STUDIES OF CASEIN

V. THE ACTION OF RENNIN ON CASEIN

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[*Manuscript received January* 30, 1959]

Summary

The liberation of non-protein nitrogen, soluble in 12 per cent. trichloroacetic acid, from various milk protein fractions by rennin (0·1 μg rennin N/ml) at pH 6·7 and 25°C is examined. 1·0 per cent. nitrogen is rapidly released from whole casein, 3·4 per cent. from second-cycle casein—fraction S, and 6·7 per cent. from κ-casein. None is split from other milk protein fractions under the same conditions.

On treatment with rennin, κ-casein is converted into insoluble para-κ-casein and a soluble fraction containing approximately 23 per cent. of the total nitrogen. Preliminary analyses indicate that this "soluble nitrogen" contains the "glyco-macropeptide" of molecular weight 6000–8000 (cf. Nitschmann, Wissmann, and Henzi 1957). It is therefore suggested that the primary action of rennin on casein is to release this glyco-macropeptide from κ-casein thus destroying the micelle-stabilizing properties of the latter. End-group analyses of κ-casein and para-κ-casein suggest that this process does not involve the rupture of peptide bonds.

I. INTRODUCTION

The mechanism of milk clotting by rennin can be broadly resolved into two stages: (*a*) the enzymic conversion of casein into paracasein, and (*b*) the subsequent coagulation in the presence of calcium ions. There has been much controversy as to which of the components of casein functions as the "protective colloid" of Linderstrøm-Lang (1929). Until very recently most of the evidence has pointed to α-casein as acting in this capacity, and as being the site of primary attack by rennin.

Nitschmann and his co-workers (Alais *et al.* 1953; Nitschmann and Keller 1955) have shown that during the rennet curdling of milk, the amount of non-protein nitrogen (NPN) which is not precipitated by 12 per cent. trichloroacetic acid (TCA) increases markedly before visible clotting occurs. They concluded that the NPN originates from the α-casein, a negligible amount being split from β-casein under the same conditions. The specific splitting reaction, which sets free the small amount of NPN very quickly, was considered to be directly responsible for milk clotting.

Alais (1956) and Nitschmann, Wissmann, and Henzi (1957) have made a study of the chemical and physical characteristics of the NPN split from whole casein by rennin. Approximately 1·5 per cent. NPN (soluble in 12 per cent. TCA) and 4 per cent. NPN (soluble in 2 per cent. TCA) were rapidly split from whole casein by rennin at 25°C. The NPN soluble in 12 per cent. TCA proved to be mainly a "glyco-

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macropeptide" with a molecular weight of 6000–8000 and a very unusual composition. On precipitating the protein in the reaction mixture by adjustment to pH 4·7, between 4 and 5 per cent. NPN remained in solution; this also consisted of the glyco-macropeptide together with smaller peptides which formed comparatively slowly. These results therefore suggest that the *primary* action of rennin on casein is to set free the glyco-macropeptide.

The discovery of κ-casein (Waugh and von Hippel 1956), its isolation, and the demonstration of its micelle-stabilizing properties in the present series throw doubt on the identification of α-casein as the protective colloid. As discussed earlier in this series the so called "α-peak" obtained on electrophoresis of skim milk or whole casein actually represents an α-κ complex. The alteration of the "α-component" of whole acid casein on rennin treatment (Nitschmann and Lehmann 1947) could therefore result from a primary attack on κ-casein and would not necessarily represent a change in α-casein. Also, the protective colloid properties attributed to "α-casein" and its behaviour under the action of rennin could be due to contamination of the α-casein with κ-casein.

In this paper it will be shown that the NPN which is split from whole casein during rennin treatment stems from the κ-casein. An examination of the free α-amino end-groups before and after the action of rennin is also included, as well as some observations on the nature of the material split from κ-casein.

A preliminary report on part of this work has been presented previously (Wake 1957).

