiding foaming. After the salt bridge and rotating platinum electrode were immersed in the solution and the rotating electrode had been rotated for 30 seconds, 0.2 ml. aliquots of 0.001 N AgNO₃ were added through a 5-ml. microburette, each spaced 30 seconds apart. The microammeter responses were noted just before each successive addition. In this way a series of readings were obtained and plotted against the volume
of 0.001 N AgNO₃ used. A straight line drawn through four or five of these points plotted on graph paper and extrapolated to the initial straight line gives the end-point of the titration. The sulfhydryl content in the sample was calculated as m.eq./l. of cysteine. Performance of the platinum electrode was checked before each series of determinations by titration of cysteine-HCl which was prepared according to Murayama's method (112). At least three or four "blank" titrations should be performed from known concentration in order to "warm up" the apparatus.
RESULTS

Table 1 shows the yield and specific activity of different rennin fractions. The final product contained 59.4 r.u. per 1 mg. protein nitrogen, which is ten times that of Hansen's rennet extract. The over-all yield was 62%.

There was no loss of activity of rennin powder dried under vacuum at room temperature for 14-48 hours as shown in Table 2.

Table 3 indicates that the activity of rennin powder stored for four months at temperatures of -10°F (-23.3°C) to 98°F (36.7°C) was 84 to 93% of the fresh powder; the best results were obtained at storage temperatures of -10°F and 34°F (1°C). There was approximately 14% loss when stored at fluctuating room temperature.

Table 4 shows that when distilled water was used as solvent for Fraction I the activity was increased by freezing, the longer the storage the greater the increase in activity. When 5% NaCl was used as solvent, there was a slight increase of activity but 50% of activity was lost when 0.04N HCl was used as solvent after three months of freezing and 91% after seven months of freezing.

Table 5 indicates that the lower the concentration of NaCl solution used as solvent the better the activity recovery. The activity recovery by freezing rennin
concentrate diluted with 2.5% NaCl solution was equal to that with distilled water. A 9% loss of activity occurred with 0.04 N HCl solution as solvent.

Table 6 shows better activity recovery when the frozen rennin was thawed with 2.5% NaCl or distilled water as the solvent in an ice water bath than when thawed at room temperature. When, however, 0.045 N HCl was used as solvent, the reverse effect of the two different methods of thawing was found.

When commercial rennet extract, diluted to approximately 9%, 4.5%, and 2.3% of NaCl was frozen at 0°F, the activity with the 2.3% NaCl dilution was 101% of the initial which was higher than those of 9% and 4.5% NaCl dilution (Table 7).

Two lots of Cheddar cheese were made with rennin powder of Fraction I, IV, and V. The soluble protein of two-month old cheese made from commercial rennin was slightly higher than those made from rennin powders. The soluble protein of Cheddar cheese made from rennin powder of Fraction I was slightly higher than that of Fraction V. However, when cheeses were 130 days old, the cheese made with rennin powder appeared to have a cleaner flavor than those made from commercial rennet extract as scored by four judges (Table 8). Three out of four judges preferred the cheese made from rennin powder.

When the rennin powder solution, adjusted to
coagulate 10 ml. of reconstituted skim milk in five minutes, was added to milk, its proteolytic action had not reached a maximum just prior to coagulation as shown in Table 9. Proteolysis was expressed in terms of the increase in soluble nitrogen in the filtrate from a mixture of 10 ml. skim milk plus 1 ml. rennin dilution plus 10 ml. of 1 N trichloroacetic acid.

Table 10 indicates the effect of length of dialysis against running tap water at 60°F on rennet activity. There was a slight loss of activity when dialyzed up to 13 hours. The longer the dialysis, the higher the loss of activity.

Table 11 shows that the activity of precipitated rennin when diluted with 5% NaCl solution was about 8% higher than that with 0.045 N HCl solution.

Table 12 indicates that the activity of the rennet in the dialyzing sac floating on top of the running tap water in the half-gallon beaker was about 10% lower than that in the sac immersed at the bottom of the beaker.

When the ethanol concentration used to precipitate the dialyzed rennet at 2°C to obtain Fraction I was 77.8%, the recovery of rennet was essentially 100 per cent; but, when the concentration was increased to 85.0%, about 16% activity was lost (Table 13).

When various organic solvents other than ethanol were used to precipitate the rennin protein from commercial rennet extract, the yields before drying are shown in
Table 14 and Figures 2 and 3. The total yields from methanol, propanol-1, propanol-2, acetone and dioxane were 85, 63, 81, 64 and 11.0% respectively.

Table 15 indicates that drying caused no loss of activity of the rennin precipitated by methanol, acetone or dioxane, but did cause losses of about 20% and 30% in those precipitates by propanol-1, propanol-2, respectively.

Table 16 shows the sulfhydryl content of some milk proteins, milks produced at different stages of lactation, rennin and pepsin. The sulfhydryl content of the colostrum milk was several times higher than the other milks and there was some indication that milk of advanced lactation was higher than those of middle lactation. The sulfhydryl content of the unheated reconstituted skim milk agreed well with that of the latter milk, but was greatly reduced by heating. No reactive sulfhydryl group was found in casein, paracasein, alcohol soluble protein from casein or paracasein, rennin, or pepsin.
The paper electrophoresis experiments were not entirely satisfactory, especially in complex systems. The electropherograms were often difficult to interpret. When commercial rennet extract was analyzed, anodewise migration occurred with barbiturate buffer, pH 8.6, u, 0.015, and with phosphate buffer, pH 7.4, u, 0.16, but cathodewise migration occurred with phosphate buffer, pH 6.6, u, 0.12 (Fig. 4).

The migration of Fraction I is shown in Figure 5. With phosphate buffer at pH 7.4 migration occurred anodewise with the formation of a single peak. At pH 6.6 two peaks appeared one on either side of the point of application. With phosphate buffer at pH 4.5 both anodewise and cathodewise migration also occurred, indicating two oppositely charged components. The migration of Fraction IV is shown in Figure 6. Migration occurred anodewise at pH 8.6 and 7.00, two prominent peaks appearing. Both cathodewise and anodewise migration occurred with phosphate buffer at pH 6.6, 5.3 and 4.5. In a preliminary experiment by the moving boundary method, however, Fraction IV with phosphate buffer, pH 6.6, u., 0.12, appeared to contain four components. Because of the lack of availability of the instrument, this approach could not be continued.
The migration of Fractions V and VI is shown in Figures 7 and 8 respectively. In both cases migration occurred anodewise with the formation of a single peak with the pH 8.6 buffer.

Commercial rennet, dialyzed about 10 hours, against cold tap water (60°F) and 3 hours against cold distilled water (34°F) and adjusted to pH 5.4, was subjected to precipitation by different concentrations of ethyl alcohol. The precipitated rennins from 20%, 50%, 60%, 66.7% and 80% of alcohol were analyzed by paper electrophoresis using phosphate buffers of pH 4.5, 5.0 and 6.6. The results are shown in Figures 10-14. It is seen that migration was nil or nearly so at pH 5.0, but that at pH 4.5 and 6.6 two-directional migration occurred.

