MATERIALS AND METHODS
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The fistulation technique for rennet extraction

Operations were performed for insertion of cannulae in the aboma of two or three weeks old cow calves, buffalo calves and goat kids according to the techniques reported by Berridge et al (1943) with modifications suggested by Nair et al (1965) and Nath et al (1969). Calves and kids recovered after operation within four or five days and were found to be suitable for experimental use.

Types of whey - Different types of milk whey fed to fistulated cow calves include (a) whey prepared from unboiled skim milk by coagulating casein with 10% hydrochloric acid at isoelectric point, (b) whey prepared from boiled skim milk by precipitating casein with 10% hydrochloric acid at iso-electric point, (c) whey from boiled skim milk prepared like (b), but half diluted with water, (d) whey from boiled skim milk prepared like (b), but 3/4th diluted with water, (e) rennet whey obtained from skim milk after rennet coagulation at 37°C for 30 minutes.

Whey with additives - Whey prepared like (d), but incorporated with following additives (i) sodium chloride (2%), (ii) glutamic acid (0.1%), (iii) aspartic acid (0.1%) and (iv) cysteine hydrochloride (0.1%, neutralised before use).
Ten feeding trials were carried out using each type of whey. The abomasal juice obtained was tested for coagulation activity and for its protein content using the modified method of Berridge (1952 a, b) and Lowry et al (1951), respectively.

Since it was not possible to use the same calf for all types of whey feeding, individual calf was fed with one type of whey and after 10 to 12 days feeding it was switched over to other type of whey.

**Volume of whey** - One calf was selected for such study. Different volumes of whey (3/4th diluted boiled skim milk whey (type d) 1000 ml, 1500 ml and 2000 ml were fed (10 days for each type) for evaluating rennet secretion in abomasal juice.

**Retention time** - A calf was fed with fixed volume of whey (1000 ml) and abomasal juice was tapped at different intervals of retention from five to thirty minutes. Five trials on every retention period were performed. The volume and the coagulation activity of abomasal juice tapped were determined for each trial.

All feeding trials were conducted with calves during their age period between 2 to 4 months when maximum rennet secretion was observed. The above mentioned conditions were standardised for cow calves only. However, these were found applicable to buffalo calves and goat kids as well.
Extraction of rennet from living calves

Fistulated calves were fed with 3/4 diluted whey prepared from boiled skim milk by coagulating the proteins at pH 4.5 with 10% hydrochloric acid. The stomach contents (abomasal juice) of the calf was collected within 15 to 25 minutes by unscrewing the caps of cannulae. This abomasal juice contained suspended materials along with the enzyme rennin. So it was centrifuged at 2500 rpm in a refrigerated centrifuge at 0 to 8°C for 15 minutes in order to remove the suspended material. A clear greenish yellow supernatant was obtained and its coagulation activity and protein content were determined using the methods of Berridge (1952 a,b) and Lowry et al (1951), respectively. This supernatant was then adjusted to pH 4.5 with 0.1N hydrochloric acid and the enzyme rennin was precipitated from it by saturating with sodium chloride. The precipitate was removed by centrifugation in a refrigerated centrifuge at 3000 rpm for 30 minutes. The supernatant was discarded and the precipitate was dissolved in 0.03M sodium lactate solution (one tenth in volume of the original abomasal juice taken). It was then stored in refrigerator after the addition of 0.5% thymol as preservative. The coagulation activity and protein content of this preparation were determined again. In order to get more quantity of rennet, two times collection of abomasal juice was also done, one in the morning while the other in the evening.
FLOW DIAGRAM FOR PREPARATION OF RENNEN

Boiled skim milk

' 10% Hydrochloric acid added

Precipitate (casein), discarded

Whey, diluted with water (1:3) fed to calves and tapped within 15-25 minutes.