II. Materials and Methods

(*a*) *Crystalline Rennin*

At the suggestion of Berridge (personal communication), "Sterren" cheese-making rennet powder (Benger's Ltd., Holmes Chapel, Cheshire, England) was used as starting material for the preparation of crystalline rennin. The first attempt, using essentially the procedure of Berridge and Woodward (1953), without seeding, was unsuccessful. In later experiments the rennin solution was clarified by centrifuging for 90 min at 5°C and 90,000 *g* in a "Spinco" preparative ultracentrifuge, model L (rotor 30; 28,000 r.p.m.), and reprecipitating once more before setting aside for crystallization. The final solution, light straw in colour, failed to yield crystals after 2 days in the refrigerator. On seeding, however, crystals appeared overnight and continued to increase in quantity for several days. Crystals were also obtained on seeding a solution of a freeze-dried preparation from Australian rennet. Approximately 1 g wet crystals were obtained from 500 g of the "Sterren" powder. The rennin was recrystallized by dissolution in a minimum quantity of 0·1M phosphate buffer (0·1M Na_2HPO_4+0·1M KH_2PO_4) at pH 6·8 and 2°C and readjustment to pH 5·4 with 0·1N HCl. Seeding at this stage was not necessary and crystals appeared overnight. However, the yield was rather low. Because of this and the difficulty in redissolving the crystals to prepare stock solutions, the once-crystallized material was further purified by dissolving in phosphate at pH 6·8 and 2°C, adjusting to pH 5·4, and reprecipitating at room temperature by direct saturation with NaCl.

The precipitated material was obtained by centrifuging for 40 min at 5°C and 13,000 r.p.m. in a M.S.E. type 92110 high-speed refrigerated centrifuge using a type S775 rotor, and stored in the moist condition at 2°C in the presence of thymol. On dissolving this material in a minimum quantity of iced water crystallization occurred very readily. The crystalline material had the same form as that described by Berridge (1955).

The once-crystallized and precipitated rennin gave a single peak on electrophoresis in phosphate buffer (Miller and Golder 1950) at pH 6·0, ionic strength 0·1, with a mobility of $-2{\cdot}95\times10^{-5}$ cm^2 V^{-1} sec^{-1} at 0·7 per cent. protein concentration. In agreement with Schwander, Zahler, and Nitschmann (1952), the material showed heterogeneity (abnormal spreading) on sedimentation in the pH 6·0 buffer at 2°C. The S_{20} value was approximately 4·4S, which is near the value reported by Schwander, Zahler, and Nitschmann (1952).

(*b*) *NPN Liberation Experiments*

(i) *Total Whey Protein.*—The supernatant, obtained after removal of the casein from the skim milk by the method of von Hippel and Waugh (1955), was used as a source of the total whey protein. It was filtered through Whatman No. 3 paper at 2°C to remove most of the fat, centrifuged at 90,000 *g* and 5°C for 90 min in a "Spinco" preparative ultracentrifuge, and dialysed exhaustively against 0·1M NaCl at 2°C. It was then freeze-dried and stored at 2°C.

(ii) *Experimental Procedure.*—Stock solutions of milk protein fractions were prepared by dissolving the freeze-dried material in 0·1M NaCl and dialysing against a large volume of the same solvent at 2°C overnight. After nitrogen estimations, solutions of required concentration were made up by first adjusting to pH 6·7 with 0·05N HCl or 0·05N NaOH, and then diluting in the correct proportion with 0·1M NaCl. Stock rennin solutions were prepared by dissolving the once-crystallized and reprecipitated material in ice-cold 0·1M NaCl. The nitrogen content of this stock solution was also determined.