The electrophoretic patterns of casein, paracasein from rennin, 'paracasein' from pepsin and a mixture of paracaseins from rennin and pepsin were very similar (A, B, C, and D of Fig. 15). The electrophoretic patterns of alcohol-soluble protein from casein (Fig. 15, F) and from paracasein (Fig. 15, G) were slightly different in that the latter appears to have lower mobility. The mobilities were not sufficiently different, however, to produce two distinct bands (Fig. 15, H). All milk proteins mentioned above were run with phosphate buffer at pH 6.6. However, the patterns for pepsin and commercial rennet at pH 6.6 were quite similar (Fig. 15, E and I).
The electrophoretic patterns (Fig. 16) of raw skim milk to which had been added boiled rennin, active rennin, boiled pepsin, or active pepsin were very similar as to the mobility of the major component, assumed to be the caseinate or paracaseinate. Likewise, those of dialyzed skim milk to which the same enzymes had been added were similar. This was to be expected since pH 8.6 is above what is considered effective for rennin action. No definite conclusion can be drawn for it is recognized that paper electrophoresis of skim milk gives erratic results. An improved technique is needed.

Figures 18-21 show the effect of rennin and pepsin action on the -SH groups as determined by amperometric titration. The rennin or pepsin was diluted to cause coagulation in pre-determined times, and at intervals in the pre-coagulation periods, samples of the milk-enzyme mixture were cooled in ice water to arrest enzyme action before titration. When the coagulation time of skim milk by rennin or pepsin was adjusted in less than 10', the -SH groups increased rapidly within 30''-1'30" after the addition of enzyme and then decreased gradually to a minimum just before coagulation would have occurred (Fig. 19, A, B, C and Fig. 21, A). When, however, the coagulation time was adjusted beyond 10', the times required to increase or decrease the -SH groups were irregular (Fig. 18, A, B;
19, D; 20, A, B; and 21, B.). It was very interesting to note that when a dialyzed skim milk was used, and the coagulation time of its equivalent non-dialyzed skim milk by rennin was adjusted to 6'20", the -SH groups increased at first and then decreased. This was followed by another cycle of increase and decrease to a constant value corresponding to the initial value prior to the addition of the enzyme (Curve C, Figure 20).

When reconstituted skim milk, diluted to 1⁄3 with 0.01 N CaCl₂ and spread over the collodion film, previously fixed on the grids, was observed under the electron microscope, many spherical caseinate particles were visualized (Fig. 9, A). Progress of coagulation of this milk by rennin, adjusted to coagulate in 1'50" was followed in the electron microscope. The caseinate particles were seen to agglomerate, first into a network of fibrils, probably one particle wide, and then into condensed bundles. This latter had occurred in 1'30".
DISCUSSION

The over-all yield of the final fraction (Fraction V, Table 1) in the procedure developed for the purification of rennin was about 62% as compared to 5.0, 27.3, and 17-50% obtained by De Baun and Berridge; Hankinson, and Oheda and Kasai, respectively. The procedure is simpler than the others. It takes about 15 hours to finish one experiment not including drying, the chemicals and equipment used are standard and the cost less than that of the other methods. The recovery of activity in powder form from Fraction I was essentially 100% of that in the original commercial rennet extract. This compares with 50% by Tauber's method. When the powder was stored at 98°F for four months, about 16% of the activity was lost, but when stored at -10°F about 7% was lost.

When the precipitated rennin was dissolved in NaCl solution, distilled water, and frozen, the activity was increased. This increase was greatest when distilled water was used as the solvent. The longer the time frozen the higher increase of activity. The higher the concentration of NaCl solution the lower was the increase. A loss of activity was extensive when 0.04 N HCl solution was used as solvent. These results may be due to the destruction by freezing of some kind of inhibitor or inhibitors detrimental to rennin activity;
or the rennin denatured during fractionation is restored or partially restored by freezing in distilled water or weak NaCl solution (Tables 4 and 5).

When commercial rennet extract was dialyzed against running tap water, the longer the dialysis the higher the loss of activity (Table 10). This loss of activity may be due to temporary denaturation. After freezing, the denatured rennin may regain its activity as indicated in Tables 4 and 5. However, the precipitated rennin frozen in higher concentration of NaCl solution seems unable to regain its activity after denaturation. Furthermore, when it was frozen in 0.04 N HCl solution, it not only lost ability for reactivation, but it was further denatured.

When the rennet extract was dialyzed in a cellophane tube against running tap water, the activity of the rennin in the cellophane tube floating on top of the water was about 10 per cent lower than that submerged at the bottom of the beaker. This loss of activity may be due to oxidation by light and air (Table 12).

When crystalline rennin prepared according to Berridge's method was used to make Cheddar cheese, soft curd and defective flavor resulted (2). The cheeses made with rennin powders from Fractions I, IV, and V. were similar to that made from commercial rennet extract except that the soluble protein in the former was slightly lower
than in the latter. Three judges out of four preferred the cheese flavor made from rennin powder to that made from commercial rennet extract.

Moquot et al found that when rennet or crystalline rennin was added to milk, the amount of nitrogen soluble in 12% trichloroacetic acid increased progressively, reaching a maximum before coagulation began. Table 9 shows that proteolysis did not increase to a maximum before coagulation occurred.

When commercial rennet extract and Fraction I and IV were subjected to paper electrophoresis with barbiturate buffer, pH 8.6 and with phosphate buffers, pH 7.4 and 7.0, anodewise migration occurred, but two-directional migration occurred at pH 4.5, 5.3, and 6.6. The same was true of the precipitated rennin from 20, 50, 60, 66.7 and 80% of ethanol at pH 4.5 and 6.6. These results suggest that there may be two components in commercial rennet, one being isoelectric between pH 4.5-5.3, the other between 6.6-7.0.

This may account for the difference of opinion regarding the isoelectric point of rennin, or rennins, held by various workers such as Hankinson (61, 62), Berridge (16, 19), Tauber (156, 157), Richardson (137, 129) and Oheda (123-126), etc.

One peak appeared in the patterns of Fractions V and VI at pH 8.6. This indicates that in the additional purification to obtain Fractions V and VI one component
was lost or changed. As shown in Table I, these Fractions possessed slightly higher specific activity than the other fractions. Additional studies at various pH levels will be required before final conclusion can be drawn. The moving boundary electrophoretic patterns of Fraction IV in phosphate buffer at pH 6.6 were too indefinite to warrant interpretation.

The results of the amperometric titration (Table 16 and Figures 18-21) offer evidence that rennin coagulation of milk may be similar to blood coagulation as visualized in modern theories. The protein, pre-keratin, is soluble and readily digestible and contains mainly cysteine. When cysteine is oxidized in vivo to cystine, the insoluble, indigestible protein, keratin, is formed. On the other hand, the hormone, insulin, is devoid of cysteine but contains a very appreciable quantity of cystine. If only a very small proportion of the cystine is reduced to cysteine, the biological effects of insulin are lost (172). As early as 1934, Kuhnau and Morganstern made the interesting observation that -SH compounds, such as glutathione and cysteine, inhibited the coagulation of fresh guinea pig and human blood, whereas oxidized glutathione accelerated it. Baumberger (12) suggested that the conversion of fibrinogen into fibrin was due to oxidation of the -SH groups of fibrinogen. He believed that thrombin is made
up of two components, thrombin A and thrombin B. Thrombin A catalyzes the liberation of sluggish -SH groups from the fibrinogen molecule, rendering them freely reacting. Thrombin B is thought to be a conjugated protein, perhaps conjugated with vitamin K. Thrombin B, through vitamin K, would then oxidize the -SH groups of fibrinogen activated by thrombin A and convert it into fibrin. Fibrin is considered to be aggregates of fibrinogen molecules linked together through -S-S- bridges. Blocking the -SH groups with alkylation agents would make impossible the formation of these -S-S- bridges (10).