Abomasal juice, centrifuged at 2800 rpm for 15 minutes

Sediments, discarded

Supernatant, saturated with sodium chloride and centrifuged at 3000 rpm for 20 minutes

Precipitate, dissolved in 0.03M sodium lactate, centrifuged or filtered

Supernatant, discarded

Precipitate, discarded

Supernatant, stored in refrigerator after addition of 0.8% thymol
Purification of rennet

a) Liquid rennet obtained from fistulated cow calves was adjusted to pH 4.5 with 1N hydrochloric acid and saturated with sodium chloride. The precipitate formed (precipitate I), was separated by centrifuging for 30 minutes at 3000 rpm in refrigerated centrifuge.

b) The precipitate (I) was dissolved in one half of the original volume of 0.03M sodium lactate solution. The solution was filtered through Whatman filter paper No.1 and adjusted to pH 4.5 with 0.1N hydrochloric acid.

c) Steps (a) and (b) were repeated until six precipitations had been done (precipitate I, II, III, IV, V and VI). Precipitate VI was dialysed against distilled water for 48 hours in refrigerator and then dissolved in 20 ml distilled water, filtered and adjusted to pH 5.5 with hydrochloric acid.

The specific activities of rennet solutions obtained by dissolving the precipitates, I, II, III, IV, V and VI, were determined. Paper electrophoresis at each step was also conducted.

Preparation of lyophilized and vacuum dried rennet powders

1. Preparation of rennet powder by lyophilization - Rennet was precipitated from liquid fistulated calf rennet by saturating with sodium chloride. Precipitate
was separated out by centrifugation in a refrigerated centrifuge at 2800 rpm for 30 minutes and frozen in a deep freezer at a temperature \(-10^\circ\text{C}\) for 24 hours. The frozen precipitate was lyophilized at a pressure of \(1 \times 10^{-4}\) mm of Hg and a temperature of \(-40^\circ\text{C}\) to a moisture content of about 1%. The process of lyophilization usually took about 2 to 3 hours. The dry material was triturated and packed in an amber colour bottle and stored in refrigerator.

2. Preparation of rennet powder by vacuum drying in cold - Rennet was precipitated from liquid fistulated calf rennet in the same way as in the lyophilization procedure. The precipitate was spread on a Petridish and placed in a vacuum desiccator containing fused calcium chloride and phosphorus pentoxide as desiccant. After sufficient vacuum was attained (when bubbling commenced in the rennet paste), the desiccator was placed in refrigerator at 4 to 10°C. In about four to six days the dry material was obtained which was then ground to powder form, packed in amber colour bottles and stored in refrigerator.

Isolation of prorennin

Prorennin was prepared essentially using the methods of Foltmann (1959) and Rand and Ernstrom (1964) with some modifications. Fresh abomasal juice obtained after feeding 3/4 diluted boiled skim milk whey to
fistulated calf was immediately centrifuged in Servall centrifuge at 4700 rpm for 15 minutes and coagulation activity of the supernatant was determined. Sodium bicarbonate at a rate of 2% was added to the supernatant followed by 2% potash alum as clarifying agent. The mixture was allowed to stand for 15 minutes and then pH was adjusted to 8.0 with saturated disodium hydrogen phosphate solution and centrifuged at 4700 rpm for 15 minutes. The proenzyme of rennin present in the supernatant was precipitated by saturating with sodium chloride after adjusting pH to 8.0 with 0.1N sodium hydroxide and placed overnight in cold at refrigerator temperature. The precipitate was separated by centrifugation at 4700 rpm for 30 minutes and dissolved in 20 ml distilled water. The above procedure was repeated once more and finally the precipitate was either dissolved in 10 ml distilled water or vacuum dried over phosphorus pentoxide.

Procedure for preparation of anti-rennet serum

Two adult rabbits of good health were subjected to immunization with rennet from fistulated cow calves prepared as detailed previously. Injections were given according to the method described by Nair et al. (1965b) for milk immunization.

The dosage of antigen (rennet from fistulated cow calves 50 mg/ml) injection was gradually increased from
0.5 ml to 5 ml. These injections were given in a week of which, the first injection in all weeks except the first one, were given into the peritoneal cavity and rest of the injections were given directly into the bloodstream. The peritoneal route was resorted to minimise the anaphylactic shock caused by the administration of the enzyme protein into the bloodstream. For intravenous injections, the external marginal vein of the left ear was chosen since it was found to be the most convenient place. Three consecutive days in a week were chosen for injection according to convenience. Subsequent immunisations of the ensuing weeks were carried out on the same day as that of the first week's injections.

After completing three consecutive weeks of immunisation, rabbits were bled from right ear into sterile, dry test tubes and the serum was separated for testing and stored in the refrigerator after adding 3% of 5% phenol solution (volume/volume), as a preservative.