20 ml milk protein solution, contained in a 50-ml conical flask, were warmed in a water-bath at 30°C for 20 min. Approximately 0·1 ml rennin solution was pipetted into the protein solution with thorough mixing to give a final concentration of 0·1 μg rennin N/ml. Samples (2 ml) were removed at suitable intervals and pipetted quickly into 4 ml 18 per cent. TCA at room temperature. After mixing, the solution was allowed to stand for 10 min before filtering through a small Whatman No. 42 paper. The nitrogen content of a sample of the clear filtrate was then estimated. NPN values at zero time were obtained from samples taken just prior to the addition of the rennin.

(iii) *Nitrogen Estimations.*—These were obtained by direct Nesslerization of a sample, after digestion under conditions suggested by McKenzie and Wallace (1954) but without the metal catalyst. The procedure used afforded results reproducible to within 5 per cent. over the range 40–150 μg N. Tryptophan (100 μg N) gave 98 per cent. recovery even in the presence of 12 per cent. TCA.

(c) *α-Amino End-group Analysis*

(i) *Preparation of Para-κ-casein.*—Approximately 50 ml 1 per cent. κ-casein in 0·1M NaCl at pH 6·7 was allowed to react with rennin (0·1 μg rennin N/ml final concentration) at 30°C for 30 min. The reaction mixture was cooled in an ice-bath and centrifuged for 50 min at 13,000 r.p.m. in a M.S.E. high-speed refrigerated centrifuge. The clear supernatant was poured off and the sediment treated with water at 80°C for 10 min to inactivate the enzyme. It was then washed with alcohol, ether, and dried in a vacuum over P_2O_5. The supernatant, on adjustment to pH 4·6 with 0·05N HCl, gave only a slight cloudiness. It was heated to 80°C for 10 min, cooled, and readjusted to pH 7 with 0·05N NaOH. It was then freeze-dried. Approximately 400 mg para-κ-casein and 100 mg "soluble nitrogen" (after correcting for NaCl) were obtained.

(ii) *End-group Analysis Procedure.*—Fluorodinitrobenzene (FDNB) (L. Light & Co., England) was used throughout. Most of the dinitrophenyl (DNP)-amino acids were prepared by the methods of Fraenkel-Conrat, Harris, and Levy (1955) or Sanger (1945). 2-DNP-lysine was prepared according to Porter and Sanger (1948). The DNP-derivatives had melting points close to those reported in the literature, and all moved as well-defined spots at the correct rates in the paper chromatographic systems used.

The paper chromatographic systems found most useful were the "toluene"–phosphate, and *tert.*-amyl alcohol systems described by Fraenkel-Conrat, Harris, and Levy (1955). Ethylene chlorhydrin, toluene, and *tert.*-amyl alcohol (laboratory grade) were distilled before use. Solvents used for washing and extracting the DNP-derivatives were also distilled. Peroxide-free ether was used throughout. All other chemicals were A.R. grade.

The DNP-derivatives of κ- and para-κ-casein were prepared as follows: 0·6 g κ-casein were dissolved in 40 ml water and adjusted to pH 9·2 with 0·5N NaOH. 0·6 ml FDNB in 5 ml ether were added with vigorous stirring. The pH was maintained at 9·2 for 2½ hr by the addition of dilute NaOH from a burette. The solution was extracted four times with an equal volume of ether to remove excess FDNB. 1N HCl was added to pH 3·8 and the yellow suspension extracted again with ether to remove dinitrophenol. The precipitate was then centrifuged out, and washed with water, acetone, and finally ether. It was dried in a vacuum over P_2O_5. Para-κ-casein (0·4 g) was dissolved at pH 12 and readjusted to pH 9·2 before reaction with FDNB. This protein aggregates very readily near neutral pH so it was necessary to use the high pH, 9·2, for reaction with FDNB.

