ISOLATION AND CHARACTERIZATION OF SOME ACTIVE PRINCIPLES FROM COMMERCIAL RENNET EXTRACT

by

UMANATH YESHWANT REGE

A THESIS

submitted to

OREGON STATE UNIVERSITY

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

June 1961
APPROVED:

Professor of Dairy Technology and Dairy Chemistry
In Charge of Major

Head of Food and Dairy Technology

Chairman of School-Graduate Committee

Dean of Graduate School

Date thesis is presented 16th May 1961

Typed by Glenda Lindsay
ACKNOWLEDGEMENT

Sincere appreciation is expressed to Dr. G. A. Richardson for his guidance and constant encouragement during the course of this study. Grateful appreciation is due Dr. J. O. Young for his numerous suggestions and valuable assistance.

Acknowledgement is made to the Department of Food and Dairy Technology for the Graduate Assistantship without which this work would not have been possible.

Thanks are also extended to fellow students, Mr. R. P. Bhatt and Mr. S. S. Kerwar, for their co-operation in developing some of the analytical technics employed.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Review of Literature</td>
<td>3</td>
</tr>
<tr>
<td>Commercial Rennet Extract</td>
<td>3</td>
</tr>
<tr>
<td>Prorennin</td>
<td>4</td>
</tr>
<tr>
<td>Rennin Versus Pepsin</td>
<td>5</td>
</tr>
<tr>
<td>Fractionation, Purification and Crystallization of Rennin</td>
<td>6</td>
</tr>
<tr>
<td>Heterogeneity of Crystalline Rennin</td>
<td>12</td>
</tr>
<tr>
<td>Factors Affecting the Clotting Process</td>
<td>13</td>
</tr>
<tr>
<td>Hydrogen Ion Concentration</td>
<td>13</td>
</tr>
<tr>
<td>Concentration of Calcium Ions</td>
<td>13</td>
</tr>
<tr>
<td>Concentration of Calcium Caseinate and of Colloidal Calcium Phosphate</td>
<td>14</td>
</tr>
<tr>
<td>Temperature</td>
<td>14</td>
</tr>
<tr>
<td>Action of Rennin on Milk</td>
<td>15</td>
</tr>
<tr>
<td>Liberation of Non-Protein-Nitrogen</td>
<td>18</td>
</tr>
<tr>
<td>Electrophoretic and Chromatographic Changes</td>
<td>21</td>
</tr>
<tr>
<td>Experimental</td>
<td>23</td>
</tr>
<tr>
<td>Clotting Activity of Rennin</td>
<td>23</td>
</tr>
<tr>
<td>Agar Electrophoresis</td>
<td>24</td>
</tr>
<tr>
<td>Proteolytic Activity of Rennin Preparations</td>
<td>26</td>
</tr>
<tr>
<td>Fractionation of Commercial Rennet</td>
<td>27</td>
</tr>
<tr>
<td>Section</td>
<td>Page</td>
</tr>
<tr>
<td>---------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>Results</td>
<td>29</td>
</tr>
<tr>
<td>Discussion</td>
<td>38</td>
</tr>
<tr>
<td>Summary and Conclusion</td>
<td>42</td>
</tr>
<tr>
<td>Bibliography</td>
<td>45</td>
</tr>
</tbody>
</table>
Rennin was one of the first enzymes used in the manufacture of food for mankind. For centuries it has been known that milk coagulates when brought in contact with the stomachs of certain young animals. As early as 1050 B.C. the Hebrews and later the old Romans employed the stomachs of young rabbits and goats to make cheese. Homer makes reference to cheesemaking in his Odyssey.

A great number of proteolytic enzymes of both plant and animal origin can coagulate the calcium caseinate-calcium phosphate micelle in milk. The clotting of these micelles by rennin is of special interest in the dairy field. The action of rennin on milk has been investigated for nearly a century, but the precise mechanism by which it acts remains an intriguing problem. It is generally accepted that the calcium caseinate of milk is changed to calcium paracaseinate by rennin. Casein and paracasein have almost the same properties except that paracasein has greater alkali binding capacity and that it is more sensitive to precipitation by cations, such as; manganese, nickel, cobalt, iron, calcium et cetera.

Whether or not rennin is a proteolytic enzyme is a
moot question. Commercial rennet is an extract from the fourth stomachs of young suckling calves. It is usually contaminated with pepsin and other impurities. Pepsin is a proteolytic enzyme. In general, all proteolytic enzymes possess milk clotting power.

The task of determining the real mechanism of rennin coagulation of milk has been further complicated by the fact that none of the purified rennin preparations reported by different investigators is free of other impurities. A number of workers such as Berridge (10, p. 179-186), Hankinson (33, p. 53-62), Alais (3, p. 26-34), Debaun (21, p. 324-338), Foltmann (27, p. 1927-1935) and others have obtained crystalline rennin from commercial rennet extract. When examined by electrophoretic methods, however, these preparations show a heterogeneity in their electrical mobility. A number of other workers have isolated different active principles from commercial rennet extract by various methods. But none of these fractions is free of contamination. These considerations prompted this attempt to isolate and characterize various active principles from commercial rennet extract.
Commercial Rennet Extract

Several methods for the preparation of a sanitary commercial rennet extract are mentioned in the literature (3, p. 26-34 and 8, p. 1087-1088). Veils (trade name for abomasa or fourth stomach), usually dried by inflating or salting, are cut into strips and extracted with a 5-10 per cent sodium chloride solution for not more than five days. Boric acid (2-4 per cent), thymol or glycerol may be added as a preservative. Decantation followed by clarification, filtration or sedimentation, after increasing the salt concentration, complete the process. The prorennin that remains in the filtrate is slowly activated in the presence of hydrogen ions and counters the normal storage losses. The addition of potassium alum followed by its equivalent of potassium phosphate forms a precipitate which carried down all the suspended particles in the extract.