Preparation of casein and its fractions

a) Preparation of acid casein - Acid casein was prepared by following the procedure of Gupta and Ganguli (1965a). Milk (cow, buffalo or goat) after separation of fat was diluted by adding equal quantity of water and casein was precipitated by dropwise addition of 10% acetic acid with continuous stirring till the pH reached 4.6. The precipitated casein was allowed to
settle for some time and subsequently the whey was removed by filtering through a muslin cloth. The casein was then washed several times with distilled water to free it from acid and other soluble materials. The washed and wet casein was blended in the presence of excess acetone in order to remove moisture and traces of fat. Three washing with acetone were enough to make the casein moisture free. The acetone treated casein was then washed with ether to remove acetone and last traces of ether were removed by filtering the casein in Buchner funnel using suction. The fine particles of casein thus obtained were spread over a filter paper and air dried. Finally it was powdered into fine grains and preserved.

b) Preparation of k-casein - k-Casein was prepared from acid casein in the same manner as described by Zittle and Custer (1963) with some modification as follows:

The acid casein was prepared in the usual manner. About 350 g of the wet casein (60 to 90 g of protein) was dissolved in one litre of 6.6M urea. This solution was acidified with 200 ml of 7N sulphuric acid. After acidification, two litres of water was added giving it a final pH between 1.3 and 1.5. No precipitate was apparent at first, but gradually there was an increase in turbidity and a flocculent precipitate was formed. After two hours standing, the precipitate was filtered using
Whatman filter paper No. 1 and discarded. It was observed in the case of cow milk casein that the precipitate was flocculent and filtration was much quicker, while in the case of buffalo milk casein the precipitate was jelly like and filtration rate was relatively slow, whereas goat milk casein behaved exactly in the same way as cow milk protein. The k-casein in the filtrate was precipitated by addition of 132g(1M) ammonium sulphate to each one litre of filtrate. The precipitate was collected by draining the supernatant carefully and dialysed in a cellophane bag against several changes of distilled water for 48 hours at 5°C. k-Casein thus obtained was dried with acetone and ether in the same manner as acid casein.

Preparation of buffers

Various buffers used in the study (except those used for electrophoresis) such as phosphate, tris, maleate, and acetate, etc. of different pH were prepared according to the compositions given by Gomori (1955).

Other materials

Sephadex G-100 was purchased from Pharmacia, Uppsala, Sweden. Hansen rennet powder was a preparation of Hansen Lab., Copenhagen, Denmark. N-acetyl neuraminic acid and 2-thiobarbituric acid used were gift samples from Sigma Chemical Co., U.S.A. Whereas
Dansyl Chloride was gift from Dr. G.C. Cheeseman, NIRD, Shinfield Reading, England. Other chemicals and reagents used were of analytical grade.

**Determination of activity of rennet**

The milk coagulation activity of rennet was determined by the method of Berridge (1962 a,b). Ten ml of reconstituted milk (12 g spray dried skim milk powder in 100 ml of 0.01M calcium chloride) was incubated at 30°C in a water bath. As soon as the milk sample attained the temperature, 0.2 ml of rennet solution or abomasal juice was added and simultaneously time was recorded. It was stirred by means of a glass rod till the curd particles were visible. The time taken for the appearance of these curd particles was noted and recorded as the rennet coagulation time.

Unit of rennet activity is the amount of rennet needed to clot 10 ml of reconstituted milk in 100 second at 30°C.

$$\text{Activity} = \frac{100 \cdot d}{C.T.}$$

Where $d$ is the dilution of the original solution of rennet when 1 ml of the diluted rennet is added to 10 ml of substrate or is the amount in ml of original solution added to 10 ml when the solution used is without dilution. C.T. is coagulation time in seconds.
In these experiments:

\[
\text{Activity/ml} = \frac{100 \times 5}{\text{C.T.}} = \frac{500}{\text{C.T.}}
\]

and

\[
\text{Specific activity} = \frac{\text{Activity/ml}}{\text{mg protein/ml}}
\]

**Assay of prorennin content in abomasal juice**

Prorennin content of abomasal juice was determined according to the method of Rand and Ernstrom (1964). The fresh abomasal juice was centrifuged in a refrigerated centrifuge at 2500 rpm for 15 minutes at 0° to 5°C. The milk-clotting activity of the juice was determined immediately after adjusting pH to 4.5. This juice was kept in the refrigerator for 12 hours and milk-clotting activity was determined again. The increase in the coagulation activity accounted rennin formed due to conversion of prorennin into rennin.

