STUDIES ON CASEIN

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SUMMARY

Casein occurs in skim milk as colloidal casein-calcium phosphate complexes termed "micelles". Casein itself is a mixture of at least three separate proteins, α-, β-, and γ-casein.

The electrophoretic splitting of the "α-component" of acid casein has been widely observed. In Section I of this thesis it is shown that this heterogeneity is due primarily to the presence of a recently discovered fourth component, κ-casein (Waugh & von Hippel, 1956), present as an α-κ-complex. Pure α-casein (free from κ-casein) can be prepared by suitable fractionation of acid casein with 50 per cent alcohol. This material exhibits no marked heterogeneity on electrophoresis over the pH range 3.0 - 8.2 ($\underline{I}$ = 0.02 and 0.1). Results on various casein fractions support the suggestion that κ-casein is "the most important single factor responsible for micelle stabilization". The identification of three additional minor components in whole casein is also described.

The marked tendency of α- and β-casein to form aggregates is well established. In Section II a brief study is made of the extent of these processes under various conditions. Sedimentation and diffusion studies on these two proteins under conditions favouring disaggregation indicate monomer molecular weights in the vicinity of 28,000 and 20,000 for α- and β-casein respectively.

In Section III a method is described for the isolation of essentially pure κ-casein. It is a mucoprotein containing

phosphorus, neuraminic acid, and probably other sugars. No free α-amino end-groups can be detected by the fluorodinitro-benzene method of Sanger (1945). At neutral pH, in the presence of salt, it is highly aggregated, and gives a single peak on electrophoresis. It can be dissociated into monomers with a molecular weight of approximately 31,000 at pH 12.

During the rennin clotting of milk only the casein fraction is altered. Some studies on the mechanism of this process are made in Section IV. The primary action of rennin on casein involves the rapid release of a small amount of nitrogen (NPN) which is not precipitated by 12 per cent trichloracetic acid. It is shown that this NPN stems only from the κ-casein, and previous claims involving α-casein were due to contamination of the latter with the κ-component. During rennin action κ-casein is rapidly split into insoluble para- κ-casein and a soluble fraction containing approximately 23 per cent of the total nitrogen. This splitting process does not involve the rupture of peptide bonds. The soluble nitrogen released is probably a macropeptide, of molecular weight near 7,000, containing phosphorus, neuraminic acid, galactose, and a high proportion of acidic and hydroxylic amino acids and proline.

PREFACE

The investigations described in this thesis were carried out in the C.S.I.R.O. Physico-Chemical Unit, Biochemistry Department, University of Sydney, under the joint supervision of Professor J. L. Still and Dr. H. A. McKenzie. Grateful acknowledgement is due to Professor Still for his assistance and permission to carry out the work in his Department; and to Dr. McKenzie I am greatly indebted for his considerable advice and discussion, and constant interest.

The studies were initially undertaken during the tenure of a C.S.I.R.O. Australian Studentship. Financial support, in the form of a University Fellowship, from funds made available by C.S.I.R.O., enabled completion of the work. It is a pleasure to acknowledge the assistance and interest of Mr. G. Loftus Hills of the C.S.I.R.O. Dairy Research Section, and his negotiations which made the latter grant possible.

A small portion of the work presented earlier in the candidate's M.Sc. thesis (Sydney University, 1955) has been incorporated here, and this has been mentioned where appropriate. All analytical ultracentrifuge runs were carried out by Dr. McKenzie or Mr. M. B. Smith. Also, to Mr. Smith I am sincerely grateful for his many suggestions and much helpful discussion throughout the course of this work.

A copy of a "preliminary communication" concerning results obtained during studies on the mechanism of the urea

denaturation of ovalbumin and bovine serum albumin, and published in conjunction with Mr. A.N. Glazer and Dr. H. A. McKenzie, is included as a "supporting paper" to this thesis.

SUMMARY

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The marked tendency of α- and β- casein to form aggregates is well established. In Section II a brief study is made of the extent of these processes under various conditions. Sedimentation and diffusion studies on these two proteins under conditions favouring disaggregation indicate monomer molecular weights in the vicinity of 28,000 and

20,000 for α- and β-casein respectively.

In Section III a method is described for the isolation of essentially pure κ-casein. It is a muco-protein containing phosphorus, neuraminic acid, and probably other sugars. No free α-amino end-groups can be detected by the fluorodinitro-benzene method of Sanger (1945). At neutral pH, in the presence of salt, it is highly aggregated, and gives a single peak on electrophoresis. It can be dissociated into monomers with a molecular weight of approximately 31,000 at pH 12.

During the rennin clotting of milk only the casein fraction is altered. Some studies on the mechanism of this process are made in Section IV. The primary action of rennin on casein involves the rapid release of a small amount of nitrogen (NPN) which is not precipitated by 12 per cent trichloracetic acid. It is shown that this NPN stems only from the κ-casein, and previous claims involving α-casein were due to contamination of the latter with the κ-component. During rennin action κ-casein is rapidly split into insoluble para- κ-casein and a soluble fraction containing approximately 23 per cent of the total nitrogen. This splitting process does not involve the rupture of peptide bonds. The soluble nitrogen released is probably a macropeptide, of molecular weight near 7,000, containing phosphorus, neuraminic acid, galactose, and a high proportion of acidic and hydroxylic amino acids and proline.

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GENERAL INTRODUCTION

The average protein composition of cow milk has been given by Macey, Kelley & Sloan (1950) as casein 2.8 per cent; globulin 0.2 per cent; and albumin 0.4 per cent. The predominant protein, casein, was first isolated by Mulder in 1838, using acid precipitation. In this method the casein was separated by filtration and purified by repeated solution in water with the aid of the smallest quantity of alkali possible, and reprecipitation with acid. The fact that the composition and properties of such casein preparations did not change during this purification process led to the idea that casein was a pure protein. However, in 1925 Linderstrøm-Lang & Kodama showed by solubility studies in acid solution that casein is heterogeneous. This work led to various attempts to separate the casein components (Linderstrøm-Lang, 1929; Cherbuliez & Meyer, 1933; Groh, Kardos, Denes & Serenyi, 1934). Mellander (1939) found that on electrophoresis, casein showed three components which he designated α, β and γ in decreasing order of mobility.

Warner (1944) devised a method for separating α- and β-casein based on the higher solubility of the latter at pH 4.4 and 2°. Subsequently Hipp, Groves, Custer & McMeekin (1952) devised two methods for the fractionation of casein into its three components. The first procedure is based on differences in the solubility of α-, β-, and γ-casein in 50

per cent alcohol in the presence of salt, as well as in water, with changes in temperature and pH. The second method depends on the differential solubility of the casein components in aqueous urea solutions at the isoelectric point. Hipp et al. suggested that whole casein contains approximately 75 per cent α-casein, 22 per cent β-casein and 3 per cent γ-casein.

Numerous attempts (see McMeekin, 1954) have been made to characterize the physical and chemical properties of casein. These have been made on both unfractionated acid casein and the components separated by the methods of Warner or Hipp et al. There have been considerable differences in the results obtained by various workers, particularly in the case of α-casein. This arises from (a) the marked tendency of the casein components to aggregate and interact with one another, these processes are highly pH and temperature dependent; and (b) the heterogeneity of at least one of the casein fractions. In Section I of this thesis a number of observations are made on the heterogeneity of casein fractions with particular reference to α-casein. Section II includes the results of an investigation on the molecular size of α- and β-casein.

The casein in skim milk occurs as a colloidal casein-calcium phosphate micelle. During rennet clotting, only the casein fraction is altered. Linderstrøm-Lang (1929) suggested

that the activity of rennin, the active enzyme in commercial rennet, is due to its ability to destroy or inactivate a "protective colloid" component whose function it is to keep the other insoluble calcium caseinates in "solution". Most of the evidence has pointed to α-casein as acting in this capacity. However, Waugh & von Hippel (1956), while purifying casein obtained from the micelles which had been centrifuged out from skim milk, discovered a fraction S containing approximately 70 per cent of a new component, κ-casein. Waugh & von Hippel have claimed that " κ-casein is the most important single factor responsible for micelle stabilization and is the protein on which rennin acts immediately." The two viewpoints may be reconciled if the conclusions concerning the "protective colloid" activity of α-casein had been based on results obtained using material contaminated with κ-casein.

In Section IV of this thesis the mechanism of the action of rennin on casein is examined. When this work began the existence of κ-casein had not been realized, and attention was directed primarily towards α-casein. The demonstration by Waugh & von Hippel (1956) of the existence of κ-casein had particular bearing on this problem. Results obtained prior to this have been re-examined in the light of this finding, and the significance of κ-casein in the problem under investigation has been critically studied. In the course of this work a method for the separation and purification of κ-casein was developed. It is described in Section III.

SECTION I

SOME OBSERVATIONS ON THE HETEROGENEITY OF CASEIN FRACTIONS WITH PARTICULAR REFERENCE TO α-CASEIN

INTRODUCTION

Since Mellander (1939) first observed the resolution of acid casein by moving boundary electrophoresis, this method has been used widely to characterize the casein complex. Mellander noted the existence of three components, which were named α-, β- and γ-casein in the order of decreasing mobility, in maleate (pH 6.1), phosphate (pH 7) and borate (pH 8.6) buffers. Krejci, Jennings & Smith (1941) reported three peaks on electrophoresis of whole casein at pH 7 (0.01 M-phosphate buffer, 0.15 M-sodium chloride), and presented evidence for the interaction of the α- and β-components.

Later, Warner (1944) was successful in isolating the major components, α- and β-casein. He found no evidence for the γ-component during his extensive electrophoretic studies. The relative amounts of α- and β-casein as determined from areas of the descending pattern in a phosphate buffer of pH 7 ($\underline{I}$ = 0.1, 0.05 M-sodium chloride) were 80.7 and 19.3 per cent respectively. There were only two single peaks observed in this phosphate buffer. In veronal buffer at pH 7.8 ($\underline{I}$ = 0.1, 0.08 M-sodium chloride)

the descending pattern was similar to that for phosphate buffer. However, in the ascending pattern the α-boundary showed two peaks when the protein concentration was 0.6 per cent. When this was increased to 1.0 per cent only one peak was observed. The same effect was noted with purified α-casein. β-casein showed only one peak. In acetate buffer of pH 5.6 ($\underline{I}$ = 0.1), the descending patterns for whole casein, and α- and β-casein were similar to those obtained in phosphate and veronal buffers. However, a small additional peak was present in the ascending pattern in each case. It moved at an intermediate rate to that of the α- and β-components. Both α- and β-casein, as well as whole casein, showed complex electrophoretic patterns in lactate buffer at pH 3.1 ($\underline{I}$ = 0.1, 0.05 M-sodium chloride). The relation between the peaks in the patterns of the fractions and those in the whole casein pattern was not clear.

Warner confirmed the interaction between α- and β-casein noted by Krejci et al. (1941, 1942). He observed an abnormal distribution in the areas of the peaks of whole casein obtained in both phosphate and veronal buffers. Information on this interaction was given by an experiment on "reconstituted casein" prepared from 21.6 per cent purified β-casein and 78.4 per cent purified α-casein in veronal buffer. Here the areas of the patterns indicated 15.7 and 16.4 per cent β-casein from the descending and ascending

patterns respectively. The α,β interaction increased with decreasing pH, from 7.8 to 5.6. Also, the mobility of the α-casein decreased as the $\beta:\alpha$ ratio was increased.

Nitschmann & Lehmann (1947) found only the α- and β- peaks for whole casein in veronal buffer at pH 7.35 ($\underline{I}$ = 0.08). Since the total protein concentration was 1.3 per cent, it is not surprising that only one α-peak was observed (cf. Warner, 1944; above). Later Cherbuliez & Baudet (1950[a]) obtained two peaks on electrophoresis of α-casein in veronal at pH 7.8 ($\underline{I}$ = 0.1) when the protein concentration was less than 1.0 per cent. The α-casein was prepared by a method similar to that described by Warner (1944).

Kondo, Yonezawa & Morita (1950) could distinguish only the α- and β- components on electrophoresis of whole casein in phosphate buffer at pH 8.0 ($\underline{I}$ = 0.1). They confirmed that both α- and β-casein prepared by the method of Warner showed single peaks on electrophoresis under these conditions. However, in glycine-hydrochloric acid, glycine-sodium hydroxide, boric acid-sodium carbonate, and ammonium chloride-ammonium hydroxide buffers (pH 2.5 - 9.4, $\underline{I}$ = 0.007 - 0.1) the ascending and descending boundaries of α-casein were heterogeneous under various conditions of pH, ionic strength, electric field and protein concentration.

Electrophoretic studies by Nitschmann & Zurcher (1950) on whole casein in M/30 phosphate buffer at pH 7.3

have confirmed the interaction between α- and β-casein. The extent of interaction was markedly dependent on the total protein concentration. γ-Casein could not be distinguished.

Hipp et al. (1950) obtained three peaks on electrophoresis of whole casein in veronal buffer at pH 8.4 ($\underline{I}$ = 0.1). These corresponded to the α-, β- and γ-components. They developed a method for the isolation of γ-casein, which gave a single peak on electrophoresis in alkaline solutions but was heterogeneous below the isoelectric point. Later, Hipp et al. (1952) reported improved methods for the separation and purification of α-, β- and γ-casein, and noted that α-casein (1.0 per cent) gave a single peak on electrophoresis in veronal buffer at pH 8.4 ($\underline{I}$ = 0.1, 0.05 - M sodium chloride). However, the ascending electrophoretic pattern of the α-component from individual cows occasionally showed two peaks (McMeekin, Della Monica & Custer 1947). Hipp et al. (1952) suggested that whole casein contains approximately 75 per cent α-casein, 22 per cent β-casein and 3 per cent γ-casein.

Both Slatter & van Winkle (1952) and Tobias, Whitney & Tracy (1952) have made an electrophoretic study of diluted skim milk, protein concentration less than 1.0 per cent, at different pH values. The former workers did not distinguish the γ-component. They showed that α-casein migrated as two distinct components, α_1- and α_2-casein, over the pH range 5.4 - 8.4 at various ionic strengths (borate phosphate and

acetate buffers; $\underline{I}$ = 0.02, 0.1 and 0.5). In addition, an interaction occurred between the α- and β-caseins and this increased in degree as the pH was lowered from 8.4 to 5.4 at ionic strength 0.02. The interaction at ionic strength 0.1 was not noticeable. Tobias et al. noted the peaks corresponding to α-, β- and γ-casein in veronal buffer at pH 8.7 ($\underline{I}$ = 0.1). In the ascending pattern the α-component split into three peaks. With acetate buffer at pH 5.6 ($\underline{I}$ = 0.1) the patterns were complex.

Heyndrickx & De Vleeschanwer (1952) have also reported three casein components from electrophoresis of dialysed skim milk in veronal buffer at pH 8.0 ($\underline{I}$ = 0.1). Two of these corresponded to α- and β-casein, while the third migrated just behind the α-component and did not correspond to γ-casein.

Assessment of this earlier work in the electrophoresis of casein fractions is difficult owing to the following: (a) unknown variations in the state of purity of the fractions; (b) the ready tendency for α- and β-casein to form polymers with themselves and with one another, processes which in the case of casein, are highly pH, ionic strength and temperature dependent; and (c) the tendency for α- and β-casein to interact with other proteins and with ions other than the hydrogen ion, this is particularly true in the case of skim milk.

Considerable light has been thrown on the problem of casein heterogeneity by recent work in Waugh's laboratory. Von Hippel & Waugh (1955) observed that all the micelles could be sedimented from skim milk after treatment with 0.06 M-calcium chloride. The casein obtained, after removal of calcium, was referred to as soluble casein since "it is in equilibrium with the casein in the casein micelles of milk and is readily soluble at neutral pH". A comparison of its properties with acid casein suggested that the latter contained "irreversibly aggregated α-casein". These aggregates were dissociated by treatment at pH 12. The scheme for the preparation of soluble casein, now known as first cycle soluble casein, and the further fractionation of this material by Waugh & von Hippel (1956) is set out in Figure 1. Waugh & von Hippel reported the existence of a new component, κ-casein, which they claim is present in skim milk and first cycle soluble casein as an α-κ-complex. They suggested the distribution of components in whole casein as 55 per cent α-casein, 15 per cent κ-casein and 30 per cent β-casein. The small amount of γ-casein in the various fractions was presumably not detectable.

Treatment of first cycle soluble casein (1st c.s. casein) with calcium (0.07M) led to the formation of stable micelles. Under similar conditions, second cycle soluble casein (2nd c.s. casein) gave a heavy precipitate, while

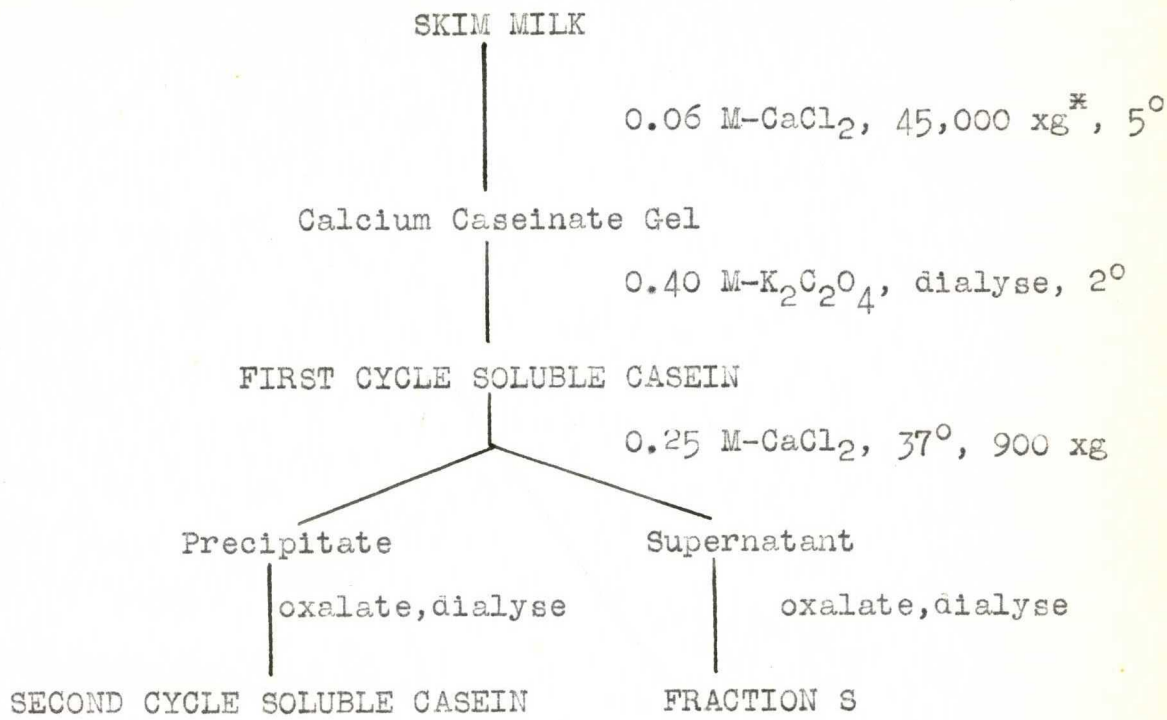


FIGURE 1. Preparation and Fractionation of Soluble Casein.

* xg = times gravity.

fraction S, containing κ-casein, remained perfectly clear. However, on mixing the last two fractions, stable micelles could be obtained. κ-Casein was considered a stabilizing factor required for micelle formation. An examination of the properties of acid casein suggested that κ-casein is present in the "irreversibly aggregated α-casein" mentioned above.

The presence of κ-casein, its normal interaction with α-casein to give the α- κ -complex, as well as the formation of "irreversibly aggregated" material with α-casein on acid treatment, and other possible interactions with β- and γ-casein, would make the interpretation of the electrophoretic patterns of acid casein and the skim milk proteins even more difficult. In addition, Waugh & von Hippel have suggested (a) that the " α-caseins obtained from acid precipitates are in fact α- and κ-caseins", and (b) that "the α_1- α_2-split described by Warner and others" in both acid casein and "purified" α-casein is possibly due to the presence of κ-casein.

In this Section of the present thesis, a study is made of the moving boundary electrophoresis, micelle forming properties, sedimentation, and paper electrophoresis, of various casein fractions. All results described here support the existence of κ-casein, and suggest that it is a major factor contributing to the heterogeneity commonly described

in the "α-peak" of acid casein. Special attention is paid to α-casein prepared from the alcohol fraction B by the method of Hipp et al. (1952), see Figure 2. It will be shown that this α-casein is free from κ-casein. (Results presented in Section IV show that α-casein prepared by the urea method is in fact a mixture of α- and κ-casein). The possibility of further components in casein is considered in the last part of this Section.