The above reactions have led to the development of several additional theories of blood coagulation (10, 60, 67, 72, 73, 75, 76, 81, 83, 92, 101, 102). They may have application to the clotting of milk by rennin. Of these theories the most important ones were those suggested by Hospelhorn, Cross and Jensen (72); Huggins, Tapley and Jensen (76); Lyons (101) and Mardones, Iglesias, Lipschutz (102). Hospelhorn stated that blood coagulation consists of two reactions. The first was considered to involve an unfolding of the protein molecule and the second an aggregation of the unfolded molecules. The second but not the first reaction, depends on the presence of free -SH groups either in the protein molecules or in simpler organic compounds.
Huggins (76) stated that the aggregation of denatured protein to form a three-dimensional network requires cross-linking. A free -SH group of one albumin molecule reacts with a disulphide group in a neighboring molecule to form an intermolecular disulphide bond at the same time generating a new -SH group. This new -SH group then repeats the process either by reaction with another disulphide group in the first albumin molecule or with a similar group in a third molecule; and so on. Mercaptan interchange reactions of this type are believed (53) to be intermediate in the reversible reduction of disulphides by excess mercaptans. Non-protein sulfhydryl compounds should be equally as effective as albumin in initiating the reaction chain.

Lyons (101) suggested the following scheme for the reaction between thrombin and fibrinogen which is essentially the same theory of Baumberger (12):

\[
\text{Fibrinogen} \rightarrow \text{Thrombin component A} \rightarrow \text{Fibrinogen -SH} \rightarrow \text{Thrombin component B} \rightarrow \text{Fibrin gel}
\]

(Fibrinogen -S-S- fibrinogen)

Mardones et al (102) suggested the equations for a disulphide interchange reaction as follows:
R^1-S-S-R^2+R^3-S-S-R^4 \rightarrow R^1-S-S-R^3+R^2-S-S-R^4
R^1-SH+R^2-S-S-R^3 \rightarrow R^2-SH+R^1-S-S-R^3. In this case, the R^2-SH formed would be available to react with a further disulphide, so that only a catalytic amount of a -SH group could bring about considerable rearrangement.

The milk coagulation by rennin phenomenon observed with the electron microscope and the changes in -SH groups during rennin coagulation as shown amperometrically may be considered similar to that of blood coagulation. If it is so, the rennin may, first, open up or unfold the calcium caseinate, and second, release the -SH groups. When the -SH groups oxidize to the -S-S- bridge, the milk coagulates.

When skim milk had been dialyzed overnight against running distilled water to prevent clotting with rennin, the conditions were standardized so that the same amount of rennin would clot in six minutes and twenty seconds at 39-40°C with the same aliquot of undialyzed skim milk. Amperometric titration for -SH groups showed that the -SH groups increased from .0014 m.eq. in 10 ml. skim milk (.14 m.eq./l.) to .002 m.eq. (0.2 m.eq./l.) expressed as cysteine in 1'30", followed by a decrease to 0.0015 in 5'. This was followed by an increase to 0.0017 in 6', followed in turn by a decrease to original in 11', after which a constant value was maintained (Curve C of Figure 20). From this it seems justified to account for rennin
coagulation after the modern theory of blood coagulation. The results suggest an over-all scheme for milk coagulation by rennin as follows:

\[
\text{Calcium caseinate} - S \text{ (blocked)} + \text{Rennin 'A'} \\
\text{I} \quad \downarrow \\
\text{Calcium caseinate} - SH \\
\text{II} \quad \downarrow \quad \text{Rennin 'B'} \\
\text{Calcium paracaseinate gel}
\]

In reaction I the calcium caseinate molecule has been unfolded so that the -SH groups increase; in reaction II, an aggregation of unfolded calcium caseinate takes place, the -SH groups would be decreased.
SUMMARY AND CONCLUSIONS

1. A simple, time-saving procedure with a minimum of equipment has been developed to partially purify rennin concentrate and reduce it to rennin powder. Six fractions were isolated, the fifth and the sixth being very similar. Fraction V contained 59.4 rennin units per mg nitrogen. This was ten times that of the initial rennet extract. The over-all yield was 62% of the original which is higher than any previously reported. A powdered rennin (Fraction I) having 99.9% of the activity of the initial commercial rennet extract was made.

2. Repeated fractionation resulted in some loss in total activity, the final fraction having only 62% of the original. This activity loss was partially recovered by freezing at -17.8°C and -23.0°C for three months or longer.

3. Rennin powder when stored for four months lost 16% of activity at 36.6°C, 14% at fluctuating room temperature, 10% at 1°C, and 7% at -23°C.

4. Distilled water was found to be the best solvent for the rennin precipitate for freezing; 2.5% NaCl solution was also satisfactory as a solvent. Frozen rennin had higher activity when thawed at ice water temperature
than at room temperature.

5. Although Cheddar cheese made from rennin powder and aged 4 months had a slightly lower soluble protein content than those made from commercial rennet extract, its flavor was superior.

6. The progress of milk coagulation by rennin as followed by the electron microscope shows an agglomeration of caseinate particles, first into a network of fibrils, and then into condensed bundles just before coagulation would occur.

7. Commercial rennet extract dialyzed against running tap water at 15.6°C for 10 hours, and against distilled water for another 3 hrs at 1°C lost none of its activity; beyond that, the longer the dialysis the greater the loss of activity.

8. Rennin appears to be sensitive to light and air oxidation.

9. A concentration of 74% ethanol may be used in the purification procedure without reducing the activity, but when the concentration is increased to 81%, a loss of activity results.

10. Various organic solvents other than ethyl alcohol, such as methanol, propanol-1, propanol-2, acetone and dioxane, etc., may be used to precipitate the rennin; of these methanol was found most satisfactory.
11. Paper electrophoretic patterns of commercial rennet extract using barbiturate buffer at pH 8.6 or phosphate buffer at pH 7.4 revealed two peaks with anode-wise migration and at pH 6.6 only one peak migrated cathodewise.

12. With barbiturate buffer at pH 8.6, and with phosphate buffer at pH 7.0 and 7.4, the migration of Fractions I and IV was anodewise with two prominent peaks appearing. With phosphate buffers at pH 4.5 and 6.6, two components migrated toward the opposite direction. Fractions V and VI, using barbiturate buffer pH 8.6, showed only one peak which migrated anodewise.

13. When rennins precipitated from 20, 50, 60, 66.7 and 80% ethanol were analyzed by paper electrophoresis, using phosphate buffers pH 4.5 and 6.6, a two-directional migration occurred, but the migration was nil or nearly so at pH 5.0.

