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An Electrophoretic Investigation of the Effect of pH on the Degradation of α-Casein by Crystalline Rennin

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The enzymatic degradation of electrophoretically homogeneous α-casein by crystalline rennin breaks down the casein into a few high molecular weight components. These are degraded further in various ways depending on the pH of the solution. In both the primary and most of the later degradation reactions the parent substance is split into two components with electrophoretic mobilities above and below, respectively, that of the original substance. In addition to the simple fission of α-casein and its primary degradation products there was also observed a slow loss of low molecular weight substances (NPN). The pH optima for the fission and for the NPN formation do not coincide. These results provide a simple explanation of the earlier contradictory properties ascribed to the different components of α-casein and "para-α-casein". Most of these components can now be described as enzymatic cleavage products of the α-casein and of the κ-casein and λ-casein which also contribute to the α-peak.

Although a number of electrophoretic investigations of the enzymatic degradation of α-casein, β-casein and whole casein have been carried out [1–8], only a few of these have dealt with the action of the rennet enzyme (rennin or chymosin) as a proteolytic enzyme in casein hydrolysis [2,4,5]. From these reports it is not possible to draw more than limited conclusions about the groups in the casein that can be split off and about the factors that govern the enzymatic processes. Particularly, little work has been done on the effect of pH on the degradation of α-casein and β-casein by pure rennet enzyme. Several extensive investigations have, however, been carried out without the use of electrophoresis. Nitschmann and coworkers, for instance, have made a detailed study of the primary reaction in the attack of rennin on the casein [9–11] and Ågren [12] and others have separated and identified several components of enzymatic hydrolysates of casein.

In the present investigation it has been found that the enzymatic hydrolysis of casein can proceed in a number of different ways depending on the pH. This explains the somewhat problematic nature of the results of earlier investigations on casein degradation.

METHODS

Preparation of α-casein. α-Casein was prepared by a method essentially the same as the urea method developed by Hipp and coworkers [13]. Great importance was attached to carrying out the preparation rapidly and under mild conditions. In the preparation of the acid casein, only two hours passed between precipitation and the dissolution of the casein in the urea solution. To make this possible the washing and the reprecipitation of the casein was done once only. If a longer time is taken for the preparation there may be enzymatic destruction of the casein and there is a risk of oxidation of sensitive groups in the protein. Three reprecipitations from the urea solution were usually sufficient to give an electrophoretically pure preparation. The casein was obtained in an enzyme-free state by dissolving in dilute alkali and heating at 80° for 30 min. To prepare a suitable form for storage the casein was precipitated with acid, washed and freeze dried. When a very pure preparation was required the casein solution was freed from fat and colloidal material by sedimentation in a Spinco model L ultracentrifuge at 30 000 rpm for 1 hour. 0.5 % thioglycol was added to all the solutions used in the preparation in order to reduce oxidation by atmospheric oxygen, and toluene was added as a bacteriostatic agent.

Preparation of rennin. Crystalline rennin was prepared from "Chr. Hansens Standardlöpe" in powder form by the method of Berridge [14]. The preparation was followed by microscopic examination and by electrophoresis and only preparations with pure, typical crystals and a well defined single peak in the electrophoresis curve were used. It was usually necessary to recrystallise from a magnesium acetate buffer to get crystals that dissolved to give a colorless, clear solution.

The experimental work was done in sets of three parallel runs about 0.2 pH units apart which took between two and three weeks depending on the speed of the degradation. The following conditions are typical of those used for a three week run:

α-Casein (72 g), which was checked electrophoretically immediately before use to ensure that it had not been affected enzymatically during storage, was dispersed in distilled water (*ca.* 1 500 ml) and the pH was adjusted to between 7 and 9 by dropwise addition of 10 % sodium hydroxide solution with vigorous stirring. After a few hours when the casein had dissolved, the pH was adjusted to 9.0 with 1 N NaOH using a Beckman Automatic Titrator and the solution was made up to 1 800 ml. The 4 % casein solution obtained was then heated to 80° for 10 min to destroy any micro-organisms and any enzymes remaining and then cooled after adding 1 % toluene.

Three buffered rennin solutions were prepared containing:

16 g NaCl
8.4 g NaH_2PO_4
x ml 1 N HCl or NaOH (0—30 ml)
y ml rennin solution (*ca.* 1 ml with 300—400 Ru/ml *)
distilled water to 200 ml.

