REVIEW OF LITERATURE
1. EXTRATION OF RENNET

The unique capability of the extract obtained from dried abomasum (vells) of young ruminants to bring about curdling of milk was recognized since the dawn of history. With passage of time, methods were developed to obtain such preparations.

One of the earliest reports of Leitch (41) describes a method wherein dry slices of vells were extracted with 5 to 10% sodium chloride containing 2-4% boric acid as preservative which was followed by clarification and filtration.

Vander-Burg and Vander-Scheer (77) obtained rennet extract on macerating the stomach tissues for two days with 10% sodium chloride containing 2% boric acid. After raising the pH to 6.3, with disodium hydrogen phosphate (1.8%), the liquid was filtered.

Stem (72) prepared a sterilized solution of rennet by lowering and raising the pH with intervals of incubation at 40°C. Microorganisms that grew under favourable conditions were subsequently destroyed by lowering of pH.

Keil and Stout (39) patented a method of rennet extraction from animal stomach using dilute hydrochloric acid as extracting solvent. Rennin was salted out from the crude extract after filtration.
The same workers (36) patented another procedure. Dilute hydrochloric acid was used to extract the enzyme from the wells and the resultant liquid kept at 42°C for 45 minutes. Ice cold water was added to lower the temperature of the material to 18°C and filtered through a bag containing wet wood. The filtrate was adjusted to pH 4.5 and rennet precipitated as floating curd which was then dried.

Vitoria (78) suggested a method of preparing liquid commercial rennet. The extracting liquid consisted of a mixture of 10% sodium chloride, 5% glycerine and 2% boric acid. The extract obtained did not have good appearance, but possessed good coagulating activity and storage capacity.

Marshall's Food Products Limited (47) patented a method of manufacturing rennet. The extract was divided into two or three parts, and substances like calcium chloride and sodium hydrogen phosphate were added in them. Protein content of these solutions was adjusted to 4% and pH between 4.0 and 5.0. On mixing the solution, the active principles of rennet were absorbed on the surface of inert, easily filterable precipitate formed due to the reaction of added substances. After filtration, the pH of precipitate was adjusted between 7.0 and 7.5 so that the rennet became soluble. The solution was centrifuged and rennet was precipitated with sodium chloride. The precipitate was then removed from the solution by centrifugation or filtration.
Krystyna (20) prepared rennet from dried stomach of milk fed calves by extracting with 12% calcium chloride solution which contained wood shavings. The extract was purified further by conventional methods.

Fomin (26) was the first worker to prepare rennet from gastric juice of living calves. The gastric juice was obtained through fistula made in the abomasum. Diluted whey (about 1.5 to 3.5 litres) was fed to fistulated calves. The abomasal fluid was removed from stomach after about 30 min. by unscrewing the cap of cannula. The fluid was filtered and saturated with sodium chloride to precipitate rennin. The rennet thus obtained could be dried at 35° - 45° C.

Berridge et al. (7) repeated Fomin's experiment and described details of surgical operation. Cheese made from this rennet was of satisfactory grade. However, the method was found to be uneconomical under the experimental conditions in their country and so any further work was abandoned.

Mikhailov (35) compared the composition and properties of rennet obtained from fistulated calves with that obtained from calves by slaughtering. It was observed that fistulated rennet contained more protein and less salt. Further, it appeared to be more stable and had greater coagulation power.

Henshel et al. (33) compared the content of rennin and pepsin in abomasal juice of three fistulated milk fed calves before and after feeding. It was observed
that at 6-8 weeks of age both rennin and pepsin are secreted. No correlation could be established between the age of animals and the secretion of these enzymes.

Nair et al. (30) described a method of collecting rennet from living calves. The surgical operation for fitting the cannula in the abomasum was the same as described by Berridge et al. (7).

Bryzgin et al. (15) repeated the work of Formin (25) and Berridge et al. (7). A fistulated calf of 7-10 days of age was fed pasteurized milk serum and the abomasal juice tapped out within 30-40 minutes of feeding. Rennet was precipitated from this fluid with addition of 20\% sodium chloride.

Mathur et al. (48) following Berridge’s method collected rennet from both cow and buffalo calves. The abomasal fluid was obtained after allowing about 30 min. of retention of whey fed to the calves on empty stomach. Buffalo fistulated calves on an average gave less rennet. The gross amount of rennet recovered from each calf was approximately 30 times the quantity recovered by slaughtering a calf.