Excess units of rennin represent the units of prorennin. Percentage of excess rennin units in total rennin units of juice represents the percentage of rennin present as prorennin.

**Determination of proteolytic activities of abomasal juice of fistulated cow calves**

a) **Proteolytic activity** - Casein (2g) used as substrate was dissolved in 5 ml of 0.1N sodium hydroxide by warming on a water bath and finally the volume was made up to 100 ml with phosphate buffer pH 7.0. The pH
of this solution remained between 6.9-7.0. Casein solution (5ml) was incubated with one ml of abomasal juice for half an hour at 30°C. After the incubation, the reaction was terminated by adding 10 ml of 5% trichloroacetic acid and the precipitate formed was removed by filtration using Whatman filter paper No.42. Tyrosine in the filtrate was estimated by the method of Lowry et al (1951) as follows:

To 0.5 ml of filtrate, 5 ml of alkaline copper sulphate was added followed by 0.5 ml of Folin’s reagent after 10 minutes. The mixture was kept for 30 minutes and intensity of the developed colour was measured in Klett-Summerson colorimeter using red filter. The readings were compared with a standard tyrosine curve and results were expressed in term of mg tyrosine liberated per g casein. A blank was run by stopping reaction of similar mixture at zero time and measuring the tyrosine liberated.

b) Peptic activity - The procedure followed for peptic activity of abomasal juice was according to Ganguli et al (1964). The complete assay system contained 5 ml of 2% solution of cow acid casein in 0.06N hydrochloric acid and 1 ml of abomasal juice of fistulated calf. The mixture was incubated at 30°C for half an hour, after which reaction was stopped by adding 10 ml of 5% trichloroacetic acid solution. The precipitated protein was filtered using Whatman filter
paper No. 42 and assayed for tyrosine as in case of proteolytic activity. Results were expressed as mg of tyrosine liberated per g of casein.

Analysis of rennet powder from fistulated calves along with Hansen rennet

a) Milk-clotting activity - Milk-clotting activity was determined according to the method of Berridge (1952 a, b) using 0.2 ml of rennet solution having 50 mg powder/ml.

b) Protein content - Protein content of rennet powder was determined by microkjeldahl method (Mathur, 1969).

c) Salt content - Salt content was determined by taking 0.1 ml of rennet solution (50 mg/ml) in 25 ml conical flask, adding 2 ml distilled water and then titrating it against 0.05N silver nitrate solution using potassium chromate as indicator. Results were expressed as percentage of sodium chloride.

Estimation of sialic acid in caseins

Sialic acid was estimated from acid and k-casein of cow, buffalo and goat milk using the thiobarbituric acid assay method of Warren (1959) with certain modifications described by Gupta and Ganguli (1965 a). The method is essentially as follows:
Acid casein (200 mg) or k-casein (40 mg) from cow, buffalo or goat milks was wetted with 4.5 ml of distilled water to which 0.5 ml of 1N sulphuric acid was added. The sample was then hydrolysed for 45 minutes at 80°C in water bath. After cooling, 0.45 ml of 0.1N sodium hydroxide was added. The tube was then shaken and centrifuged for 5 minutes. The supernatant was transferred into a separate tube. The residual casein was washed with 5 ml of acetate buffer pH 4.5 and again centrifuged for 5 minutes. This supernatant was combined with the previous supernatant. To this solution 0.1 ml of chloroform was added in order to clarify the solution. Finally, this solution was used for sialic acid estimation.

A known quantity, 0.2 ml of above solution was taken in a centrifuge tube with the help of a micropipette and 0.1 ml of sodium periodate solution (1.07g of sodium metaperiodate powder dissolved in 3 ml of warm distilled water and made to 25 ml with concentrated orthophosphoric acid of 89% strength) was added. The tube was incubated for 20 minutes at room temperature and 1 ml of sodium arsenite solution (10 g of sodium arsenite dissolved in 100 ml 0.1N sulphuric acid containing 7.25 g/100 ml of sodium sulphate) was added. The tube was shaken immediately after addition till the brown precipitate disappeared and then 3 ml of 2-thiobarbituric acid (0.6 g of 2-thiobarbituric acid in 100 ml of warm distilled water containing 7.25 of
sodium sulphate) was added. After proper shaking the tube was immersed in a boiling water bath for 15 minutes, taking care that the tube was covered with glass marbles. The tube was then cooled in ice cold water for 5 minutes. The colour was extracted with equal volume (4.3 ml) of cyclohexanone by mixing thoroughly and centrifuging at 2800 rpm for 5 minutes. The cyclohexanone layer containing the developed pink colour was removed with the help of a dropper. The intensity of colour was then read in Klett-Summerson colorimeter at 540 nm using green filter. Finally sialic acid values were calculated with the help of a standard curve of N-acetylneuraminic acid.