Another method for obtaining rennet extracts from calves' stomachs was described recently by Placek (56, p. 2-8). De-fatted, dried calf stomachs were ground in a hammer mill and extracted for two days with 10 per cent sodium chloride at 35-45° F. at a pH of 5.95-6.05. The prorennin is activated by the addition of 18 per
cent HCl to the extract. This is followed by the addition of propylene glycol, sodium benzoate or propionate as a preservative. Diatomaceous earth is added and the extract is filtered. The bacteriologically-controlled extract is packaged in propylene bags.

**Prorennin**

Very few investigations have been published concerning prorennin, the inactive precursor of the milk-clotting enzyme, rennin.

A method for isolation of prorennin was described by Kleiner (40, p. 755-756). Seventy-five grams of washed and minced mucosa of the fourth stomach of the calf are extracted with 150 ml. of 2 per cent calcium carbonate solution. After filtering, the extract is carefully adjusted to pH 9 to 10 and held at 22° C. for about 40 minutes to destroy any active rennin and pepsin. It is then saturated with MgSO₄. The resulting precipitate is removed by centrifuging, and dissolved in 0.25 per cent MgSO₄. It is re-precipitated in the same way as above. This precipitated prorennin is dried under vacuum over sulfuric acid at room temperature. A water solution of this substance shows milk-clotting properties on activation with hydrogen ions.
Foltmann (26, p. 344) recently described a method for obtaining prorennin. Finely-cut calf stomachs are extracted with 2 per cent NaHCO$_3$ at room temperature with continuous stirring. The tissue is separated by centrifugation and the extract is purified by constant precipitation of impurities with Al$_2$(SO$_4$)$_3$ and Na$_2$HPO$_4$. The preformed activity of this extract was 3.6 RU$^a$/mg N and the activity after activation, presumably by hydrogen ions, was 500 RU/mg N.

**Rennin Versus Pepsin**

Commercial rennet extracts are usually contaminated with pepsin (20, p.324). These enzymes show a similarity in the clotting of milk (63, p. 647). Pepsin is recognized as a proteolytic enzyme (6, p. 79). The optimum pH for proteolytic activity of pepsin is about 2, and its activity ceases above a pH of 4 (10, p. 185). Pepsin has an isoelectric point of approximately 1.7. Rennin differs from pepsin in many respects. The isoelectric point of rennin has been reported as 4.5 (34, p. 277), and the optimum pH for proteolytic activity as at 3.7 (10, p. 186). A typical substrate for

---

$^a$This unit may be defined as the rennin activity which clots 10 ml. of reconstituted skim milk in 100 seconds at 30° C.
pepsin as shown by Laidler (41, p. 38) is carbobenzoxy-L-glutamyl-L-tyrosine and that for rennin (41, p. 184) is glycyl-L-glutamyl-L-tyrosine. Crystalline rennin can also be distinguished from pepsin by Rf values on paper chromatography (51, p. 378). Tsugo et al. (63, p. 647), however, showed that, out of the eight proteolytic enzymes studied, only pepsin behaved like rennin on the basis of Ca/N ratio, P/N ratio, curd tension and electrophoretic experiments. Tauber and Kleiner (62, p. 745-753) demonstrated that crystalline pepsin completely digests highly purified rennin. Trypsin also digests rennin, but not erepsin (54, p. 215). On the basis of the above findings Palmer (54, p. 215) rejected the belief that pepsin is transformed into rennin as the calf grows older.

**Fractionation, Purification and Crystallization of Rennin**

The Berridge method of preparing crystalline rennin (12, p. 255-257) consists of salting out impurities from a commercial rennet extract by saturation with sodium chloride. The supernatant liquid is filtered through large, fluted Whatman number 3 filter paper. The filters are pulped and exhaustively extracted with distilled water. The combined extracts are filtered, adjusted to a pH 5.4, and again saturated with sodium chloride.
The precipitate is centrifuged down and dissolved in water. On standing in the refrigerator a good crop of crystals is obtained.

Berridge reported that the crystals contained 13 per cent Kjeldahl-N and were more soluble in cold salt solutions than in warm salt solutions. They were able to clot, in 10 minutes at 37°C, approximately 10^7 times their dry ash-free weight of reconstituted milk (9, p. 474). The crystals could be dried without decomposition (10, p. 186). They had a high proteolytic activity on hemoglobin as the substrate (10, p. 185). The crystals obtained were flat, block-shaped plates (9, p. 474).

Hankinson (34, p. 277-283 and 33, p. 53-62) prepared crystalline rennin by adjusting the pH of commercial rennet extract to 5.0 and saturating with sodium chloride. The precipitated rennin was removed by centrifugation and dissolved in water using half the volume of the original extract. The pH was adjusted to 5.7-6.0 and the resulting precipitate filtered off. The rennin was re-precipitated from the filtrate by adjusting the pH to 5.0 and saturating with sodium chloride. The whole process of precipitation and dissolving was repeated for four times. The last precipitate was suspended in water and dialyzed against distilled water.
until free of sodium chloride. The dialyzed solution was adjusted to 0.05 per cent solids with distilled water and its pH was brought to 4.5. The crystals obtained were needle-shaped. The activity of the crystalline rennin was 18 to 21 times higher than that of the commercial extract on the basis of total nitrogen content. It was 99.77 per cent "pure" of peptic activity using egg albumin as the substrate. Its isoelectric point was close to pH 4.5. It was reported that the crystals were high in sulfur content, low in phosphorous and copper and free of iron.