Nain et al. (34, 57, 58) at this Institute have standardized a procedure for extracting rennet from fistulated cow and buffalo calves and goat kids. The surgical operation involved was modified by Math et al. (60). The whey fed (3/4 diluted) was tapped out between 15-20 min. and subjected to filtration to remove extraneous substances. The juice was next treated with
sodium chloride, final concentration ranging between 25 to 30%, thereby causing precipitation of the enzyme remain. The supernatant was decanted off as much as possible and precipitate centrifuged at 3,000 r.p.m./15 min. It was finally dissolved in 0.03 M sodium lactate in which its stability was found to be satisfactory over a long period. Such preparation could be purified 3-4 folds with a recovery of about 50 percent based on turbidimetric method of estimation of rennet activity. No regular pattern was observed in the activity profiles of pepsin and trypsin with the age of animals. Rennet activity appeared to decrease considerably during the last phase of collections (at the end of 3 months).

Cisneros et al (18) reported production of rennet from fistulated calves over a period of six months. The technique of Nair et al (59) for fixation of cannula was followed. The author observed that the yield of rennet increased with age. The total amount of rennet obtained over a period of six months with two collections per day, was equivalent to the amount of rennet from 70 dried vells of slaughtered calves. The ten calves studied showed variation in the volume of abomasal juice tapped and in also the concentration of rennin in the fluid.

Hagyard and Davey (30) report that age and starvation adversely affect rennin yield when expressed as units of rennin/g dry, fat free vell, with a 49%
decrease between 1-5 and 20-24 day old calf groups, and 33% decrease after 3 day's starvation. There appears to be no change with age when results are expressed as rennin units/vell or vell/litre of the New Zealand Standard strength rennet. This suggests that although vell weight increases with calf age, the rennin content remains constant, within the age limits studied. With starvation, however, the vell weight change is not significant, and loss of activity expressed as either rennin units/g dry, fat-free solids, rennin units/vell or vell/litre of New Zealand Standard strength rennet is significant.

Garnet et al (22) fed three groups of calves with either milk proteins, whey proteins or a 50:50 mixture of these two diets. In a separate experiment, another group of animals was fed milk proteins. After extraction of enzyme from dried vells at acid pH, agarose-acrylamide gel electrophoresis was done. The enzymes detected were rennin and bovine pepsin II. Statistical analysis of qualitative enzyme determination by DEAE-cellulose chromato-graphy indicated a trend for the vells from the calves fed diets containing casein to be richer in total activity and in rennin, while the level of pepsin remained approximately constant. It was felt that casein may induce the secretion of rennin. Important differences were observed between the 2 groups of vells from calves given the same diet, but grown in slightly different conditions.
2. PURIFICATION AND CHARACTERIZATION OF RENNIN

Attempts have been made in the past years to obtain a purified preparation of rennin with a view to characterize and study its physico-chemical attributes.

Hammerstern (31) seems to have been the first to separate rennin from pepsin by sodium chloride precipitation. Fengler (31) precipitated rennin from salt-free extract of calf mucosa at acidity just below neutrality. The precipitate was finally dissolved in an acid solution.

Taubert and Kleiner (73) extracted rennin from fresh mucosa with 0.04M hydrochloric acid. The extract was dialyzed against distilled water until the pH reached 5.4. The enzyme was precipitated with ethyl alcohol; reprecipitation was done and the material finally dissolved in 0.04M HCl. The solution was able to coagulate 4.8 x 10^6 times of its dry weight of the substrate.

Hankinson and Palmer (32) prepared a strong solution of enzyme rennin from commercial rennet extract by repeated precipitation at pH 4.8 with sodium chloride solution at pH 6.0 and reducing the volume of solution 80% each time.

Berridge (6) isolated rennin in the form of flat crystalline plates of near triangular outline from commercial rennet extract. The procedure involved precipitation of rennin with sodium chloride at pH 5.4, followed by its adsorption on alumina. After settling out
a number of times, magnesium sulphate was added to cause precipitation. Fractional crystallisation led to the growth of plates of rennin crystals. Reid (66) purified rennin using ion-exchange chromatography.

Berridge (13) prepared crystalline rennin from the extract of fresh calf stomach. The extract was purified by electrophoresis using the method of Tiselius (78). The slow moving homogeneous component was separated and adjusted to pH 5.4 and then treated with sodium chloride. Rennin crystals appeared after a few days of storage at 3°C.

Feltman (23) fractionated crystalline rennin into four components on DEAE-cellulose using phosphate buffer, pH 5.7, as an eluting solvent. The concentration of buffer was increased from 0.2M to 0.3M. Three fractions, C, B and A possessed milk clotting activity and comprised 20, 55 and 22% of applied protein, respectively.

