ACTA CHEMICA SCANDINAVICA 13 (1959) 1927–1935

# Studies on Rennin

## II. On the Crystallisation, Stability and Proteolytic Activity of Rennin

BENT FOLTMANN

*Chr. Hansen's Laboratory, Ltd., Copenhagen, Denmark*

Rennin has been crystallised from commercial preparations as well as from laboratory extracts from calf stomachs after clarification with $Al_2(SO_4)_3$ and $Na_2HPO_4$ followed by 2 precipitations with NaCl.

Solutions of crystalline rennin are shown to have optimum stability at pH 5.5 to 6.0. The stability is also good at pH 2, whilst at pH around 3.5 and above 6.5 the enzyme is unstable.

Proteolytic experiments have shown that casein is digested by rennin over a wide range of pH (2–6.5), whereas bovine serum albumin is digested only in a rather narrow interval around pH 3.4.

### 1. INTRODUCTION

Crystallisation of the milk-clotting enzyme, rennin, has been described by several authors [1–9], but only few systematic studies have been published on the chemistry and properties of rennin. Alais [5] has stated that crystalline rennin is homogeneous on free electrophoresis in phosphate buffer 0.1 M pH 6.8, whilst Ernstrom [9] found that crystalline rennin on electrophoresis in phosphate buffer, pH 6.8, ionic strength 0.2, consisted of a main component and a component of slower mobility representing about 3.8 % of the total protein. According to Schwander *et al.*[10], crystalline rennin prepared by Berridge [1] was electrohomogeneous on paper electrophoresis. The preparation was found to be not quite homogeneous in the ultracentrifuge and by diffusion measurements. Schwander *et al.*[10] also confirmed Hankinson's [2] observation that the isoelectric point of rennin is about pH 4.6.

No investigations concerning the stability of rennin * solutions in relation to pH have been published, but it has been stated earlier that rennet is rapidly inactivated by alkali, and that its maximum stability lies between pHs 5.3 and 6.3 (Holwerda [11], Porcher [12], Ege and Lundsteen [13]).

* Here and in the following rennin means the enzyme purified by crystallisation, and rennet means a preparation containing rennin and other proteins.

Having isolated the crystalline rennin, Berridge [1] proved that it was a proteolytic enzyme with pH optimum at 3.7 against hemoglobin as substrate. Using the same substrate, de Baun *et al.*[3] have confirmed Berridge's statement. The proteolytic degradation of casein by rennin from pH 2 to pH 7 has been studied by Nitschmann and Bohren [14]. However, these investigations have mostly concerned the primary proteolytic action which, according to Alais *et al.*[15], is thought to be the essential change for the coagulation of casein with calcium as observed in the ordinary clotting of milk by rennet.

In the course of the present work, 19 preparations of crystalline rennin have been made. The raw materials for the crystallisations have been Chr. Hansen's commercial rennet preparations, as well as extracts made in the laboratory from calf stomachs directly. On electrophoresis by the moving-boundary method, the preparations appeared electrohomogeneous at pH 5.19, 5.78 and 6.35, ionic strength 0.2. The stability experiments have shown that solutions of rennin have optimum stability in the same interval of pH as stated earlier for rennet. The experiments on proteolytic degradation of casein and serum albumin with rennin have shown maximum proteolytic activity at pH 3.4 to 4.0.

## 2. METHODS AND RESULTS

### 2.1. Determination of the milk-clotting activity

The activity is expressed in rennin units (RU) according to Berridge [1]. This unit is defined as the rennin activity which clots 10 ml of reconstituted skim milk in 100 sec at 30°C. The substrate consists of 12 g spray-dried skim milk powder reconstituted in 100 ml 0.01 M $CaCl_2$.

Though the differences in clotting ability of various skim milk powders are diminished to some extent by reconstitution in 0.01 M $CaCl_2$, the powders may still show differences from batch to batch. Instead of using correction factors to overcome this difficulty, as suggested by Berridge [16], it was considered preferable to carry out the routine milk-clotting tests as relative tests. The activity of unknown samples was compared with that of a sample of liquid rennet as reference standard. The stability of this reference standard was checked at intervals by means of a basic standard consisting of freeze-dried rennin crystals stored at −15°C.