Debaun et al. (21, p. 326) prepared crystalline rennin using commercial cheese rennet as the starting material, by a modification of Haninson's technique. The pH of commercial cheese rennet was adjusted to 5.0 and saturated with sodium chloride. The precipitate obtained was dissolved in water using half the volume of the original extract. The rennin was re-precipitated from the solution by adjusting the pH to 5.0 and saturating with sodium chloride. This process of precipitation and dissolving was repeated for four times. The final precipitate obtained this way was dissolved in water and to it 0.1 per cent potassium alum was added followed by an equivalent amount of sodium hydroxide. The resulting Al(OH)₃ precipitate
was centrifuged out. The supernatant was dialyzed against running tap water and filtered. The filtrate was adjusted to 0.1 per cent total solids and allowed to stand overnight in presence of thymol. The crystals obtained resembled those described by Hankinson.

The presence of three components was revealed when the crystalline enzyme was subjected to electrophoretic analysis. The major component comprised 75 per cent of the total and exhibited both milk-clotting power and proteolytic activity with hemoglobin as the substrate. The minor component did not clot milk. Crystalline rennin exhibited an optimum activity for proteolysis against hemoglobin at pH 3.9.

Hostettler and Stein (37, p. 40-46) prepared crystalline rennin from commercial rennet powder. The method was quite similar to that of Debaun et al. (29, p. 324-338). The crystals contained 76.0 to 79.8 per cent of protein. They concluded that the nature of the starting material as well as the method of purification had a marked influence on the properties of crystalline rennin.

Foltmann (27, p. 1927-1935) prepared crystalline rennin by a slight modification of the Berridge method (10, p. 179-186). The yield on recrystallization was 90 per cent. The activity of the recrystallized
rennin varied from 880 RU/mg N to 1000 RU/mg N. The rennin crystallized from these preparations in rectangular plates (27, p. 1930). Two smaller samples, however, crystallized in needles. These latter preparations were stored in a freezer at -15°C and after four months the needle-form crystals had been transformed into rectangular plates. Attempts were made to reproduce the needles but without success. The crystals were reported electrophoretically homogeneous. Solubility tests (28, p. 1936) showed that the enzyme was free of significant contamination.

Ernstrom (23, p. 1663-1670) purified and crystallized rennin from commercial rennet extract by a modification of the method of Debaun et al. (29, p. 324-338). Block-shaped crystals were obtained.

Oeda and Kasai (52, p. 90 and 53, p. 372) obtained crystalline rennin by extracting rennet powder with 13-16 per cent calcium chloride. The crystals showed slight proteolytic activity against alpha-casein, were readily soluble in water and their isoelectric point was pH 4.8. These authors (51, p. 376-378) claimed that the crystals contained a single protein fraction. The electrical mobility of this rennin was similar to that of alpha-globulin obtained from the blood serum of the calf from which the rennin was prepared.

Alais (3, p. 26-34) recently obtained block-shaped
rennin crystals by a method similar to that of Berridge. The crystals were reported as electrophoretically homogenous.

Chuan (18, p. 12-14) purified rennin by a modification of the method of Tauber and Kleiner (62, p. 745-763). Commercial rennet extract was dialyzed against running distilled water. The resulting precipitate was removed by centrifugation and the filtrate was then subjected to various concentrations of alcohol. Five fractions were thus obtained. Fraction number V which was obtained by precipitation with highest concentrations of alcohol from the rennet extract subjected to various treatments, had the highest activity per mg. of N.

Payens (55, p. 595-596) demonstrated by electrophoresis that commercial rennets contain five protein components, the most important of which was chymosine (55%). It was the only one taking part in milk coagulation.

Jirgensons et al. (39, p. 96-102) crystallized rennin by a method similar to Hankinson's (33, p. 53-62). The crystallized rennin was purified and fractionated on diethylaminoethyl cellulose ion exchange column. Of the three major fractions obtained only two showed proteolytic action.
Heterogeneity of Crystalline Rennin

In spite of the fact that nonpurified enzymes cannot be crystallized and that quite small quantities of some impurities prevent crystallization (10, p. 179), the crystalline form is not of itself complete and final evidence of either purity or true crystallinity (9, p. 474). Schwander et al. (59, p. 553) claimed that Berridge's crystalline rennin (7, p. 195) did not behave homogeneously in the ultracentrifuge and diffusion measurements. It, however, showed no heterogeneity in paper electrophoresis experiments.

Ernstrom (23, p. 1663-1670) reported that a solution of crystalline rennin in sodium phosphate buffer, pH 6.8, ionic strength 0.20, showed a single electrophoretic boundary which represented 96 per cent of the total protein. At least four components were evident in the same buffer at 0.033 ionic strength. He concluded that the crystalline form of rennin is not homogeneous.

As previously mentioned, Jirgensons et al. (39, p. 96-102) fractionated crystalline rennin into three major fractions on a diethylaminoethyl cellulose ion exchange column. Of the two fractions that showed any proteolytic activity the fraction with greater
proteolytic activity was homogeneous in the ultracentrifuge.

**FACTORS AFFECTING THE CLOTTING PROCESS**

**Hydrogen Ion Concentration** (8, p. 109; 38, p. 316 and 54, p. 225-228)

In rennin coagulation of milk as the pH is lowered below that of normal milk, the clotting rate increases and a firmer clot is formed. This may be due to the effect of hydrogen ion concentration on the activity of the rennin, the increased dissociation of calcium salts, or to a reduced stability of the caseinate-phosphate complex.

**Concentration of Calcium Ions**

The rate of clot formation can be easily affected by very small changes in the calcium ion concentration in the milk (54, p. 229). Any metallic cation can substitute for calcium in the performance of its functions. Cations of higher valence, however, are more affective (54, p. 233).