These quantities were chosen so that a 3:1 mixture of casein solution and buffer solution would have the following concentrations:

NaCl	2 %
NaH_2PO_4	0.0675 M
Rennin	400 Ru/ml

The following volumes of HCl or NaOH solution were added in making up the rennin buffer solutions.

The 4 % casein solution was measured out in 15 ml portions into three sets of twelve 25 ml test-tubes with glass stoppers and in 60 ml portions into three sets of six 100 ml conical flasks with glass stoppers. Buffer solution (5 and 20 ml, respectively) was added to each of these casein solutions and the test-tubes were immediately shaken strongly to give a mixture as homogeneous as possible. Toluene (0.2 and 0.8 ml, respectively) was added to each mixture and they were again shaken.

* Ru indicates rennin activity units according to Berridge[14]

Table 1. Volumes of acid or alkali solution added to the buffer solution and corresponding pH values in the substrate (see text).

1 N HCl or NaOH, ml	pH of casein-buffer solution
17.5 NaOH	7.0
12.5 »	6.7
7.5 »	6.4
0 »	5.89
1.5 HCl	5.81
3 »	5.75
5 »	5.45
10 »	5.21
15 »	4.85
20 »	4.79
25 »	4.48
30 »	4.21

A sticky precipitate was formed when the solutions were mixed, occluding part of the buffer salts and enzyme. Full equilibrium inside and outside this gel was not attained immediately but was gradually reached in about a day. Flasks from each mixture were withdrawn for analysis of the contents every Monday, Wednesday and Friday.

Analyses. To determine the distribution of nitrogen the flasks containing 80 ml of mixture were shaken lightly, trichloroacetic acid (TCA, 5 ml, 25 %) was added and the flasks were shaken again vigorously and then centrifuged for 15 min at 3 500 rpm. The supernatant was then decanted through a filter and a nitrogen determination was made on a 20 ml sample of the filtrate by the macro-Kjeldahl method. The amount of nitrogen soluble in 2 % TCA as found by this method was corrected for the amount of soluble nitrogen in the original casein before the rennin was added. The $NPN_{2\%\ TCA}$ (non-protein nitrogen soluble in 2 % TCA) was then expressed as a percentage of the total nitrogen in the original mixture.

The electrophoreses were run on the mixture in the 25 ml tubes. The contents of each tube were first treated with EDTA (0.4 g) to bind any traces of calcium remaining in the casein and with 0.2 ml of 30 % NaOH to raise the pH. After rapid mixing by shaking the pH of the mixture had risen to about 9. This destroyed the rennin and stopped the enzyme reaction. At the same time the casein began to dissolve and after shaking for 2 h the casein was completely dissolved. The protein solution was then dialysed against two changes of the electrophoresis buffer in a rotary dialyser, first for 3 h and then for 16 h. The ratio of sample to buffer during the dialysis was about 1:100.

After dialysis the protein solution was slightly opaque due to the presence of globules of fat and toluene and particles of other material in suspension. After sedimentation in a Spinco model L ultracentrifuge * at 30 000 rpm for 1 h the solution gave a very small bottom layer (*ca.* 0.1 ml), a clear middle layer, and an opaque top layer. A 15 ml sample of the clear layer was taken for the electrophoresis. Centrifuging gave optically clear solutions much more rapidly and conveniently than any of the methods used previously and gave much better reproducibility.

The electrophoreses were run in a Spince model H instrument at 1°C using a phosphate buffer, μ 0.1, pH 7.3, containing 0.02 % merthiolate as preservative. Standard conditions of 120 min at 12 mA were used throughout the investigation.

The sensitivity of the optical recording device and the protein concentration in the sample were kept constant and the electrophoresis curves were therefore directly comparable. The pH of the electrophoresis samples was measured before the addition of EDTA and sodium hydroxide. The variations between tubes were usually about 0.03—0.04 pH units; the values given below are means of the values found for each series.

The conductivity of the buffer and of the electrophoresis sample were measured with an LKB conductivity bridge with the electrodes immersed in the electrophoresis bath.

* Centrifuge purchased with grants from the SMR fund for research in dairy science.