**Assay system for rennet action by estimation of the released sialic acid from casein**

The release of bound sialic acid from acid casein and k-casein by rennets from fistulated cow calves, buffalo calves, goat kids and Hansen rennet was evaluated following the method of Gupta and Ganguli (1985 b). Acid casein (200 mg) or k-casein (40 mg) from cow, buffalo or goat milks was dissolved in 2 ml of 0.02N sodium hydroxide by mild heating at a temperature not exceeding 60°C. The pH of the mixture was brought down to 7.0 by the addition of 3 ml of phosphate buffer, pH 7.0 and 0.1 ml of 0.1M calcium chloride was added. Then 0.1 ml of rennet (50 mg/ml) was added to the casein solution and mixture was
incubated for 20 minutes at 30°C in a water bath. The reaction was terminated by adding 80% trichloroacetic acid (TCA), final concentration of TCA in the mixture being 12.8%. The precipitated protein was filtered through Whatman filter paper No. 42 and the filtrate after measuring its volume was used for free sialic acid by the method of Warren (1959).

Another aliquot of the filtrate was hydrolysed with equal volume (1 ml each) of 0.2N sulphuric acid in a water bath at 80°C for 45 minutes for the estimation of bound sialic acid. The hydrolysates were cooled and 0.18 ml of sodium hydroxide added to bring up the pH to 4.5 and 0.2 ml of this solution was used for sialic acid estimation as described above for casein. The release of bound sialic acid was calculated after correcting the value for free sialic acid.

**Casein-agar plate assay of rennets**

The action of rennets prepared from fistulated cow calves, buffalo calves, goat kids and Hansen rennet were studied on acid caseins from cow, buffalo and goat milks, using the casein-agar gel diffusion technique. Acid casein was prepared from fresh milk (cow or buffalo or goat) according to method described by Ganguli et al. (1964). Particular attention was paid to such preparations, as the solubility of casein
samples in sodium acetate for plating with agar was found to be dependent on the method of preparation.

The casein-agar plate was prepared according to the method of Cheeseman (1963) with modifications suggested by Ganguli and Bhulela (1965). A hole of 12 mm diameter was made at the centre of casein-agar plate with the help of a cork borer. Rennet sample (0.18 ml to 0.2 ml) was then poured into the hole and allowed to diffuse. The incubation was carried out at 30°C for 48 hours. The increase in the diameters of two consecutive precipitated zones and clear transparent zone following the second zone was then recorded by placing a millimeters scale on the plate and taking average of three individual measurements on zone diameters from different positions in same plate. The diameters were recorded at a definite interval for 48 hours. Rennet samples having same coagulation activity were used for such studies.

**Assay system for development of turbidity by rennet in k-casein**

a) **Optimum pH** - Cow or buffalo k-casein (0.3%) was first dissolved in 5 ml of N/50 sodium hydroxide and then the required volume was made up by using maleate buffer of different pH from 5.5 to 7.5. Hansen or fistulated calf rennet (0.2 ml and having same coagulating activity) was added to 5 ml of k-casein solution of known pH and the mixture incubated
at 37°C. The turbidity developed in solution was measured in a Photochem colorimeter (using red filter 680 nm) after 5 minutes. The turbidity as optical density, was measured against a blank containing k-casein solution at same pH.

b) **Optimum concentration of casein and time** - Cow or buffalo k-casein (0.1 to 0.5% casein concentration) was first dissolved in 5 ml of sodium hydroxide and then the required volume was made up by using maleate buffer pH 6.8. Hansen or fistulated calf rennet (0.2ml) was added to 5 ml of k-casein solution of known concentration and the mixture was incubated at 37°C and turbidity developed was measured in Photochem colorimeter (using red filter 680 nm) after one minute interval upto 5 minutes and subsequently every 5 minutes. Casein solution without addition of rennet served as the blank.