The hydrolysis and extraction procedure was carried out as follows: Approximately 20 mg DNP-protein were hydrolysed with 1 ml constant-boiling HCl (glass-distilled) at 105°C in a sealed tube, usually for 16 hr. After cooling it was diluted with 4·7 ml water and centrifuged to remove the brown insoluble humin, which was only formed in any quantity in the case of DNP-κ-casein. The solution was extracted four times with 5 ml ether (only a faint yellow colour was transferred to the ether layer in each case). The combined extracts were evaporated in a vacuum at room temperature. The water layer was evaporated to dryness in a vacuum at 50°C. Both layers were then examined for DNP-amino acids by the chromatographic systems mentioned.

The ether layer could be transferred to the paper with small portions of acetone. The water layer, however, proved rather difficult to handle. This was due to the large amount of ϵ-DNP-lysine always present. Acetone, containing a trace of concentrated HCl, was generally used to extract the yellow colour. In some cases it was extracted directly from the water layer, before evaporation, with *n*-butanol.

Quantitative estimations of the DNP-amino acids were carried out on the hydrolysates from approximately 40 mg DNP-protein using the procedure of Fraenkel-Conrat, Harris, and Levy (1955). The simplified procedure for the identification and estimation of DNP-arginine described by Wissmann and Nitschmann (1957) was applied to the water layer in each case.

Details of other materials and methods have been given in Parts I and II of this series (McKenzie and Wake 1959*a*, 1959*b*).

III. Results

(*a*) *The Liberation of NPN from Milk Protein Fractions by Rennin*

The results of an investigation of the liberation of NPN (soluble in 12 per cent. TCA) from various milk protein fractions by crystalline rennin are summarized, in Figure 1. The first-cycle casein, second-cycle casein—fractions P and S, and the total whey protein were all prepared from the one milk sample. With first-cycle casein 1·0 per cent. NPN is released within 20 min while none is split from the total whey protein. There is no significant release of NPN with casein from which the κ-component has been removed (second-cycle casein—fraction P) while a marked increase to 3·4 per cent. is evident with second-cycle casein—fraction S. With pure κ-casein approximately 6·7 per cent. NPN is released rapidly. No significant splitting occurs with pure α-casein.

Aggregates form during the action of rennin on the pure κ-casein. On addition of the enzyme to a clear solution of κ-casein the latter begins to appear cloudy. This cloudiness increases in intensity, and the reaction mixture has the appearance of skim milk by the time the maximum value for NPN is reached. These aggregates can be centrifuged out to leave a clear supernatant, containing 23 per cent. of the total nitrogen, which gives no significant precipitate on adjustment to pH 4·6. (The 23 per cent. value could be slightly high due to the trace of β-casein in the κ-casein preparation.) The aggregated material, which will be referred to as para-κ-casein, appeared to move as a single component on paper electrophoresis in veronal buffer at pH 9·0, ionic strength 0·1 (Fig. 2). Extensive adsorption of para-κ-casein makes the identification of traces of other components difficult.

The "soluble nitrogen" (23 per cent.) gives a deep red colour when boiled with Bial's reagent and this is due to the presence of neuraminic acid. It also contains phosphorus (molybdate), and gives a positive sulphuric acid–cysteine test (Dische 1953, 1954) for galactose.

The NPN values obtained at zero time might be of some significance in indicating a small amount of TCA-soluble nitrogen in first-cycle casein and second-cycle casein—fraction S. The zero time values for the various casein fractions were as follows: first-cycle casein, 1·4 per cent.; second-cycle casein—fraction P, 0·6 per

cent.; second-cycle casein—fraction S, 3·1 per cent.; κ-casein, 1·2 per cent. A certain zero time value was expected in each case as a result of slight hydrolysis caused by the TCA at room temperature—lower values would almost certainly have been obtained had the TCA been cooled to 0°C before addition of the protein sample. It is possible that there is approximately 0·6 per cent. TCA-soluble nitrogen in