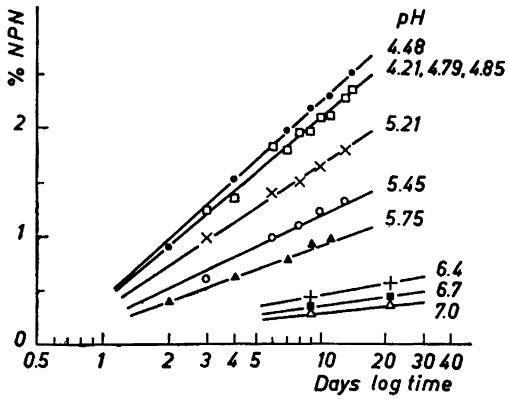

Fig. 1. The release of NPN from α-casein by rennin.

RESULTS

A. Formation of soluble nitrogen

The soluble nitrogen values obtained, when plotted against a logarithmic time scale, gave a linear set of curves with a common origin at 0 % NPN. This point lies rather high on the logarithmic time scale — at approximately 0.6 days (Fig. 1). The slope of the curves which is of course a measure of the rate of the enzymatic process, has a pH optimum at 4.48. At pH 7.0 the reaction rate is very low.

Plotting the NPN value at 15 days as a function of the pH gives curves of the type shown in Fig. 2. To decide whether the curve is symmetrical around the pH 4.5 ordinate would require measurements further down the pH scale

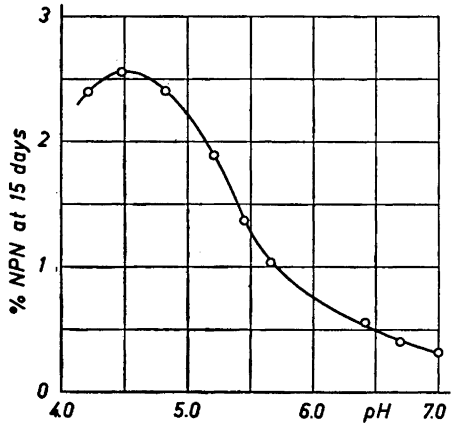

Fig. 2. NPN liberated from α-casein in 15 days.

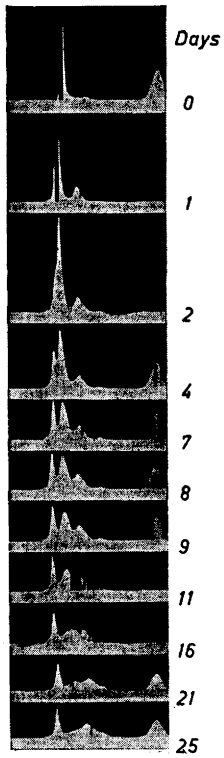

Fig. 3. Electrophoresis curves showing the course of degradation of α-casein by rennin at pH 5.81.

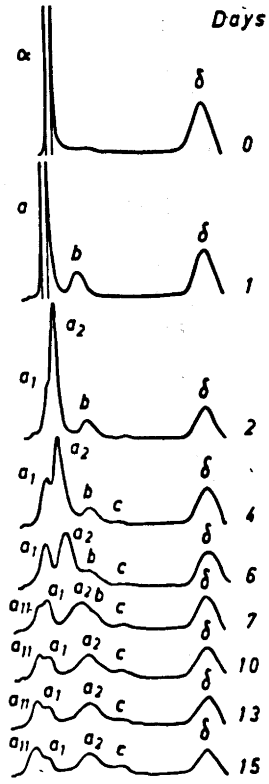

Fig. 4. Electrophoresis curves showing the course of degradation of α-casein by rennin at pH 5.21. Tracings from the photographic curves.

B. Course of the degradation

More than 200 electrophoreses were run in order to elucidate the course of the degradation and approximately 75 of these would be essential to show the phenomena observed completely. For practical reasons only a few will be given here. The curves selected have been supplemented by diagrams showing the course of the more important phenomena occurring during the degradation.

Fig. 3, for example, shows the degradation of α-casein at pH 5.81. The sequence of changes shown is typical of that occurring at pH values between 5 and 7. It shows clearly the successive breakdown of the α-casein into a few components which then undergo further degradation at different rates.

Fig. 4 shows the degradation at pH 5.21. By using only tracings of the curves it has been possible to get the curves close enough to each other to show successive changes more clearly. In this case the degradation is more compli-

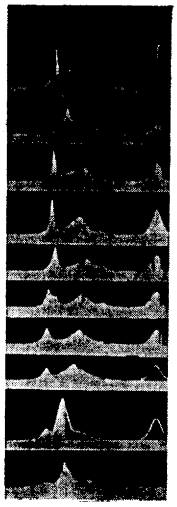

Fig. 5. Typical electrophoresis curves at an intermediate stage of the degradation for different pH values.

cated than at pH 5.81 and some of the components formed first are split again into two components before the subsequent degradation.