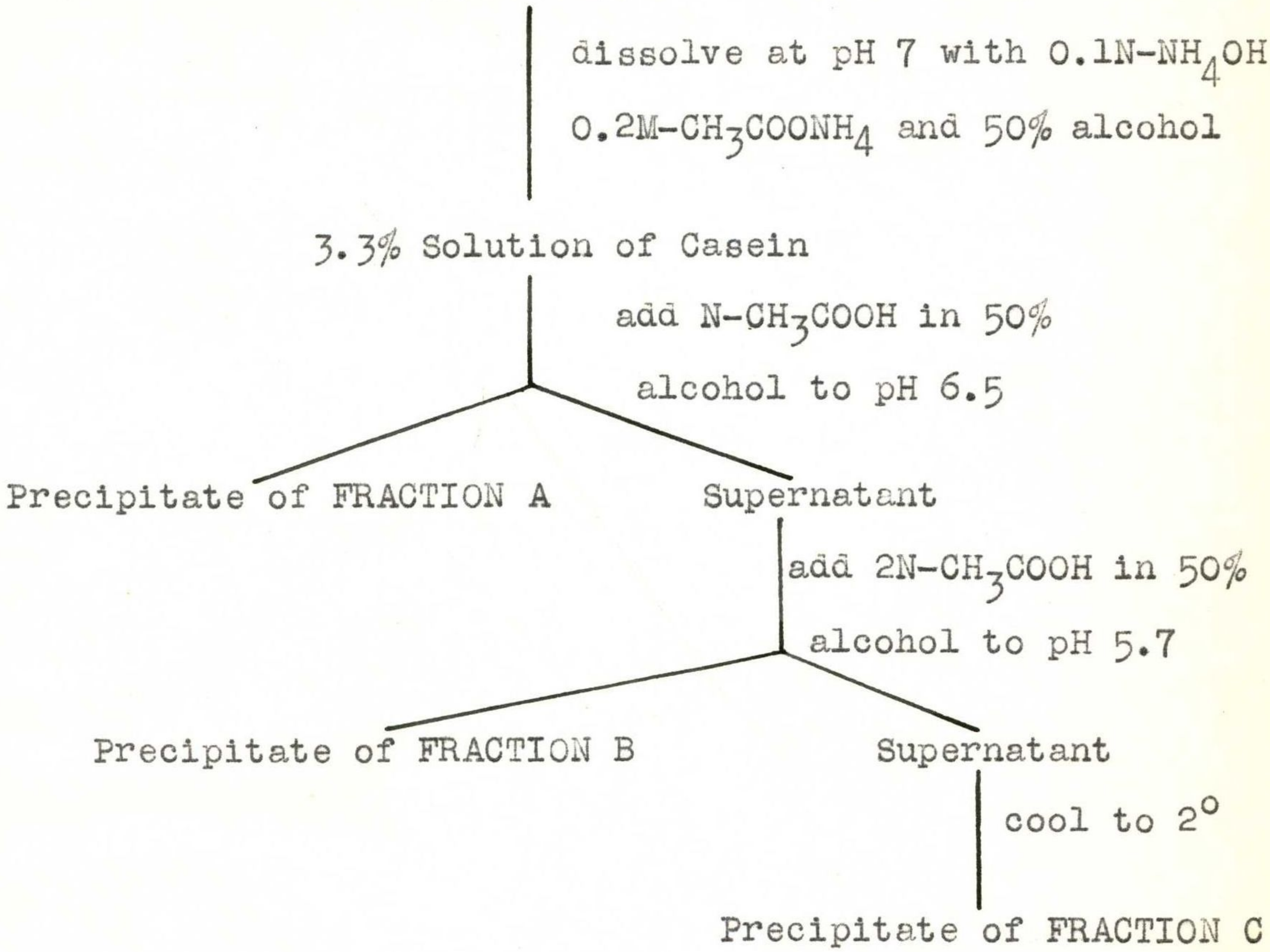


FIGURE 2. Alcohol Fractionation of Acid Casein.

MATERIALS AND METHODS

Protein Fractions. In all fractionation procedures, fresh unpasteurized pooled samples of whole milk were used as starting material.

Acid Casein. This was prepared at room temperature by the method of Hipp et al. (1950) except that, after the first dissolution, the milky solution was clarified by filtration through a Whatman No. 1 paper before reprecipitation. This gave a final product which was completely soluble as a clear solution at pH 7.0.

α- and β-Casein. These were obtained from acid casein by the alcohol method of Hipp et al. (1952). Only the crude fraction B, which is free from the proteolytic enzyme of milk, was used as a source of α-casein.

Alcohol Fractionation of Casein for Micelle Studies etc. Acid casein was dissolved in water at pH 7.0 by the slow addition of N-NH_4OH to give an approximately 6 per cent solution. This was fractionated by the alcohol method of Hipp et al. (1952) to give three preliminary fractions A, B and C. The proportions of A, B and C have usually been different from those obtained by Hipp et al. and, in particular, the yield of fraction A has been near 55 per cent with only 15 per cent fraction B. Each of these fractions, as well as

a sample of the acid casein, was dissolved in dilute NaOH to give an approximately 3 per cent solution and then adjusted to pH 12.0. After standing for three hours at 1° the solutions were dialysed against phosphate buffer to pH 7.0 and then against water to remove most of the salts. At this stage fractions B and C were perfectly clear while the others were slightly opalescent. The pH values of all solutions were between 6.5 and 6.7.

Soluble Caseins. 1st and 2nd c.s. caseins, and fraction S were prepared according to Waugh & von Hippel (1956) except for the use of NaCl throughout instead of KCl.

Total Milk Protein. 1 l. skim milk was treated with 200 ml. $K_2C_2O_4$ at constant pH (von Hippel & Waugh, 1955) filtered through Whatman No. 3 paper, centrifuged at 90,000 xg for 60 min. (Spinco Preparative Ultracentrifuge Model L, Rotor 30, 28,000 rev./min., manufactured by Beckman Instruments Inc., Palo Alto, California, U.S.A.), and dialysed exhaustively against 0.1 M-NaCl. The whole procedure was carried out at 2°.

Storage of Protein Fractions. Acid casein, and α- and β-casein were dissolved at pH 7.0 by the addition of N-NaOH. The solutions were dialysed exhaustively against water at 2°. These, and all other solutions of protein fractions, were freeze-dried and stored at 2°.

Protein Concentrations. Protein concentrations of stock solutions were obtained by drying samples to constant weight at 105°.

Reagents and Buffers. A.R. chemicals were used unless indicated. pH measurements were carried out with a type 1199-44 Leeds & Northrup glass electrode, using a Leeds & Northrup type 7666 pH indicator (Leeds & Northrup Co., Philadelphia, Pa., U.S.A.). For pH's > 10 a Doran type M4969 alkacid glass electrode (L.S.B. Components Ltd., Stroud, Glos., England) was used. Values of standard buffer solutions and general procedure were in accordance with the recommendations of Bates (1954).

The pH values given in the moving boundary electrophoresis and sedimentation results are those of the protein solutions at room temperature and after dialysis. The various buffers with which the stock protein solutions were mixed were made up as in Table 1.

Moving Boundary Electrophoresis. These measurements were made at 0.6° in a modified Tiselius cell (Alberty, 1949) in a water bath controlled to within 0.03°. After the boundary had been formed, it was brought into view by means of an electrolytic gas compensator, modified from Johnson & Shooter (1949). The optical system was of the Philpot cylindrical lens type. A glass fibre was used instead of the inclined knife edge. The light-source, horizontal slit, camera lens

TABLE 1

Composition of Buffers

Buffer	pH	Composition (per litre)
Veronal (1)	9.0	40 ml. 0.5M-NaV*, 5.2 ml. 2N-HCl
Veronal (2)	8.5	40 ml. 0.5M-NaV, 2.65 ml. 2N-HCl
Veronal (3)	8.0	40 ml. 0.5M-NaV, 1.0 ml. 2N-HCl
Phosphate (1)	7.0	1.18g. $NaH_2PO_4.2H_2O$, 2.02g Na_2HPO_4, 1.18g.
($\underline{I}$ = 0.1)		NaCl
Phosphate (2)	7.0	11.35 ml. 0.5M-Na_2HPO_4, 0.8 ml. 4M-NaH_2PO_4
Phosphate (3)	6.5	8.3 ml. 0.5M-Na_2HPO_4, 1.85 ml. 4M-NaH_2PO_4
Acetate	5.5	10.0 ml. 2M-NaAc, 0.6 ml. HAc
Glycine	3.0	15.8 ml. M-glycine + M-NaCl, 2.1ml. 2N-HCl

* The sodium veronal (NaV) was B.P. grade (May & Baker, Dagenham, England).

All buffers except phosphate (1) have $\underline{I}$ = 0.02. For $\underline{I}$ = 0.1, 16 ml. 5M-NaCl were added before making up to 1 l.

and cylindrical lens were of the standard Hilger type (Hilger & Watts Ltd., London, England). The Hilger camera was modified by removal of the bi-prism and slits in front of the plate holder, the latter was altered to take a $3\frac{1}{4}$ x $4\frac{1}{4}$ in. photographic plate. A Siemens type MB/D, 125 W, low-pressure mercury arc was used as light-source. The Schlieren lenses were kindly made available by Dr. R. G. Giovanelli, C.S.I.R.O. Division of Physics, National Standards, Laboratory, Sydney. They were carefully chosen to be free from defects such as spherical aberration, coma etc. The boro-silicate crown glass windows of the water bath were 2.5 cm. thick to minimize distortion due to water pressure. They were polished flat to Rayleigh tolerances and tested in the Optics Section, C.S.I.R.O. Division of Physics. The power was supplied by a constant current power supply which would deliver 50 mA at 500 V. The potential drop across a standard 20 ohm resistor was measured on a Leeds & Northrup portable potentiometer at various intervals during each run to obtain an accurate value of the current.

Mobilities were calculated from the descending boundaries using the following formulae,

$$\mu = \frac{\Delta x}{tE}$$

where μ = mobility in $cm.^2\ sec.^{-1}\ V^{-1}$

Δx = distance of migration in cm.

t = time in sec.

E = potential gradient in V cm.$^{-1}$

$= i/\kappa A$

where i = current in amperes

κ = specific conductivity of protein soln.

A = cross-sectional area in cm.^{2} of the optical channel

The distance to the centroid of each peak was obtained directly from the photographic plate by means of a travelling microscope measuring to within 0.01 mm.

Solutions for electrophoresis were prepared by dialysing the protein solution, made up with buffer, against a relatively large volume of the same buffer with stirring for 16 hr. at 2°. Conductivities of protein solutions were measured at 0° in a Shedlovsky type conductivity cell. Area measurements were made from enlarged tracings by means of a planimeter. Corrections for the and effects were made as suggested by Longsworth, Curtis & Pembroke (1945). Mobilities (at 0°) are given in c.g.s. units multiplied by 10^5.

Micelle Forming Properties. These were investigated using the rapid mixing technique of Waugh & von Hippel (1956).

Ultracentrifugation. Measurements of sedimentation rates were carried out in a Spinco Model E Analytical Ultracentrifuge at a nominal speed of 59,780 rev./min. An analytical cell with a 12 mm. thick centrepiece was used, and an inclined wire replaced the bar in the Philpot cylindrical lens optical

system. Rotor temperature was measured at the beginning and end of a run and the mean value, after application of the Waugh & Yphantis (1952) correction, used in subsequent calculations.

Sedimentation coefficients were calculated by use of the equation,

$$S = \frac{1}{\omega^2 x} \cdot \frac{\Delta x}{\Delta t}$$

where S = sedimentation coefficient in cm./sec.

ω = angular velocity

x = radial distance from the peak to the centre of rotation at time t (sec.)

A travelling microscope measuring to within 0.01 mm. was used to determine the distance x. The distance from the axis of rotation to the inner reference line was taken as 5.725 cm. at 60,000 rev./min. (Taylor, 1952). Values of S have been corrected to standard conditions by the method of Svedberg & Pedersen (1940), and are given in c.g.s. units multiplied by 10^{13}.

Paper Electrophoresis. These measurements were made at 2° using an LKB type 3276 Paper Electrophoresis Apparatus (LKB - Produkter Co., Stockholm, Sweden). In all cases approximately 0.01 ml. 5 per cent protein solution was applied as a spot to the paper. The protein was dyed with bromphenol blue (Kunkel & Tiselius, 1951) after drying at 110° for 30 minutes.

RESULTS

Moving Boundary Electrophoresis

The electrophoretic behaviour of acid casein has been examined over a limited range of pH, ionic strength and protein concentration in an attempt to explain some of the apparent inconsistencies observed by other workers using similar conditions. A comparison with the behaviour of the soluble casein fractions and pure α-casein, as well as with acid casein by other workers, is sufficient to explain most of these discrepancies.

Acid Casein. The results of electrophoresis on two different samples of acid casein under varying conditions are shown in Figure 3 (a and b) and Table 2. Preparation I appears to contain a higher proportion of β-casein. Although γ-casein can be detected in some cases, its presence has been ignored in determining the approximate proportions of the major components.

In the pH range 7.1 - 8.8 ($\underline{I}$ = 0.1 and 0.02) the ascending " α-peak" shows obvious signs of heterogeneity only after prolonged electrophoresis (No. 5, 6 and 8). The slight skewness, particularly in some of the ascending peaks here and later on, $\underline{I}$ = 0.1, is possibly due to boundary distortion during compensation. The results in veronal at pH 7.8 differ from those of Warner (1944) and Cherbuliez & Baudet (1950[a]) who observed a more distinct splitting in the " α-peak" after

TABLE 2

Electrophoresis of Acid Casein

Casein	No.	Concn. (g./100 ml.)	pH and Buffer	$\frac{1}{2}$	V/cm.	$\mu_x 10^5$ (cm.^{2} V^{-1} sec.$^{-1}$)		Relative Concn. (%)			
								"α"		β	
						"α"	β	Desc.	Asc.	Desc.	Asc.
I	1	0.6	8.8 V.*(1)	0.1	3.68	- 6.68	- 3.40	74	70	26	30
I	2	0.6	8.3 V. (2)	0.1	3.81	- 6.97	- 3.40	73	70	27	30
II	3	1.0	8.2 V. (2)	0.02	7.39	- 8.50	- 4.46	80	81	20	19
II	4	1.0	8.1 V. (2)	0.1	3.67	- 6.62	- 3.16	79	78	21	22
II	5	0.6	7.8 V. (3)	0.1	3.55	- 6.75	- 3.30	77	75	23	25
I	6	0.6	7.8 V. (3)	0.1	3.76	- 6.85	- 3.20	73	71	27	29
II	7	0.5	7.2 P.φ(2)	0.02	8.66	- 9.92	- 4.44	80	79	20	21
II	8	0.5	7.1 P. (2)	0.1	3.67	- 6.84	- 2.99	79	75	21	25
I	9	0.6	6.8 P. (2)	0.02	8.81	- 8.71	- 3.58	82	71	18	29
I	10	1.0	6.4 P. (3)	0.1	3.66	- 6.39	- 2.46	81	70	19	30
II	11	1.0	6.3 P. (3)	0.02	9.30	- 7.50	- 2.81	89	82	11	18

* V. = Veronal. φ P. = phosphate.

FIGURE 3 (a and b). Electrophoresis of acid casein. See Table 2 for details. The times after starting the electrophoresis are given below, as well as the angle, θ, made by the inclined glass fibre with the horizontal. The values given in brackets are those for the leading peak at a later time.

1, 251 min., 20° (339 min., 10°); 2, 133 min., 20° (246 min., 15°); 3, 57 min., 40°; 4, 147 min., 30° (asc.), 20° (desc.); 5, 252 min., 20°; 6, 245 min., 20° (300 min., 15°); 7, 72 min., 20°; 8, 209 min., 10°; 9, 66 min., 30°; 10, 242 min., 15° (339 min., 10°); 11, 41 min., 40° (84 min., 20°).

Peaks: 1, "α"-component; 2, β-casein; 3, γ-casein.

(over)

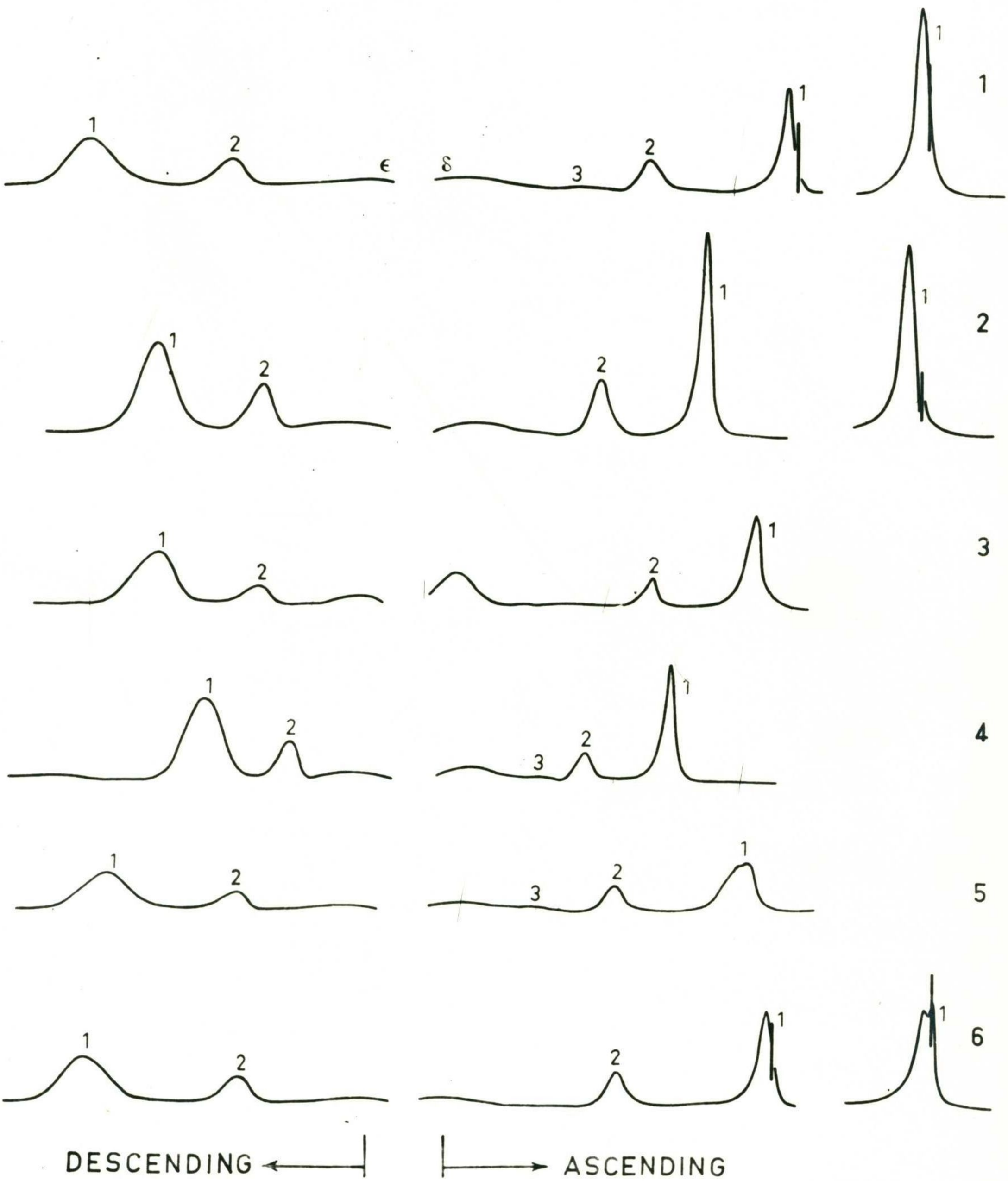
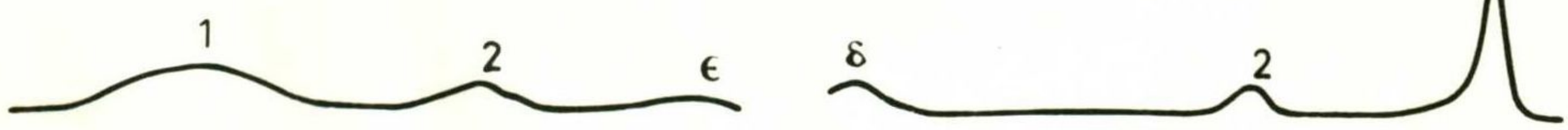
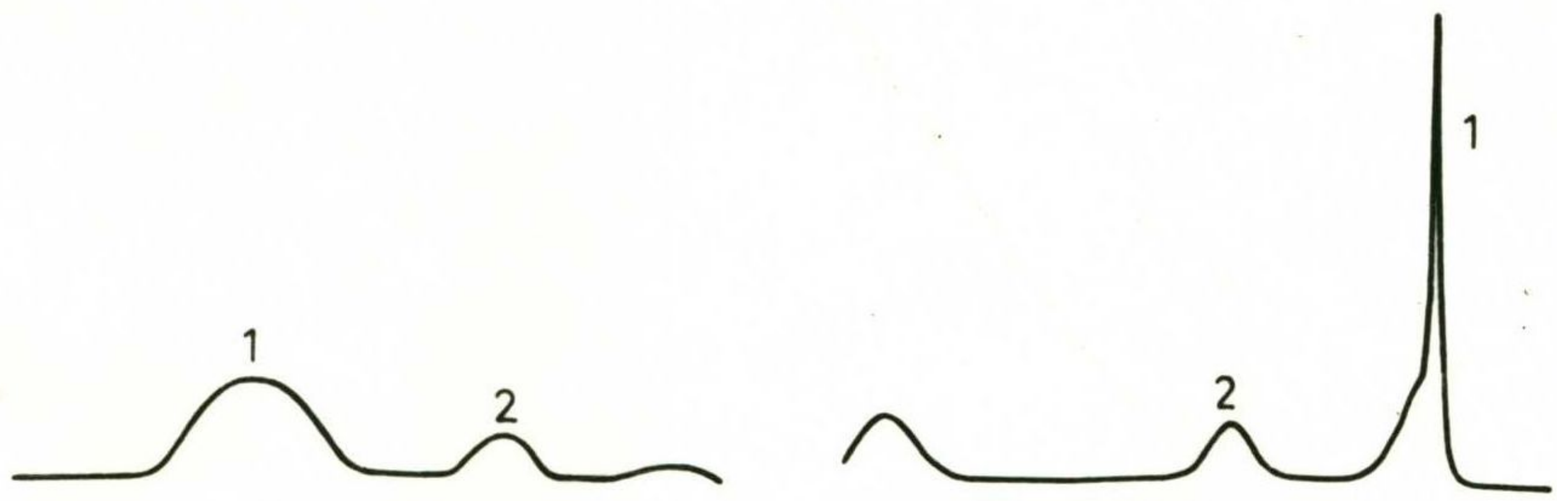