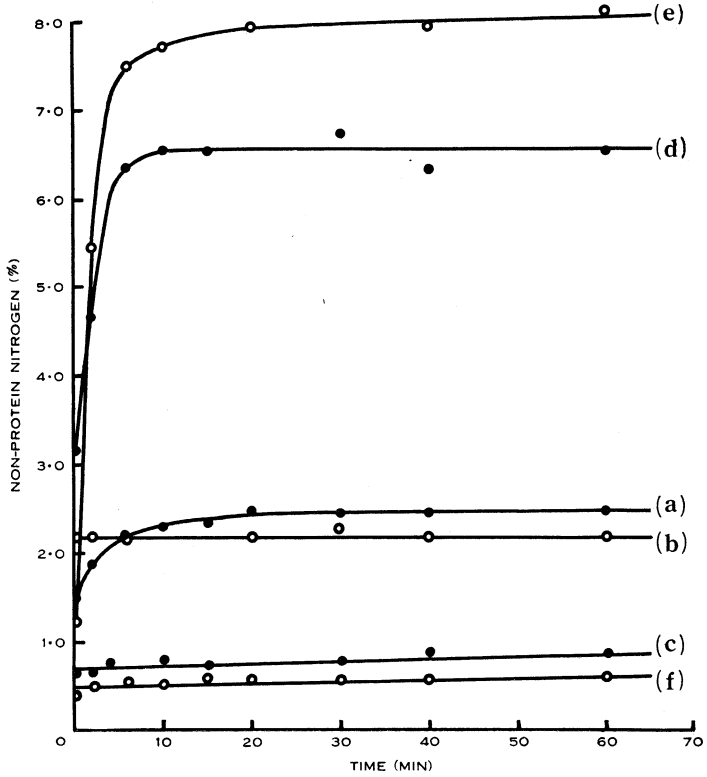

Fig. 1.—Liberation of non-protein nitrogen (soluble in 12 per cent. TCA) from milk protein fractions by rennin. All studies were made at pH 6·7 and 30°C using a preparation containing 0·1 μg rennin N/ml. (*a*) 2 per cent. first-cycle casein; (*b*) 1 per cent. total whey protein; (*c*) 2 per cent. second-cycle casein—fraction P; (*d*) 1 per cent. second-cycle casein—fraction S; (*e*) 1 per cent. κ-casein; (*f*) 2 per cent. α-casein.

first-cycle casein and this remains in the supernatant (second-cycle casein—fraction S) on treatment with 0·25M $CaCl_2$ to give the precipitated second-cycle casein—fraction P. All or most of this TCA-soluble nitrogen is removed during the isolation of pure κ-casein. It is certainly not any of the whey proteins which give a zero time value of only 2·2 per cent.

(*b*) *α-Amino End-groups in κ-Casein and Para-κ-casein*

Examination of the ether layer, obtained from the hydrolysis of DNP-κ-casein by the two-dimensional toluene–phosphate and *tert.*-amyl alcohol systems indicated the presence of dinitroaniline, dinitrophenol, small amounts of DNP-aspartic and

DNP-glutamic acids, and traces of DNP-serine and DNP-threonine. Also present was an orange-coloured derivative which moved just ahead of threonine in the *tert.*-amyl alcohol system, and in the valine area in the two-dimensional system. Like dinitrophenol, it was decolorized by HCl, and therefore did not represent a DNP-amino acid. It is most likely identical with one of the "orange-coloured artefacts" observed by Redfield and Anfinsen (1956) during their studies on the structure of ribonuclease. No traces of additional DNP-amino acids could be identified in the ether layer with short hydrolysis periods of 2 and 10 hr. It is unlikely that DNP-proline, DNP-glycine, and bis-DNP-cystine would have been *completely* destroyed