The crystals of the basic standard were prepared as described below. After crystallisation, most of the salt from the mother liquor was removed by rapid washing with ice cold water. After centrifugation, the crystals were suspended in water and freeze-dried as a suspension. Using a sample of good-quality spray-dried skim milk powder, the activity of the basic standard was determined using Berridge's [16] method, 900 RU/mg N being obtained.

The routine assays were performed at 35°C, and took place in a water thermostat similar to that described by Sommer and Matsen [17]. 50 ml of fresh skim milk were pipetted into wide-necked, 125-ml glass bottles. The bottles were placed on rollers in the thermostat. They had a 20° inclination, and the opening was just above the surface of the water. During the experiments, the bottles were slowly rotated (18 r.p.m.). 1 ml of rennin dilution was added to each bottle and thoroughly mixed with the contents. The clottingpoint,

indicated by the appearance of the first grains in the film flowing down the sides of the bottles, was noted. It was arranged that the rennin concentration chosen would give a clotting-time of 6—8 min. This could generally be achieved by using rennin concentrations of about 4 RU/ml.

The dilutions were made so that the difference between the clotting-time of the unknown sample and that of the reference standard was less than 20 sec. The rennin activity of the unknown sample was then calculated on the assumption of inverse proportionality between the clotting-time and rennin concentration (Storch and Segelcke[18]).

Care was taken that the unknown sample and the reference standard used in the milk-clotting test had the same buffer composition, as even small amounts of buffers with pH different to that of the skim milk could influence the clotting-time.

## 2.2. Preparation of crystalline rennin

28 dried calf stomachs (450 g) were finely cut and extracted at room temperature under continuous stirring in 7 litres 1 N NaCl. After 4 h, the suspended tissue was separated by passing through a coarse strainer. 5.5 litres of raw extract was obtained (pH 5.8, pre-formed activity 9.8 RU/ml).

Clarification and activation were carried out in one process: 150 ml of 1/3 M $Al_2(SO_4)_3$ was added to the raw extract; pH after addition, 3.65. Immediately after, 150 ml of 1 M $Na_2HPO_4$ was added and the resulting pH was 4.6. In order to diminish the dilution caused by clarification, concentrated solutions of alum and phosphate, temperature 40°C, were used. The preparation was left to stand 24 h at room temperature, after which the suspension was neutralised to pH 5.6 with 70 ml 1 M $Na_2HPO_4$, and the precipitate formed was discarded after centrifugation. The last traces of turbidity disappeared after filtration. The volume of the clarified extract was 5 400 ml. Yield and activity of this extract and of the extract in the following steps of the preparation are illustrated in Table 1.

The clear yellow extract was precipitated by saturation with NaCl. After 24 h, the precipitate was collected by means of centrifugation. It was re-dissolved in 450 ml 1/20 M phosphate buffer, pH 6.3. The volume of the resulting solution was 500 ml, pH 5.5. The second precipitation took place in a refrigerator (2°C) as a slow salting-out according to McMeekin[19]. A dialysing bag

*Table 1.* Crystallisation of rennin.

| | ml | RU/ml | mgN/ml | RU/mgN | RU thousands | % recovery |
|---|---|---|---|---|---|---|
| Clarified extract | 5 400 | 164 | 2.40 | 68.4 | 886 | 100 |
| Dissolved 1st precipitation | 500 | 1 240 | 2.88 | 431 | 620 | 70 |
| » 2nd » | 140 | 3 830 | 7.06 | 542 | 536 | 60 |
| » 1st crystallisation | 90 | 3 880 | 4.30 | 902 | 349 | 39 |
| » 2nd » | 75 | 3 640 | 3.84 | 948 | 273 | 31 |
| Suspension 3rd » | 50 | 4 300 | 4.59 | 937 | 215 | 24 |

(Visking cellulose) with solid NaCl was slowly rotated whilst submerged 2 cm in the solution. 130 g of NaCl was dissolved in the course of 5 days. The bag was removed, and the globular precipitate was collected by centrifugation. It was dissolved in 115 ml 1/20 M phosphate buffer pH 6.3, and after one day in the refrigerator, a little insoluble residue was removed by means of centrifugation.

The clear solution (140 ml, pH 5.4) was left in the refrigerator. After 3 days, the first traces of crystals appeared, and the following week a rapid crystallisation took place. After 18 days, the suspension was centrifuged. The activity of the supernatant was 930 RU/ml. The crystals were washed in distilled water (2°C). The activity of the washings (100 ml) was 236 RU/ml. The crystals were dissolved in distilled water, and left to stand overnight in the refrigerator. A small, insoluble residue was discarded.