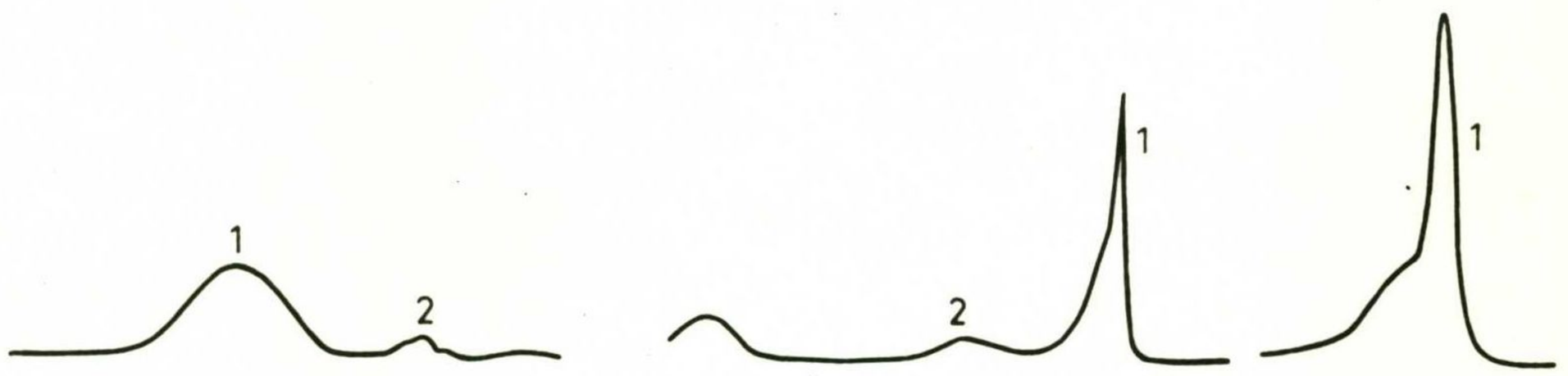
FIGURE 3a


1
2
ε
δ
2
1
7


1
2
2
1
1
8


1
2
2
1
9


1
2
2
1
1
10


1
2
2
1
1
11


DESCENDING
ASCENDING

FIGURE 3b

a relatively short time under similar conditions (protein < 1.0 per cent). The heterogeneity observed here is still evident with 1.0 per cent protein and is unlike the α_1-α_2 split described by these workers. A sharp spike is invariably present on the leading side of the " α- peak" in acid casein I. Its position, relative to the " α-peak", changes throughout the electrophoresis run. In agreement with Krejci et al. (1941, 1942) and Warner (1944) the relative concentration of β-casein is usually greater in the ascending than in the descending limb. The difference is not more than 20 per cent. The usual explanation has been on the basis of an interaction between the "α-" and β- components. The marked dependence of this interaction on protein concentration is well established (Nitschmann & Zurcher, 1950).

In the lower pH range (6.3 - 6.8; No. 9, 10 and 11) heterogeneity in the ascending " α-component" is more readily distinguishable. Of greater extent too is the difference in relative concentrations of the "α-" and β-components in the ascending and descending limbs, approximately 60 per cent for the β-component in each case. The relative concentrations of the " α-component" in the ascending limb, however, are not significantly different from those observed at the higher pH values. There is presumably an enhanced interaction as the " α-component" migrates down through the medium containing β-casein under these conditions. There is a marked split in the descending β-peak of casein B at pH 6.3 and 7.2 with

$\underline{I}$ = 0.02.

On electrophoresis under various conditions the two preparations of acid casein used here behave somewhat differently from each other as well as from that examined by Warner (1944). This could be due to (a) differences in composition and/or (b) different conditions used in separating and purifying the acid caseins.

The enhanced interaction between the "α-" and β-components at the lower pH values observed here and by other workers, and the more readily distinguishable heterogeneity in the ascending "α-peak" under the same conditions suggest a connection between the two effects. However, electrophoresis on the soluble caseins where marked interaction is evident, will suggest that, although such a relationship could exist, there is probably something additional and distinct for <u>acid</u> casein which is primarily responsible for this commonly described heterogeneity.

<u>Soluble Casein</u>. The results of an electrophoretic examination of the total milk protein, lst and c.s. casein, acid casein, and fraction S, all prepared from the one milk sample, are shown in Figure 4 (a and b) and Table 3. All these investigations were made with 1.0 per cent protein at pH 7.0 (phosphate, $\underline{I}$ = 0.1 and 0.02). In no case is there any marked abnormality in the shape of the leading ascending peak. However, heterogeneity is obvious in the descending

TABLE 3

Electrophoresis of Soluble Casein Fractions

Protein	No.	Concn. (g./100ml.)	pH and Buffer	μ	V/cm.	$\mu \times 10^5$ ($cm^2 V^{-1} sec^{-1}$)		Relative Concn. (%)			
								α-κ, α or κ		β	
						α-κ, α or κ	β	Desc.	Asc.	Desc.	Asc.
T.M.P.*	12	1.0	7.0 P.(1)	0.1	4.93	- 6.24	- 2.52	-	-	-	-
1st c.s.casein	13	1.0	7.0 P.(1)	0.1	5.19	- 6.04	- 2.43	84	78	16	22
2nd c.s.casein	14	1.0	7.0 P.(1)	0.1	4.87	- 6.41	- 2.57	84	70	16	30
T.M.P.	15	1.0	7.0 P.(2)	0.02	6.85	- 8.15	- 3.02	-	-	-	-
1st c.s.casein	16	1.0	7.0 P.(2)	0.02	7.16	- 7.62	- 2.61	85	78	15	22
2nd c.s.casein	17	1.0	7.0 P.(2)	0.02	6.90	- 8.05	- 2.88	87	72	13	28
Acid casein	18	1.0	7.0 P.(2)	0.02	7.06	- 8.13	- 2.86	84	72	16	28
Acid caseinϕ	19	1.0	7.0 P.(2)	0.02	7.34	- 8.08	- 3.18	-	-	-	-
Fraction S	20	1.0	7.0 P.(1)	0.1	4.92	- 4.88	- 2.46	79	80	21	20
Fraction S	21	1.0	7.0 P.(2)	0.02	7.20	-	-	-	-	-	-
α-casein	22	0.75	7.0 P.(2)	0.02	7.24	- 8.39	-	-	-	-	-
β-casein	23	0.5	7.0 P.(2)	0.02	7.14	-	- 3.49	-	-	-	-
75% α-casein + 25% β-casein	24	1.0	7.0 P.(2)	0.02	7.10	- 7.62	- 2.60	-	-	-	-
1st c.s.casein	25	0.6	7.9 V.(3)	0.1	4.08	- 6.24	- 2.99	-	-	-	-

* T.M.P. = total milk protein. ϕ Acid casein treated at pH 12.

FIGURE 4 (a and b). Electrophoresis of soluble casein.
See Table 3 for details.

12, 173 min., 40° (315 min., 30°); 13, 174 min., 40° (340 min., 40°); 14, 176 min., 40° (303 min., 40°); 15, 103 min., 30°; 16, 37 min., 60° (91 min., 40°); 17, 47 min., 60° (88 min., 40°); 18, 42 min., 60° (73 min., 40°); 19, 50 min., 60° (121 min., 40°); 20, 151 min., 40°; 21, 91 min., 40°; 22, 91 min., 40°; 23, 106 min., 40°; 24, 117 min., 40°; 25, 271 min., 40° (331 min., 40°).

Peaks: 1, α-κ-, α-, or κ-casein; 2, β-casein; 3, γ-casein; 4, β-lactoglobulin.

(over)

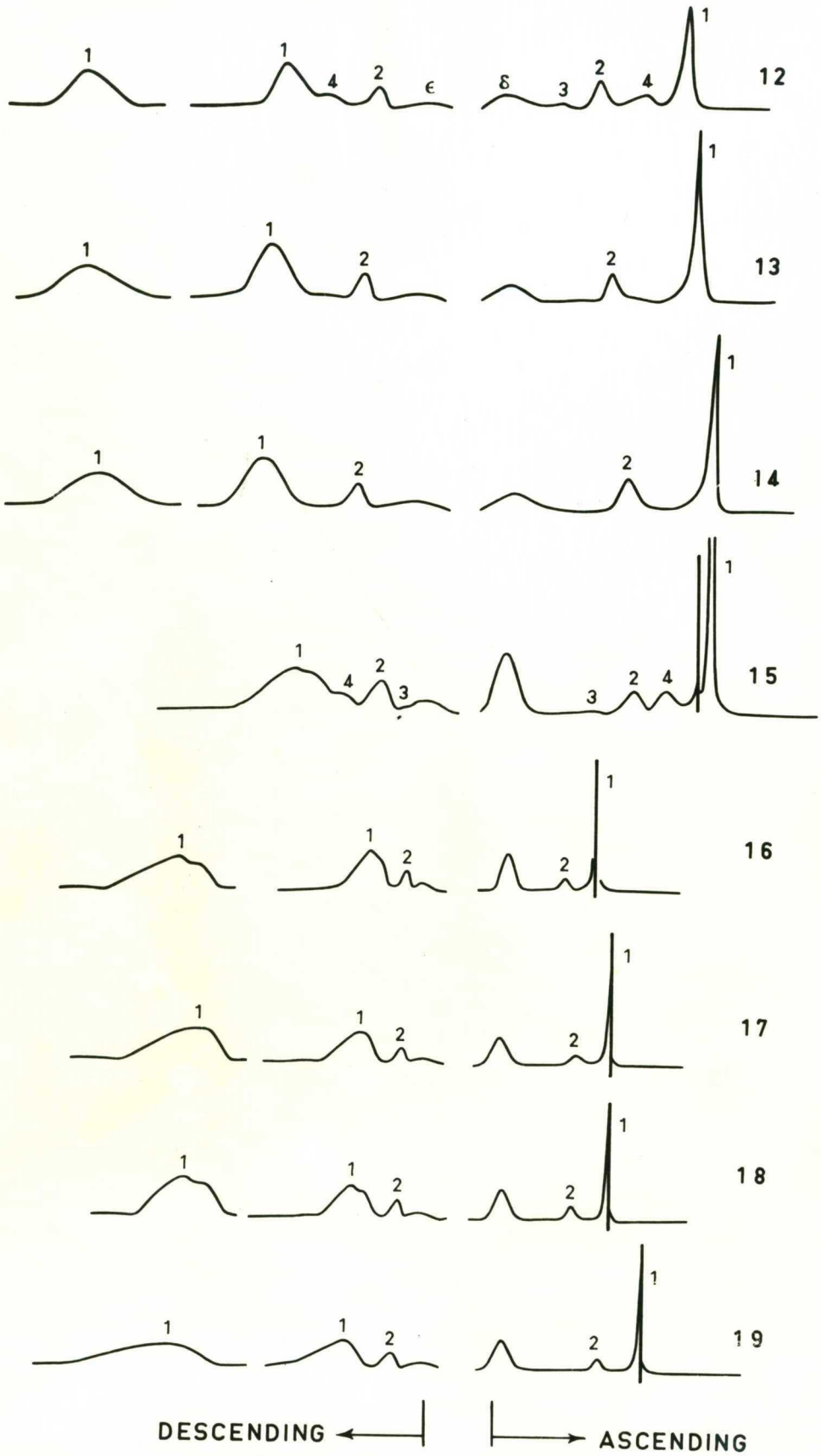


FIGURE 4a

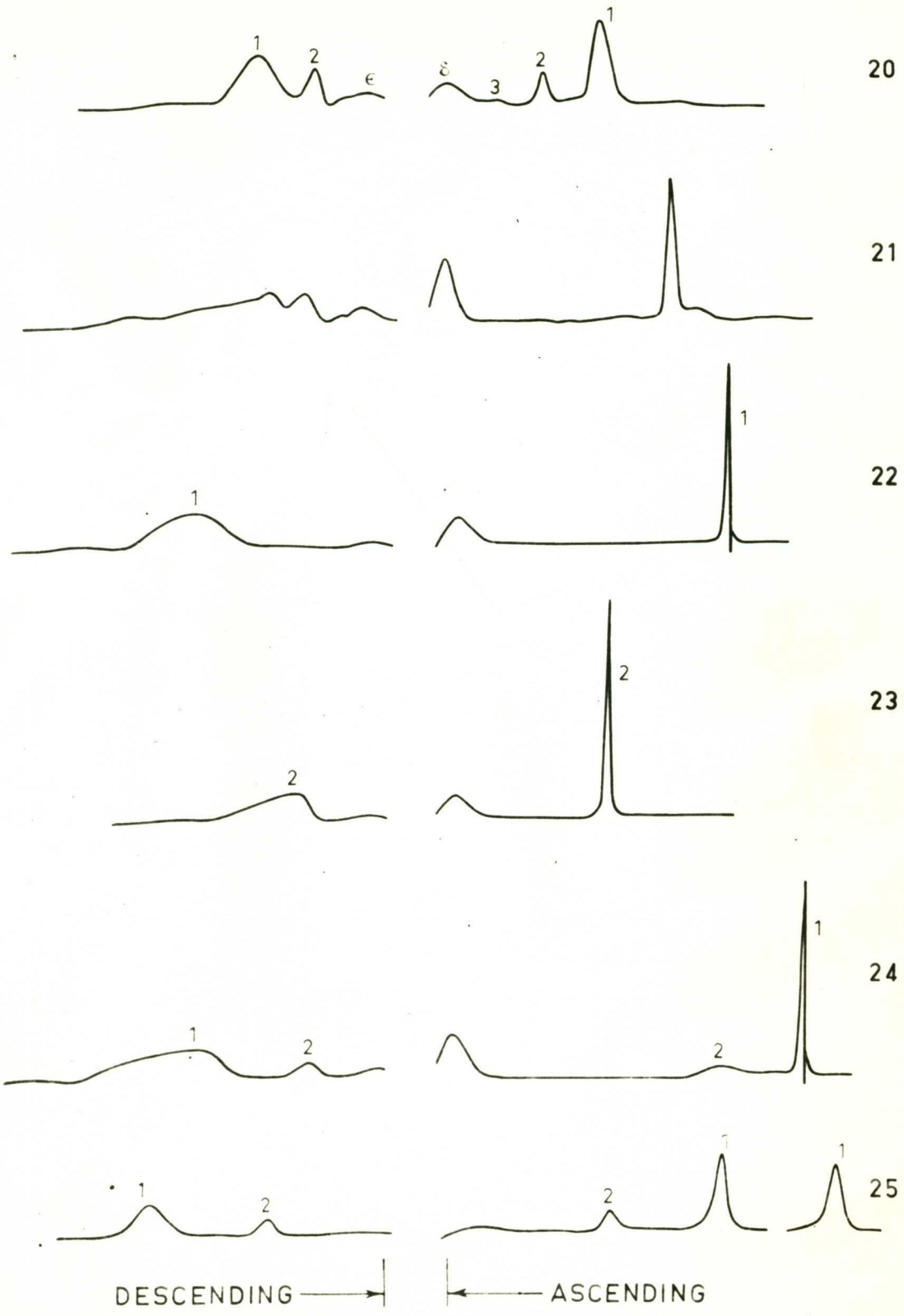


FIGURE 4b

patterns. At the higher ionic strength, 0.1, the asymmetry in the leading peak of the total milk protein and 1st c.s. casein (No. 12 and 13) stands out in comparison with that of 2nd c.s. casein (No. 14). At $\underline{I}$ = 0.02 a discontinuity appears and the difference is more marked (cf. No. 15, 16 and 17). These results support the existence of κ-casein and its presence in the total milk protein and 1st c.s. casein. At both ionic strengths the mobility of the leading peak in 1st c.s. casein is significantly lower than that in 2nd c.s. casein, and is probably due to the presence of the $\alpha - \kappa$-complex in the former. An abnormal distribution of concentrations is again evident, that of the ascending β-component being higher than that in the descending limb, by approximately 40 per cent in 1st c.s. casein and 100 per cent in 2nd c.s. casein. These results suggest a much greater interaction between β- and α-casein than between β-casein and the $\alpha - \kappa$- complex under these conditions. The area distributions in the ascending patterns are in accordance with the absence of κ-casein from 2nd c.s. casein. Electrophoresis results with pure α-casein, β-casein, and a mixture of the two are also included (No. 22, 23 and 24). The last mentioned gives a pattern similar to that of 2nd c.s. casein, although the mobilities are somewhat lower.

From a study of the properties of 1st c.s. casein and acid casein Waugh & von Hippel (1956) suggested that

the " α -peak" in acid casein represents the two distinct components, α - and κ -casein, involved to a certain extent in some type of difficulty reversible aggregation. A comparison has therefore been made of the electrophoretic behaviour of acid casein and 1st c.s. casein both from the one sample of milk at pH 7.0 and $\underline{I}$ = 0.02. Under these conditions acid casein (No. 18) shows a distinct splitting in the leading descending peak similar to that obtained with the total milk protein and 1st c.s. casein. However, it could be further complicated by the presence of difficulty reversible aggregates. A difference from 1st c.s. casein can be noted in the higher mobility of the leading peak possibly brought about by such aggregation. It was expected that dissaggregation of complexes involving α - and κ -casein by titration of acid casein to pH 12 and subsequent dialysis to pH 7 would result in a pattern still resembling that of 1st c.s. casein but with a lower mobility for the " α -peak". The pattern obtained (No. 19) however, resembles that of 2nd c.s. casein in that no splitting in the leading descending peak is apparent and in addition, the mobility is still significantly higher than in 1st c.s. casein. It is likely that different types of α - κ -complexes are formed depending on whether both are mixed as monomers (at pH 12) and then allowed to polymerize (by dialysis to pH 7), or whether they are formed from the aggregates of both at neutral pH (as in milk). This could also explain the lowering in the sedimentation rate of

the α - κ -complex in 1st c.s. casein, after treatment at pH 12, observed by Waugh & von Hippel.

The distribution of areas in the ascending and descending electrophoretic patterns of 1st and 2nd c.s. casein at pH 7 (phosphate, $\underline{I}$ = 0.1 and 0.02) indicates marked interaction as the leading component migrates down through a medium containing β -casein. It occurs to a greater extent with 2nd c.s. casein and in this case can be assumed to involve α- and β- casein. The tendency towards interaction here does not result in any noticeable heterogeneity or splitting in the α-casein component. However, it does not follow that β-casein present in 1st c.s. casein is not involved in the heterogeneity observed in the peak representing the α- κ - complex. Results on the electrophoresis of fraction S (below) and observations made during the separation and purification of κ-casein (Section III) indicate an extensive and possibly unique interaction between β - and κ-casein. This effect could be extended to involve β-casein with the α - κ - complex. The suggestion presented by the electrophoretic results on acid caseins I and II, particularly the pH dependence, that interactions involving β-casein with the other components are responsible for the heterogeneity observed in the ascending " α -peak" is quite possible. However, electrophoresis of 1st c.s. casein in veronal at pH 7.9 ($\underline{I}$ = 0.1, No. 25) under conditions where splitting or heterogeneity in the leading ascending peak has

been most commonly described in acid casein shows no such effect. It must therefore be concluded that the procedures used to separate and purify the acid casein bring about some alteration which is mainly responsible for the heterogeneity of the " α-peak" in veronal at pH 7.8 ($\underline{I}$ = 0.1). The most likely factor is the formation on acid precipitation, of the difficultly reversible aggregates involving α- and κ-casein. Since interactions involving the casein components with themselves and with each other, are highly pH, temperature and ionic strength dependent (see later), it is only to be expected that the exact nature of the interactions involving α- and κ-casein will be very sensitive to, and dependent upon the experimental conditions used to acid precipitate the casein. The "apparent" discrepancies between the results on the two acid caseins used here and those of other workers can be explained on this basis. Variations in the relative abundance of the components could also be involved. Further support for the suggestion that the heterogeneity in the " α-component" of acid casein is due to the α- κ-complex will be found in the results of electrophoresis on pure α-casein.