14. It appears that rennin may include two main components, one has an isoelectric point between 4.5-5.0, the other between 6.6-7.0.

15. Amperometric titration indicates that neither casein, paracasein, nor the alcohol-soluble protein from casein or paracasein, has free -SH groups.

16. The electrophoretic patterns of paracasein from rennin and 'paracasein' from pepsin were similar.
17. The changes in -SH group during milk coagulation by rennin and by pepsin were similar.

18. The mechanism of milk coagulation by rennin may be considered similar to the modern concept of blood coagulation in which a sulphydryl-disulphide chain reaction is involved. It is postulated that two main reactions may be involved in milk coagulation:

The first consists of a rapid unfolding of the calcium caseinate molecules; the second involves slow aggregation with colloidal calcium phosphate to form a gel structure with milk serum as the continuous phase. The serum is later released as whey as the curd shrinks. The over-all scheme for milk coagulation by rennin is suggested as follows:

\[
\text{Calcium caseinate-S (blocked) + Rennin 'A'} \quad \text{I} \\
\downarrow \\
\text{Calcium caseinate-SH \quad II} \\
\downarrow \\
\text{Rennin 'B'} \\
\downarrow \\
\text{Calcium paracaseinate gel} \\
(\text{Calcium caseinate-S-S-Calcium caseinate})
\]

**ADDENDUM:** The following interesting reference was discovered after this thesis was completed. McKerns, K. W. Paper chromatography of casein and rennin. Canadian journal of medical sciences 29:59-62. 1951. The author postulated that rennin appears to cause an unfolding and breaking of the alpha casein molecule with the appearance of several large polypeptides.
### TABLE 1. Activity of Fractions Obtained in Rennin Purification

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Activity /ml.</th>
<th>Total N r.u.</th>
<th>Total N mg.</th>
<th>Specific activity (per mg. N)</th>
<th>Yield or recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hansen's rennet</td>
<td>35</td>
<td>32.7</td>
<td>1144</td>
<td>5.9</td>
<td>206</td>
<td>5.5</td>
</tr>
<tr>
<td>Hansen's rennet after dialysis for 10-13 hrs.</td>
<td>60</td>
<td>16.2</td>
<td>972</td>
<td>1.2</td>
<td>72</td>
<td>13.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I.</td>
<td>60</td>
<td>15.70</td>
<td>942</td>
<td>.4</td>
<td>24</td>
<td>39.3</td>
</tr>
<tr>
<td>Hansen's rennet after dialysis for 10-13 hrs.</td>
<td>50</td>
<td>16.2</td>
<td>810</td>
<td>1.2</td>
<td>60</td>
<td>13.5</td>
</tr>
<tr>
<td>II.</td>
<td>50</td>
<td>12.3</td>
<td>613</td>
<td>.3</td>
<td>14</td>
<td>42.3</td>
</tr>
<tr>
<td>Hansen's rennet after dialysis for 10-13 hrs.</td>
<td>40</td>
<td>16.2</td>
<td>648</td>
<td>1.2</td>
<td>48</td>
<td>13.5</td>
</tr>
<tr>
<td>III.</td>
<td>40</td>
<td>12.6</td>
<td>502</td>
<td>.28</td>
<td>11</td>
<td>44.9</td>
</tr>
<tr>
<td>Hansen's rennet after dialysis for 10-13 hrs.</td>
<td>30</td>
<td>16.2</td>
<td>486</td>
<td>1.2</td>
<td>36</td>
<td>13.5</td>
</tr>
<tr>
<td>IV.</td>
<td>30</td>
<td>11.8</td>
<td>354</td>
<td>0.2</td>
<td>7</td>
<td>53.6</td>
</tr>
<tr>
<td>Hansen's rennet after dialysis for 10-13 hrs.</td>
<td>20</td>
<td>16.2</td>
<td>324</td>
<td>1.2</td>
<td>24</td>
<td>13.5</td>
</tr>
<tr>
<td>V.</td>
<td>20</td>
<td>10.1</td>
<td>202</td>
<td>0.17</td>
<td>3</td>
<td>59.4</td>
</tr>
</tbody>
</table>

\[
\text{r.u./ml} = \frac{100d}{t}
\]

Where r.u. = rennin units, d = dilution of the rennin sample, and t = clotting time in seconds.
TABLE 2. Activity of rennin powder dried under vacuum at room temperature from 14-48 hours.

<table>
<thead>
<tr>
<th>No. of fraction</th>
<th>Wt. of powder</th>
<th>Equivalent volume of concentrate</th>
<th>Total activity in powder</th>
<th>Total activity in equivalent concentrate</th>
<th>Total activity of powder as % of equivalent concentrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.</td>
<td>209.9 mg.</td>
<td>22 ml.</td>
<td>1164 r.u.</td>
<td>1164 r.u.</td>
<td>100</td>
</tr>
<tr>
<td>III.</td>
<td>61.4 mg.</td>
<td>22 ml.</td>
<td>319 r.u.</td>
<td>315 r.u.</td>
<td>101</td>
</tr>
<tr>
<td>IV.</td>
<td>376.0 mg.</td>
<td>61.5 ml.</td>
<td>2151 r.u.</td>
<td>2151 r.u.</td>
<td>100</td>
</tr>
<tr>
<td>V.</td>
<td>99.5 mg.</td>
<td>40.0 ml.</td>
<td>514 r.u.</td>
<td>513 r.u.</td>
<td>100</td>
</tr>
<tr>
<td>VI.</td>
<td>102.5 mg.</td>
<td>40.0 ml.</td>
<td>658 r.u.</td>
<td>656 r.u.</td>
<td>100</td>
</tr>
</tbody>
</table>

TABLE 3. The effect of different temperatures of storage on rennin powder from Fraction I of Lot XXXI.

<table>
<thead>
<tr>
<th>No. of sample</th>
<th>Storage temperature °C</th>
<th>Coagulation* time before storing Seconds</th>
<th>Average of 20, 90, and 120 days in storage Coagulation time Seconds</th>
<th>Activity recovery %</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>98</td>
<td>95</td>
<td>113</td>
<td>84</td>
</tr>
<tr>
<td>7</td>
<td>86</td>
<td>95</td>
<td>108</td>
<td>88</td>
</tr>
<tr>
<td>1</td>
<td>70</td>
<td>95</td>
<td>110</td>
<td>86</td>
</tr>
<tr>
<td>2</td>
<td>58-62</td>
<td>95</td>
<td>110</td>
<td>86</td>
</tr>
<tr>
<td>3</td>
<td>45</td>
<td>95</td>
<td>105</td>
<td>90</td>
</tr>
<tr>
<td>4</td>
<td>34</td>
<td>95</td>
<td>102</td>
<td>93</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>95</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>-10</td>
<td>95</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*The rennin powder dilution was prepared by dissolving 4.8 mg. of rennin powder in 25 ml. of 5% NaCl. 1 ml. of this rennin powder dilution was added to 10 ml. of 10% reconstituted skim milk (10 g. low-heat skim milk powder dissolved in 90 ml. of distilled water) with a wide-tip 1 ml. pipette, at 39-40°C. Then the coagulation time was taken as described under rennin coagulating activity.
TABLE 4. The effect of different solvents on freezing of Fraction I* of Lots III, IV, and V. at 0°F.