Fig. 5 shows a cross section of the electrophoresis curves through the whole pH scale. These have been selected to show the typical sequence at eacn pH. It is apparent that at a pH greater than 6 or less than 4.5 the electrophcresis curves are much simpler than in the interval between. The appearance and degradation of some of these main components will be discussed below.

The two components first formed are designated here as a and b. Component a splits into a_1 and a_2 and then a_1 is further degraded to a_{11}. Component a_2 is degraded more or less continuously without forming any definite degradation product. In some cases where indications of degradation products of a_2 were observed they have been numbered 1, 2 and 3. No special designation has been given to minor substances that could not be traced back to any parent substance except in the case of a component labelled c.

General description of the degradation. At all pH values between 4 and 7 the α-casein is split immediately after adding the enzyme (within a few hours) into two components, a main component (a) with a slightly higher migration rate than the α-casein and a component present in smaller amount (b) with a lower migration rate. The course of the subsequent degradation varies considerably depending on the pH. The b component appears to break down comparatively slowly at a rate independent of the pH and its migration rate changes little once the component has been formed. The formation of the final form of the b component proceeds more slowly the higher the pH and at a pH above 6.5 it takes more than 25 days under the conditions used here. At a pH lower than this it reaches a stable migration rate in two to three days.

Fig. 6 which illustrates the changes in the migration rates of all components shows that there is a strong reduction in the mobility of the b component after 7 to 10 days at pH 5.8—6.4. This may, however, be only an artifact due

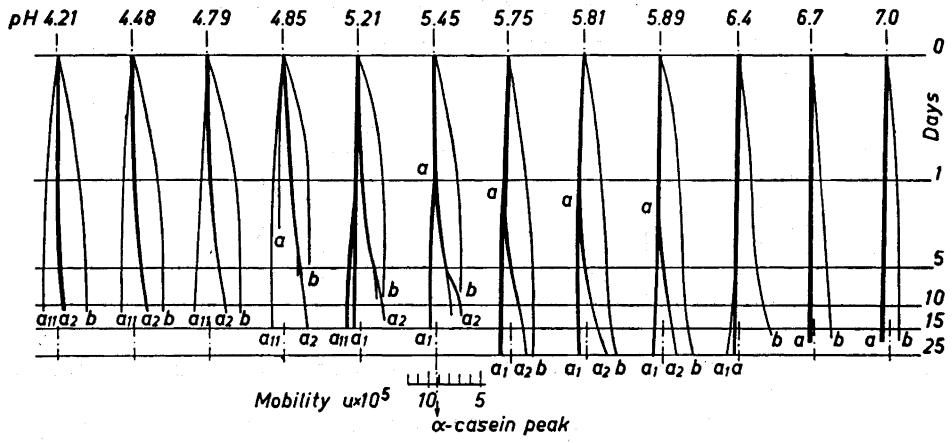

Fig. 6. Changes in electrophoretic mobility during degradation at different pH values. The dotted line shows the position of the α-casein peak at the start of the experiment. The full lines show the changes of mobility of those peaks that can be followed. The thickness of the lines gives a rough indication of the amount of the component present in the mixture.
The electrophoretic migration is from right to left; mobilities can be read from the scale under the pH 5.45 curve.

to the movement through the b component peak of peaks due to other components as their speed changes in the course of the degradation. This would either displace the apparent position or the b peak or form an asymmetric peak with a displaced centre of gravity.

The a-component. The migration rate of the a component is only slightly higher than that of the α-casein (9.3×10^{-5} as aginst 9.1×10^{-5}). The degradation of the a-casein becomes apparent quite soon after it is formed but the rate of degradation depends strongly on the pH. At pH 6.4 it can be observed after 10 days and at pH 5.2—5.3 the degradation is so fast that the a-casein is completely changed after only a day. At pH values lower than this the a-casein is not observed and only its degradation products, a_1 and a_2, appear.