Waugh & von Hippel suggested that fraction S contains 70 per cent κ-casein and 30 per cent β-casein, and that the former has an electrophoretic mobility close to that of α-casein. Electrophoresis of fraction S at pH 7.0 in phosphate, $\underline{I}$ = 0.1 (No.20) indicates the presence of approx-

imately 80 per cent of a faster moving component, with a mobility of - 4.88, in both the ascending and descending limbs. Sedimentation and paper electrophoresis of the same fraction S suggest a much lower proportion of κ-casein (see later). Results on the action of rennin on casein fractions (Section IV) also indicate the presence of nearer 50 per cent of this component. The indication of 80 per cent of a faster moving component on electrophoresis of fraction S is apparently due to interaction between κ- and β-casein. The apparent discrepancy between the sedimentation and moving boundary electrophoresis results is difficult to explain. The pattern obtained for fraction S at pH 7.0 in phosphate, $\underline{I}$ = 0.02 (No. 21) is complex and probably a result of various types of aggregations and interactions involving κ-casein and other components (the minor components in fraction S will be discussed in the last part of this Section).

Mention should be made here of the recent work of Payens (1958). He has reported briefly the results of an electrophoretic study on the action of commercial rennet on 1st and 2nd c.s. casein. He draws the conclusion that the commonly described " α_2-casein" of the α_1-α_2 split is closely related to κ-casein and is possibly identical with it. In veronal buffer at pH 7.3 ($\underline{I}$ = 0.1) 1.7 per cent 1st c.s. casein "occasionally" exhibits the α_1-α_2 split in the ascending limb. His results showing the presence of 27 per

cent α_2-casein in 1st c.s. casein indicates that α_2-casein is not "identical" with κ-casein. It will be shown in Section IV of this thesis that results on the liberation of non-protein-nitrogen from casein fractions during the action of rennin, support Waugh & von Hippel's estimation of only 15 per cent κ-casein in 1st c.s. casein. Also it will be apparent that 2nd c.s. casein contains essentially no κ-casein. The appearance of 13 per cent α_2-casein (unresolved) in the ascending limb during electrophoresis of Payens' 2nd c.s. casein, therefore, cannot be explained by the presence of κ-casein; and the increase in the α_1-α_2 split after rennet treatment of this fraction is certainly unexpected.

α-Casein. Warner (1944), Kondo et al. (1950) and Cherbuliez & Baudet (1950a) have all reported on the electrophoretic heterogeneity of "pure α-casein". All three preparations were obtained by a method similar to that described by Warner. This is based on the relatively high solubility of β-casein at pH 4.4 and 2° in comparison with α-casein. Later in this Section it will be shown that α-casein obtained from the alcohol fraction B of Hipp et al. (1952) is free from κ-casein. This is because of the preliminary separation of acid casein into three crude fractions, A, B and C, the first of which contains all of the κ-casein. The method proposed by Warner involves no

such preliminary fractionation, and the α-casein separated using this procedure probably contains κ-casein.

Results of an electrophoretic study on α-casein, free from κ-casein are described here, Figure 5 (a and b) and Table 4. Acid casein II was used as a source of this α-casein. Over the pH range 3.0 - 8.2 ($\underline{I}$ = 0.1 and 0.02), the material shows no marked splitting as was evident in acid casein under similar conditions. The asymmetries in the peaks and the tailing, which are more noticeable at the lower pH values above the isoelectric point, are probably due to α-α-interaction and the presence of relatively large aggregates which form as the pH is lowered (see Section II). A preparation of the α-casein described here in glycine - HCl buffer at pH 3.0, $\underline{I}$ = 0.1, showed extensive aggregation even at 0.5 per cent protein. The solution was quite "milky" and it is likely that the heterogeneity described by Warner for " α-casein" in lactate buffer under similar conditions was due to a similar effect.

The results presented here support the view that the marked heterogeneity observed in the ascending " α-peak" of acid casein is due primarily to the presence of κ-casein as an α- κ-complex.

Sedimentation

The behaviour of various casein fractions on sedimentation has been examined, in conjunction with micelle

TABLE 4

Electrophoresis of α-Casein

No.	Concn. (g./100 ml.)	pH and Buffer	I	V/cm.	$\mu \times 10^5$ ($cm^2 V^{-1} sec.^{-1}$)
26	0.5	8.2 V.(2)	0.1	3.74	- 7.17
27	0.6	8.0 V.(3)	0.1	3.93	- 6.95
28	0.6	7.8 V.(3)	0.1	4.73	- 6.60
29	0.6	7.6 V.(3)	0.1	3.84	- 6.60
30	1.0	6.2 P.(3)	0.1	4.81	- 6.52
31	1.0	6.2 P.(3)	0.02	8.68	- 8.22
32	1.0	5.6 acetate	0.02	7.55	- 8.56
33	1.0	5.5 acetate	0.1	4.82	- 5.99
34	0.5	3.0 glycine	0.02	6.31	+ 9.88

FIGURE 5 (a and b). Electrophoresis of α-casein. See Table 4 for details.

26, 223 min., 12°; 27, 294 min., 40°; 28, 236 min., 40°; 29, 327 min., 30°; 30, 256 min., 50°; 31, 96 min., 40°; 32, 112 min., 60°; 33, 257 min., 40°; 34, 103 min., 40°.

(over)

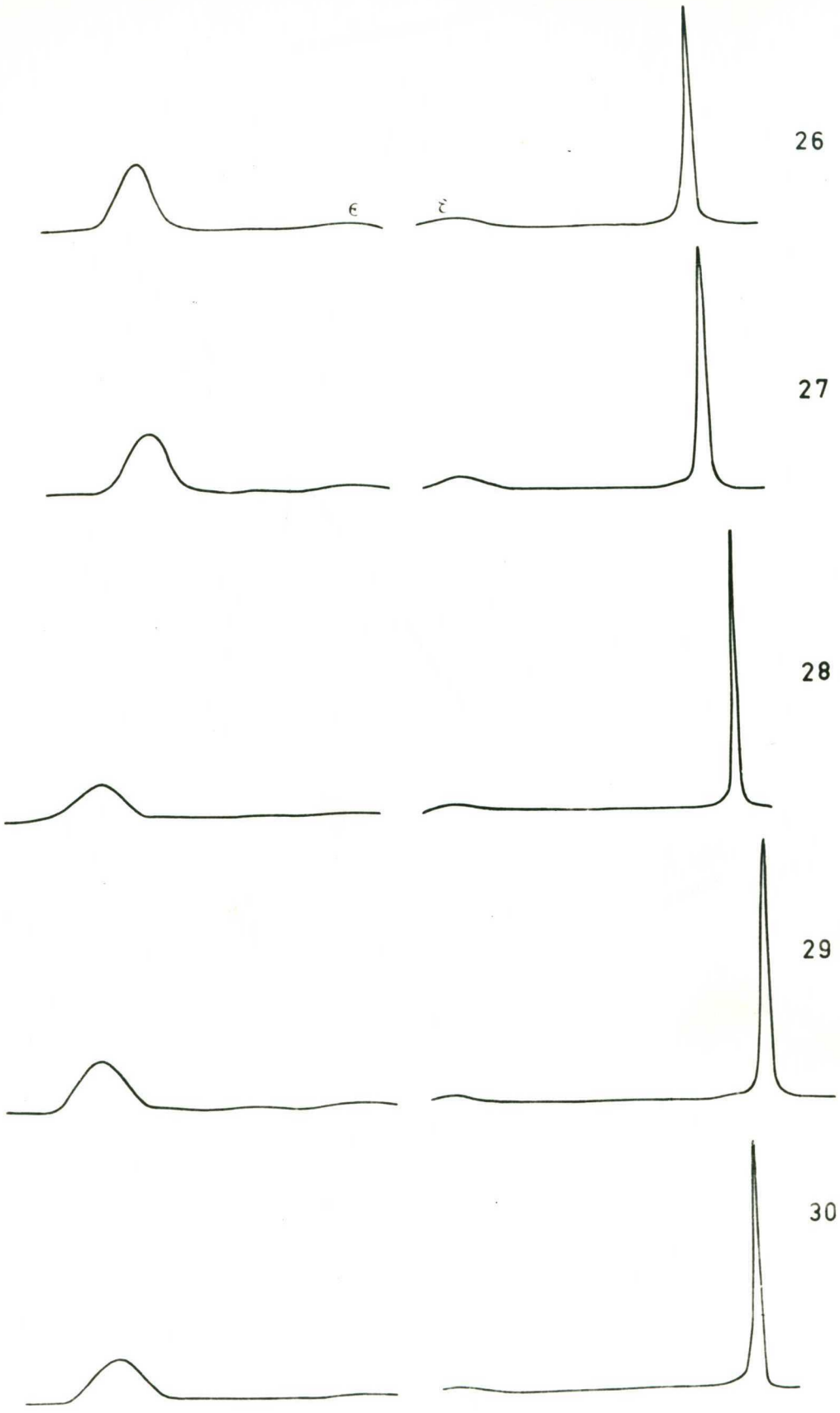


FIGURE 5a

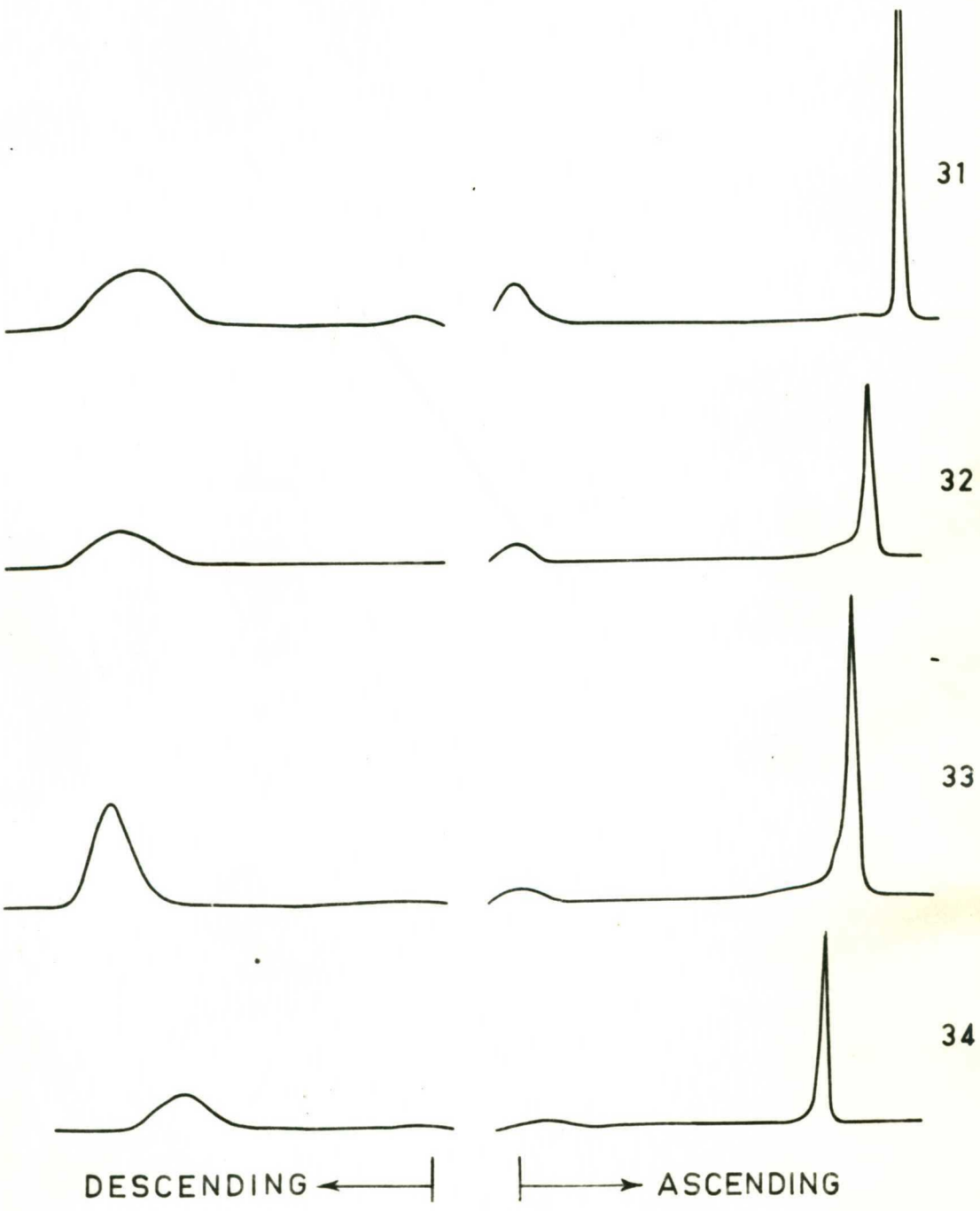


FIGURE 5b

forming properties and paper electrophoresis, to show conclusively that during the preliminary fractionation of acid casein into the crude alcohol fractions, A, B and C, by the method of Hipp et al. (1952) all of the κ-casein is concentrated in fraction A. Thus, it has been possible to prepare pure α-casein, i.e., free from κ-casein, by using fraction B as starting material. The product thus obtained is also free from the proteolytic enzyme of milk.

Sedimentation patterns obtained for 2nd c.s. casein, fraction S, and fractions A and B in phosphate at pH 7.0, $\underline{I} = 0.1$, are shown in Figure 6. S_{20} values are indicated in Table 5. Fractions A and B had been treated at pH 12 to disperse any "difficultly reversible aggregates" which could have been present. The results obtained for the soluble caseins are in general agreement with those described by Waugh & von Hippel (1956), see Table 5.

The minor, slow moving component in fractions A and B represents β-casein. The presence of a diffuse and very fast moving component in fraction A with S_{20} approximately 14 indicates the presence of excess κ-casein. The faster and well-defined peak in fraction B, 1.0 - 1.5 per cent, has $S_{20} = 3.4$, and this is near the value for α-casein in 1st c.s. casein, 4.2 at 1.0 per cent protein. Certainly there is no faster moving material which could indicate the α- κ-

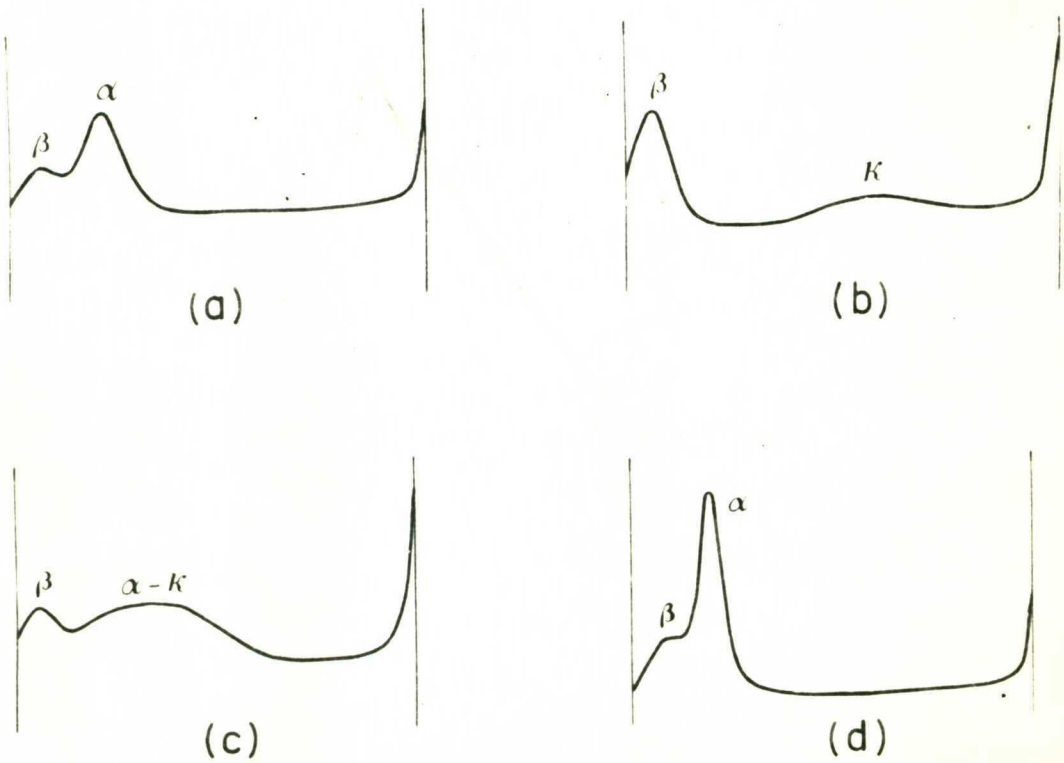


FIGURE 6. Sedimentation of casein fractions in phosphate (1) at pH 7.0, $\underline{I}$ = 0.1, and 2° - 5°. Sedimentation is from left to right.

(a) 1.0 per cent 2nd c.s. casein, 67 min., Θ = 70°;
(b) 1.0 per cent fraction S, 76 min. Θ = 65°;
(c) approx. 1.5 per cent fraction A, 71 min., Θ = 60°;
(d) approx. 1.5 per cent fraction B, 68 min., Θ = 70°.

TABLE 5

Approximate S_{20} Values for Casein Fractions

Protein	Peak*				
	I	II	III	IV	V
1st c.s. casein	(1.3)		(7.5)		
2nd c.s. casein	1.4 (1.3)	4.2(4.5)			
Fraction S	1.3 (1.3)			10 (13.5)	
Fraction A	1.2				14
Fraction B	1.6	3.4			

All runs were carried out in phosphate (1) at pH 7.0, $\underline{I}$ = 0.1, at 2° - 5°. The total protein concentration with fractions A and B was approximately 1.5 per cent. Others were 1.0 per cent. The values given in brackets are those obtained by Waugh & von Hippel (1956) under similar conditions.

* The compositions of the peaks are largely: I, β-casein; II, α-casein; III, α- κ-complex; IV, κ-casein; V, κ-, plus α-casein.

complex or free κ-casein.

Micelle Forming Properties

Waugh & von Hippel (1956) have claimed that " κ-casein is the most important single factor responsible for micelle stabilization" and that it "exerts its primary stabilization in the formation of the α- κ- complex."

Table 6 shows the results of an examination of fractions A, B and C, treated at pH 12, for micelle forming properties. The "degree of cloudiness" produced in the presence of calcium has been taken to indicate the micelle forming capacity of a particular fraction. The absence of a precipitate and decreased cloudiness in tube 2 indicates the presence of excess κ-casein in fraction A, while the complete precipitation in tube 3 supports the absence of κ-casein from fraction B. Tubes 5 and 6 show that micelles can be formed with fraction B in the presence of added κ-casein. The behaviour of fraction C, tube 4, at 37° and 2° is consistent with the large proportion of β-casein - it is well known that calcium β-caseinate forms a colloidal suspension at room temperature but is completely soluble at 2°.

α-Casein, prepared from fraction B, was completely precipitated by 0.05 M-$CaCl_2$, but gave stable micelles when mixed with fraction S prior to the addition of $CaCl_2$.

Paper Electrophoresis

Zweig & Block (1953) were the first to report the

TABLE 6

Micelle Forming Properties of Casein Fractions

Tube	Soln. Casein Fraction (ml.)					Observations			
	Acid casein	A	B	C	S	After 30 min., 37°		After 12 hr., 2°	
						Cloudiness	Ppt.	Cloudiness	Ppt.
1	2.0					xxxx	o	xxxx	o
2		2.0				xx	o	xx	o
3			2.0			o	xxxx	o	xxx
4				2.0		slight	xxxx	o	o
5			2.0		1.0	xxxxx	x	xxx	xx
6					1.0	slight	o	slight	slight

All protein solutions used were 1.0 per cent at pH 7.3. Each tube contained 0.12 ml. 4M-NaCl, and the volume was made up to 3.1 ml. with water before adding 0.15 ml. M-$CaCl_2$ at 37°.

results of a paper electrophoretic examination of acid casein and its components. More recently Hofmann (1958) has examined the acid casein obtained from goat's milk by this method. The present author has found the technique very convenient and efficient in characterizing casein fractions. Because of the marked interaction between casein components, particularly at room temperature, all runs have been carried out at 2°.

The results of an examination of the total milk protein and soluble casein fractions in phosphate at pH 7.0, $\underline{I}$ = 0.1, are shown in Figure 7. The α- κ- complex, present in both the total milk protein and 1st c.s. casein, moves at an intermediate rate between that of α-casein, in 2nd c.s. casein, and κ-casein, in fraction S. This is consistent with the mobilities observed by moving boundary electrophoresis under the same conditions.

The results on acid casein and the alcohol fractions A, B and C, treated at pH 12, under the same conditions as above, are shown in Figure 8. The α- κ- complex, present in acid casein, moves at an intermediate rate between the fastest moving component in fractions A and B. The fact that the leading spot in fraction B moves just ahead of that in acid casein and still further ahead of that in fraction A suggests that κ-casein is concentrated in the last fraction. The high concentration of β-casein in fraction C is evident.