Re-crystallisation took place after addition of 5.4 g NaCl, and after 3 weeks in the refrigerator, the activity of the clear supernatant had fallen to 180 RU/ml. The crystals were once more collected, dissolved, and recrystallised as above. After the third crystallisation, the crystals were washed three times in 2 N, 3 N and 4 N NaCl respectivly. The crystals were stored in 4 N NaCl at 2°C.

Regarding the shape of rennin crystals, various statements have been published by different authors. Berridge [1], Alais [5] and Ernstrom [9] have published photos of rectangular crystals which appear to be identical with most of the preparations made in this laboratory. Hankinson [2] and Oeda [6] describe their preparations as needles. De Baun *et al.*[3] state that their crystals resemble Hankinson's, whilst Hostettler [7] has published a photo of some crystal fragments.

Nearly all our preparations of rennin have crystallised in rectangular plates or blocks, but two smaller samples crystallised in needles or long thin rods. The preparations were stored in a freezer at −15°C, and after 4 months, the needle-formed crystals had been transformed into rectangular plates. In spite of several attempts, it has not been possible to reproduce the needles, whether by re-crystallisation or in other preparations.

The homogeneity of the preparations was controlled by means of moving-boundary electrophoresis in a Tiselius apparatus equipped with the Svensson

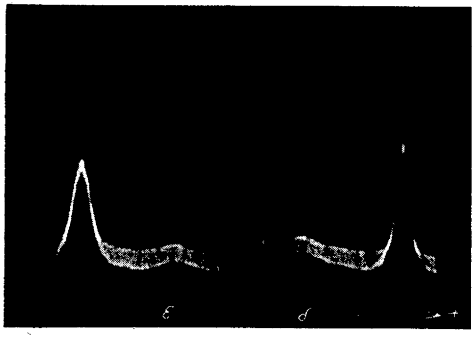

*Fig. 1.* Electrophoretic pattern of twice re-crystallised rennin. Concentration 0.29 % in phosphate buffer pH 5.78, ionic strength 0.1 + 0.1 M NaCl. The pattern is shown after 31 440 sec of migration, field strength 4.1 V/cm. Temperature in the bath 1.3°C. Svensson's optical system with parallel slit was used.

*Table 2.* Stability of rennin in solutions.

| | pH | Ionic strength | Initial activity | Activity after: 2 | 4 | 7 | 11 | days |
|---|---|---|---|---|---|---|---|---|
| *2°C* | | | RU/ml | RU/ml | | | | |
| Citrate buffer | 3.5 | 0.05 | 196 | 160 | 150 | 137 | 130 | |
| Phosphate buffer | 7.0 | 0.05 | 196 | 184 | 181 | 169 | 158 | |
| *25°C* | | | | 6 | 24 | 48 | 96 | hours |
| Citrate buffer | 3.5 | 0.05 | 196 | 164 | 133 | 112 | 92 | |
| Phosphate buffer | 7.0 | 0.05 | 196 | 176 | 147 | 113 | 95 | |

optical system. The runs were made at 1.3°C in phosphate buffers, ionic strength 0.1, to which was added NaCl to a total ionic strength of 0.2, pH 5.19, 5.78 and 6.35. The runs were made with a field strength of 3.7 to 4.4 V/cm. The mobility of the descending boundary was determined and found to be $-2.05$, $-3.44$ and $-3.53$ $10^{-5}$ cm$^2$ volt$^{-1}$ sec$^{-1}$. Under these conditions, the rennin appeared to be electrohomogeneous, Fig. 1. A solubility test also showed that the enzyme was free of significant contamination with other proteins (Foltmann [20]).

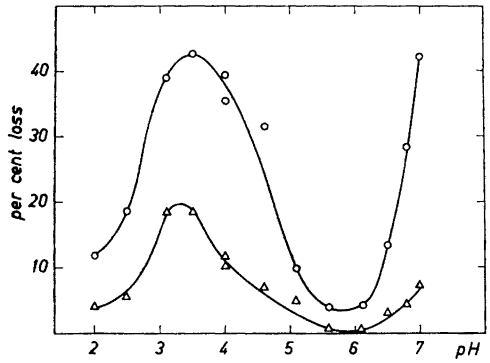


*Fig. 2.* The influence of pH on the stability of rennin expressed as per cent loss of initial activity after 48 h at 25°C (○) and 48 h at 2°C (△). Ionic strength of all solutions 0.05, pH 2—4 citrate buffers, pH 4—5.1 acetate buffers, pH 5.1—7 phosphate buffers. The upper points at pH 4 represent loss in citrate buffers, and the lower loss in acetate buffers.