Pyne (57, p. 532-535) has shown that the concentration of ionic calcium in the system affects the enzymatic as well as the clotting stage of the reaction.
Concentration of Calcium Caseinate and of Colloidal Calcium Phosphate

The concentration of calcium caseinate is significant at least in determining the character of the clot, a higher concentration of calcium caseinate particles resulting in a firmer curd (54, p. 233). For clot formation on the addition of rennin to a calcium caseinate sol it is essential to have colloidal calcium phosphate present in the system; otherwise only coagulation takes place (54, p. 227). The presence of colloidal calcium phosphate has been considered to sensitize the rennet-altered caseinate to precipitation by calcium ions (45, p. 410).

Temperature

The optimum temperature for rennin coagulation is 38° C. to 40° C. No coagulation occurs below 10° C. or above 65° C. (38, p. 317). Differences observed in rennin clotting time due to variations in temperature may be due to differences in the concentration of calcium ions and of the colloidal calcium phosphate (54, p. 230). If milk is heated at or above 65° C., cooled and allowed to clot with rennin a decrease in the firmness of clot and the rate of coagulation is observed. Milk held at
cold temperatures shows a further decrease in the rate of coagulation (38, p. 317).

**Action of Rennin on Milk**

It has been conclusively proved that rennin does not affect the constituents of milk other than the casein (54, p. 217). Since the pioneer experiment of Hammarsten (32, p. 1-4) it has been generally accepted that rennin acts in two independent stages in the clotting process. The first stage is the rennin action itself or the enzymatic conversion of caseinate to paracaseinate; the second is the precipitation of the altered caseinate by ions such as calcium.

Casein and paracasein are similar in many respects. The differences between casein and paracasein lie in their solubility, acid- and base-binding capacity as indicated by the titration curves with acid and base, and the sensitivity to cations (54, p. 228). Calcium paracaseinate-calcium phosphate sols are not coagulable by rennin.

In the last half-century numerous theories regarding rennin action on milk have been proposed but no one theory is generally accepted.

Hammarsten (32, p. 1-4) as early as 1870 postulated that rennin splits off a small peptide from the caseinate
particle; the residual altered casein being calcium sensitive coagulates. Claesson and Mitschmann (19, p. 341-360) consider that the rapid liberation of non-protein-nitrogen (NPN) by rennin from casein supports Hammarsten's postulation. Cherbuliez (17, p. 959-961) reports that the peptide split from casein is not a degradation product but an integral constituent of casein.

Holter (36, p. 160-188) and Linderstrom-Lang (43, p. 76-78 and 41, p. 1-116) proposed the protective colloid theory. Rennin was supposed to cleave off a particular entity from the caseinate particle which served as a protective colloid for the entire particle. The paracasein so formed is susceptible to coagulation by calcium ions. Gerbullis and Zittle (15, p. 418-420) found that the more calcium insensitive their alpha-casein was, the more acid-soluble nitrogen and phosphorous were liberated during rennin action. These findings led them to support the protective colloid theory.

Richardson and Palmer (58, p. 557-576) showed that rennin caused a lowering of the zeta-potential of the caseinate micelle to a potential critical at the cation concentration of milk. A slow coagulation conducive to clotting results.

Berridge (11, p. 194-195) advanced the denaturation
theory. He showed that the enzymatic stage progressed with a temperature coefficient similar to known enzymatic reactions. The high temperature coefficient of the second stage, however, was similar to that found in heat denaturation of protein. He believed, therefore, that rennin altered the normal folded configuration of the casein molecule by the breaking of secondary bonds. A more definite chemical reaction was proposed by Holter and Li (35, p. 1321) and D'Yachenko (22, p. 629-635) who postulated that rennin cleaves phosphoamide bonds in casein. Fish (24, p. 345) agreed that the phosphoamidase activity was shown by commercial rennet but not by crystalline rennin. On the other hand, Anderson and Kelley (5, p. 182) claimed that there are no phosphoamide bonds (O = P - N) in casein. It may be significant that commercial rennet, but not crystalline rennin, shows phosphatase activity (63, p. 698).

Nitschmann et al. (49, p. 1421-1430 and 44, p. 687-698) suggested that in the enzymatic stage rennin alters certain exposed molecules on the surface of the caseinate particle. Later findings, however, disproved this theory (4, p. 1955-1968).

Mellanby (47, p. 116) suggested that the rennin converts the emulsoid casein into a suspensoid paracasein. This is in agreement with the later report of Glagolev
Chuan (18, p. 2) theorized on the basis of electronmicrographs and amperometric titrations for -SH groups that the mechanism of milk coagulation by rennin may be considered similar to the modern concept of blood coagulation in which a \(-\text{SH} \leftrightarrow -\text{S-S-}\) chain reaction is involved. From electronmicrographs caseinate particles were seen to agglomerate into particle-wide fibrils, which in turn condensed into bundles of fibrils. He further postulated that there was a rapid irreversible unfolding of the caseinate particle in the first stage, followed by a slow aggregation, probably in association with the colloidal calcium phosphate, in the second stage to form a gel structure with milk serum as the external phase.

**Liberation of Non-Protein-Nitrogen**

This aspect of the rennin coagulation of milk was developed from the modern concept of the poly-state nature of casein as it exists in milk. The different fractions which can be obtained from casein by various methods were recently named by Brunner et al. (13, p. 901-911).