It will be noted that in all cases the distance moved by β-casein depends upon that travelled by the leading

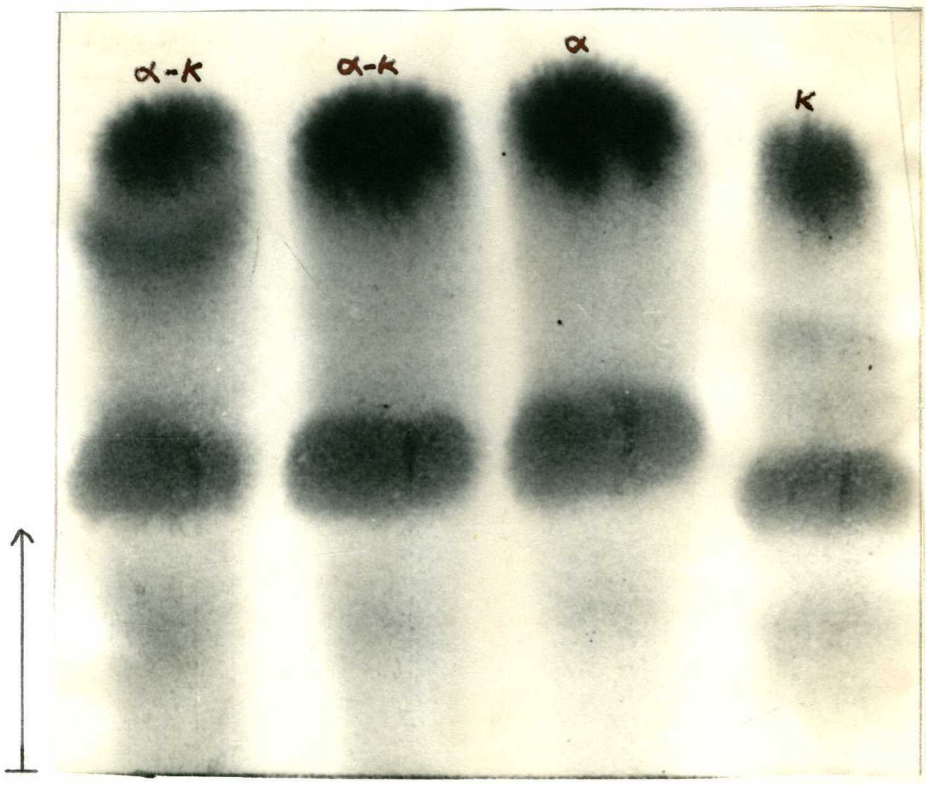


FIGURE 7. Paper electrophoretic patterns obtained for (a) total milk protein, (b) 1st c.s. casein, (c) 2nd c.s. casein, and (d) fraction S. The run was carried out in phosphate (1) at pH 7.0, $\underline{I}$ = 0.1, and 2° on Whatman No. 1 paper for 20 hr. at 200 V.

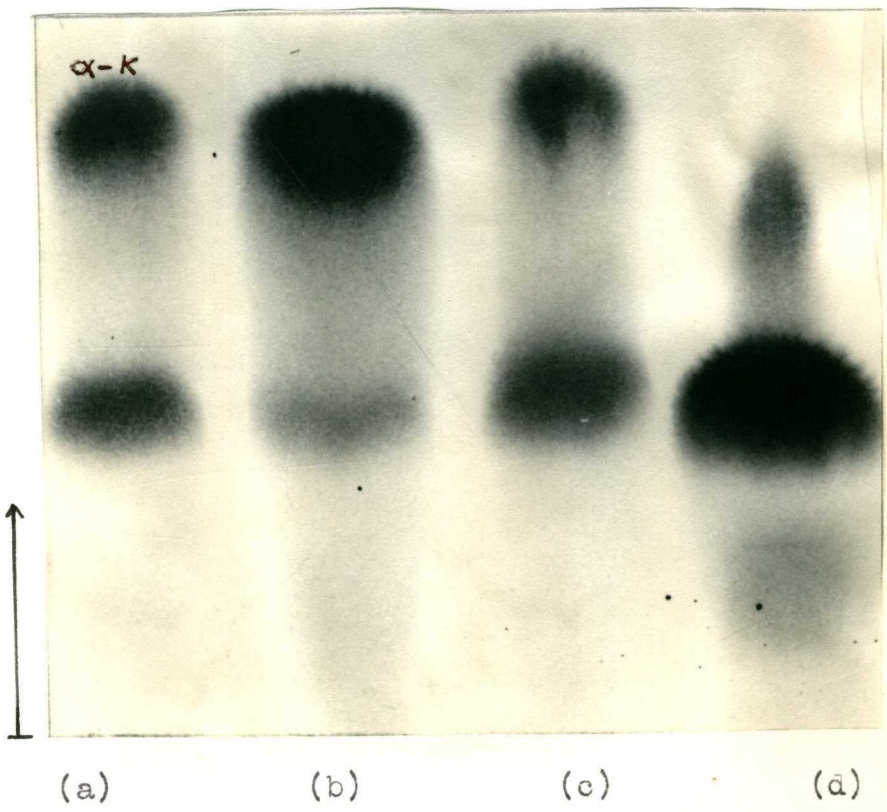


FIGURE 8. Paper electrophoretic patterns obtained for (a) acid casein, (b) fraction A, (c) fraction B, and (d) fraction C; conditions as in Figure 7.

component. This is also generally true for the mobilities determined by moving boundary electrophoresis and is due undoubtedly to interactions between β-casein and the other components. The effect on paper electrophoresis could be reproduced by mixing samples of pure α-, β- and κ-casein (see Section III for preparation of this fraction). β-Casein had no noticeable effect on the rate of migration of α- and κ-casein. α-Casein, however, greatly enhanced the rate of movement of β-casein, while κ-casein had no obvious effect. The result was intermediate when all three components were mixed. It is likely that the distance moved by β-casein depends primarily upon the proportion of α-casein, with which it so readily interacts, present in the fastest moving material. The formation of the α- κ-complex, moving at an intermediate rate between those of the individual components was clearly demonstrable on mixing the two caseins.

The results presented above, together with those on sedimentation and micelle forming properties of casein fractions, clearly indicate that during the alcohol fractionation of acid casein all of the κ-component is concentrated in fraction A, so that fraction B can be used as a source of pure α-casein.

During the paper electrophoretic examination of various casein fractions a number of additional and minor protein components have been observed. Invariably present

in preparations of acid casein and soluble casein fractions has been a component moving more slowly on electrophoresis than γ-casein and present to approximately the same extent as it. It was noted that during the preparation of γ-casein by the alcohol method Hipp et al. obtained a fraction which contained 44 per cent γ-casein, 12 per cent β-casein, and 44 per cent of "a component of casein moving more slowly than γ-casein." The results of paper electrophoresis of this fraction along with a sample of acid casein and 2nd c.s. casein in veronal at pH 8.3, $\underline{I}$ = 0.1, are shown in Figure 9. It is most likely that the component observed here is identical with that noticed by Hipp et al. Moving boundary electrophoresis of their fraction under the same conditions is shown in Figure 10. The γ-casein component has a mobility of -2.02 which agrees well with published values. The slow moving material has a mobility of -0.94. It could be detected also in preparations of fraction S, but was not always identifiable, even in whole casein. γ-Casein itself was difficult to detect in many cases (cf. Zweig & Block, 1953).

Paper electrophoresis of fraction S indicates more than κ- and β-casein. γ-Casein is also present and, in addition, there are two components, both moving between κ- and β-casein. Exhaustive dialysis of fraction S against water at 2° causes precipitation of most of the protein as the pH drops from 7.0 to 5.2. Paper electrophoresis of this precipitate and the supernatant (after freeze-drying),

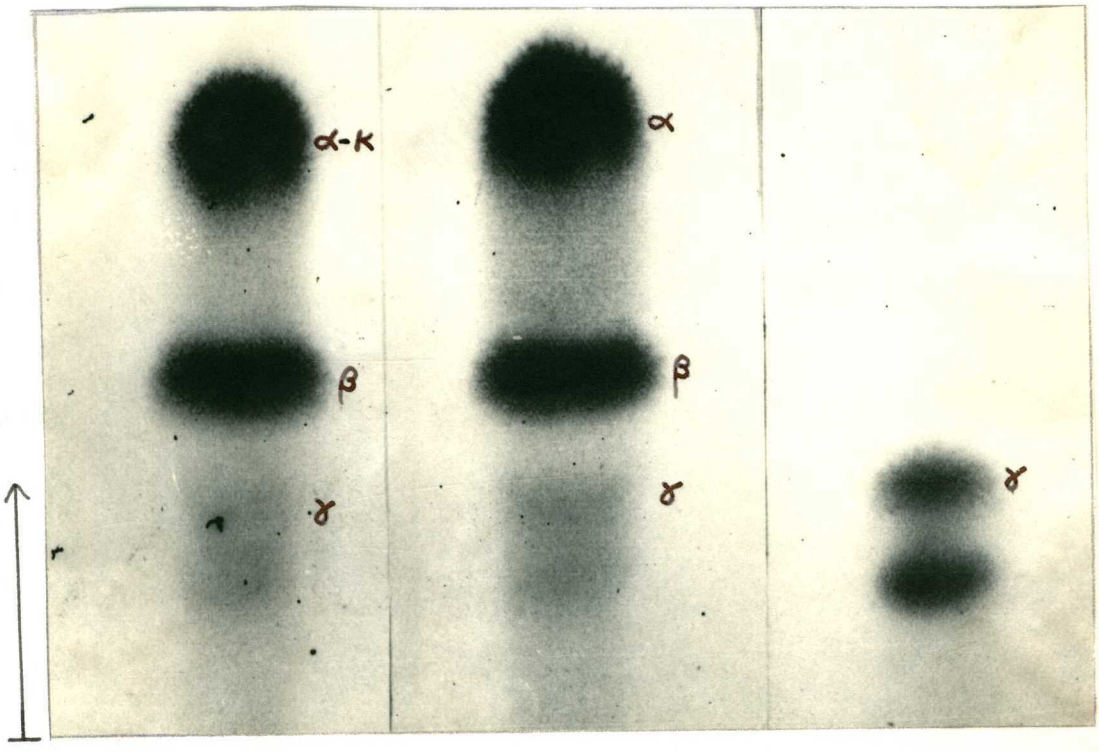


FIGURE 9. Paper electrophoretic patterns obtained for (a) acid casein, (b) 2nd c.s. casein, and (c) alcohol fraction containing component moving more slowly than γ-casein. The run was carried out in veronal (2) at pH 8.3, $\underline{I}$ = 0.1, and 2° on Schleicher & Schull, No. 2043 B paper for 16 hr. at 170 V.

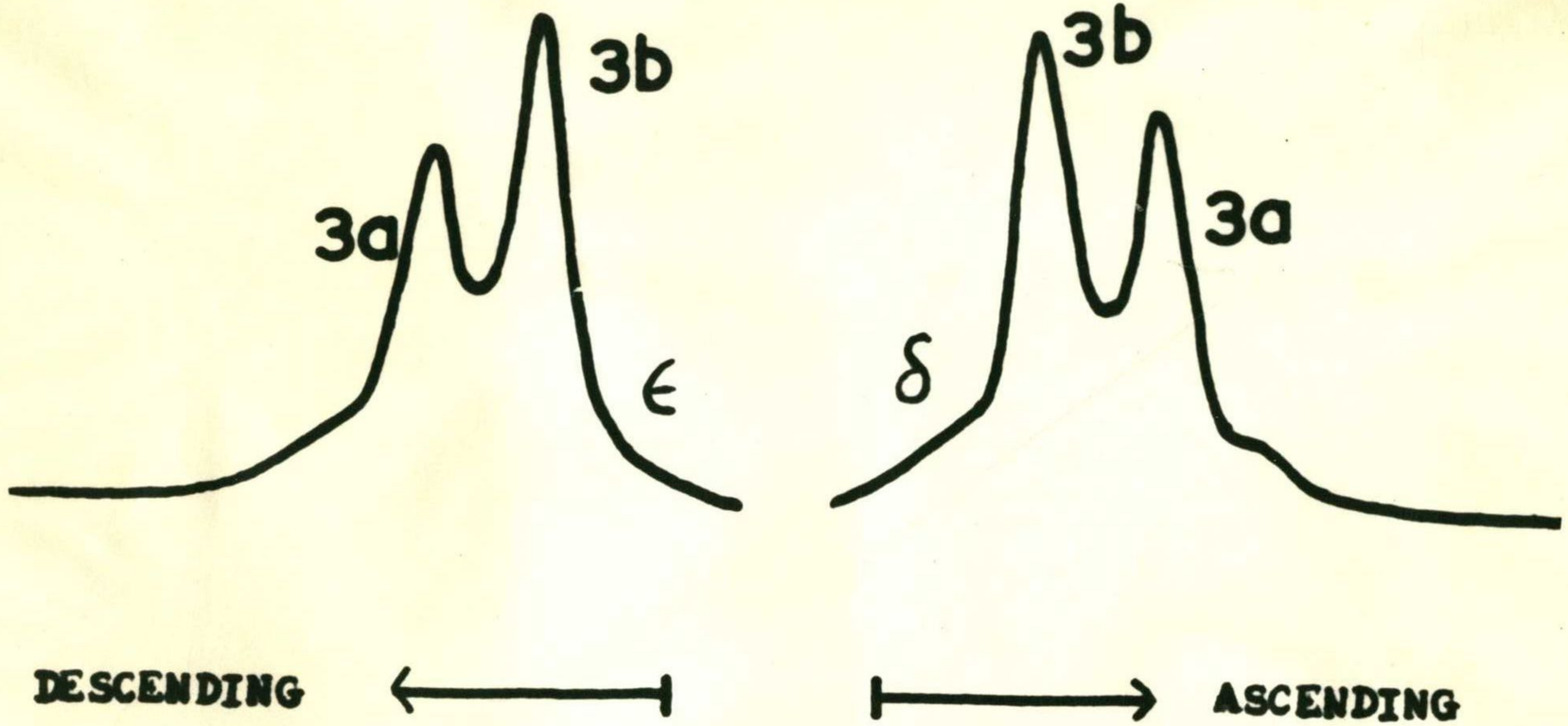


FIGURE 10. Moving boundary electrophoresis of alcohol fraction of casein containing component moving more slowly than γ-casein; 3(a) γ-casein, 3(b) additional component. The run was carried out with 0.5 per cent protein in veronal (2) at pH 8.3, $\underline{I}$ = 0.1, and 4.12 V/cm; 178 min., 20°.

together with fraction S and β-lactoglobulin, in veronal at pH 8.3, $\underline{I}$ = 0.1, is shown in Figure 11. The two additional components are concentrated in the supernatant and move more slowly than β-lactoglobulin under these conditions. They can be detected also on close examination of paper electrophoretic patterns of 1st c.s. casein. Precipitation of acid casein with 0.25 M - $CaCl_2$ at constant pH causes their appearance in the supernatant, although no κ-casein is removed. They are also concentrated into a single fraction during the large scale preparation of pure κ-casein (Section III), approximately 4 g. material being obtained from 4 gallons raw milk. It is unlikely that they are identical with any of the whey proteins. They possibly correspond to the fastest moving material observed on paper electrophoresis of the alcohol fraction C, obtained from acid casein (see Figure 8), and also to an "unidentified component with an electrophoretic mobility midway between α- and β-casein" observed in small amounts by Larson & Gillespie (1957) during the fractionation of acid casein by urea.

It is not known whether the three additional minor components observed here can be classed as integral parts of the casein complex. Perhaps they are merely adsorbed onto the surface of the casein micelles and separated along with them when soluble or acid casein is prepared.

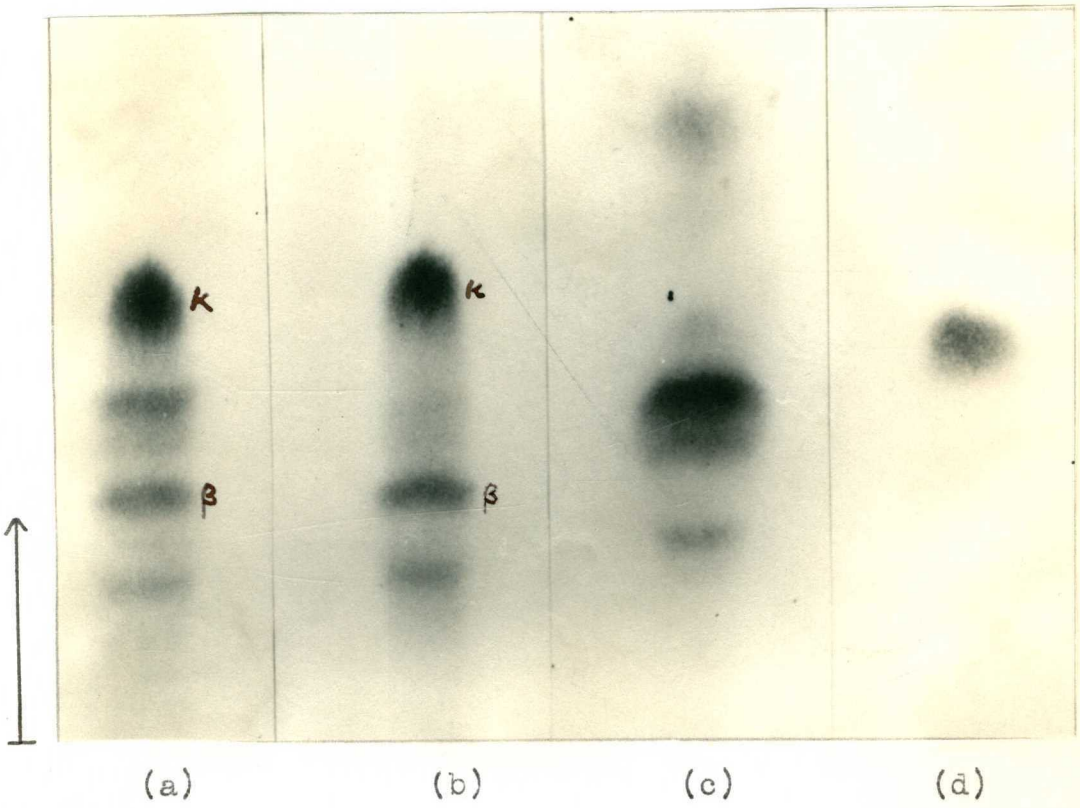


FIGURE 11. Paper electrophoretic patterns obtained for (a) fraction S, (b) precipitate and (c) supernatant obtained by exhaustive dialysis of fraction S, and (d) β-lactoglobulin; conditions as in Figure 10.

It will be noted that with the paper electrophoretic patterns presented here, those obtained using veronal buffer at pH 8.3, $\underline{I}$ = 0.1, are more clear-cut and distinct than those for which phosphate buffer at pH 7.0, $\underline{I}$ = 0.1, has been used. This has been generally true for most runs carried out on casein fractions.

GENERAL DISCUSSION

It is now known that in whole casein there are at least four distinct components, α, κ, β and γ. The first two are normally present as an α - κ - complex, and, as suggested by Waugh & von Hippel (1956), preparations of " α-casein" obtained by methods worked out so far are probably a mixture of the two. α-Casein prepared from the alcohol fraction A of acid casein (Hipp et al., 1952) gives a slightly turbid solution when dissolved and this suggests the presence of κ-casein, probably as difficultly reversible aggregates with α-casein. The behaviour of " α-casein" prepared by Cherbuliez & Baudet (1950a), particularly its relative "solubility" in the presence of calcium, indicates that this material also contains κ-casein. However, it has been shown here that the alcohol fraction B of Hipp et al. is free from κ-casein and can be used as a source of pure α-casein. This α-casein shows no marked splitting in the peaks on electrophoresis over the pH range 3.0 - 8.2,

ionic strength 0.1 and 0.02, as is evident in the leading component of acid casein from which it was prepared. However, the ascending peak particularly is asymmetric and shows some tailing at the lower pH values above the isoelectric point. This is probably due to α- α interaction and the presence of relatively large aggregates under these conditions (see Section II).

The heterogeneity in the ascending " α-peak" of acid casein over the pH range 6.2 - 8.6, ionic strength 0.1 and 0.02, is due primarily to the presence of κ-casein. However, the commonly described heterogeneity in the " α-peak" of whole casein in veronal buffer at pH 7.8, ionic strength 0.1, is only obvious in acid precipitated casein in contrast to 1st c.s. casein, although both contain the α-κ- complex. It is suggested that the difficultly reversible aggregates involving α- and κ-casein are responsible for this heterogeneity, and possibly for that observed under all conditions examined. The tendency of β-casein to interact with the α-κ-complex could be a factor contributing to the extent of this heterogeneity. The overall electrophoretic pattern of acid casein changes from one preparation to another and can be explained on the basis of variations in the conditions used to separate and purify the material. The interactions of the casein components with themselves and with each other are highly pH, temperature, and ionic strength dependent (see Section II) and it is not unexpected that differences in

these conditions, which arise during preparative procedures, will result in interactions varying in electrophoretic behaviour. The relative abundance of the casein components in different milk samples could also add to such effects.

Heterogeneity in the leading component of the total milk protein and 1st c.s. casein can be detected on electrophoresis in phosphate buffer at pH 7.0, ionic strength 0.1 and 0.02. This can be explained in terms of κ-casein present as an $\alpha - \kappa -$ complex. The fact that it is possible to detect such heterogeneity, particularly at the higher ionic strength, suggests the presence of more than one type of $\alpha - \kappa$-complex, although all might contain the same proportion of $\alpha -$ and κ-casein. Observations on pH 12 treated casein also suggest that different types of $\alpha - \kappa -$ interaction occur depending upon whether both components are mixed as monomers or as polymers.