<table>
<thead>
<tr>
<th>Lot number</th>
<th>Volume used for freezing</th>
<th>Solvent</th>
<th>Total activity before freezing</th>
<th>Total activity after 3 months at 0°F</th>
<th>Total activity after 7 months at 0°F</th>
</tr>
</thead>
<tbody>
<tr>
<td>III</td>
<td>10 ml.</td>
<td>distilled water</td>
<td>272 r.u.</td>
<td>362 r.u. +33</td>
<td>385 r.u. +42</td>
</tr>
<tr>
<td>IV</td>
<td>10 ml.</td>
<td>distilled water</td>
<td>135 r.u.</td>
<td>189 r.u. +40</td>
<td>202 r.u. +49</td>
</tr>
<tr>
<td>V</td>
<td>10 ml. 5% NaCl</td>
<td>199 r.u.</td>
<td></td>
<td>211 r.u. +6</td>
<td>213 r.u. +7</td>
</tr>
<tr>
<td>V</td>
<td>10 ml. 0.04 N HCl</td>
<td>184 r.u.</td>
<td></td>
<td>92 r.u. -50</td>
<td>16.8 r.u. -91</td>
</tr>
</tbody>
</table>

* Before the rennin activity was tested, the frozen product was thawed at room temperature.

Fraction I of Lots III and IV was obtained by fractionating at room temperature and its yield was 41% of the initial rennet extract before freezing.

Fraction I of Lot V was obtained by fractionating at 2°C. Its yield was 85% of the initial rennet extract before freezing.

TABLE 5. The effect of various concentrations of sodium chloride on the activity of rennin of Fraction IV*, Lot XXXVIII, held at 0°F for 96 days when the frozen rennin was thawed in ice water bath.

<table>
<thead>
<tr>
<th>Level of NaCl</th>
<th>Volume used</th>
<th>Total activity before freezing</th>
<th>Total activity after 96 days of freezing Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>%</td>
<td>10 ml.</td>
<td>r.u.</td>
<td>r.u. %</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>127</td>
<td>131.9 +4</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>127</td>
<td>134.1 +6</td>
</tr>
<tr>
<td>2.5</td>
<td>10</td>
<td>127</td>
<td>138.5 +9</td>
</tr>
<tr>
<td>Distilled water</td>
<td>10</td>
<td>127</td>
<td>138.5 +9</td>
</tr>
<tr>
<td>0.045 N HCl</td>
<td>10</td>
<td>127</td>
<td>115.5 -9</td>
</tr>
</tbody>
</table>

* Fraction IV was obtained by fractionating at 2°C and its yield was 70% of the original rennet extract before freezing.
TABLE 6. The effect of two different methods of thawing the frozen rennin, Fraction I.*, Lot XXXI., stored at 0°F for 96 days.

<table>
<thead>
<tr>
<th>Solvent used</th>
<th>Volume used</th>
<th>Total activity</th>
<th>Total activity</th>
<th>Thawed at</th>
<th>Thawed in</th>
<th>Change</th>
<th>Thawed at</th>
<th>Thawed in</th>
<th>Change</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0°F</td>
<td>-10°F</td>
<td></td>
<td>0°F</td>
<td>-10°F</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ml.</td>
<td>r.u.</td>
<td>r.u.</td>
<td>%</td>
<td>r.u.</td>
<td>%</td>
<td>r.u.</td>
<td>%</td>
<td>r.u.</td>
</tr>
<tr>
<td>5% NaCl</td>
<td>2</td>
<td>52.6</td>
<td>48.8</td>
<td>-7</td>
<td>51.3</td>
<td>-2</td>
<td>49.3</td>
<td>-6</td>
<td>53.5</td>
</tr>
<tr>
<td>Distilled water</td>
<td>2</td>
<td>52.6</td>
<td>49.0</td>
<td>-7</td>
<td>54.4</td>
<td>+3</td>
<td>51.2</td>
<td>-3</td>
<td>55.8</td>
</tr>
<tr>
<td>0.045 N HCl</td>
<td>2</td>
<td>52.6</td>
<td>42.8</td>
<td>-19</td>
<td>42.4</td>
<td>-19</td>
<td>45.0</td>
<td>-14</td>
<td>41.4</td>
</tr>
</tbody>
</table>

* With exception of Lots I to IV, all fractions were fractionated at 2°C.
Table 7. The effect of freezing on commercial rennet extract at 0°F.*

<table>
<thead>
<tr>
<th>No.</th>
<th>Vol. rennet extract added</th>
<th>Distilled water added</th>
<th>Total Volume</th>
<th>Approximate concentration NaCl</th>
<th>Activity before freezing</th>
<th>Activity after 18 days of freezing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ml.</td>
<td>ml.</td>
<td>ml.</td>
<td>%</td>
<td>r.u.</td>
<td>r.u.</td>
</tr>
<tr>
<td>1</td>
<td>5</td>
<td>5</td>
<td>10</td>
<td>9</td>
<td>47.4</td>
<td>237</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>46.7</td>
<td>233.5</td>
</tr>
<tr>
<td>2</td>
<td>2.5</td>
<td>7.5</td>
<td>10</td>
<td>4.5</td>
<td>46.8</td>
<td>117</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>44.9</td>
<td>112.5</td>
</tr>
<tr>
<td>3</td>
<td>1.25</td>
<td>8.75</td>
<td>10</td>
<td>2.3</td>
<td>45.4</td>
<td>58.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>45.8</td>
<td>57.3</td>
</tr>
</tbody>
</table>

* The frozen rennet had been thawed in ice water before the rennet activity was taken.
TABLE 8. Analysis of two-months old cheese.

<table>
<thead>
<tr>
<th>Lot number</th>
<th>Moisture %</th>
<th>T.N. %</th>
<th>T.P. %</th>
<th>S.N. %</th>
<th>S.P. %</th>
<th>S.P. as % of T.P.</th>
<th>Flavor score</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. (1)</td>
<td>36.0</td>
<td>6.1</td>
<td>39.3</td>
<td>1.1</td>
<td>7.3</td>
<td>18.7</td>
<td>36</td>
</tr>
<tr>
<td>I. (2)</td>
<td>36.5</td>
<td>6.3</td>
<td>40.2</td>
<td>1.1</td>
<td>7.0</td>
<td>17.5</td>
<td>37</td>
</tr>
<tr>
<td>II. (1)</td>
<td>37.2</td>
<td>5.7</td>
<td>36.4</td>
<td>1.2</td>
<td>7.4</td>
<td>20.4</td>
<td>35</td>
</tr>
<tr>
<td>II. (2)</td>
<td>36.7</td>
<td>5.8</td>
<td>36.8</td>
<td>1.0</td>
<td>6.4</td>
<td>17.5</td>
<td>36</td>
</tr>
<tr>
<td>II. (3)</td>
<td>37.0</td>
<td>5.7</td>
<td>36.3</td>
<td>0.9</td>
<td>5.8</td>
<td>16.0</td>
<td>36</td>
</tr>
</tbody>
</table>

* I. (1) 13 ml. Hansen's rennet added to 172 pounds of pasteurized whole milk.