Nitschmann and coworkers (44, p. 687-698) reported that in the enzymatic stage of coagulation of milk by rennin about 2 per cent of the total nitrogen was
liberated from alpha-casein. They also reported that the liberated nitrogenous material consisted of more than one peptide and was soluble in 12 per cent trichloroacetic acid (TCA). The curve plotted with the amount of nitrogenous material soluble in 12 per cent TCA against rennin contact time in a caseinate system, showed two distinctly different types of slopes. The initial steep rise in soluble nitrogen levelled off as soon as coagulation of the caseinate system took place. This indicated that after the initial action of rennin little or no TCA soluble nitrogen was liberated.

Using ion exchange chromatographic methods, Alais (1, p. 671-678) found that the nitrogenous material, soluble in 12 per cent TCA, that is liberated from alpha-casein by the action of rennin was not the same as that liberated from whole casein under the same conditions.

Garnier (29, p. 249) demonstrated that the slopes of the NPN/Time curves obtained from studies on the milk of the cow, the ewe and the goat were similar before and after coagulation. The NPN liberated was higher with ewe than with goat milk and with goat than with cow milk.

Waugh and von Hippel (68, p. 4576-4582) reported that \( \kappa \)-casein is the primary target of rennin action on casein. They also reported (68, p. 4582) that
k-casein lost 20 per cent of its molecular weight during rennin action.

Wake (66, p. 148) supported Waugh's claim. He concluded that k-casein is the primary target of attack when rennin acts on whole casein and that the specific splitting reaction which liberates a small amount of NPN in the initial stage is directly responsible for milk-clotting.

Garnier (30, p. 1453) showed that 5 per cent of the total nitrogen is liberated by the initial action of rennin on k-casein. Cerbulis and Zittle (15, p. 418-420) determined soluble nitrogen and phosphorous following rennin action on calcium-sensitive and calcium-insensitive alpha-casein. More soluble nitrogen and phosphorous were released from the calcium-insensitive alpha-casein presumably because it contained more of the "protective colloid" than the calcium-sensitive alpha-casein. Oeda (51, p. 378) claimed that rennin yields polypeptides and no free amino acids from alpha-casein. Verma et al. (65, p. 654) postulated that rennin causes aggregation of casein particles in the enzymatic phase. Formol titration did not show any -NH₂ groups to be freed as a result of rennin action.

Alais (2, p. 834) showed that a 12 per cent TCA extract following rennin action in the sodium-caseinate
sol contained a single macropeptide which did not
dialyze through a cellulose membrane. The macro-
peptide closely resembled the d-casein as reported
by Cherbuliez (16, p. 1673 and 17, p. 959-961).
Cherbuliez considered the s-casein to be the same as
Hammarsten's proteose (32, p. 4), which the latter
regarded as a preformed constituent rather than a
degradative product of rennin proteolysis of casein.
Since the macropeptide is released very quickly from
casein by rennin and since they consider that rennin
is slightly proteolytic, Nitschmann and Varin (49,
p. 1421-1430) postulated that the nitrogenous material
liberated may not be bound by primary valencies. The
macropeptide liberated from alpha-casein by rennin
action was reported (50, p. 76-77) to be monodisperse
in the ultracentrifuge. The molecular weight was
reported to be 6000 to 8000. Alais (4, p. 1955)
showed that it consisted of more than one peptide and
that it comes from alpha-casein only. Brunner analysed
the macropeptide and reported it contained 15.2 per
cent galactose, 4.3 per cent glucosamine and 11.4 per
cent neuraminic acid.

**Electrophoretic and Chromatographic Changes**

Nitschmann and Lehman (48, p. 153-154) showed
that electropherograms of casein and paracasein differed considerably. The alpha-casein peak of paracasein was split into two which they referred as $\alpha_1$- and $\alpha_2$- casein, with $\alpha_1$-casein having greater mobility. Sasaki (59, p. 240 and 60, p. 209) and Tsugo (64, p. 588) have confirmed these results. Tsugo and Yamauchi (63, p. 643 and 64, p. 588) reported that the $\beta$-casein electrophoretic peak was split over a prolonged rennin contact time. McKearns (46, p. 59) demonstrated that whole casein acted upon by rennin showed new protein fractions on a chromatogram. Chromatographic observations in several organic solvent solutions showed no amino acid appearing as a result of rennin action.

Sasaki (60, p. 209) claimed that rennin and pepsin showed similarity in their action in causing heterogeneity in $\alpha$-casein peak. Electrophoretic patterns showed a greater decomposition of casein by the action of papain and ficin.
EXPERIMENTAL

Clotting Activity of Rennin

The clotting activity of the enzyme preparation was determined by a modification of the method of Debaun (21, p. 324-338). Twelve grams of low-heat skim milk powder were dispersed in 100 ml. of N/50 CaCl₂ solution and stored at 34° F. for at least six hours before use. This served as the substrate solution. One ml. of the enzyme preparation was mixed with 10 ml. of substrate, each having been forewarmed to 35-37° C. A stop watch was started simultaneously with the mixing. By observing the sudden breaking up of the milk film into discrete particles while slanting the test tubes from time to time, clotting time was recorded. The average of two duplicate tests was taken as the clotting time when a difference of not more than 5 seconds was observed between the two. The clotting activity was then expressed in terms of seconds per mg. of nitrogen of the enzyme.

Agar Electrophoresis

The method and apparatus employed for the agar electrophoresis were essentially those of Das and Giri (20, p. 67-73), with some minor modifications.
Apparatus:

The diagram for the agar electrophoresis apparatus is shown in figure 3. A rectangular electrophoretic cell (B) (10" x 3" x 1½"), made of plastic, rests on two electrode vessels (E) on either end. On either side of the rectangular cell depressions are made (1/8") to accommodate filter paper strips. Direct current (115V) is obtained through a rectifier unit (DC) and is connected to the plastic electrode vessel containing buffer solution in contact with the platinum wire electrodes through a milliammeter (A). The filter paper strips on each side of the cell maintain contact between the agar gel in the electrophoretic cell and the buffer solution in the electrode vessels.