e) **Optimum concentration of rennet** - A solution of 0.3% k-casein from cow or buffalo milk was prepared in maleate buffer pH 6.8. To each 5 ml of casein solution different quantities of fistulated calf or Hansen rennet (from 0.1ml to 0.5ml) was added and volume adjusted with distilled water. Turbidity developed after incubation of the mixture at 37°C for 3 minutes was measured in Photochem colorimeter in similar manner as in the case of optimum pH.
Activation of prorennin in presence of additives

The prorennin solution was adjusted to pH 4.5 and 2.0 by addition of 0.1N hydrochloric acid for activation studies. The additive such as cysteine (0.1%), aspartic acid (0.1%), glutamic acid (0.1%) and sodium chloride (2%), was added to prorennin solutions separately after adjusting the pH. The milk coagulation activity of these solutions was determined after 2 hours of incubation in cold. The prorennin solutions at pH 2.0 were adjusted to pH 4.5 with disodium hydrogen phosphate before determining the milk-clotting activity. The activity of prorennin preparations without additives served as control. Stimulation or inhibition of prorennin transformation into rennin was calculated by differences in milk-clotting activity.

Paper electrophoresis

Rennets, prorennin, activated prorennin, milk whey, and abomasal juice were subjected to paper electrophoresis according to the method of Sabarwal and Ganguli (1968b). Paper strips (40 cm x 4 cm) were cut from 3MM Whatman filter paper sheets. A line was drawn with lead pencil on every strip (8 cm apart from one end). They were then soaked in veronal buffer pH 8.6 containing 10% urea. Excess buffer was removed by blotting the strips between the sheets of filter paper.
and then were placed on the bridge horizontally folding the ends of the strips on both sides of bridge. About 0.05 ml of sample was applied in centre of line previously drawn leaving some space on both the sides. The bridge was placed in the migrating cells dipping its two ends in veronal buffer of pH 8.6 contained in the cell and sample side near the anode. The migration cell was then covered properly and run was conducted at 300 volts for 6 hours at room temperature.

After the completion of the run, the strips were taken out and dried in the oven at 60°C. Dried strips were stained with 0.1% bromophenol blue in alcohol saturated with mercuric chloride for 15 minutes. Excess of dye was removed by giving three successive washing with 0.5% acetic acid for 10 minutes, 5 minutes and 5 minutes. The stained strips were again dried at 60°C and protein spots were made prominent by ammonia vapour exposure.

**Starch gel electrophoresis of rennet**

Starch gel electrophoresis used for resolution of different rennets was the Petridish technique devised by Ganguli and Majumder (1967). The procedure employed was as follows:

1. **Preparation of rennet sample for electrophoresis**

Rennet (50 mg/ml) was dissolved in veronal buffer pH 8.6. The solution was applied on a strip of 3MM filter paper of 1.0 x 15.0 cm size, after leaving 1.0 x 3.0 cm space
on the top for writing the particulars of the sample. The solution was applied with the help of a pipette, excess solution being soaked out with a filter paper and dried with the aid of a hair dryer by hanging the strips on a piece of thread. After drying again the sample was applied and strip was dried in the same manner. The sample strips thus prepared were preserved in a desiccator over fused calcium chloride.

2. Preparation of starch gel plate - The following composition was used for the preparation of starch gel:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrolysed starch</td>
<td>2.085 g</td>
</tr>
<tr>
<td>Water</td>
<td>12.72 ml</td>
</tr>
<tr>
<td>Veronal buffer pH 8.6</td>
<td>2.28 ml</td>
</tr>
<tr>
<td>Urea</td>
<td>2.45 g</td>
</tr>
<tr>
<td>2-Mercaptoethanol</td>
<td>0.12 ml</td>
</tr>
</tbody>
</table>

This composition was used for each Petridish of 8.6 cm diameter. Veronal buffer (2.28 ml) and 12.72 ml of distilled water were mixed in a conical flask. A small portion of this mixture was then taken in a 250 ml suction flask and 2.085 gm of hydrolysed starch was added. The flask was shaken continuously to ensure a free suspension. The other portion of buffer-water mixture was heated to boil and then added to suction flask containing starch. The solution thus obtained was heated over a flame with continuous shaking till the mixture became viscous and clear. Then urea was
added and mixed in the solution over a flame till the whole solution again became clear. The solution was made free from entrapped air bubbles formed due to shaking by means of suction. After adding 0.12 ml of 2-mercaptoethanol in the hot solution, the solution was immediately poured in Petridish of 8.0 cm diameter. The plate was kept in a uniform place to spread the gel uniformly and was kept overnight in the refrigerator so that the gel solidifies.