I. (2) 0.1084 g. of Fraction IV powder equivalent to 14 ml. of Hansen's rennet was added to 168 pounds of pasteurized whole milk.

II. (1) 16 ml. Hansen's rennet added to 170 lbs. of pasteurized whole milk.

II. (2) 0.1243 g. of Fraction I powder equivalent to 15 ml. of Hansen's rennet was added to 170 pounds of pasteurized milk.

II. (3) 0.0995 g. of Fraction V powder equivalent to 13 ml. of Hansen's rennet was added to 170 pounds of pasteurized whole milk.

All the rennin powders were dissolved in about 200 ml. of 5% NaCl solution before adding to milk.

T.N. = total nitrogen
T.P. = total protein
S.N. = soluble nitrogen
S.P. = soluble protein
### TABLE 9. Proteolytic activity of rennin powder from Fraction IV.*

<table>
<thead>
<tr>
<th></th>
<th>4 minutes</th>
<th>30 minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total nitrogen (T.N.) in original 10 ml. skim milk, mg.</td>
<td>60.2</td>
<td>60.2</td>
</tr>
<tr>
<td>Nitrogen not precipitated with trichloroacetic acid (TCA) in the filtrate from rennin flask, mg.</td>
<td>3.81</td>
<td>4.22</td>
</tr>
<tr>
<td>Nitrogen not precipitated with TCA in the filtrate from control, mg.</td>
<td>3.63</td>
<td>3.78</td>
</tr>
<tr>
<td>Increase in nitrogen due to proteolysis, mg.</td>
<td>0.18</td>
<td>0.44</td>
</tr>
<tr>
<td>Increase in nitrogen due to proteolysis, %</td>
<td>0.29</td>
<td>0.73</td>
</tr>
</tbody>
</table>

* Rennin solution was prepared by dissolving 1.5 mg. of rennin powder of Fraction IV in 25 ml. of 5% NaCl. 1 ml. of this dilution, containing 0.66 mg. nitrogen, was added to 10 ml. of 10% reconstituted skim milk. At 40-41°C, the coagulation time was 4'58". At the end of 4 and 30 minutes, the protein in the samples was precipitated by adding 10 ml. of 1 N trichloroacetic acid (TCA) to the well-mixed sample. The nitrogen of the filtrate as determined by the Kjeldahl method was considered as nitrogen, not precipitated by TCA. In the calculations, allowance was made for nitrogen contained in the rennin.
### TABLE 10. The effect of length of dialysis against running tap water at 60°F on the rennet activity

<table>
<thead>
<tr>
<th>Volume and activity of commercial rennet extract after dialysis for</th>
<th>0 hours</th>
<th>13 hours</th>
<th>17 hours</th>
<th>22 hours</th>
<th>33 hours</th>
<th>43 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ml.</td>
<td>r.u.</td>
<td>r.u.</td>
<td>%</td>
<td>ml.</td>
<td>r.u.</td>
</tr>
<tr>
<td>50</td>
<td>21.0</td>
<td>1050</td>
<td>0</td>
<td>50</td>
<td>20</td>
<td>1000</td>
</tr>
</tbody>
</table>

V. = Volume  
T.A. = Total activity  
A. = Activity  
r.u. = Rennet unit
TABLE 11. The effect of 5% NaCl and of 0.045 N HCl used as diluents on rennet activity

<table>
<thead>
<tr>
<th>No. of fraction</th>
<th>Volume</th>
<th>Amt. of solvent added</th>
<th>Final volume and concentration</th>
<th>Activity / ml.</th>
<th>Total activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ml.</td>
<td>NaCl</td>
<td></td>
<td>r.u.</td>
<td>r.u.</td>
</tr>
<tr>
<td>V (3)</td>
<td>35</td>
<td>35 ml of 10% NaCl</td>
<td>70 ml of 5% NaCl</td>
<td>19.90</td>
<td>1393</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V (3)</td>
<td>35</td>
<td>35 ml of 0.09 NHCl</td>
<td>70 ml of 0.045 NHCl</td>
<td>18.38</td>
<td>1287</td>
</tr>
</tbody>
</table>

TABLE 12. The effect of position of dialyzing sac in a 2-l. beaker against running tap water at 60°F on the rennet activity

<table>
<thead>
<tr>
<th>Position</th>
<th>Final volume</th>
<th>Activity/ml</th>
<th>Total activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ml</td>
<td>r.u.</td>
<td>r.u.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Experiment I.</td>
</tr>
<tr>
<td>Bottom</td>
<td>50</td>
<td>40.6</td>
<td>2028</td>
</tr>
<tr>
<td>Top</td>
<td>50</td>
<td>36.2</td>
<td>1812</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Experiment II.</td>
</tr>
<tr>
<td>Bottom</td>
<td>100</td>
<td>22.4</td>
<td>2240</td>
</tr>
<tr>
<td>Top</td>
<td>100</td>
<td>20.8</td>
<td>2080</td>
</tr>
</tbody>
</table>
TABLE 13. The effect of the concentration of 95% alcohol used in precipitating the rennin on the activity of the rennin.

<table>
<thead>
<tr>
<th>No. of fraction</th>
<th>Volume</th>
<th>Conc. of alcohol used for precipitation</th>
<th>Total activity</th>
<th>Total activity as % of dialyzed extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dialyzed rennet extract</td>
<td>45</td>
<td>%</td>
<td>706</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>85.2</td>
<td>595</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>77.8</td>
<td>706</td>
<td>100</td>
</tr>
<tr>
<td>Fraction</td>
<td>Vol-</td>
<td>Activity</td>
<td>Total</td>
<td>Total</td>
</tr>
<tr>
<td>--------------</td>
<td>------</td>
<td>----------</td>
<td>-------</td>
<td>-------</td>
</tr>
<tr>
<td></td>
<td></td>
<td>per to-</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ml tal</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>r.u.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dialyzed rennet extract</td>
<td>40</td>
<td>17.9</td>
<td>717</td>
<td>44</td>
</tr>
<tr>
<td>Precipitates from methanol*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50%</td>
<td>10</td>
<td>13.3</td>
<td>132</td>
<td>2.2</td>
</tr>
<tr>
<td>66.7%</td>
<td>10</td>
<td>35.5</td>
<td>355</td>
<td>4.4</td>
</tr>
<tr>
<td>77.8%</td>
<td>10</td>
<td>12.1</td>
<td>121</td>
<td>9.2</td>
</tr>
<tr>
<td>Precipitates from propanol-1*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50%</td>
<td>10</td>
<td>2.2</td>
<td>22</td>
<td>4.3</td>
</tr>
<tr>
<td>66.7%</td>
<td>10</td>
<td>24.3</td>
<td>243</td>
<td>6.6</td>
</tr>
<tr>
<td>77.8%</td>
<td>10</td>
<td>18.7</td>
<td>187</td>
<td>7.2</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Precipitates from propanol-2*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50%</td>
<td>10</td>
<td>10.8</td>
<td>108</td>
<td>7.4</td>
</tr>
<tr>
<td>66.7%</td>
<td>10</td>
<td>29.4</td>
<td>294</td>
<td>7.6</td>
</tr>
<tr>
<td>77.8%</td>
<td>10</td>
<td>17.9</td>
<td>179</td>
<td>4.3</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Precipitates from acetone*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50%</td>
<td>10</td>
<td>2.6</td>
<td>26</td>
<td>7.0</td>
</tr>
<tr>
<td>66.7%</td>
<td>10</td>
<td>38.5</td>
<td>385</td>
<td>6.2</td>
</tr>
<tr>
<td>77.8%</td>
<td>10</td>
<td>4.6</td>
<td>46</td>
<td>2.3</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Precipitates from dioxane*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50%</td>
<td>10</td>
<td>0.7</td>
<td>7</td>
<td>5.6</td>
</tr>
<tr>
<td>66.7%</td>
<td>10</td>
<td>1.3</td>
<td>13</td>
<td>3.2</td>
</tr>
<tr>
<td>77.8%</td>
<td>10</td>
<td>5.9</td>
<td>59</td>
<td>3.6</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The detailed procedure for fractional preparation had been described (p. 12). 40 ml. of dialyzed rennet extract (continued)
TABLE 14. (continued)