Component a_2. The a_1 and a_2 caseins have mobilities higher and lower, respectively, than that of the parent a-casein in the same way as the a and b caseins have higher and lower migrations rates, respectively, than that of the α-casein. This simultaneous formation of fission products with migration rates higher and lower than that of the parent substance appears to be a general rule in this type of casein degradation.

The mobility of the a_2 component gradually falls in the same way as that of the b-casein. The final product with a stable migration rate is reached most rapidly at a pH of about 5.2. Both at lower and at higher pH values the change is much slower. The final migration rate coincides at optimal pH values with that of the b component but at other pH values is usually higher.

In the pH range 4.8—5.5 there is a tendency for a_2 to split up in the intermediate stages into several components. Later, however, these are largely

Table 2. Composition (%) of the casein degradation product.

Preparation		a		b	c
pH	days	a_1	a_2		
5.75	2	83.2		15.0	1.7
5.45	1	83.3		15.0	1.7
5.75	4	25.5	53.2	19.3	2.0

broken down and only one or two remain. The final migration speed of a_2 coincides even in this case more or less with that of the b-component but varies somewhat depending on the amount and type of these intermediate components left. The degradation of the a_2 subcomponents apparently does not stop but continues as long as there is any material left in the sample.

Component a_1. The a_1 component like the a component is broken down very rapidly at certain pH values. However, within the pH range 4.85—6.4 the ratio of the rate of formation to the rate of degradation is such that it can be observed during relatively long period. At a pH of 5.21 or lower it changes to a form with a slightly higher migration rate as shown in Figs 7 and 8; this has been designated here as a_{11}. At a pH of 4.8 or less a_1 is broken down so rapidly that a appears to be transformed directly into a_{11} and a_2. Like other components a_{11} gradually attains a fairly stable migration rate.

Quantitative measurements. The electrophoresis curves obtained were suitable for quantitative estimates only during the first part of the degradation. The rates of breakdown of a into a and b and the rate of formation of the component c, which is discussed below, can be measured fairly readily. In some cases it was also possible to measure the splitting of a into a_1 and a_2 but the subsequent breakdown of a_2 is rapid and gives components which coincide with b so that the measurements are then much less certain. Table 2 gives estimates of the proportions of the different degradation products.

DISCUSSION

Even though the α-casein and the crystalline rennin used in these experiments were electrophoretically pure they cannot be regarded as homogeneous. Waugh and von Hippel [15] have reported the presence in α-casein of up to 12 % of a component which they named $\varkappa$-casein and Long, Winkle and Gould [16] have isolated from electrophoretically homogeneous α-casein an additional substance, which they named λ-casein, that forms about 3 % of the α-casein. The degradation products observed here cannot therefore be referred immediately to any particular one of the three substances in the mixture formerly regarded as α-casein.

Crystalline, electrophoretically pure rennin has been found by Schwander *et al.*[17] to contain a small amount of a substance which, according to the

results of sedimentation in an ultracentrifuge and of diffusion measurements, is of higher molecular weight. Ernstrom [18] has found, moreover, by electrophoretic examination of crystalline rennin, that the single rennin peak obtained under normal conditions is split into several components at low ionic strengths. It cannot be excluded that one or more of these components may have proteolytic properties different from those of the main component of the rennin.

It is thus apparent that the present investigation, no more than any other hitherto published on the enzymatic degradation of casein, cannot satisfy the requirements set out by Ogston for a defined biochemical process [19]. Nevertheless, while awaiting the development of methods for the preparation of absolutely homogeneous casein fractions and chemically pure rennin, the results obtained give some perspective to the investigations that have been made on the structure and properties of the phosphopeptones and polypeptides obtained from casein. The results of the present investigation show more directly that it is possible to find experimental conditions under which the degradation products have simpler electrophoretic properties than are found under other conditions. It now seems possible on the basis of the present observations to give a fairly simple explanation of some older observations on "paracasein".

Enzymatic processes in a heterogeneous mixture of the type concerned here do not lend themselves to rate determinations nearly as readily as when they occur in a homogeneous phase. In the present case there was strong gel formation and precipitation of α-casein as soon as the pH came below 5.3. However, by adapting the technique used to these conditions it was found possible to obtain more or less reproducible results, as is apparent from Figs. 1 and 2. At pH 5.45 and higher pH values where the α-casein is in solution the curves obtained had approximately the same origin on the time scale as when the pH was less than 5.45 and the casein insoluble. This shows that the rate and the course of the processes occurring were more or less the same in both cases. The delay of 0.6 days that was observed in all these experiments may be due to the presence of an inhibitor of the type mentioned by Dixon [20]. The results of Amundstad's investigations [21], when plotted on a semi-logarithmic scale as in Fig. 1 show a similar delay. In this case the delay varied rather more than in the present investigation but was of approximately the same order. Amundstad worked with total casein and technical rennet and the chances of some poisoning effect occurring are rather different from the present case. An alternative explanation of the delay could be that the NPN is not liberated in the first stages of the degradation but only after certain key links are broken by the rennin.