Procedure:

A three hundred ml. 1 per cent agar solution was prepared by mixing the proper buffer solution with 2 per cent sterilized agar solution in water. Whatman number 3 filter strips were introduced at either end of the cell at an angle of about 60°. A rectangular piece of perspex (F) (2" x 7/8" x 3/32") was placed at the center of the trough without touching the sides of the cell. The melted agar solution was poured in
the cell (B) and the gel was allowed to set for about 20-30 minutes. The buffer solution in the electrode vessels was properly diluted with distilled water to give the same ionic strength as that in the agar gel. A glass plate was placed over the cell. The side of the plate exposed to agar was covered with Whatman number 3 filter paper. The paper absorbs the moisture evaporated from the agar gel during electrophoresis. A current of about 10 ma. was allowed to run for about 30 minutes and stopped. The perspex piece was slowly removed and the narrow slit formed in the gel was filled with the sample by means of a capillary pipette. Care was taken to see that no air bubble was introduced in the sample during the application. The sample was prepared by dialyzing commercial rennet (10 ml.) in running distilled water for ten hours and then pervaporated at room temperature to approximately 1 ml. The electrophoresis was carried out at 115 V and 10 ma. for 24 hours at 45° F. The electric circuit was then switched off. A narrow strip of Whatman number 3 paper was placed on the gel for 3 seconds and removed. It was then stained by the usual methods employed for dyeing electrophoretic strips (bromphenol blue dye). The different fractions in the gel were thus located. The agar gel was cut with
a spatula and each fraction corresponding to the band on the dyed filter strip was collected in separate test tubes and triturated well with a stirrer. The test tubes were left in a refrigerator at -10° F. for 3 to 4 hours. The frozen gel was thawed at room temperature. The solution that thaws out of the agar was filtered through a sintered glass funnel into separate clean dry test tubes corresponding to each fraction.

Proteolytic Activity of Rennin Preparations

The method employed in determining the proteolytic activity of rennin preparations on a substrate is a modification of that of Garnier (29, p. 245-256). Several test tubes containing 10 ml. of 0.089 per cent sodium-caseinate sol, pH 5.5, were brought to 35-37° C. in the same water bath used for rennet activity tests. These served as the substrate solutions. One ml. of the enzyme preparation previously brought to a temperature of 35-37° C. was added to each tube, and mixed thoroughly. After the desired length of time, 10 ml. of 25.2 per cent TCA were added to precipitate the protein and to stop the reaction. The extract was filtered through a Whatman number 42 filter paper, 30 minutes after TCA was added. The NPN that is soluble
in 12 per cent TCA was estimated by determining the optical density at 280 mmc in a Beckman DU spectrophotometer. A blank test in which rennin was added after the addition of the TCA was run for each test tube.

Another set of experiments was carried out to determine the extent of proteolysis at various hydrogen ion concentrations. Hemoglobin solutions (2.5 per cent) from a pH of 1.0 to 5 served as the substrates. Five ml. of these substrate solutions were allowed to react with 1 ml. of rennin preparations, at 35-37°C, for one hour. Following this, 5 ml. of 26.40 per cent TCA were added to stop the reaction. After standing for 10 minutes the precipitated proteins were removed by filtration. The extent of proteolysis was determined by measuring the optical density at 280 mmc in a Beckman DU spectrophotometer. A blank test in which water was added instead of rennin preparation was run at each pH.

**Fractionation of Commercial Rennet**

Fractionation of commercial rennet was done by isoelectric precipitation and salting out techniques. Commercial rennet was adjusted to pH 4.5 with N/10 HCl and allowed to stand overnight at 45° F. The precipitate formed was centrifuged out at 2000 rpm for 5 minutes, dissolved in 0.5 per cent NaCl and labelled
as Fraction I. The supernatant from above was saturated with NaCl using a dialysis membrane. The precipitate formed was filtered out and treated as above and was labelled as Fraction II. Fraction III was obtained by adjusting the pH of the filtrate to 1.5 and saturating with NaCl. The resulting precipitate was dissolved in 0.5 per cent NaCl. The supernatant obtained from above was labelled as Fraction IV.
RESULTS

The 'proteoclastic' activities of a commercial rennet extract (Hansen's) and of various rennin fractions isolated therefrom, using a 2.5 per cent hemoglobin solution as substrate, at pH values ranging from 1 to 5, are shown in Figure 1.

The maximum activity of the commercial rennet was found at pH values of 2 and 4. According to Debaun (21, p. 338) that which occurred at pH 2 is probably due to pepsin and that at pH 4 to rennin. The steep slope of the curve representing the 12 per cent TCA-soluble NPN split off by Fraction I, with hemoglobin as the substrate, occurring at pH 2 indicates that this fraction is contaminated with pepsin. In contrast to commercial rennet no dip is obtained at pH 3.

The peak in the reaction curve for Fraction II on hemoglobin appearing at pH 3 may be due to rennin action (21, p. 338).

Fraction III had slight 'proteoclastic' activity at pH 2 compared to that at pH 1. The activity at pH 4 predominated.

The activity of Fraction IV at pH 4, was not

"'Proteoclastic,' as used herein, signifies a protein cleavage not necessarily hydrolytic in nature."
pronounced as with other fractions. The activity at pH 2 was higher than that at pH 4.

The reaction curve for the fraction that migrated to the negative pole (Fraction V) at pH 5.8 in the agar electrophoresis experiment showed a maximum but weak 'proteoclastic' activity in the pH range of 2 to 3. The high 'proteoclastic' activity at pH 2 cannot be attributed to pepsin since pepsin having a negative charge at this pH migrates to the anode, unless of course the pepsin is associated with the positively-charged rennin in a positively charged complex.