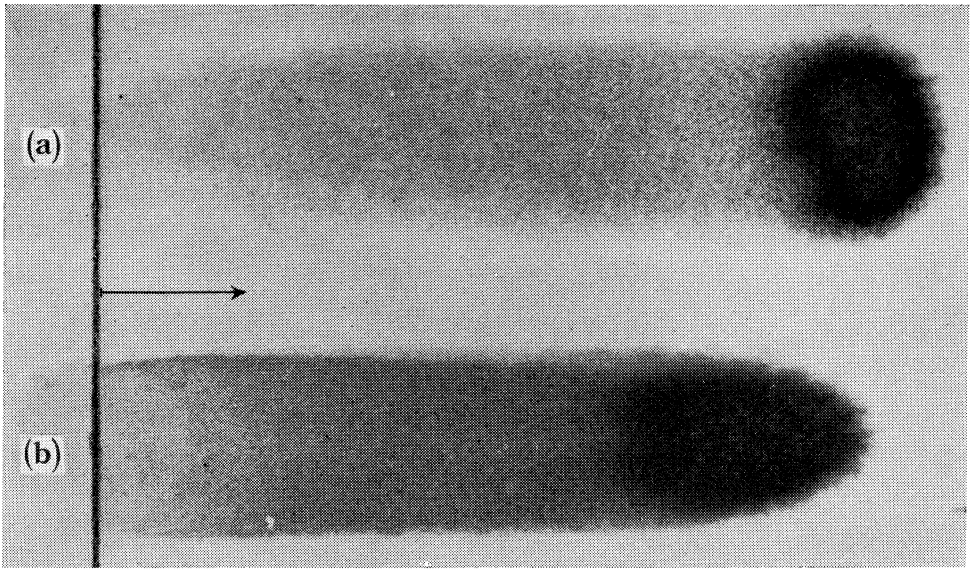

Fig. 2.—Paper electrophoresis patterns of (*a*) κ-casein and (*b*) para-κ-casein in veronal, pH 9·0, ionic strength 0·1, at 2°C, using Whatman No. 1 paper, and run for 14 hr at 140 V.

under these conditions. Certainly the two breakdown derivatives of DNP-proline (Fraenkel-Conrat, Harris, and Levy 1955) were absent. The absence of cysteine from whole casein (Gordon *et al.* 1949) made it unnecessary to check for the presence of this amino acid as an end-group.

Examination of the ether layer from DNP-para-κ-casein indicated the presence of dinitroaniline, dinitrophenol, small amounts of DNP-aspartic and DNP-glutamic acids, and traces of DNP-serine, DNP-threonine, bis-DNP-lysine, DNP-leucine or DNP-isoleucine or both, and DNP-phenylalanine. The orange-coloured artefact was again present.

In both cases only DNP-aspartic and DNP-glutamic acids were present in measurable amounts. A quantitative evaluation of these DNP-amino acids was made after chromatographing the ether-soluble derivatives, from a 16-hr hydrolysate, in the *tert.*-amyl alcohol system which gave the best separation of DNP-aspartic and DNP-glutamic acids. The absorption curves of the DNP- derivatives were checked

over the range 335–390 mμ and found to be typical of a DNP-amino acid. A breakdown of 30 per cent. for these DNP-amino acids under the hydrolysis conditions used (Porter 1951) was assumed in evaluating the results of this quantitative study as shown in Table 1. Taking 23 per cent. nitrogen as being split from κ-casein, a molecular weight of 18,000 has been assumed for para-κ-casein. It is obvious that DNP-aspartic and DNP-glutamic acids are themselves present only in trace amounts, and there is essentially no difference between κ-casein and para-κ-casein in this respect.

The only DNP-amino acids of significance which could have been present in the water layer were ε-DNP-lysine, DNP-arginine, and bis-DNP-histidine. ε-DNP-lysine, is very frequently present in large excess and this renders the identification and estimation of the other two very difficult. No trace of the bis-DNP-histidine could