Some support for this hypothesis is obtained from a comparison of the changes in the electrophoresis curve with the change in the amount of NPN. At a given degree of degradation, for instance 1 % NPN, the electrophoresis curves are different for different pH values (Fig. 7). The primary degradation thus cannot have any direct effect on the formation of NPN. This supposition was supported by the very slow formation of NPN at pH 7 and the simultaneous relatively rapid fission of the α-casein at this pH into a and b components (Figs. 5 and 7). Another criterion supporting this is that the optimum pH

Fig. 7. Electrophoresis curves at NPN 1 %. The absence of any relationship between the NPN content and the appearance of the curve is an indication that these concern two independent processes.

for NPN formation is 4.5, as shown in Fig. 2, but is 5.2—5.5 for the degradation of a_2 as is visible in Figs. 6 and 7.

These phenomena are parallelled by the rapidity with which a glyco-macropeptide is split off during the primary reaction observed by Nitschmann *et al.*[10] in the coagulation of casein with rennet and the slowness of the subsequent, general proteolytic action noted by Nitschmann[9] in the tertiary stage. There is as yet no proof that any of our components is identical with Nitschmann's glyco-macropeptide nor, on the other hand, is there any contradictory evidence.

Nitschmann and Lehmann[2] found that the paracasein which is formed when casein is coagulated with rennet gave two peaks in place of the α-casein peak. They called these rennet α_1- and rennet α_2- caseins and recorded mobilities in phosphate buffer at pH 7.35, μ 0.077, 2°C of

$$\alpha_1 : 9.55 \times 10^{-5} \quad \text{and} \quad \alpha_2 : 8.83 \times 10^{-5}$$

The α-casein had a mobility of 9.30×10^{-5}. This is in good agreement with our observations where the α-casein had a mobility in phosphate buffer at pH 7.3, μ 0.1, 1°C of 9.09×10^{-5}. The mobilities of the a and a_1 components fall within the range $9.2—10.8 \times 10^{-5}$ while a_2 has a mobility between 9.1 and 7.0 depending on the extent of the degradation of the casein.

It seems quite possible that Nitschmann's rennet α_1-and rennet α_2-caseins are identical with the a or a_1 components and with a_2 respectively, with Nitschmann's material representing forms of an intermediate degree of degradation.

More difficult to explain is the observation of Cherbuliez[22] that casein that has not been treated with rennet also gives two α peaks which he called

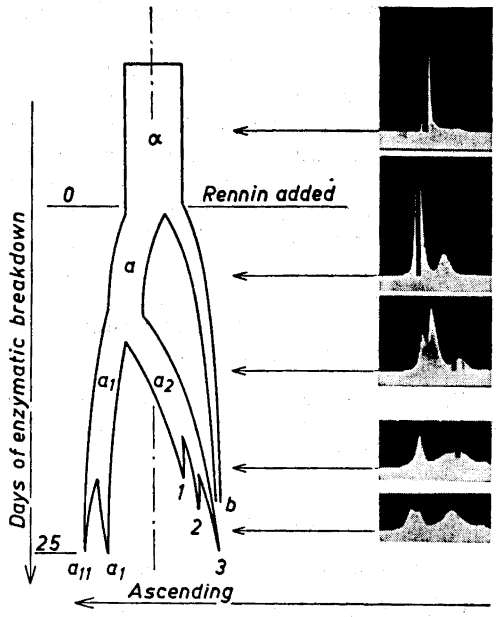

Fig. 8. The curves in Fig. 7 slightly idealized to cover the pH range 5.0—5.5 and to illustrate the major changes taking place during the α-casein decomposition.