The curve for the fraction of the rennet that migrated to the positive pole (Fraction VI) at pH 5.8 in the agar electrophoresis experiment was peculiar in that it shows a progressive rise in activity as the pH is increased from 1 to 5.

Figure 2 shows the relationship between concentrations of tyrosine in TCA extract and the optical density measured at 280 mm wavelength. The TCA extract was obtained by adding 12 per cent TCA to a 0.89 per cent casein sol, allowing to stand for 30 minutes, and filtering. A weighed quantity of tyrosine was dissolved in this extract and serially diluted with the extract. The curve obtained served as the standard curve for the
tyrosine content and the optical density at 280 mm wavelength. It obeys the Beer-Lambert law.

The 'proteoclastic' activities of the various rennet fractions on casein sols at pH 5.2, in terms of grams of tyrosine per 100 ml. TCA extract, are illustrated in Figures 4 and 5. All show an initial steep slope which later level off in varying degrees. The commercial rennet does not exhibit significant activity after the initial action. All the fractions of the commercial rennet, however, did show activities.

The rennet fractions that migrated to the anode (Fraction VI) or to the cathode (Fraction V) in the agar electrophoresis experiment showed considerable activity. The anode-migrating fraction (Fraction VI) shows a steep and progressive rise in 'proteoclastic' activity, presumably due to pepsin content. The cathode-migrating fraction (Fraction V) shows an initial steep rise in 12 per cent TCA-soluble NPN followed by a decreased rate.

The clotting activities per mg. rennin nitrogen of the various rennet fractions are shown in Table 1. That of commercial rennet was higher than any of the fractions except those obtained by agar electrophoresis. Of the three fractions obtained by agar electrophoresis
FIGURE I. EFFECT OF pH ON THE RELEASE OF 12 PER CENT TCA-SOLUBLE-NPN IN 2.5 PER CENT HEMOGLOBIN SOLUTION BY RENNET AND RENNET FRACTIONS.

- COMMERCIAL RENNEN
- FRACTION I
- FRACTION II
- FRACTION III
- FRACTION IV
- FRACTION V
- FRACTION VI
FIGURE 2. STANDARD CURVE FOR THE ABSORBANCY OF TYROSINE AT 280 nm, CELL DEPTH, 1 cm.
FIGURE 3. AGAR ELECTROPHORESIS APPARATUS.

C. PLASTIC COVER.
F. PERSPEX PIECE.
D. FILTER PAPER.
E. ELECTRODE VESSEL.
G. AGAR GEL.
L. ELECTROPHORETIC CELL.
A. MILLIAMETER.
DC. DIRECT CURRENT.
Figure 4. The effect of time of incubation of rennin fractions on the release of nitrogenous material (calculated as tyrosine) in casein sol at pH 5.2, temperature 35°-37°c.
TABLE 1

Milk Clotting Activities of Commercial Rennet and Various Rennin Fractions Determined at 35-37° C.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Clotting Time In Seconds</th>
<th>mgN/ml</th>
<th>^°RU/ml</th>
<th>RU/mgN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Commercial Rennet</td>
<td>671</td>
<td>5.90</td>
<td>149.2</td>
<td>25.2</td>
</tr>
<tr>
<td>Fraction I</td>
<td>190</td>
<td>0.01137</td>
<td>0.5263</td>
<td>46.2</td>
</tr>
<tr>
<td>Fraction II</td>
<td>408</td>
<td>0.0804</td>
<td>0.2450</td>
<td>3.0</td>
</tr>
<tr>
<td>Fraction III</td>
<td>430</td>
<td>0.01086</td>
<td>0.2325</td>
<td>21.4</td>
</tr>
<tr>
<td>Fraction IV</td>
<td>140</td>
<td>0.27512</td>
<td>0.7142</td>
<td>2.5</td>
</tr>
<tr>
<td>Cathode-wise Migrating</td>
<td>1331</td>
<td>0.00133</td>
<td>0.0751</td>
<td>56.4</td>
</tr>
<tr>
<td>Anode-wise Migrating</td>
<td>425</td>
<td>0.00416</td>
<td>0.2352</td>
<td>56.5</td>
</tr>
<tr>
<td>Non-migrating</td>
<td>68</td>
<td>0.0150</td>
<td>1.470</td>
<td>98.0</td>
</tr>
</tbody>
</table>

^°Rennin units (RU) is defined as the rennin activity which clots 10 ml. of reconstituted skim milk in 100 seconds at 35-37° C.
the fraction that did not migrate at pH 5.8 has the highest activity, while the activities of the other two fractions which migrated in opposite directions were almost identical.
DISCUSSION

Commercial rennet was fractionated by precipitation and salting out techniques. Commercial rennet was adjusted to pH 4.5 and allowed to stand overnight. The precipitate, removed by centrifugation, was dissolved in 0.5 per cent sodium chloride. The solution was labeled as Fraction I. The supernatant was saturated with sodium chloride and the resulting precipitate removed by filtration was dissolved in 0.5 per cent sodium chloride. This solution was labeled Fraction II. Fraction III was obtained by adjusting the pH of the filtrate to 1.5, and saturating with sodium chloride and dissolving the precipitate in 0.5 per cent sodium chloride. The supernatant constituted Fraction IV. The fraction of commercial rennet that migrated to the negative pole at pH 5.8, in the agar electrophoresis experiment, was labelled Fraction V; that which migrated to the positive pole was labeled Fraction VI.