Purified rennin solution was prepared by Krueger et al (30) using gel filtration technique. Gastric juice after removing fat and mucous, was passed through a dextran gel. Rennin was eluted with either distilled water or disodium hydrogen phosphate. The largest molecule which emerged first with the eluate was collected.

Payens (61) analysed rennet obtained from abomasum of calves, cows and commercial rennet by zone electrophoresis on a column of cellulose. Some samples appeared to be homogeneous while others heterogeneous,
The content of pepsin was higher in rennets from stomachs of older calves and cows.

Yoshino et al. (79) performing DEAE-cellulose column chromatography of commercial rennet reported five fractions.

Gel filtration of commercial rennet on a column of Sephadex G-75 was performed by Ritter and Schilt (88). Sodium chloride, 0.1 M, was used for elution and eluents examined continuously in u.v. light at 226 m/λ. Three peaks were observed. Rennet activity was associated only with the intermediate peak.

Melachouris (51) achieved separation of calf rennin into 9 or 10 fractions by horizontal discontinuous polyacrylamide gel electrophoresis.

Castle and Wheeler (10) have described a method for the purification of rennin. Proteins were precipitated from commercial rennet by saturation with sodium chloride at 20°C, filtered, suspended in water and dialysed against tap water to remove Cl ions, and precipitated iso-electrically by adjustment to pH 4.5 with 0.1 M HCl. After washing with acetate buffer, pH 4.5, the precipitate is resuspended in water and rendered soluble by increasing the pH to 6.8 with sodium hydroxide. The whole process is repeated and thereafter the material is subjected to gel filtration at 4°C on Sephadex G-200. Rennin was crystallized from the fraction containing milk clotting activity.
Proskurovskov and Sushkevskaya (63) obtained a highly purified rennin preparation from calf rennet extracts and rennet powder by sodium chloride treatment, followed by chromatographic separation on CM-cellulose of the material precipitating at sodium chloride concentration of 2-10%. Degree of purification attained was 23 fold.

Nain (58) was able to achieve 10 fold purification of rennet preparation obtained from fistulated cow calves. Electrophoresis of rennet from fistulated calves on paper, starch and polyacrylamide gel revealed the presence of a single protein band at pH 3.6 in veronal buffer. Further, it had greater mobility than Hansen rennet. Molecular sieving on Sephadex G-100 resolved rennet from fistulated animals into two protein peaks compared to three of Hansen rennet, thus signifying the molecular heterogeneity of these enzyme preparations. Immunological studies on rennets highlighted the immunological homogeneity of fistulated calf rennet preparation and heterogeneity of Hansen rennet. Such endeavour offered a useful tool for differentiation of fistulated cow calf rennet from fistulated buffalo calf and goat kid rennets.

3. EFFECT OF ADDITIVES

Nain and Nain (58) while studying the influence of certain additives observed that the presence of Mn, Fe, Zn, Co, Cu and Ni, as their chlorides having 0.001M concentration in rennet did not effect the rate of milk clotting by rennet.
Sattler (69) reported that the absence of preservative like boric acid and ethanol in rennet preparation reduced the storage life of rennet considerably.

Shorebats et al. (80) studied the effect of drugs on the digestive juice secretion from the glands of lesser curvature of abomasum of fistulated calf. It was observed that an injection of acetylcholine (1 mg/kg of body weight) increased rennin activity, whereas injections of pilocarpine, atropine and ephedrine had no effect on rennin activity of the juice.

Mikhailis (83) examined the effect of glutamic acid (40 to 200 mg/100 ml) on rennets from living calves and from the abomasum of slaughtered calves. The reduction in milk clotting time of rennets from living calves was by 1.8 to 7.3% at 19-22°C and 9.4 to 19.1% at 38°C whereas corresponding reduction in case of rennet from slaughtered calves was found to be 4.2 - 9.1% and 5.8 to 10.3%, respectively.

Mikhailis (84) further reported an increase in milk clotting activity of rennets from living calves on treatment with aspartic acid (27 to 100 mg/100 ml). In the case of rennet from slaughtered calves no such effect was noticed.

Bakman et al. (3) exposed commercial rennet preparation to \( \gamma \)-radiation. No loss in enzymatic activity was detected with doses below 3.5 Mrad. However, a loss of about 10% in activity occurred with 3.5 Mrad strength radiation.
On fortification of whey with certain additives like glutamic acid, aspartic acid and cysteine (0.1%) and sodium chloride (2%), Nair (55) did not observe any beneficial effect either on total rennin secretion or its specific activity in abomasal juice. Of the various chemicals added to fistulated calf rennet, it was found that addition of BaCl₂, FeCl₂, NaCl₂ and KCl stimulated rennet activity, whereas CaCl₂, NiCl₂, NiSO₄, CoSO₄, FeSO₄ and ZnSO₄ inhibited, while addition of NaCl, NH₄Cl and (NH₄)₂SO₄ were without effect on the rennet activity.