α_I and α_{II}. However, he used a rather time-consuming method to obtain ash-free and fat-free preparations and it is quite possible that proteolytic enzymes originally present in the milk, which would accompany the α-casein in the preparation, may easily have given rise to a degradation similar to that produced by the rennet. The components later found by Cherbuliez [23] in para α-casein may very well have been rather degraded products formed according to the scheme described here for the further degradation of a_1 and a_2. In this case it would not be possible to compare the mobilities since the experimental conditions would be quite different.

Fig. 6 summarises all the electrophoresis results obtained as branched curves showing how the mobilities of the different degradation products are related to the mobilities of the parent substance, which is marked with a dashed line. As is shown in the figure the rates of the secondary, tertiary and further degradation reactions, that is the branching points of the curves, are very strongly dependent on the pH. A diagram showing the full course of the degradation for the pH range 5.0—5.5 is given in Fig. 8. The course of the degradation at pH values outside this range can be seen from a comparison of Figs. 7 and 8. The most striking point about these diagrams is that components such as a_{11} and a_1 and b are either extremely stable to rennin or else their migration rates do not change at all on further degradation while a_2 on the other hand is rapidly broken down forming substances of lower mobility.

This must of course be dependent on the amino-acid composition of the fission products.

Since the a and b produced in the first step of the degradation of the α-casein have a higher and a lower mobility, respectively, than the parent substance it is probable that a contains relatively more serine-threonine bound phosphoric acid and more glutamic acid and aspartic acid than the α-casein, while b must contain more basic amino acids than the α-casein or a. The large changes in the mobility of a_2 during the degradation must be ascribed to the presence of relatively greater amounts of easily removed acidic amino-acids and of difficultly removable basic amino-acids. The distribution of the amino-acids in a_1 and a_{11} on the other hand must be such that the neutral amino-acids predominate or that any acidic and basic amino-acids present neutralise each other and, moreover, are not too readily accessible to proteolysis.

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REFERENCES

1. Mellander, O. *Upsala Läkareförens. Förh.* **52** (1947) 107.
2. Nitschmann, H. and Lehmann, W. *Experientia* **3** (1947) 153.
3. Perlmann, G. E. *Discussions Faraday Soc.* **1953** 67.
4. Hagberg, E. C. and Sullivan, R. A. *J. Dairy Sci.* **36** (1953) 569.
5. Tsugo, T. and Yamauchi, K. *XIV International Dairy Congr. Rome 1956. Proc.* Vol II, part II, 588.
6. Lillevik, H. A. and Anwar, R. A. *J. Dairy Sci.* **40** (1957) 597.
7. Petersson, R. F., Naumann, L. W. and Mc. Meekin, T. L. *J. Am. Chem. Soc.* **80** (1958) 95.
8. Groves, M. L., Hipp, N. J. and Mc Meekin, T. L. *J. Am. Chem. Soc.* **80** (1958) 716.
9. Nitschmann, H. and Keller, W. *Helv. Chim. Acta* **38** (1955) 942.
10. Nitschmann, H., Wissmann, H. and Henzi, R. *Chimia* **11** (1957) 76.
11. Wissmann, H. and Nitschmann, H. *Helv. Chim. Acta* **40** (1957) 356.
12. Ågren, G. and Glomset, J. *Acta Chem. Scand.* **7** (1953) 1071.
13. Hipp, N. J., Groves, M. L., Custer, J. H. and Mc Meekin, T. L. *J. Dairy Sci.* **35** (1952) 272.
14. Berridge, N. J. and Woodward, C. *J. Dairy Research* **20** (1953) 255.
15. Waugh, D. F. and von Hippel, P. H. *J. Am. Chem. Soc.* **78** (1956) 4576.
16. Long, J., van Winkle, Q. and Gould, J. A. *J. Dairy Sci.* **41** (1958) 317.
17. Schwander, H., Zahler, P. and Nitschmann, H. *Helv. Chim. Acta* **35** (1952) 553.
18. Ernstrom, C. A. *J. Dairy Sci.* **41** (1958) 1663.
19. Ogston, A. G. *Ann. Rev. Biochem.* **24** (1955) 181.
20. Dixon, M. *Enzymes*, London 1958, p. 94.
21. Amundstad, O. *Statens Mejeriförsök, Alnarp 1950 (Sweden)*, Medd. No. 28.
22. Cherbuliez, E. and Baudet, P. *Helv. Chim. Acta* **33** (1950) 398.
23. Cherbuliez, E. and Baudet, P. *Helv. Chim. Acta* **33** (1950) 1673.

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