TABLE 1

NUMBER OF FREE α-AMINO END-GROUPS DETECTED IN κ-CASEIN AND PARA-κ-CASEIN BY THE FDNB METHOD

End-group	DNP-κ-casein		DNP-para-κ-casein	
	Mole/10^5g	Mole/Mole	Mole/10^5g	Mole/Mole
Aspartic acid	0·13	*c.* 0·03	0·16	*c.* 0·03
Glutamic acid	0·12	*c.* 0·03	0·13	*c.* 0·02

be detected in the ether layer, even after exhaustive extraction with ether. It was, therefore, unlikely to appear in significant quantities in the water layer, and could not be detected in any of the chromatographic systems used. The phosphate system proved most useful as bis-DNP-histidine moved very slowly in comparison with the others. DNP-arginine moves rather close to ε-DNP-lysine in the chromatographic systems used and cannot be separated from the large excess of the latter—an attempt to use the benzene system of Mellon, Korn, and Hoover (1953) resulted in considerable tailing of the spots. However, no DNP-arginine could be detected on chromatograms of the water layers on spraying with Sakaguchi's reagent.

Because of the presence of arginine as a free α-amino end-group in both α- and β-casein (Mellon, Korn, and Hoover 1953; Wissmann and Nitschmann 1957) and the difficulty in detecting and estimating it by the procedures described above, the simplified procedure of Wissmann and Nitschmann was applied once to the water layer in an attempt to clarify the position. The results indicated the presence of 0·6 and 0·5 arginine α-amino end-groups per 10^5 g DNP-κ-casein and DNP-para-κ-casein respectively. Interfering factors, such as the incomplete removal of free arginine, could give rise to a false estimate of DNP-arginine by this method, and it cannot be said with certainty that κ-casein and para-κ-casein contain free α-amino arginine. It is obvious, however, that there is no significant difference between κ-casein and

para-κ-casein in this respect. It is interesting to note that Lea and Hannan (1950) and Schwartz and Lea (1952) reported only trace amounts of glutamyl, lysyl, phenylalanyl, and valyl α-amino end-groups in whole casein. Terminal arginine residues were not found to be present and the results contrast with those of Mellon, Korn and Hoover (1953) and Wissmann and Nitschmann (1957). Seno, Murai, and Shimura (1955), during a study of the lysyl peptides in α-casein by the FDNB technique, could not detect any terminal arginine residues in this component.

IV. Discussion

The results on the liberation of NPN from various milk protein fractions clearly support the conclusion that κ-casein is the site of primary attack when rennin acts on whole casein (Waugh and von Hippel 1956). They are also in accordance with the assumption that the specific splitting reaction, which sets free a small part of NPN very quickly, is directly responsible for milk clotting (Alais *et al.* 1953; Nitschmann and Keller 1955). The NPN/time curves obtained by the latter workers for "α-casein" prepared by the urea method of Hipp *et al.* (1952) can be explained on the basis of contamination with κ-casein.

The release of 1·0 per cent. NPN from first-cycle casein and 6·7 per cent. NPN from κ-casein indicates the presence of approximately 15 per cent. of the latter component in whole casein (cf. Waugh and von Hippel 1956). κ-Casein on rennin treatment is rapidly converted into an insoluble fraction, referred to here as para-κ-casein, and a soluble fraction which accounts for approximately 23 per cent. of the total nitrogen.

The difference between the 6·7 per cent. NPN value obtained when the protein is precipitated with 12 per cent. TCA and the 23 per cent. "soluble nitrogen" remaining in solution when the para-κ-casein is removed by centrifugation suggests that the "soluble nitrogen" is not a single component. However, Alais (1956) has shown that the maximum NPN value (soluble in 12 per cent. TCA) obtained when rennin acts on whole casein depends largely upon the method of adding the TCA to the reaction mixture, and it is almost certain that 12 per cent. TCA causes precipitation of an appreciable portion of the glyco-macropeptide described by him.