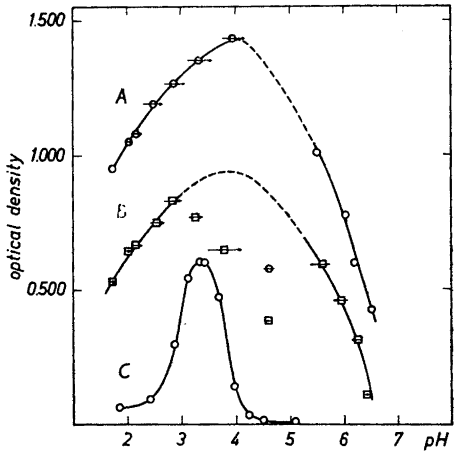


*Fig. 3.* Proteolytic activity of rennin. Substrate: A and B, 0.89 % casein; C, 0.86 % bovine serum albumin. Enzyme activity per ml reaction mixture: A, 5.33 RU; B, 1.33 RU; C, 14 RU. All experiments, 1 h reaction at 32°C. Ordinate, optical density at 275 mμ of supernatant after precipitation with TCA.

### 2.3. The influence of pH on the stability of rennin solutions

A 10-ml suspension of rennin crystals was centrifuged. The crystals were washed with cold, distilled water, and after suspension in water, were dialysed in a Visking bag against 3 × 2 litres distilled water at 2°C (6 + 20 + 24 h). By conductivity measurement, it was established that the final concentration of NaCl was less than $10^{-4}$ N. The activity of the solution was, after dilution to 200 ml, 392 RU/ml, pH 6.0. Equal volumes (5 ml) of buffers, ionic strength 0.1, and rennin solutions were mixed, and to each tube were added three drops of toluene. The tubes were stoppered and divided into two series, one being placed in a water thermostat at 25°C, and the other being in a refrigerator at 2°C. The result of the experiment, expressed as the per cent loss of the initial activity after two days, is illustrated in Fig. 2. Table 2 shows the course of the inactivation at pH 3.5 and 7.0.

The stability experiments were performed in citrate buffers, pH from 2.0 to 4.0. Acetate buffers were used from 4.0 to 5.1, and phosphate buffers for pH over 5.1. At pH 4.0, acetate and citrate buffers were tested in parallel. In all experiments, citrate buffers gave slightly higher losses in activity than acetate buffers at pH 4.0. As the inactivation of rennin at pH below 5 may be considered to be an autolysis, it is of interest to note that similar results have been obtained with digestion of serum albumin in citrate and acetate buffers. The former gave a more rapid degradation than the latter, indicating that citrate has an activating effect upon the proteolytic activity of rennin. At pH 5.1, experiments were made in parallel with acetate and phosphate buffers, but no difference in stability was noted.

### 2.4. The proteolytic activity of rennin

*Digestion of casein by rennin.* The casein was precipitated from fresh skim milk by adding 0.1 N HCl to pH 4.6, redissolved in water by slowly adding 0.1 N NaOH to pH 6.7, and was then re-precipitated twice. After washing with distilled water, the casein was dried with alcohol and ether. For the experiments, the casein was dissolved by slowly adding 0.1 N NaOH to pH 6.7. Two ml of a 2 % casein solution was mixed with 2 ml of diluted HCl (the concentration of HCl required to give a suitable pH was determined in preliminary experiments). Digestion took place in a water thermostat at 32°C. The casein mixtures were stirred for 60 min by mechanical inversions of the tubes (three inversions per min). 0.5 ml of rennin was added to each tube, and after a further 60 min, during which time the inversions were continued, 3 ml of the reaction mixture was precipitated with 3 ml 1 M trichloroacetic acid (TCA). The precipitate was removed by centrifugation, and the optical density of the supernatant was determined at 275 mμ. A blank test, in which rennin was added after addition of TCA, was run at each pH. The pH was measured at the beginning and at the end of the reaction. The results are illustrated in Fig. 3 A and B. In most of the tubes the pH was displaced during the reaction as illustrated by the length of the arrows.