was adjusted to pH 5.4 with 0.1 N HCl precipitated with 50% of organic solvent (about 40 ml.) and centrifuged at 2000 r.p.m. at 10°C. The precipitate (ppt.1) was dissolved in 10 ml. of distilled water and tested for activity. The supernatant (sup.1) was adjusted to pH 5.4 again and precipitated with 66.7% of organic solvent (about 40 ml.) to obtain precipitate 2. The supernatant (sup.2) was adjusted to pH 5.4 and precipitated with 77.8% of organic solvent (about 60 ml.). The precipitate (ppt. 3) was centrifuged and dissolved in 10 ml. of distilled water and tested for activity.

Precipitates 1, 2 and 3 were precipitates from 50, 66.7 and 77.8% of organic solvent respectively.
TABLE 15. The yield of precipitated rennin from dialyzed rennet extract with various organic solvents, before and after drying.

<table>
<thead>
<tr>
<th>Fraction Volume</th>
<th>Before drying Activity per ml</th>
<th>T.A.</th>
<th>Yield %</th>
<th>After drying Activity per ml</th>
<th>T.A.</th>
<th>Yield %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dialyzed rennet extract</td>
<td>40 ml</td>
<td>17.9</td>
<td>717</td>
<td>100</td>
<td>7.7</td>
<td>309</td>
</tr>
<tr>
<td>Precipitates from methanol*</td>
<td>50 %</td>
<td>10 ml</td>
<td>13.3</td>
<td>133</td>
<td>18</td>
<td>11.9</td>
</tr>
<tr>
<td></td>
<td>66.7%</td>
<td>10</td>
<td>35.5</td>
<td>355</td>
<td>50</td>
<td>46.3</td>
</tr>
<tr>
<td></td>
<td>77.8%</td>
<td>10</td>
<td>12.1</td>
<td>121</td>
<td>17</td>
<td>15.9</td>
</tr>
<tr>
<td>Total</td>
<td>85</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Precipitates from propanol-1*</td>
<td>50 %</td>
<td>10</td>
<td>2.2</td>
<td>22</td>
<td>3</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>66.7%</td>
<td>10</td>
<td>24.3</td>
<td>243</td>
<td>34</td>
<td>15.1</td>
</tr>
<tr>
<td></td>
<td>77.8%</td>
<td>10</td>
<td>18.7</td>
<td>187</td>
<td>26</td>
<td>12.4</td>
</tr>
<tr>
<td>Total</td>
<td>63</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Precipitates from propanol-2*</td>
<td>50 %</td>
<td>10</td>
<td>10.8</td>
<td>108</td>
<td>15</td>
<td>8.1</td>
</tr>
<tr>
<td></td>
<td>66.7%</td>
<td>10</td>
<td>29.4</td>
<td>294</td>
<td>41</td>
<td>13.8</td>
</tr>
<tr>
<td></td>
<td>77.8%</td>
<td>10</td>
<td>17.9</td>
<td>179</td>
<td>25</td>
<td>15.8</td>
</tr>
<tr>
<td>Total</td>
<td>81</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Precipitates from acetone*</td>
<td>50%</td>
<td>10</td>
<td>2.6</td>
<td>26</td>
<td>4</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>66.7%</td>
<td>10</td>
<td>38.5</td>
<td>385</td>
<td>54</td>
<td>45.7</td>
</tr>
<tr>
<td></td>
<td>77.8%</td>
<td>10</td>
<td>4.6</td>
<td>46</td>
<td>6</td>
<td>4.3</td>
</tr>
<tr>
<td>Total</td>
<td>64</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Precipitates from dioxane*</td>
<td>50%</td>
<td>10</td>
<td>0.7</td>
<td>7</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>66.7%</td>
<td>10</td>
<td>1.3</td>
<td>13</td>
<td>2</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>77.8%</td>
<td>10</td>
<td>5.9</td>
<td>59</td>
<td>8</td>
<td>4.9</td>
</tr>
<tr>
<td>Total</td>
<td>11</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The detailed procedure for fractional preparation had been described (p. 12).
TABLE 16. The sulfhydryl content of various milks, casein, paracasein, rennin, and pepsin.

<table>
<thead>
<tr>
<th>Product</th>
<th>Sulfhydryl content expressed as cysteine m.eq./l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reconstituted skim milk</td>
<td>0.12</td>
</tr>
<tr>
<td>Reconstituted skim milk after heating to 80°C for 10'</td>
<td>0.06</td>
</tr>
<tr>
<td>Casein</td>
<td>0.00</td>
</tr>
<tr>
<td>Paracasein</td>
<td>0.00</td>
</tr>
<tr>
<td>Alcohol soluble from casein</td>
<td>0.00</td>
</tr>
<tr>
<td>Alcohol soluble from paracasein</td>
<td>0.00</td>
</tr>
<tr>
<td>Rennin</td>
<td>0.00</td>
</tr>
<tr>
<td>Pepsin</td>
<td>0.00</td>
</tr>
<tr>
<td>* Colostrum from cow J281</td>
<td>0.32</td>
</tr>
<tr>
<td>* Whole milk from cow 620</td>
<td>0.16</td>
</tr>
<tr>
<td>* Whole milk from cow 325</td>
<td>0.12</td>
</tr>
<tr>
<td>* Whole milk from cow 531</td>
<td>0.12</td>
</tr>
</tbody>
</table>

* Cow 620 10½ months in lactation
* Cow 325 5½ months in lactation
* Cow 531 5 months in lactation
* J281 calved 1 day before sample was taken.
Figure 1

Paper electrophoretic apparatus (Spinco Model R)
Fig. 2. Percentage yield of rennin precipitated by various organic solvents at pH 5.4 from 40 ml. of dialyzed rennet extract. (13 hrs. dialysis against running tap water.)
FIG. 3. Yield in terms of mg. of nitrogen and mg. of total solids from 40 ml. dialyzed rennet extract precipitated with different concentrations of various organic solvents at pH 5.4.
**Fig. 4.** Paper electrophoretic patterns of commercial rennet extract.
A, Barbiturate buffer pH 8.6, $u = 0.05$, 8 ma, 16 hrs.
B, Phosphate buffer pH 7.4, $u = 0.16$, 5 ma, 16 hrs.
C, Phosphate buffer pH 6.6, $u = 0.12$, 8 ma, 16 hrs.