4. STATUS OF PRORENNIN

The inactive form of rennin, known as prorennin, also determines the milk clotting process. As a matter of fact it is the prorennin which resides in abundance in the abomasum. The mechanism involved in the activation of prorennin to rennin appears to stimulate other enzymes of the digestive tract, namely pepsin and trypsin.

Tempor and Kleiner (75) were the first to isolate and study the properties of prorennin from gastric mucosa. Gastric extract, of pH 4.7, with low milk clotting activity was prepared and proteins precipitated with magnesium sulphate. After reprecipitation, the material was dried in vacuum over sulphuric acid. Such preparation did not coagulate milk but upon its treatment with hydrochloric acid, acquired milk clotting activity. Thus
revealing that the preparation contained prorennin which was activated by hydrogen ions.

Berridge et al. (8) reported only small amount of prorennin in the gastric juice of living calves.

Peltzman (23) prepared prorennin from dried stomachs of calves. The dried abomasum were treated with 2% sodium bicarbonate and the extract was clarified by the addition of aluminium sulphate. The resulting solution was neutralized with disodium hydrogen phosphate and prorennin was precipitated from the solution with sodium chloride. Prorennin was activated at pH 2.0. Rennin activity was assayed using milk coagulation procedure. Rennin activity of the preparation before activation was found to be 2.6 rennin units/mg, which increased to 500 rennin units/mg.

Peltzman (24) purified prorennin by chromatography on DEAE-cellulose using phosphate buffer as an eluting solvent. One minor and one major constituent designated as Prorennin-A and Prorennin-B were obtained. These two fractions on activation gave rise to chromatographically distinct fractions of rennin, which corresponded to similar fractions obtained by chromatography of crystalline rennin. Activation studies suggested splitting of the peptides from N-terminal side of proenzyme.

Rand and Erastrom (84) observed autocatalytic conversion of prorennin to rennin at pH 4.0 and 8.0. Further, the rate of conversion was very rapid below
pH 4.0, but extremely slow above pH 5.0. Sodium chloride
appeared to stimulate conversion above pH 5.0 unlike at
pH below 4.0.

Bundy et al. (14) isolated prorennin from acetone
powder of fresh calf abomasum and purified it by ion
exchange chromatography on DEAE-cellulose and gel filtrat-
tion. The product was found to be homogeneous and the
N-terminal amino acid residue was observed to be alanine.
The release of peptide on activation of prorennin, appeared
to be derived from N-terminal part of the proenzyme.

Rund and Ernstson (65) reported that pepsin
catalyzed activation of prorennin at pH 6.0 and 9.8 at
25°C with little interference from autocatalytic activ-
ivation. Presence of sodium chloride retarded the rate
of activation, but it was still faster than the rennin
catalyzed activation.

Asato and Rund (1) achieved resolution of prorennin
into 3 or 4 components on DEAE-cellulose using compound
phosphate buffer gradient. The results were confirmed
using polyacrylamide gel electrophoresis. Number of
components depend upon the method of prorennin prepara-
tion. Activation of the prorennin fractions at pH 5.0
demonstrated that each zymogen was a precursor to an
electrophoretically distinct component of rennin. Analysis
of the increase in milk clotting activity with time
showed that the mechanism of activation of whole pro-
rennin, individual prorennin components and a mixture of
prorennin fractions followed autocatalytic kinetics.

Mair et al (88) in their investigation reported the content of rennin as prorennin in the abomasal juice, averaging 9 percent. The kind of whey fed to the fistula-lated calves seemed to influence the appearance of prorennin in the abomasal juice. A maximum of 17% of prorennin was obtained with rennet whey feeding. Additives like glutamic and aspartic acids (0.1%) in whey reduced the prorennin content while sodium chloride (2%) and cysteine (0.1%) caused complete absence in the abomasal juice. Activation studies revealed that at pH 4.5, cysteine and sodium chloride stimulated conversion of prorennin to rennin unlike glutamic and aspartic acids which inhibited such a process. However, at pH 2.0, with due exception of sodium chloride, the remainder of the additives stimulated prorennin activation. Electrophoresis on paper, in veronal buffer, pH 8.0, and molecular passage through Sephadex G-100 revealed the presence of one protein band and one protein peak signifying the homogeneity of the prorennin preparation.