Due to the insolubility of casein around the isoelectric point pH 4.6, the proteolytic activity gives irregular results in this range of pH. In Fig. 3, the branches of the curves outside the range of pH in which casein is precipitated, are connected with dotted lines in order to obtain regular curves with only one maximum.

*Digestion of serum albumin by rennin.* The substrate in these experiments was "bovine serum albumin — Armour". The substrate was supplied as a 30 % solution and was diluted down to 6 % before use. 1 ml substrate solution was mixed with 5 ml citrate buffer, ionic strength 0.1. The reaction took place at 32°C. The substrate mixtures were placed in the thermostat 15 min before addition of 1 ml rennin dilution to each tube. The reaction was stopped after 60 min by adding 5 ml 1 M TCA to each tube, and after centrifugation, the optical density of the supernatant was determined at 275 mμ. A blank test was run at each pH. No displacement of pH was noticed during the reaction. The results are shown in Fig. 3 C.

## 3. DISCUSSION

As the clotting ability of re-constituted skim milk depends to a great extent upon the milk powder used, it has been very difficult to compare the enzymic activity of preparations of rennin crystals made at different laboratories, even if the activity has been expressed in rennin units according to Berridge [1].

Alais [5] made an attempt to compare his crystals to those of Berridge but came to rather divergent results, probably owing to the above-mentioned difficulties. Fortunately, the activity of several preparations of crystals has been compared against Chr. Hansen's commercial rennet, and the author has therefore been able to convert the activities to the same RU used in this work.

The author is grateful to C. Alais for personal communication of results on crystals made after salt fractionation and after preparative electrophoresis on agar gel(published [21]). The author is likewise indebted to M. T. Sode-Mogensen and J. Thomasov, and to M. T. Sode-Mogensen and N. Tofte Jespersen for permission to publish their analyses of a sample of Berridge's crystals (prepared 1945), analysed in 1951 and 1955. H. Dan of Chr. Hansen's Laboratory, Inc., Milwaukee, has independently prepared a sample of rennin crystals, which has been analysed at this laboratory. Ernstrom's result is taken from his publication [9]. The various results appear in Table 3.

*Table 3.* Comparison between the rennin activity per mg N of different preparations of crystalline rennin.

| Preparation | Analysed by | RU/mg N |
|---|---|---|
| Average this work | B. Foltmann | 944 |
| H. Dan | B. Foltmann | 1 066 |
| C. A. Ernstrom [9] | C. A. Ernstrom | 922 |
| N. J. Berridge [1] (1945) | M. T. Sode-Mogensen and J. Thomasov (1951) | 889 |
| N. J. Berridge [1] (1945) | M. T. Sode-Mogensen and N. Tofte Jespersen (1955) | 731 |
| C. Alais [5] (prepared by salt fractionation) | C. Alais | 662 |
| C. Alais [21] (prepared by electrophoresis) | C. Alais | 858 |

Though there are appreciable differences between the various preparations, the activity of all is of the same order of magnitude. It should be added that deviations in the preparations made at this laboratory have been from 880 RU/mgN to 1 000 RU/mgN. Alais, analysing a sample of our crystals, found a deviation of only 4 % from that found here.

The stability of solutions of crystalline rennin is considerably less than that of commercial liquid rennet. This is often met with in the purification of enzymes (Anson [22]). The results of the present work confirm the well-known fact that rennin is very sensitive to alkaline or even neutral reaction. Around pH 3.5, the enzyme is unstable. As this range coincides with that of the maximum of proteolytic activity, it indicates that the loss may be due to autolysis. When comparing the inactivation of rennin at 2° and 25°C it is noticed that by raising the temperature, the inactivation at pH 7 is increased considerably more than at pH 3.5.