In view of the large number of factors capable of influencing the electrophoretic behaviour of acid casein, the exact effect on which would be difficult to estimate, a more extensive investigation to cover a wider pH, ionic strength, protein concentration, and temperature range would not be warranted at the present time. Some knowledge, however, particularly insight into the mode of interaction of the casein components, could possibly be gained from such a study of the soluble caseins and mixtures of its components. Because of these interactions moving boundary and paper

electrophoresis has not given an accurate estimate of the distribution of components in casein fractions. It is quite possible that the use of dispersing agents such as concentrated urea (see Section II) in such investigations would considerably simplify the problem.

The presence of a number of minor components in casein fractions is not at all unexpected. Perhaps they are related to some of the many enzymes known to be present in milk. Morton (1953) has shown that the alkaline phosphatase in milk is associated with the casein micelles.

SECTION II

THE MOLECULAR SIZE OF α- AND β-CASEIN

INTRODUCTION

Early sedimentation studies and molecular weight estimations of casein were made on the unfractionated material. The considerable disagreement between the results of various workers is not unexpected.

Svedberg, Carpenter & Carpenter (1930) and Pedersen (1936) showed casein preparations to be heterogeneous in the ultracentrifuge. Sedimentation values reported in these two papers were markedly different. The former found that the sedimentation coefficients of the components varied with the method of preparation, and the main component of casein, prepared by the method of van Slyke & Baker (1918) had a molecular weight of 75,000 - 100,000.

Burk & Greenberg (1930) found from osmotic pressure measurements that the molecular weight of whole casein in 6.66 M-urea was 33,600. Neurath, Greenstein, Putnam & Erickson (1944) therefore concluded that urea split the casein molecule. Hipp et al. (1952), however, pointed out that the components of casein separated by means of urea had the same composition and properties as when separated by 50 per cent alcohol. This indicated that concentrated urea did not split the casein molecule. The nigh molecular weight

obtained by Svedberg et al. (1930) was due to aggregation and interaction of the casein components and the urea caused a dispersion of these aggregates.

D'yachenko & Vlodavets (1952) made molecular weight estimations on whole casein by the method of light-scattering. In particular, they studied the effect of pH , temperature, and concentrated urea solutions on the molecular weight. For whole casein it was 32,000 in potassium hydroxide at pH 9 and 25°, 31,000 in 40 per cent urea, and 27,000 in hydrochloric acid at pH 1.5 and 84°. They presumed the true molecular weight to be about 30,000.

The only molecular weights in which confidence can be placed are those determined for the individual components of casein. Unfortunately the results obtained by various workers using "pure" fractions differ somewhat. This can be explained by the ease with which α-and β-casein aggregate. In addition, the " α-casein" used by workers before 1957 could have been contaminated with the recently discovered κ-casein.

From a light-scattering study of the effect of electrolytes on α- and β-casein solutions, Halwer (1954) showed that both proteins aggregate extremely easily. The extent of aggregation was highly dependent on the electrolyte concentration, being a minimum at low ionic strengths. He was unable to obtain a suitable medium in which no aggregation occurred, and therefore it was not possible to make a

molecular weight estimation of the monomer.

From the phosphorus content of α-casein Perlmann (1954a) estimated the minimum molecular weight to be 31,000. However, her conclusions concerning the nature of the phosphorus bonds in α-casein have been criticized by Hofmann (1955).

Cherbuliez & Baudet (1950a) have estimated the minimum molecular weights of their α- and β-casein, on the basis of tyrosine, tryptophan, and phosphorus content, to be 130,000 and 48,000 respectively. However, the content of tyrosine, tryptophan, and phosphorus for their α- and β-casein do not agree very well with that of α- and β-casein prepared by other methods.

A study of the influence of temperature and electrolytes on the apparent size and shape of α- and β-casein has been made recently by Sullivan et al. (1955). Sedimentation experiments at room temperature and 8° revealed only one component for α-casein at pH 7.8. Diffusion studies under the same conditions at 1° indicated some heterogeneity. The molecular weight of α-casein was found to be 121,800. Sedimentation at room temperature revealed two distinct components in β-casein at neutral pH. Lowering the temperature produced a shift in the relative amounts of the two components, and at 15° the faster moving one was completely absent. Diffusion results presumably gave no indication

of heterogeneity at the lower temperature. From sedimentation and diffusion values the molecular weight of β-casein was calculated to be 24,100.

An examination of the sedimentation behaviour of 2nd c.s. casein, which is essentially a mixture of α- and β-casein, has been made by von Hippel & Waugh (1955). At pH 7 ($\underline{I}$ = 0.23) and 4° this material showed two components, A and B, with S_{20} = 4.4 and 1.3 respectively. The distribution of material was similar to that between α- and β-casein on electrophoresis. It was concluded that A represented α-casein, and B, β-casein. As the pH was increased from 7 to 10.8 at 8° the distribution between the A and B peaks remained constant while S_{20} for α-casein decreased progressively. The symmetrical peak suggested that α-casein at pH 7 consisted of a number of monomeric units aggregated to give polymers centering around a preferred size. At each pH, as it was increased, there was a redistribution of the subunits to give polymers centering around a particular and smaller equilibrium size. "At pH values beyond 10.9, and up to the highest pH used, (pH 12.0), all of the protein in the solution is reversibly dissociated into monomeric units with an average S_{20} of 1.25, homogeneity being indicated by the shape and behaviour of the peak." It is obvious that at low temperature and all pH values β-casein was present as the monomer. These results were in agreement with the observations of Sullivan <u>et al.</u> on the behaviour of pure β-casein

but suggested that the molecular weight estimation for α-casein by these workers did not represent a minimum value. Sedimentation and diffusion studies by von Hippel & Waugh on 2nd c.s. casein in alkaline solution gave an average monomer molecular weight of 15,000. It was suggested that the molecular weight of the α-casein monomer lies in the range 13,000 - 15, 000, and that of the β-casein monomer in the range 15,000 - 25,000.

This Section contains the results of a study of the behaviour of pure α- and β-casein during sedimentation under various conditions. α-Casein, free from κ-casein, has been used throughout. The molecular weights have been calculated under conditions favouring disaggregation of these two proteins in an attempt to determine their monomer molecular weights.

Sedimentation and diffusion studies on α- and β-casein in concentrated urea solutions were presented in the candidate's M.Sc. thesis (Sydney University, 1955). The former have now been corrected for the drop in temperature during acceleration of the rotor, and both are represented here along with the other results.

MATERIALS AND METHODS

Protein Concentrations. These were obtained from Kjeldahl nitrogen determinations carried out according to the procedure of McKenzie & Wallace (1954). The percentages of nitrogen in α- and β-casein were taken as 15.58 per cent and 15.33 per cent respectively (Hipp et al., 1952)

Reagents and Buffers. The urea (C.P.) was recrystallized once from aqueous alcohol. The stock phosphate buffer (0.5M), used with the 6M - urea solutions, was prepared by mixing 0.5M-Na_2HPO_4 and 0.5M-KH_2PO_4 in the required proportion, and the borate buffer (0.5M) from 0.5M-H_3BO_3 and 0.5N-NaOH. The phosphate buffer (pH 12.0, $\underline{I}$ = 0.19) was made by dissolving 7.1 g. Na_2HPO_4 and 1.33 g. NaOH in 1 l. water. The glycine buffer (pH 11.0-11.1, $\underline{I}$ = 0.20) was prepared by diluting the following to 1 l. with water: 10.2 ml. N-NaOH, 9.8 ml. 1.0 M-glycine - 1.0M-NaCl, and 36 ml. 5.0 M-NaCl. Details of other buffers are given in Section I.

Diffusion Measurements. These were made at 25° in a stainless steel Claesson-type diffusion cell in a water bath controlled to within 0.03°. The optical system was of the Philpot cylindrical lens type with an inclined glass fibre instead of the knife edge.

The solutions for analysis were prepared by

dialysing a small amount of protein-buffer solution against a relatively large volume of the same buffer with stirring for 24 hr. at 25°. Precautions were taken while filling the cell to prevent evaporation from the solutions; this was particularly important where concentrated urea solutions were used. The filled cell was allowed to equilibrate for at least 1 hr. before forming the boundary. Exposures were then taken at suitable intervals over a period of 3 - 4 days.

For calculations the diffusion curves were enlarged approximately 15 times and copied onto tracing paper. Ordinates were measured to within 1 mm. and weight average diffusion coefficients, D, were determined by the method of moments (Lundgren & Ward, 1951). Values of D are given in c.g.s. units multiplied by 10^7.

Molecular Weights. These were calculated from S and D at zero concentration using Svedberg's relation,

$$M = \frac{RTS^o}{D^o (1-\bar{v}\rho)}$$

where M = molecular weight

R = gas constant

T = absolute temperature

ρ = density of the solvent

$\bar{v}$ = partial specific volume

McMeekin, Groves & Hipp (1949) found no change in the partial specific volume of unfractionated casein in concentrated urea solutions. For α- and β-casein the

values were 0.728 and 0.741 at 25°, and these have been used throughout. The densities of the aqueous urea solutions were obtained from the data of Gucker, Gage & Moser (1938).

Sedimentation Equilibrium. The Archibald ultracentrifugal procedure was applied to α-casein at pH 12 using essentially the method of Smith, Wood & Charlwood (1956). The ultracentrifuge was run at a nominal speed of 12,590 rev./min. over a period of approximately 800 min. The initial and final temperatures of the rotor differed by about 1.5° and the mean value was used in subsequent calculations. For three consecutive runs the average temperatures varied between 19.3° and 19.6°. The concentration of protein at the meniscus of the cell, c_m, was calculated using the simplified procedure of Charlwood (1957). The concentration at the bottom of the cell, c_b, was then obtained from the relation,

$$c_b - c_m = \int_{r_m}^{r_b} \frac{dc}{dr} \cdot dr$$

where r is the distance from the centre of rotation. In this procedure the concentration differences were determined from area measurements on patterns enlarged approximately seven times. This was carried out by means of a planimeter. The area corresponding to a known concentration difference, with the inclined wire at different angles, was determined on an α-casein solution at neutral pH using the synthetic boundary cell.

Molecular weights have been calculated from δ_m using the relation,

$$\delta_m = \frac{1}{rc} \cdot \frac{dc}{dr} = \frac{M(1 - \bar{v}\rho)\omega^2}{RT}$$

where, ω = angular velocity in radians/sec.

All other materials and methods used have been described in Section I.

RESULTS

α-Casein

All sedimentation runs have been carried out at room temperature. At pH 6.8 α-casein (0.5 per cent, 0.1M-NaCl) sediments with $S_{20} = 4.4$. This value agrees well with that of the α-casein component in 2nd c.s. casein at pH 7 (von Hippel & Waugh, 1955). Of two preparations investigated the first, α-casein I, contains a small amount of faster moving material, $S_{20} = 9$, which is not present in the later preparation, α-casein II (Figure 12, a and b). Both preparations were electrophoretically free from α-casein. The faster moving material could be due to κ-casein which has a relatively high S_{20}, approximately 10, in 1 per cent fraction S. However, in view of the results presented in Section I, this is unlikely. The fact that as the pH is increased there is a conversion of the faster into the slower component having a definite S_{20} value supports the view that

FIGURE 12. Sedimentation of α- and β-casein under various conditions. All runs were carried out with 0.5 per cent protein at approximately 20°. The times and inclined-wire angles are indicated below. Sedimentation is from left to right.

(a) α-casein I, pH 6.8, 18 min., 75°; (b) α-casein II, pH 6.8, 17 min., 70°; (c) α-casein I, pH 7.7, 17 min., 75°; (d) α-casein I, pH 9.7, 19 min., 75°; (e) α-casein I, pH 11.0, 80 min., 75°; (f) α-casein I in 6 M-urea, pH 7.3, 82 min., 75°; (g) β-casein, pH 6.9, 18 min., 60°; (h) β-casein, pH 11.1, 86 min., 70°; (i) β-casein in 6 M-urea, pH 7.2, 90 min., 70°. See text for other details.

(over)

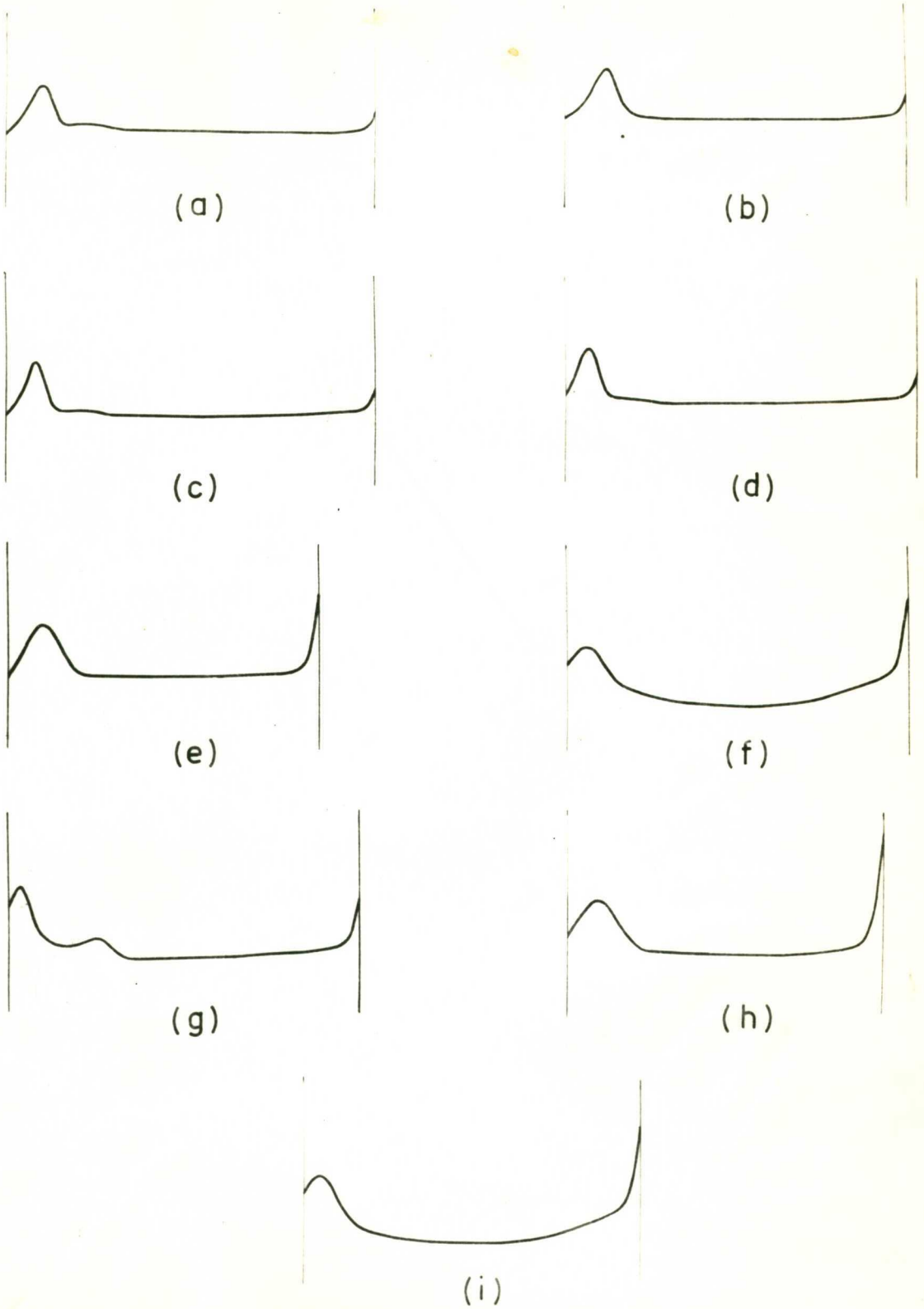


FIGURE 12

it represents mainly, if not entirely, highly aggregated α-casein (see below). Unfortunately, the studies on α-casein I were completed before the existence of κ-casein was realized. α-Casein II was completely free from this component.

As the pH is increased the sedimentation coefficient of α-casein decreases. At pH 7.7 (0.1 M-NaCl), 9.7 (0.05 M-borate, 0.05 M-NaCl) and 11.0 (glycine, $\underline{I}$ = 0.20) the corresponding S_{20} values at 0.5 per cent are 3.8, 2.7 and 1.3. The fact that mainly single, well-defined peaks are always observed, indicates that at each pH, in agreement with von Hippel & Waugh (1955), we have polymers centering around a preferred size (Figure 12, c, d and e). (These results on α-casein I at pH 6.8, 7.7 and 9.7, were presented earlier in the candidate's M.Sc. thesis but were interpreted incorrectly.)

Since no trace of a faster moving component was apparent in α-casein I at pH 11.0 it was thought that under these conditions all the protein would be present as the monomer. Sedimentation and diffusion measurements have been carried out on this material at different protein concentrations in glycine buffer at pH 11.0 ($\underline{I}$ = 0.2). These results are shown in Figure 13 and Table 7. A comparatively high concentration dependence of the sedimentation rate is apparent, the value of S_{25} increasing from 1.36 at 1.0 per cent protein to 1.99 at zero concentration. Using this latter value with

FIGURE 13. Dependence of S_{25} on concentration.

(a) α-casein I, glycine buffer, pH 11.0, $\underline{I}$ = 0.20;

(b) β-casein, glycine buffer, pH 11.1, $\underline{I}$ = 0.20;

(c) α-casein I, 6M-urea, 0.05 M-phosphate buffer, 0.05 M-NaCl, pH 7.3; (d) β-casein, 6M-urea, 0.05 M-phosphate buffer, 0.05 M-NaCl, pH 7.2.

(over)

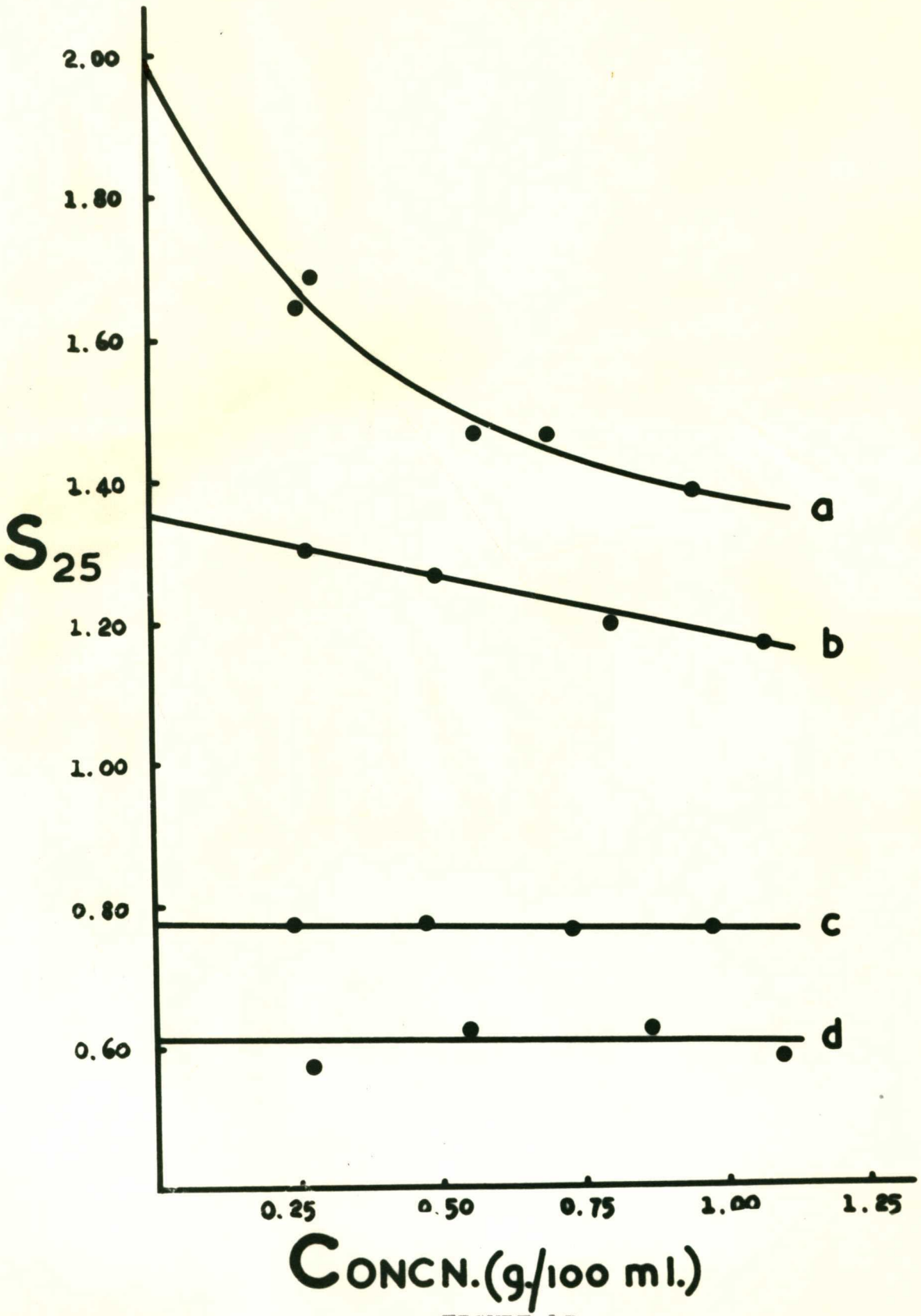

2.00
1.80
1.60
1.40
1.20
1.00
0.80
0.60
S25
a
b
c
d
0.25
0.50
0.75
1.00
1.85
CONCN.(g/100 ml.)