Although calcium caseinate is the natural substrate for rennin, modified sodium caseinate sols were used in this study for determining the 'proteoclastic' activity of various rennet fractions. The activity of crystalline rennin in milk is feeble at pH 6.5-6.7, the pH of normal milk. This is also true for sodium caseinate
sols (27, p. 1931). The proteoclastic activity of crystalline rennin on casein sols is greatest near the isoelectric point of casein (27, p. 1934). Precipitated casein is not particularly susceptible to the proteoclastic activity of whole rennet. But, as casein is very slightly soluble in the vicinity of its isoelectric point (pH 4.6), this pH is not well suited for determining the activity of rennin in this range of pH. It was decided, therefore, to employ a casein sol, made by reducing the pH of a sodium caseinate sol with HCl from its original pH of 7.5 to as near as possible the isoelectric point of casein without coagulation, as a substrate for characterizing various active components isolated from commercial rennet extract. It was found that a pH of 5.2 was minimum for a stable casein sol on the alkaline side of the isoelectric point of casein, and hence was selected for these studies.

The curves plotted with the amount of nitrogenous material liberated, against rennin contact time, indicate two distinct phases in reaction rates. The initial rapid release of 12 per cent TCA-soluble nitrogen during the first phase, was followed by a much reduced rate in the second phase. It should be borne in mind that the substrate was a casein-sodium salt suspension at a pH of 5.2. The action of rennin on a calcium
caseinate-calcium phosphate substrate at pH 6.6 might well be quite different.

According to Alais et al. (4, p. 1955) in the clotting of milk by rennin there is a rapid initial release of NPN soluble in 12 per cent TCA. After clotting occurs, the rate of release declines. Calcium phosphocaseinate and sodium caseinate give similar curves. Foltmann (25, p. 655) from his study of the enzymatic and coagulation stages reported that the release of NPN soluble in 12 per cent TCA corresponds to the enzymatic phase proper. Garnier (29, p. 249) likewise found that after the primary action of rennin on milk there was no increase in NPN soluble in 12 per cent TCA. In these studies, however, considerable NPN soluble in 12 per cent TCA was liberated in the second phase of rennet action on the casein sol. Both the agar fractions, the one migrating toward the anode and the other to the cathode at pH 5.8, exhibited high activity at pH 5.2.

Further characterization of rennet was achieved by using a 2.5 per cent hemoglobin solution as the substrate at different hydrogen ion concentrations. The individual fractions behaved differently as shown in Figures 4 and 5. Each fraction showed a high proteoclastic activity in the pH range of 3 to 4. If Debaun (21, p. 338) is
correct in attributing the peak appearing at pH 4 to proteolysis by rennin, then the activities of the various rennet fractions may be attributed to rennin and not pepsin. The fractions of commercial rennet that migrated to the positive pole in the agar electrophoresis experiment at pH 5.8, however, showed a continuous increase in the 'proteoclastic' activity as the pH of the substrate increased to 5. All the fractions isolated from commercial rennet showed activity on hemoglobin.
SUMMARY AND CONCLUSION

Commercial rennet (Hansen's) was fractionated by precipitation by pH adjustments and by salting out techniques. The first fraction was the precipitate resulting from the addition of HCl to the rennet extract to a pH of 4.5. Fraction II was the precipitate obtained by saturating the supernatant with NaCl. Fraction III resulted from adjusting the pH of the latter supernatant to 1.5. The final supernatant constituted Fraction IV.

Hemoglobin solutions ranging in pH from 1 to 5 and a casein-sodium salt suspension, pH 5.2, were used as substrates in characterizing the rennet and the rennet fractions on the basis of 'proteoclastic' activity. The 'proteoclastic' activity was determined by measuring the concentration of nitrogenous material soluble in 12 per cent TCA, using optical density values at 280 mmc. The values were calculated in terms of tyrosine.

All fractions were active on the hemoglobin substrate at all pH values studied. Greatest activity appeared in the range of pH 2 to 4. The anode-wise migrating fraction, however, showed a progressive rise in activity as the pH increased from 1 to 5. The activity at pH 2 may be due to pepsin, which is present as a contaminant in the extract; that at pH 4 is attributed
to rennin.

In the case of casein sols at pH 5.2, the enzyme preparations showed two distinct reaction rates—a rapid initial rate followed by a decreased activity. Commercial rennet exhibited the least activity in both the initial and secondary stages. The cathode-wise and the anode-wise migrating fractions had high activities.

In regard to milk clotting, commercial rennet had an activity of 25 rennin units (RU) per mg. N. The remaining fractions had activities as follows; non-migrating, 98; fraction I, 46; fraction II, 3; fraction III, 21; fraction IV, 2; cathode-wise migrating, 56; anode-wise migrating, 56 RU per mg. N. RU is defined as the activity which clots 10 ml. of reconstituted skim milk (12 gm. dry milk solids-not-fat in 100 ml. of N/50 CaCl₂) in 100 seconds at 35-37° C.

Rennet extract has at least four active fractions all of which have exhibited milk-clotting power. Each liberates nitrogenous material soluble in 12 per cent TCA in hemoglobin solutions and casein-sodium salt suspension in varying degree, depending upon the particular fraction and the pH. The term 'proteoclastic' has been used instead of proteolysis since data are insufficient to determine if there is hydrolysis or a mere release of some associated protein entities.
Commercial rennet exhibited slight 'proteoclastic' activity on casein sol at pH 5.2. The isolated rennet fractions, however, showed high activity under the same conditions. The reason for this apparent paradox is not clear unless one assumes the presence of an antiproteoclastic factor in the rennet extract. Additional studies are necessary to determine the relationship, if any, between 'proteoclastic' activity and the milk-clotting power of rennin and the various fractions.
BIBLIOGRAPHY