Nitschmann, Wissmann, and Henzi (1957) found that 4–5 per cent. of the nitrogen remained in solution when rennin-treated whole casein was precipitated at pH 4·7. Assuming this nitrogen is derived solely from κ-casein, present to the extent of 15 per cent. in whole casein, a value of 27–34 per cent. NPN would be expected on treating κ-casein with rennin. Nitschmann and his co-workers have shown that this material consists of the glyco-macropeptide as well as other smaller peptides which form only very slowly during rennin action in comparison with the glyco-macropeptide. It is therefore possible that the 23 per cent. "soluble nitrogen" split from κ-casein consists largely of the glyco-macropeptide or a product similar to it. A molecular weight of 26,000 for κ-casein (see Part III, McKenzie and Wake 1959*c*) would result in a molecular weight of 8000 for the soluble nitrogen if it were mainly a single component. This is essentially the same as the value of 6000–8000 obtained by Nitschmann *et al.* for the glyco-macropeptide. Certainly the soluble nitrogen split from κ-casein contains this material. Galactose and neuraminic acid,

as well as phosphorus, have been found in both. The large amount of humin formed on hydrolysing DNP-κ-casein with HCl is probably derived from neuraminic acid which is known to give such products on acid treatment (Blix 1936). The fact that this effect was not pronounced with DNP-para-κ-casein indicates that most of the neuraminic acid, if not all, is split from κ-casein by rennin and is found in the soluble fraction.

Para-κ-casein moves at a slower rate, when subjected to paper electrophoresis at pH 9, than does κ-casein, and this could be due to the release of an "acidic" macropeptide of the composition indicated by Nitschmann, Wissmann, and Henzi (1957). A comparatively simple procedure for the isolation of κ-casein, completely free from β-casein, has been developed, and it is intended to use this material to investigate more fully the nature of the soluble nitrogen split off by rennin.

It is proposed that the specific action of rennin on κ-casein at neutral pH is to release a glyco-macropeptide of rather unusual composition with the consequent formation of insoluble para-κ-casein. Does the enzyme rupture some specific peptide bond and thus set free a part of a polypeptide chain in κ-casein as the glyco-macropeptide? If this were the mechanism of rennin action then a new α-amino end-group would be expected to appear in either para-κ-casein or the glyco-macropeptide. However, the observations made here indicate essentially no difference between κ-casein and para-κ-casein with respect to their content of α-amino end-groups, and in addition, Nitschmann, Wissmann, and Henzi (1957) could detect no free α-amino end-groups in the glyco-macropeptide. These results suggest that the conversion of κ-casein to para-κ-casein does not rest in the ability of rennin to split peptide bonds. This result was unexpected in view of the observations of Wissmann and Nitschmann (1957) who reported the appearance of phenylalanyl α-amino end-groups to the extent of 1·8 residues/10^5 g protein in rennin-treated "α-casein", presumably contaminated with κ-casein. Their observations suggested that "the primary reaction in the rennet curdling of milk is a specific limited proteolysis". In a recent paper by Fish (1957) it has been shown that rennin has a proteolytic activity similar to that of pepsin. In the B chain of insulin it splits the following five bonds: Leu–Val, Leu–Tyr, Tyr–Leu, Phe–Phe, and Phe–Tyr. The optimum effect is at pH 4. It is possible that the appearance of the phenylalanyl α-amino end-group reported by Wissmann and Nitschmann was due to a similar proteolytic activity involving phenylalanine residues. This would have been enhanced on adjustment to pH 4·6 where the casein was precipitated.

It is generally considered that calcium is needed for the clotting of paracasein, and many workers are of the opinion that calcium acts as a bridge between different paracasein "molecules" (see Higgins and Fraser 1954). The exact mechanism of the clotting stage and the role of calcium await elucidation.

V. Acknowledgments

Grateful acknowledgment is due to Dr. H. A. McKenzie and Mr. M. B. Smith for their advice and assistance during the course of this work. Thanks are also due to Dr. N. J. Berridge who supplied the seeding crystals of rennin, and to Dr. K. Knox

who carried out the preliminary carbohydrate analyses. Other acknowledgments are given in Part I.

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