FIGURE 13

TABLE 7

Diffusion Coefficient Values for α- and β-Casein

Protein	Solvent	Concn. (g./100 ml.)	D_{25}	Mean
α-casein	glycine, pH 11.0,	0.29	6.70	
	$\underline{I}$ = 0.20	0.57	6.75	
		0.67	6.75	
		0.95	6.40	6.66
β-casein	glycine, pH 11.1,	0.25	7.65	
	$\underline{I}$ = 0.20	0.50	7.30	
		0.75	7.40	
		1.00	7.70	7.51
α-casein	6 M - urea,	0.48	3.45	
	0.05 M-phosphate	0.97	3.40	3.42
	0.05 M-NaCl,			
	pH 7.3			
β-casein	6 M - urea,	0.55	3.85	
	0.05 M-phosphate,	0.55	3.75	
	0.05 M-NaCl	0.82	4.10	
	pH 7.2	0.82	3.80	3.87

D_{25}^{o} = 6.66 a molecular weight of 27,600 is obtained. (The mean value for D_{25} has been used here, and in all other cases, since there is no obvious dependence of this property on concentration.)

The results of sedimentation and diffusion measurements on α-casein I in 6 M-urea at pH 7.3 (0.05 M-phosphate buffer, 0.05 M-NaCl), which effectively disperses the protein, are also included. The sedimentation pattern is shown in Figure 12 (f). The markedly curved base line is due to the high concentration of urea present. Using S_{25}^{o} = 0.78 and D_{25}^{o} = 3.42 the molecular weight is calculated to be 28,000.

These values for the monomer molecular weight of α-casein are approximately twice that suggested by von Hippel & Waugh (1955) from studies on 2nd c.s. casein at pH 12.0. It was decided, therefore, to investigate the possibility of a dissociation of α-casein into smaller units as the pH is raised from 11.0 to 12.0. α-Casein II has been used in this investigation. S_{25} decreases to 1.10 with 0.36 per cent protein in phosphate buffer at pH 12.0 ($\underline{I}$ = 0.19). To estimate the molecular weight under these conditions, use has been made of the Archibald ultracentrifugal procedure. Three runs have been carried out at initial protein concentrations of 0.389, 0.680 and 1.050 per cent. The results for the δ functions $(\frac{1}{rc} \cdot \frac{dc}{dr})$ and concentrations at the meniscus and bottom of the cell at corresponding times throughout the runs are shown in Table 8. In all cases the

TABLE 8

Archibald Ultracentrifugal Procedure Applied to α-Casein at pH 12.0 - Values of δ_m and δ_b

Time (min.)	Initial Protein Concentration (g./100 ml.)											
	0.389				0.680				1.050			
	δ_m	c_m	δ_b	c_b	δ_m	c_m	δ_b	c_b	δ_m	c_m	δ_b	c_b
312	0.39	0.303	0.33	0.489	0.33	0.551	0.29	0.829	0.25	0.889	0.22	1.227
513	0.41	0.284	0.34	0.522	0.32	0.526	0.27	0.870	0.24	0.857	0.21	1.292
560	0.42	0.272	0.34	0.536	0.30	0.522	0.26	0.888	0.24	0.850	0.22	1.295
596	0.43	0.271	0.34	0.536	0.30	0.519	0.27	0.884	0.25	0.835	0.21	1.311
660	0.42	0.268	0.31	0.535								
716	0.40	0.267	0.35	0.542								

Runs were carried out in phosphate buffer at pH 12.0, $\underline{I}$ = 0.19, and approximately 20°, at a nominal speed of 12,590 rev./min.

value for S_b is lower than S_m and this is a result of the rather high concentration dependence of S. Because the values at different initial protein concentrations are rather randomly distributed the concentration effect has been eliminated by plotting all values on one graph and extrapolating to zero concentration as shown in Figure 14. This gives a corrected value for S_m of 0.56. The values for S_b cannot be extrapolated with any degree of certainty but it can be seen from Table 8 that they are near the values for S_m at corresponding protein concentrations. There is thus no indication of any material heavier than that present at the meniscus of the cell. Using $S_m = 0.56$ the molecular weight of α-casein at pH 12.0 is calculated to be 29,000. Within the limits of experimental error, this value is the same as those obtained from separate sedimentation and diffusion studies at pH 11.0 and in 6 M-urea at pH 7.3. The lowering in sedimentation rate as the pH is increased from 11.0 to 12.0 is not due to a change in molecular size, and must be due to some other alteration in the physical characteristics of α-casein.

β- Casein

The sedimentation pattern for 0.5 per cent β-casein at room temperature and pH 6.9 (0.1 M-NaCl) is shown in Figure 12 (g). A considerable amount of fast moving

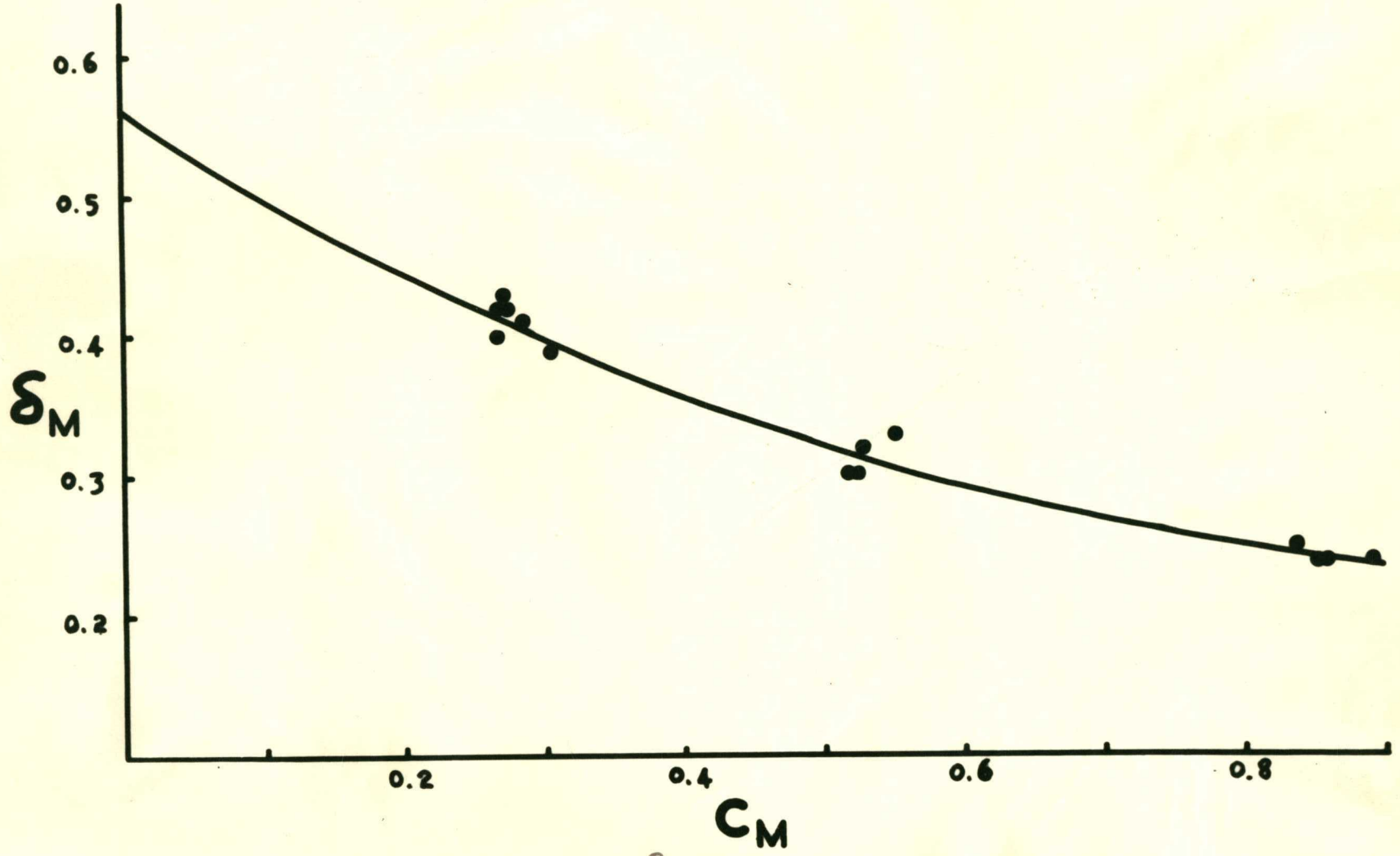


FIGURE 14. Dependence of δ_m on concentration. α-Casein II in phosphate buffer, pH 12.0, $\underline{I}$ = 0.19.

material with S_{20} = 11.5 is present. S_{20} for the major slow-moving component is 1.5 and is essentially the same as the value obtained for the monomer of β-casein (zero concentration) at reduced temperature (Sullivan et al., 1955). On increasing the pH to 11.0 (glycine, $\underline{I}$ = 0.20) only one peak, S_{20} = 1.2, separates (Figure 12, h). (The result for β-casein at pH 6.9 was presented earlier in the candidate's M.Sc. thesis.)

The results of sedimentation and diffusion experiments in glycine buffer at pH 11.1 ($\underline{I}$ = 0.20) are given in Figure 13 and Table 7. Using S_{25}^{o} = 1.35 and D_{25}^{o} = 7.51 the monomer molecular weight of β-casein under these conditions is found to be 17,500.

The sedimentation pattern of β-casein in 6 M-urea at pH 7.2 (0.05 M-phosphate buffer, 0.05 M-NaCl) shows only one slow-moving peak (Figure 12, i). Sedimentation and diffusion measurements under these conditions give S_{25}^{o} = 0.61 and D_{25}^{o} = 3.87. From these values a molecular weight of 20,800 is calculated.

DISCUSSION

In recent years a number of investigators have studied the factors affecting the molecular size of α- and β-casein. It is generally agreed that at room temperature and neutral pH, in the presence of salt, both α- and β-

casein are aggregated. β-Casein dissociates into monomers as the temperature is lowered, while this treatment has no effect on α-casein (Sullivan et al., 1955; McMeekin & Peterson, 1955). Both McMeekin & Peterson and von Hippel & Waugh (1955) have shown that these two proteins dissociate in alkaline solutions, while Peterson (1955) has reported on the presence of monomers of casein and its components in concentrated urea solutions. The decrease in the extent of aggregation as the salt concentration is lowered has been observed by Halwer (1954) and McMeekin & Peterson. All this previous work is in general agreement with the results presented here.

The values, 27,600, 28,000 and 29,000, obtained for the molecular weight of α-casein under different conditions favouring disaggregation indicate that the true monomer molecular weight lies in the vicinity of 28,000. Possibly the value of 121,800 reported by Sullivan et al. for α-casein at pH 7.8 represents a polymer consisting of four of these units. It could be a polymer similar to this which enters into the α- κ- complex to give a weight ratio of 4 : 1 (the monomer molecular weight of κ-casein is near 31,000, see Section III). The value of 28,000, however, lies far outside the range of 13,000 - 15,000 suggested by von Hippel & Waugh. Possibly the explanation for this discrepancy can be found in the rather high concentration dependence of S

exhibited by α-casein in alkaline solutions. Working at low temperatures and pH 12 von Hippel & Waugh did not observe such an effect with 2nd c.s. casein on which their estimation was made. Two of their observations, however, which lend support to the concentration effect are (a) the dependence of viscosity on protein concentration, the viscosity decreasing sharply below 0.3 per cent and (b) the increase in S_{20} as the ionic strength is increased from 0.19 to 0.68. All these could be explained on the basis of charge effects which are most marked with α-casein. The values in the vicinity of 30,000 for the "molecular weight" of whole casein observed by other workers, particularly Burk & Greenberg (1930) and D'yachenko & Vlodavets (1952), are due no doubt to the large proportion of α-casein with a monomer molecular weight near 28,000.

Sullivan et al. (1955) obtained a value of 24,100 for the monomer molecular weight of β-casein. This value was derived from sedimentation and diffusion studies carried out at low temperature. This and the lower values reported here, 17500 and 20,800, all lie within the range 15,000 - 25,000 suggested by von Hippel & Waugh. The reason for the significantly lower values reported here than that suggested by Sullivan et al. is not obvious.

The difference between the pH 11.1 and urea values in the present work could be due to significant changes in

the partial specific volume of this protein in the two solvents used. This possibility is being investigated.

SECTION III

THE ISOLATION AND SOME PROPERTIES OF PURE κ-CASEIN

INTRODUCTION

The scheme for the preparation of fraction S from skim milk has been set out in Section I, Figure 1. This material contains approximately 50 per cent κ-casein, as well as β- and γ-casein, and a number of minor components. In the present Section a method is described for the separation and purification of κ-casein. Also included are some observations on its chemical and physical properties.

The main disadvantage in the procedure of Waugh & von Hippel (1956) for preparing large quantities of fraction S is the considerable number of centrifugations at high speed necessary to spin out and wash the casein micelles. An attempt was first made to obtain fraction S from acid casein by treatment with 0.25 M-calcium chloride. This proved completely unsatisfactory, only a small amount of the two additional components with mobilities intermediate to those of κ- and β-casein, remaining in solution. Attention was therefore directed towards obtaining a simpler method for the separation of casein micelles from milk. It was found that approximately 40 per cent of the micelles could be removed from skim milk, containing 0.06-M calcium chloride, by means of a Sharples Super-Centrifuge under suitable conditions. On resuspending and washing the micelles virtually

complete recovery was obtained using the same procedure.

It was also possible to eliminate the step in the preparative procedure for fraction S where residual calcium oxalate is first removed by centrifugation at 45,000 xg for 90 minutes. Traces of calcium oxalate did not interfere with the remaining steps and could be removed with the calcium caseinate later on.

The solution of fraction S obtained contained only 0.3 - 0.4 per cent protein and was unsuitable for direct fractionation. An attempt to obtain most of the protein from solution by precipitation on exhaustive dialysis against water (see Section I) was unsuccessful with a large quantity of such a dilute solution. Adjustment of a solution of fraction S to pH 4.6 at 2^{o} produced no precipitate, but subsequent warming to room temperature caused flocculation of most of the protein. This material was completely soluble as a clear solution at pH 7.

Treatment of freeze-dried fraction S with 50 per cent alcohol to give the fractions corresponding to A, B and C of Hipp et al. (1952) caused precipitation of nearly pure κ-casein in the first two fractions. In most cases, where these were precipitated together, a small amount of β-casein, approximately 5 per cent, was also present. The same fractionation could be achieved with acid precipitated fraction S.

Finally, it was found that virtually all the β-casein could be removed by making use of its relative solubility at pH 4.4 and 2°.

MATERIALS AND METHODS

Benzene-free 95 per cent alcohol was distilled before use in the fractionation procedure.

Estimations of the distribution of protein components obtained by paper electrophoresis of the various fractions in phosphate buffer at pH 7.0, $\underline{I} = 0.1$, and 2° have been made by visual examination of the paper strips.

The Archibald ultracentrifugal procedure was applied to κ-casein at pH 12 using the procedure described in Section II of this thesis, except that the ultracentrifuge was run at a nominal speed of 15,220 rev./min. For three consecutive runs the average temperatures varied between 21.1° and 21.3°. A run in the synthetic boundary cell on approximately 1.0 per cent κ-casein at pH 9 (0.02 M-$Na_2B_4O_7$, 0.15 M-NaCl) was carried out in order to relate concentration differences with areas under the curve at various angles of the inclined wire. The absolute protein concentration was not determined, but the relationship between the concentration of this solution and those used in the equilibrium runs was obtained by comparing the extinctions of the protein (approximately 0.1 per cent) in 0.1 N-NaOH at 291 mu using a Hilger Uvispek Spectrophotometer, series 304. The protein

concentrations given in the results are therefore not absolute values.

All other materials and methods have been described in the previous Sections.

RESULTS

Large Scale Preparation of κ-Casein

The procedure for the preparation of κ-casein is shown schematically in Figure 15 and given in detail below.

The cream was removed from 4 gal. fresh, raw, pooled milk (containing toluene) by centrifuging for 30 min. at 5° and 895 xg in an International type PR-2 Refrigerated Centrifuge (International Equipment Co., Boston, Mass., U.S.A.).

60 ml. 2.0 M-$CaCl_2$ were added to each litre of skim milk (14.1) at 5°, and the casein micelles were separated from 3.1. lots, precooled to 2°, in a Sharples type T-66-24 Super-Centrifuge (The Sharples Corp., Philadelphia, Pa., U.S.A.) using a type T-9-46 clarifier bowl at 50,000 rev./min. The skim milk was allowed to run through a 1 mm. jet at the rate of approximately 1 l./5 min. Cooling of the centrifuge during operation was not necessary.

The micelles were washed twice by blending with (a) 6 l. 0.08 M-NaCl, 0.13 M-$CaCl_2$, and (b) 0.085 M-NaCl at 2°. In each case the washed micelles were collected from 2 l. lots of suspension as before. The heavier material which

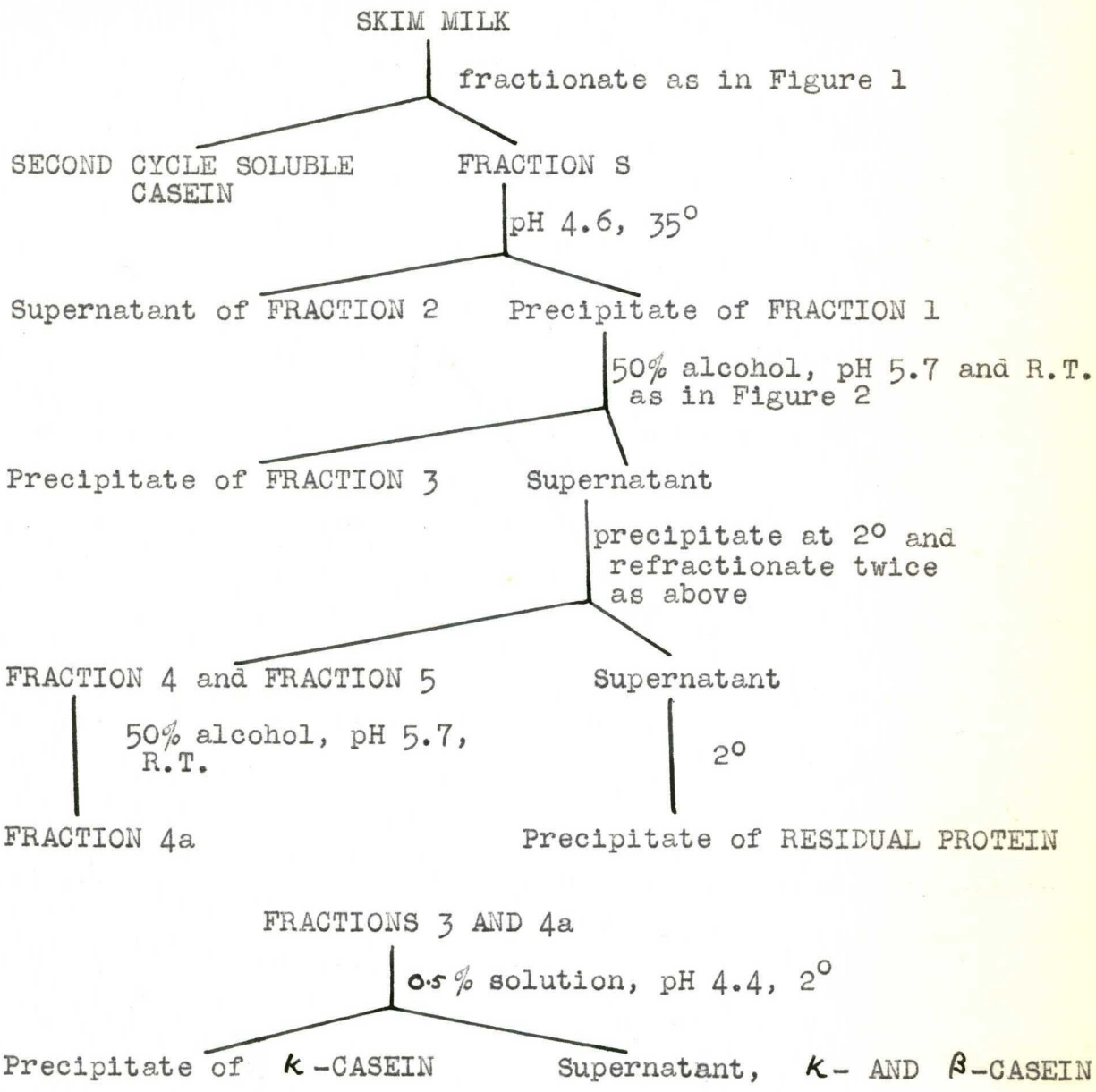


FIGURE 15. Scheme for Preparation of κ-Casein.

tended to settle out and which collected in the last litre of suspension was separated by centrifugation for 20 min. at 5° and 12,000 rev./min. in a M.S.E. type 92110 High-Speed Refrigerated Centrifuge (Measuring & Scientific Equipment Ltd., London, England) using a type S775 rotor.