3. Application of the sample -- Aliquots of the enzyme soaked filter paper strips were cut and gently inserted in the gel with the aid of a pair of forceps. The gel plate was kept for 30 minutes after insertion of sample before the electrophoretic run.

4. Electrophoretic run and subsequent drying of protein fractions -- The electrophoresis was run in veronal buffer, pH 8.6. Buffer was placed in equal volume in two beakers containing electrodes. The gel plate was kept on a stand in between the two beakers and connected with the buffer with the help of 3MM filter papers. The enzyme proteins were then allowed to migrate in the electrophoretic field for 1.0 to 2.0 hours from cathode to anode. The voltage was kept constant at 300 volt, while the current varied with the voltage.

After the completion of the run, the filter paper was gently separated from the gel and gel stained
with 0.1% amido black dye in methanol: water: acetic acid (5:5:1) for 10 minutes. The excess dye was washed by giving 4 to 5 successive washings at reasonable intervals by methanol: water: acetic acid solution (5:5:1).

Polyacrylamide gel electrophoresis

Acrylamide electrophoresis was done in Shandon disc electrophoresis apparatus according to the method described by Tombs and Akroyd (1967).

1. Preparation of acrylamide monomer - 'Cyanogum 41' a product of the American Cyanamide Company which was a mixture of 95% acrylamide monomer and 5% N,N'-methylene-bis-acrylamide (Bis) was used for gel preparation. A solution of 7.8% acrylamide gel was prepared by dissolving 1.08g 'Cyanogum 41' in 24 ml of veronal buffer, pH 8.6. To this solution, 7 ml of 0.1 to 0.3% β-methylaminopropionitrile (DMAP) was added with stirring, as catalyst-accelerator. After filtration of the solution, the polymerization was initiated in it by addition of 1.0 ml of freshly dissolved 7% (w/v) ammonium persulphate. It was then transformed to the gel tube (0.5 x 8.5 cm). During the filling of the gel tubes and polymerisation of the monomer solution, the tubes were perpendicularly held in stand with their ends closed by the polythene caps. The tubes were filled upto mark, 10 mm from the top and each tube was then over-layered with distilled water.
All this operation was completed within 10 minutes of addition of persulphate catalyst to monomer solution. After polymerization of monomer solution which usually took 20 to 25 minutes, the caps were removed from the ends of the gel tubes with care to avoid disturbing the gel by a suction effect.

2. Pre-electrophoretic run - The tubes were fixed vertically between upper and lower electrode reservoirs. The upper electrode reservoir had eight holes each fitted with a rubber gasket and a platinum electrode fixed in the centre of the vessel. Each tube was inserted underneath into one of the holes in upper electrode reservoir, any unused hole was sealed with rubber stopper. The space above the glass tubes was filled with buffer after removing water. Veronal buffer pH 8.6 was poured into upper electrode reservoir as well as lower reservoir. The lower reservoir had electrode similar to the upper. The bottom ends of tubes were dipped into buffer of lower reservoir. A pre-run of electrophoresis was carried out at a current 2 mA per tube for half an hour to one hour.

3. Preparation and application of sample - Rennet (50 mg/ml) was dissolved in 10% sucrose solution and optionally a trace of bromophenol blue was added as tracer dye. The enzyme preparation (26 µl) was layered directly on the top of gel in each tube by means of a
micro-pipette and a current of about 1 mA per tube was immediately run. After 10-15 minutes the current was increased to 2 mA per gel tube for a period of 2 to 3 hours.