Casein is the natural substrate for rennin, but as it is only very slightly soluble around its isoelectric point (pH 4.6) it is not well suited to characterise the proteolytic activity of rennin in relation to pH. The precipitated casein is not particularly accessible to the proteolytic activity of rennin, and this will cause irregular observations around pH 4.6. On deeper degradation, these irregularities will be reduced to some extent. However, if the observations plotted in Fig. 3 are connected in such a way that regular curves with only one maximum are obtained, the "maximum" of proteolytic activity is found at pH 4. As it was observed that citrate precipitates casein over a greater range of pH than does HCl, the experiments have been performed without addition of buffer, although this had the disadvantage of the pH being displaced during the reaction. On comparing the degradation of casein and serum albumin, it was noticed that, 1: serum albumin is only degraded in a rather narrow interval around pH 3.4, and 2: that the degradation of serum albumin proceeds much more slowly than that of casein. However, this cannot be regarded as a special case for casein and rennin, as casein in general is more easily digested by proteolytic enzymes than are globular proteins.

Though the general proteolytic activity of rennin is only feeble at pH 6.6—6.7, which is the normal pH of fresh skim milk, Alais *et al.*[15] have shown that the clotting of casein starts with a rapid release of non protein nitrogen (NPN) soluble in 12 % TCA. A study on the enzymatic and the coagulation stages of the clotting of milk by rennin (Foltmann [23]) has supported the point of view that the increase of NPN soluble in 12 % TCA corresponds to the enzymatic phase proper. The lack of linearity between the rennin concentration and the reciprocal of the clotting time may be explained by the time lag of the coagulation stage, when the clotting point observed occurs some time after the increase of NPN comes to an end.

*Acknowledgements.* The electrophoretic experiments were carried out at the Protein Laboratory of the University of Copenhagen by Dr. N. Harboe. The author is grateful to Dr. N. Harboe for his kind help and for many inspiring discussions. The author wishes to thank the management of Chr. Hansen's Laboratory Ltd. for permission to publish the results. Thanks are also due to the staff of the laboratory for their kind interest in the work, especially to Mrs. K. M. Djurtoft for her valuable co-operation and to Mrs. I. M.

Kristensen for skilled technical help. The author is indebted to Prof. C. Faurholt, Dr. R. Djurtoft and Dr. N. Harboe for critical review of the manuscripts. Mr. R. Gillman has kindly assisted in the translation to English.

## REFERENCES

1. Berridge, N. J. *Biochem. J.* **39** (1945) 179.
2. Hankinson, C. L. *J. Dairy Sci.* **26** (1943) 53.
3. De Baun, R. M., Connors, W. M. and Sullivan, R. A. *Arch. Biochem. Biophys.* **43** (1953) 324.
4. Berridge, N. J. and Woodward, C. *J. Dairy Res.* **20** (1953) 255.
5. Alais, C. *Lait* **36** (1956) 26.
6. Oeda, M. *XIV Intern. Dairy Congr.* Rome 1956 **2** (2) 367.
7. Hostettler, H. *Milchwissenschaft* **10** (1955) 40.
8. Foltmann, B. *Acta Chem. Scan.* **12** (1958) 343.
9. Ernstrom, C. A. *J. Dairy Sci.* **41** (1959) 1663.
10. Schwander, H., Zahler, P. and Nitschmann, H. *Helv. Chim. Acta* **35** (1952) 553.
11. Holwerda, B. J. *Biochem. Z.* **134** (1923) 381.
12. Porcher, C. *Lait* **10** (1930) 47.
13. Ege, R. and Lundsteen, E. *Biochem. Z.* **268** (1934) 164.
14. Nitschmann, H. and Bohren, H. U. *Helv. Chim. Acta* **38** (1955) 1953.
15. Alais, C., Mocquot, G., Nitschmann, H. and Zahler, P. *Helv. Chim. Acta* **36** (1953) 1955.
16. Berridge, N. J. *Analyst* **77** (1952) 57.
17. Sommer, H. H. and Matsen, H. *J. Dairy Sci.* **18** (1935) 741.
18. Storch, V. and Segelcke, Th. *Milch-Ztg.* **3** (1874) 997.
19. McMeekin, T. L. *J. Am. Chem. Soc.* **61** (1939) 2884.
20. Foltmann, B. *Acta Chem. Scand.* **13** (1959) 1936.
21. Alais, C., Grabar, P. and Cristol, G. *Intern. Symp. Enzyme Chem.* Tokyo 1957, Pergamon Press, London 1958, p. 523.
22. Anson, M. L. *Les Protéines*, 9éme Conseil de Chimie, Stoops, Bruxelles 1953 p. 213.
23. Foltmann, B. *XV Intern. Dairy Congr.* London 1959 **2**, p. 655.

Received July 15, 1959.