**Fig. 5.** Paper electrophoretic patterns of Fraction I.
A, Phosphate buffer pH 7.4, $u = 0.16$, 5 ma, 16 hrs.
B, Phosphate buffer pH 6.6, $u = 0.12$, 8 ma, 16 hrs.
C, Phosphate buffer pH 4.5, $u = 0.067$, 8 ma, 16 hrs.
FIG. 6. Electrophoretic patterns of Fraction IV.
A, Barbiturate buffer pH 8.6, \( u = 0.05 \), 8 ma, 16 hrs.
B, Phosphate buffer pH 7.0, \( u = 0.15 \), 5 ma, 16 hrs.
C, Phosphate buffer pH 6.6, \( u = 0.12 \), 8 ma, 16 hrs.
D, Phosphate buffer pH 4.5, \( u = 0.067 \), 8 ma, 16 hrs.
E, Phosphate buffer pH 5.3, \( u = 0.07 \), 5 ma, 16 hrs.
**FIG. 7.** Electrophoretic patterns of Fraction V.  
Barbitur ate buffer pH 8.6, u = 0.05, 8 ma, 16 hrs.

**FIG. 8.** Electrophoretic patterns of Fraction VI.  
Barbitur ate buffer pH 8.6, u = 0.075, 8 ma, 16 hrs.
Observations of rennet coagulation under electron microscope with coagulation time of one minute and fifty seconds both by commercial rennet and purified rennin Fraction IV. A, Control; B, 30 seconds after adding Fraction IV; C, one minute and 30 seconds after adding Fraction IV; D, 30 seconds after adding commercial rennet; E, one minute and 30 seconds after adding commercial rennet.
**FIG. 10.** Paper electrophoretic patterns of rennin precipitated by 20% of ethanol.
A, Phosphate buffer at pH 4.5, \( u = 0.067 \), 8 ma, 16 hrs.
B, Phosphate buffer at pH 5.6, \( u = 0.12 \), 8 ma, 16 hrs.

**FIG. 11.** Paper electrophoretic patterns of rennin precipitated by 50% of ethanol.
A, Phosphate buffer at pH 4.5, \( u = 0.067 \), 8 ma, 16 hrs.
B, Phosphate buffer at pH 5.0, \( u = 0.068 \), 8 ma, 22 hrs.
Paper electrophoretic patterns of rennin precipitated by 60% of ethanol.

A, Phosphate buffer at pH 4.5, 8 ma, 16 hrs, $u = 0.067$.
B, Phosphate buffer at pH 6.8, 8 ma, 16 hrs, $u = 0.12$. 
Fig. 13. Paper electrophoretic pattern of rennin precipitated by 66.7% of ethanol with phosphate buffer.

A, pH 4.5, 8 ma, 16 hrs, \( u = 0.067 \)
B, pH 5.0, 8 ma, 22 hrs, \( u = 0.068 \).

Fig. 14. Paper electrophoretic patterns of rennin precipitated by 90% of ethanol with phosphate buffer.

A, pH 6.6, 8 ma, 16 hrs, \( u = 0.12 \)
B, pH 5.0, 8 ma, 22 hrs, \( u = 0.068 \).
FIG. 15. Continued
FIG. 15. Paper electrophoretic patterns of casein, paracasein, pepsin, alcohol soluble protein from casein and from paracasein.

A, Casein with phosphate buffer at pH 6.6, 8 ma, 16 hrs, \( u = 0.12 \);
B, Paracasein from rennin with phosphate buffer at pH 6.6, 8 ma, 16 hrs, \( u = 0.12 \);
C, Paracasein from pepsin with phosphate buffer at pH 6.6, 8 ma, 16 hrs, \( u = 0.12 \);
D, Mixture of paracaseins from rennin and pepsin with phosphate buffer at pH 6.6, 8 ma, 16 hrs, \( u = 0.12 \);
E, Pepsin with phosphate buffer at pH 6.6, 8 ma, 16 hrs, \( u = 0.12 \);
F, Alcohol soluble from casein with phosphate buffer at pH 6.6, 16 hrs, \( u = 0.12 \);
G, Alcohol soluble from paracasein with phosphate buffer at pH 6.6, 8 ma, 16 hrs, \( u = 0.12 \);
H, Mixture of alcohol solubles from casein and paracasein with phosphate buffer at pH 6.6, 16 hrs, \( u = 0.12 \);
I, Commercial rennet with phosphate buffer at pH 6.6, 8 ma, 16 hrs, \( u = 0.12 \).
Paper electrophoretic patterns of raw skim milk and dialyzed, raw skim milk after adding rennin Fraction IV or commercial pepsin just before coagulation occurs, with barbiturate buffer at pH 8.6, 21 hrs, u = 0.05.

A, Raw skim milk plus boiled rennin;  
B, Dialyzed skim milk plus boiled rennin;  
C, Raw skim milk plus boiled pepsin;  
D, Dialyzed raw skim milk plus boiled pepsin;  
E, Raw skim milk plus active rennin;  
F, Dialyzed raw skim milk plus active rennin;  
G, Raw skim milk plus active pepsin;  
H, Dialyzed raw skim milk plus active pepsin.

Note. Arrow pointing to the right - indicates anodewise migration; to the left - cathodewise. Vertical arrow indicates point of application.
FIG. 17. Apparatus for amperometric titration — -SH group analysis.
A, Rotating platinum electrode;
B, Reference electrode;
C, Salt bridge;
D, No. 18 copper wire connecting to + of microammeter H;
E, No. 18 copper wire connecting to - of microammeter H;
F, Stirrer;
G, 250-ml. beaker;
H, Simson microammeter model 29 with a range of 0-25 microamperes;
I, A jar filled with saturated potassium chloride;
J, U-shaped, soft glass tubing connecting reference electrode with jar, I.
The change of -SH groups during rennet coagulation (Purified rennin Fraction IV in powder form) with pasteurized skim milk.

A, Coagulation time = 18.7 min;
B, Coagulation time = 26.25 min.
The change of -SH groups during rennet coagulation (purified rennin Fraction IV in powder form) with reconstituted skim milk.

- A, Coagulation time = 3'35"
- B, Coagulation time = 6'38"
- C, Coagulation time = 7'30"
- D, Coagulation time = 42'30"
The change of -SH group during rennet coagulation (purified rennin Fraction IV in powder form) with reconstituted skim milk after dialyzing overnight at 34°F room with 5-gallon distilled water.

A, The coagulation time of equivalent non-dialyzed skim milk = 12';
B, The coagulation time of equivalent non-dialyzed skim milk = 26'25'';
C, The coagulation time of equivalent non-dialyzed skim milk = 6'20''.
Fig. 21. The change of -SH groups during pepsin coagulation with reconstituted skim milk
A, Coagulation time = 815;
B, Coagulation time = 13'50".

Time in minutes
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