4. Staining and destaining the acrylamide gel rod -

After electrophoretic run, the buffer solution in the upper tank was discarded and the gel tube systematically detached. Each gel rod was ejected from its glass tube by inserting a fine needle 1 to 2 cm between the gel (bottom end) inside surface of the glass tube, while holding the tube under the surface of cold water, in a bowl and rotating the needle with a gentle pulling action in order to break the adherence of the rod to the glass tube at that end. The needle was then inserted at the original end of the tube and again rotated with a pushing action and as a result, the rod was slid out. The gel rods were flexible and were picked up by hand and slid into a bath containing 1% amido black in 10% acetic acid. The rods were incubated for 12 hours and washed with water. The background staining was removed by washing the rods with 10% acetic acid solution with few changes of acetic acid solution. The stained protein band remained fixed in the gel.

Gel filtration -

Rennets and prorennin samples from fistulated animals were fractionated on columns of 2.8 x 40 cms length using Sephadex G-25, G-50 & G-100.
The gel powder was soaked in excess of distilled water for a minimum period of 120 hours and the column was carefully packed with the gel up to a height of 31 cm. It was then equilibrated with Tris-hydrochloric acid buffer pH 8.0. Two ml of sample (50 mg/ml for powder sample) was applied on the gel column and separated by molecular sieving in the cold. Tris-hydrochloric acid buffer at a flow rate of approximately one ml per minute, was used as the eluting solvent and 82 fractions (each fraction of 8 ml) were collected following the procedure of Majumder et al (1970). The collected fractions were then analysed for their protein content by the method of Lowry et al (1951).

Photooxidation of rennet

Rennet solution from fistulated cow calves was adjusted to different pH by addition of 0.1N hydrochloric acid or 0.1N sodium hydroxide. The solution of known pH was taken in a conical flask and methylene blue at a rate of 20 mg/100 ml, was added. The flask was illuminated by a table lamp of 100 watt electric bulb. The flask was kept at a distance of 20 cm from electric bulb facing a particular point marked on the flask. This distance remained same in all experiments. Air was sucked through the solution with the help of a water pump for 15 hours. The loss of volume of rennet solution due to air bubbling was made up with distilled water and
coagulation activity of the solution determined. Sample which was treated in similar way without light served as the control. All experiments were performed at 30°C. The percentage loss of activity was calculated by difference in milk-clotting activity.

Dansylation of rennet

Fistulated calf rennet (50 mg/ml) was dialysed against distilled water in cold till it was free from sodium chloride. The dialysed enzyme preparation was centrifuged in a refrigerated centrifuge at a speed of 10,000 rpm for 30 minutes. Supernatant obtained was assayed for rennet activity according to the method of Berridge (1952 a,b). The enzyme solution (supernatant) was adjusted to different pH between 4 to 8. To 12 ml of enzyme solution, 50 μl of dimethylaminonaphthalene sulphonyl chloride (dansyl chloride) in acetone (6 mg/ml) was added, incubated for an hour in refrigerator and rennet activity was assayed. Enzyme solution (12 ml) containing 50 μl acetone served as control. For comparative studies Hansen rennet was also treated in the same manner.

Double diffusion-in-gel technique of Ouchterlony for antibody and antigen reaction

The double diffusion-in-gel technique by Ouchterlony (1948) modified by Wadsworth (1957) was used for immunoprecipitation analysis.
1. **Preparation of agar gel plates** - One gram agar powder (Agar-Agar, Japan) was weighed and transferred into a 500 ml beaker containing 100 ml of 0.85% sodium chloride solution. Then 0.5 ml phenol was added to solution in order to prevent the growth of undesirable mold and microorganisms. The mixture was heated with continuous stirring till agar dissolved. Melted agar was poured into a Petridish to form a uniform thin layer of agar. After solidification of the agar layer, melted agar was again poured over it to form about 2 mm thick layer over first layer and allowed to set completely. In 20 to 25 minutes, the agar was completely set.

2. **Application of sample and subsequent development of plates** - Circular wells each having a diameter of 1 cm, were made in the top layer of agar gel. The pattern of the wells made in agar gel in the present studies consisted of five wells, one in the centre and the remaining four wells around it, each having a distance of one cm from the central well. The central well was filled with 0.2 ml of anti-rennet serum prepared against fistulated cow calf rennet while other four wells with 0.2 ml of antigens (150 mg/ml) i.e. Hansen rennet, fistulated cow calf, buffalo calf and goat kid rennets, respectively. After the application of samples, the plates were incubated...
at refrigerator temperature for six days. The plates were thoroughly washed with saline solution (0.9% sodium chloride) after incubation.