The micelles were resuspended by blending in 1.5 l. 0.085 M-NaCl and solubilized by the addition of 600 ml. 1.5 M-$K_2C_2O_4$ at constant pH and 2° (von Hippel & Waugh, 1955). Most of the calcium oxalate was removed by centrifuging for 30 min. at 5° and 3,900 xg in an International Refrigerated Centrifuge.

Excess potassium oxalate was removed by dialysing against 5 x 28 l. lots 0.085 M-NaCl at 2° over 3 days. The dialysis bags were rotated continuously at 20 rev./min., and solutions were kept saturated with toluene.

The casein solution (3.1 l.) was treated with 270 ml. 3.5 M-$CaCl_2$ (0.28 M final concentration) at 2° and constant pH. A slight excess of $CaCl_2$ was used to account for any oxalate which might have remained in the solution.

The suspension was warmed to 37° and most of the calcium caseinate precipitate removed by centrifuging for 40 min. at 5° and 895 xg in an International Refrigerated Centrifuge. Residual calcium caseinate and traces of calcium oxalate were then removed by centrifuging for 120 min. at 5° and 90,000 xg in a Spinco Preparative Ultracentrifuge, Model L.

The precipitate was discarded.

The clear solution (3.5 l.) was treated with 640 ml. 1.5M-$K_2C_2O_4$ at 2° and constant pH. Most of the calcium oxalate was removed by centrifugation for 45 min. at 5° and 3,900 xg as before.

The solution, slightly turbid, was dialysed against 5 x 28 l. lots 0.15M-NaCl at 2° over 3 days. Residual calcium oxalate was then removed by centrifuging for 15 min. at 5° and 12,000 rev./min. in a M.S.E. High-Speed Refrigerated Centrifuge.

The clear solution of fraction S thus obtained was adjusted to pH 4.6 at 2° by the slow addition of 0.5N-HCl. On warming to 35° a heavy precipitate (fraction 1, 12 g.) was obtained. This was removed after 30 min. by centrifugation for 20 min. at room temperature and approximately 1000 xg in a M.S.E. laboratory centrifuge. The residual protein in the clear supernatant was precipitated by the slow addition of alcohol to 50 per cent and cooling to 2° (fraction 2, 4 g.). Fraction 1 was soluble at pH 7 and contained approximately 60 per cent κ-casein, 30 per cent β-casein, and 10 per cent minor components. A comparison on paper electrophoresis with fraction S showed κ-casein to have the same mobility in both preparations. The two additional components with mobilities intermediate to those of κ- and β-casein were at an unusually low concentration

and could only just be detected. Fraction 2, however, proved to contain mainly these components.

Fraction 1 was dissolved in 700 ml. water at pH 7.5 by the slow addition of N-NH_4OH. The clear solution was made 0.4M with ammonium acetate, and then 95 per cent alcohol was added slowly, with stirring, to a final concentration of 50 per cent. During the addition of the alcohol a precipitate formed but redissolved as the concentration approached 50 per cent. The solution was adjusted to pH 5.7 by the slow addition of 2N-acetic acid in 50 per cent alcohol. Stirring was continued for 1 hr. and the precipitate (fraction 3, 4 g.) removed by filtration on a Whatman No. 1 paper. It contained approximately 95 per cent κ-casein.

The protein in the filtrate was precipitated by cooling to 2°. After filtering off, it was redissolved in 270 ml. water and refractionated as before. This gave fraction 4 (2 g.) containing approximately 70 per cent κ-casein. Further treatment of the residual protein, after dissolution in 130 ml. water, gave fraction 5 (1.5 g.) containing approximately 50 per cent κ-casein. The final residual protein (3 g.) contained about 40 per cent κ-casein.

Fractions 4 and 5 were each dissolved in 50 ml. water and fractionated again. However, only the former yielded a precipitate on adjustment to pH 5.7 in 50 per cent alcohol. This material (Fraction 4a, 0.8 g.) contained

approximately 90 per cent κ-casein. The compositions of the various fractions are summarized in Table 9.

Fractions 1 and 4a were mixed and used as a source of pure κ-casein. (These two fractions, as well as pure κ-casein after precipitation at pH 4.4, are rather difficult to dissolve and it is necessary to adjust the suspension to pH 12 to effect solution in a reasonable time). They were dissolved in 1 l. water at pH 12 with 2N-NaOH, brought to pH 7 with N-HCl, cooled to 2°, and adjusted to pH 4.4 with 0.2 N-HCl. The precipitate (3.3 g.) was obtained by centrifugation for 10 min. at 2° and 895 xg in an International Refrigerated Centrifuge. It was essentially pure κ-casein with only a trace of β-casein. On adjusting the supernatant at 2° to pH 4.8 with 0.2 N-NaOH a small amount of material containing about 20 per cent β-casein was precipitated. An attempt to remove the last trace of β-casein, which could only just be detected on paper electrophoresis, by dissolution of the material in 600 ml. water at 2° and adjustment to pH 4.4 was unsuccessful. The yield was decreased to 2.4 g. The κ-casein was dissolved in water at pH 12, adjusted to pH 7, and freeze dried. Sedimentation patterns of fractions 1 and 3, and κ-casein, demonstrating the continual removal of β-casein are shown in Figure 16.

Properties of κ-Casein

Freeze-dried κ-casein was readily soluble in

TABLE 9

Approximate Yields and Compositions of Fractions Obtained During the Purification of κ-Casein from Fraction S.

Fraction	Yield (g.)	Composition (per cent)			
		κ	β	γ	Minor components
1	12	60	30	10	
2	4				>80
3	4	95	5		
4	2	70	30		
4a	0.8	90	10		
5	1.5	50	45	5	
Residual protein	3	40	50	20	

The above yields are based on the quantity of fraction S obtained from 4 gal. raw milk.

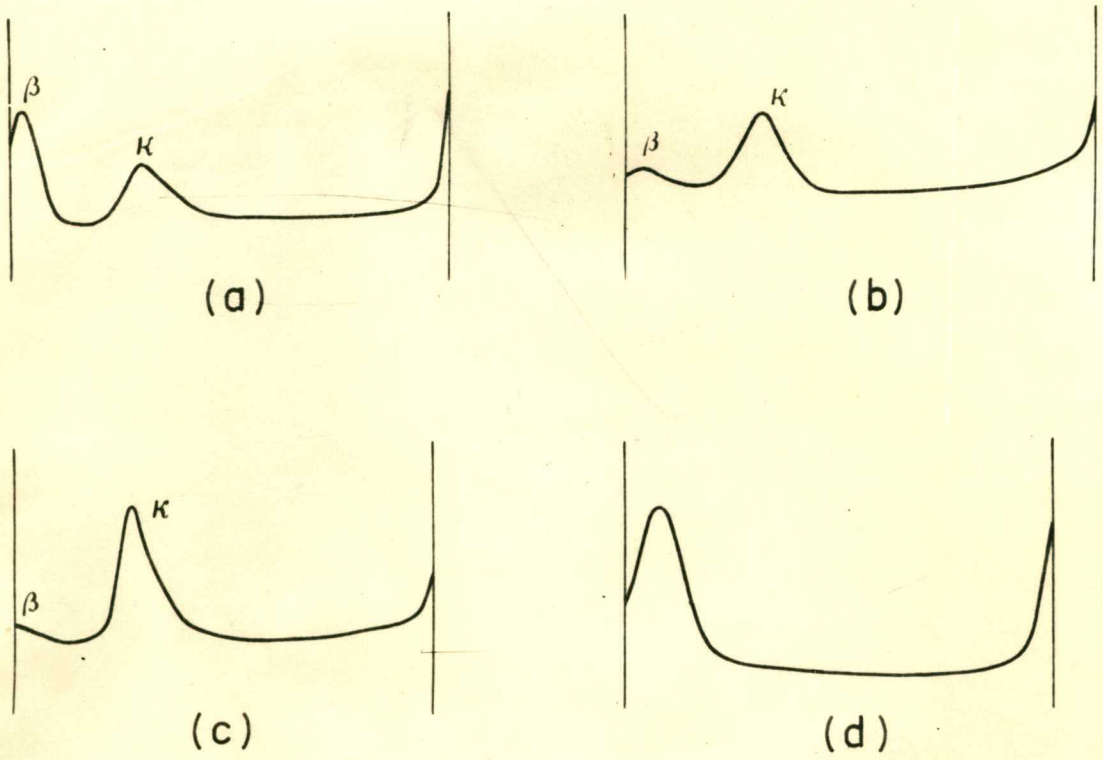


FIGURE 16. Sedimentation of fractions obtained during the purification of κ-casein from fraction S.(a), (b), and (c) were carried out in phosphate (1) at pH 7.0, $\underline{I}$ = 0.1 (0.2 for (b)) and 2° - 5° using 0.5 - 1.0 per cent protein; (d) was in phosphate, pH 12.0, $\underline{I}$ = 0.19, at room temperature with 1.0 per cent protein. Sedimentation is from left to right. (a) fraction 1, 34 min., 65°; (b) fraction 2, 36 min., 55°; (c) κ-casein, 26 min., 60°; (d) κ-casein, 59 min., 65°.

water at neutral pH and gave no precipitate on the addition of $CaCl_2$. With α-casein and in the presence of 0.06 M-$CaCl_2$ it gave stable micelles which could be clotted by rennin. A positive molybdate test, after fusion with Na_2CO_3 - KNO_3 mixture, indicated the presence of phosphorus. It gave a slight reddish colour when boiled with Bial's reagent and this is due to the presence of neuraminic acid (see SectionIV). Significant quantities of carbohydrate were indicated by the sulphuric acid - cysteine reaction of Dische (1953, 1954) - the candidate is indebted to Dr. K. Knox of this Department for this analysis. The exact nature of the carbohydrate is at present being investigated. No significant quantities of free α-amino end-groups could be detected in κ-casein by means of the FDNB technique of Sanger (1945), the possibility of arginine being present as such has not been settled (see Section IV).

Moving boundary electrophoresis of 0.7 per cent κ-casein in phosphate buffer at pH 7.1, $\underline{I}$ = 0.1, showed a single peak in both the ascending and descending limbs (Figure 17), with a mobility of - 6.80. Sedimentation of κ-casein (0.7 per cent) in phosphate buffer at 2° and pH 7.0 showed essentially a single peak with S_{20} approximately 13 (Figure 16). This peak obviously represents an aggregate of very high molecular weight, and the abnormal shape of the curve indicates that it is not completely mono-

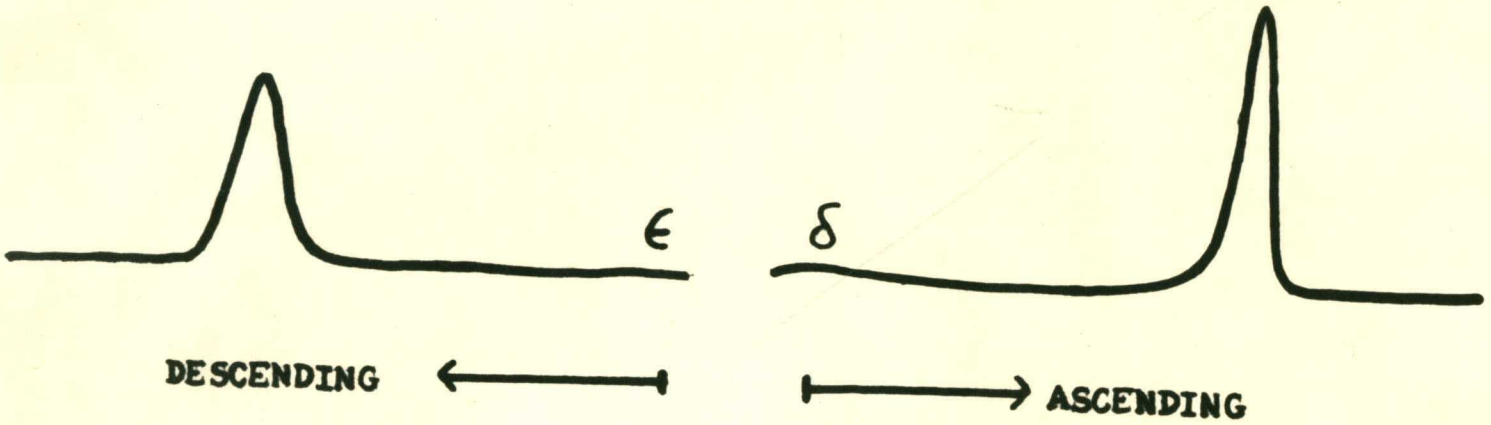


FIGURE 17. Moving boundary electrophoresis of κ-casein. The run was carried out with 0.7 per cent protein in phosphate (1) at pH 7.1, $\underline{I}$ = 0.1, and 5.02 V/cm; 168 min., 40°.

disperse. κ-Casein dissociates in phosphate buffer at pH 12.0, $\underline{I}$ = 0.19, and under these conditions showed a single well-defined peak with S_{20} = 1.0 for 1.0 per cent protein (Figure 16).

Molecular Weight of κ-Casein

The molecular weight of κ-casein in phosphate buffer at pH 12.0, $\underline{I}$ = 0.19, has been determined by application of the Archibald ultracentrifugal procedure in an attempt to estimate its minimum molecular weight. Three runs were made at initial "protein concentrations" of 0.333, 0.667 and 1.000 per cent. The results for δ_m and c_m at corresponding times throughout the runs are shown in Table 10. Because of the sharp rise in the schlieren curve at the position corresponding to the bottom of the cell δ_b could not be determined with a suitable degree of accuracy, and the values for this function are not included. This is rather different from the behaviour of α-casein under similar conditions, where the rate of change of concentration was not nearly so great in this region of the cell. The values for δ_m, obtained over the period 64 - 320 min., showed no obvious gradation during the course of the run. This indicates that the material is at least mostly uniform as regards molecular size under these conditions. As with α-casein, δ_m was concentration dependent, and this effect was eliminated by extrapolating to zero concentration as

TABLE 10

Archibald Ultracentrifugal Procedure Applied to κ-Casein at pH 12.0 - Values of δ_m

Time (min.)	Initial Protein Concentration (g./100 ml.)					
	0.333		0.667		1.000	
	δ_m	c_m	δ_m	c_m	δ_m	c_m
64	0.74	0.258	0.62	0.536	0.57	0.810
128	0.78	0.235	0.66	0.487	0.57	0.763
192	0.72	0.224	0.66	0.460	0.57	0.716
256	0.74	0.206	0.65	0.443	0.59	0.687
320	0.80	0.202	0.63	0.424		

Runs were carried out in phosphate buffer at pH 12.0, $\underline{I}$ = 0.19, and approximately 21°, at a nominal speed of 15,220 rev./min.

shown in Figure 18. Using a corrected value for $\delta_m = 0.88$, and a partial specific volume equal to that of α-casein, the molecular weight of κ-casein at pH 12.0 was calculated to be approximately 31,000.

DISCUSSION

κ-Casein can be prepared in essentially pure form from skim milk. The method which has been worked out is based on (a) the preparation of the crude fraction S of Waugh & von Hippel (1956) and (b) separation of κ-casein from this material by means of alcohol fractionation, and further purification using variations in temperature and pH. The yield of κ-casein, approximately 3 g. from 14 l. skim milk, is less than 10 per cent (using 3 per cent casein in skim milk, and 15 per cent κ-casein in the former). From observations made during the preparative procedure it is quite clear that β-casein influences markedly the behaviour of κ-casein, possibly by some type of interaction with it. Firstly, appreciable quantities of β-casein prevent the precipitation of κ-casein at pH 4.6 and 2°, under which conditions β-casein is largely soluble; on warming to 37° precipitation of both occurs readily. Secondly, β-casein diminishes the extent of precipitation of κ-casein in 50 per cent alcohol at pH 5.7 and room temperature. Finally, acid precipitated κ-casein is not soluble at pH 7 and must

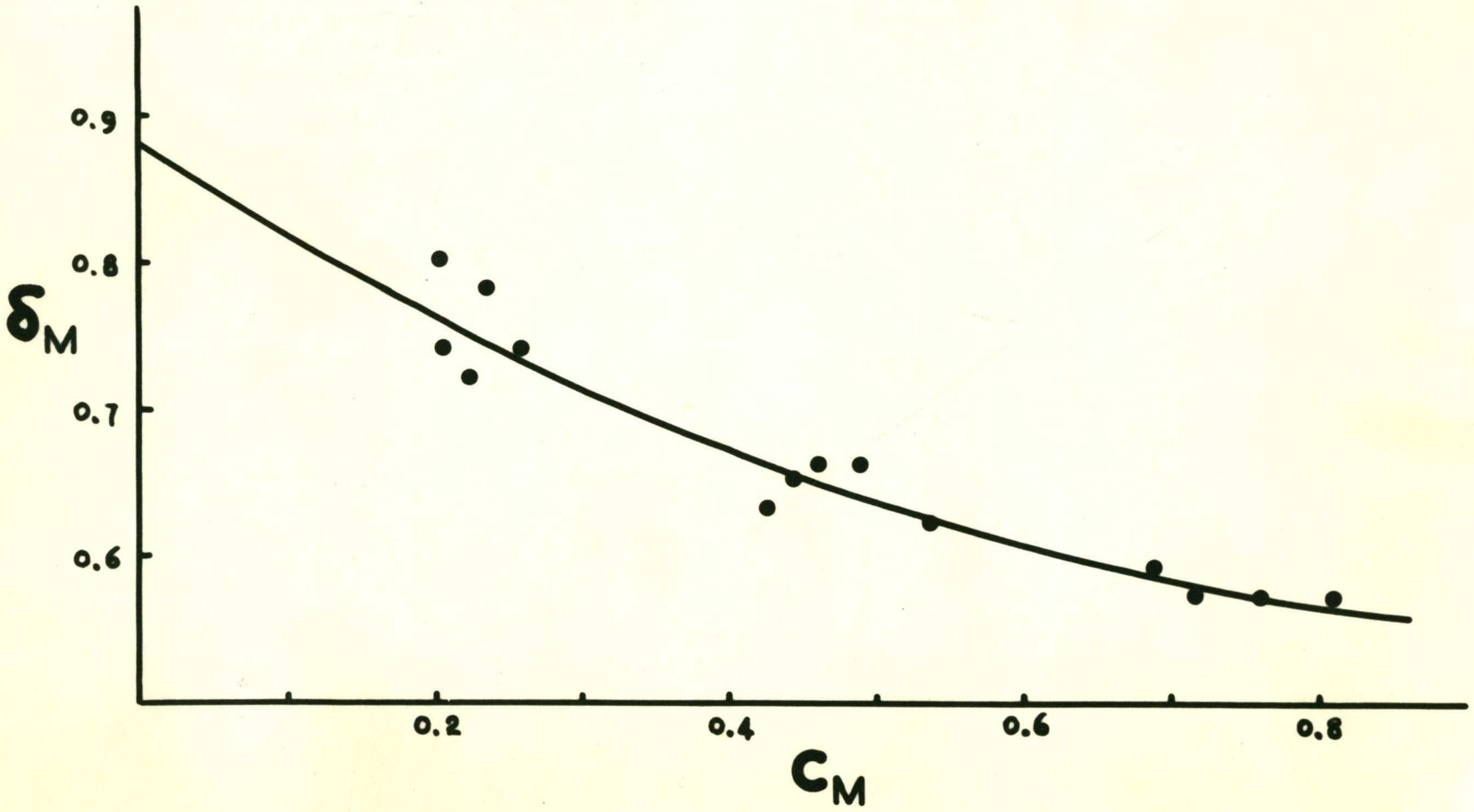


FIGURE 18. Dependence of δ m on concentration. κ-Casein in phosphate buffer, pH 12.0, $\underline{I}$ = 0.19.

be treated at pH 12 to effect solution in a reasonable time; β-casein hinders the formation of this difficultly reversible aggregate under acid conditions. Because of this strong interaction between β- and κ-casein it is extremely difficult to remove the last traces of β-casein from preparations of the latter. Also, it decreases markedly the yield of κ-casein obtained from fraction S. An improved method for the preparation of large quantities of κ-casein from acid casein is at present being developed. It is intended to use the crude alcohol fraction A obtained from acid casein by the method of Hipp et al. (1952) as a source of κ-casein. This fraction contains approximately 92 per cent of the α- κ-complex and only 8 per cent β-casein. It is hoped to be able to remove the latter before splitting the α- κ-complex, after treatment under alkaline conditions, with 0.25 M-calcium chloride.

Acid precipitated κ-casein is difficult to dissolve and requires adjustment to pH 12 to effect solution. On the other hand, acid precipitated α-casein is completely soluble as a clear solution at pH 7. This would indicate that the "difficultly reversible aggregates", involving α- and κ-casein, in acid casein are probably the result of interactions involving only the κ-component.

κ-Casein is a mucoprotein containing phosphorus, neuraminic acid, and probably other sugars [cf. bovine

submaxillary mucin (Gottschalk, 1958)]; and has no detectable α-amino end-groups. Waugh & von Hippel suggest that its phosphorus content is approximately one half or less that of α-casein. It is highly aggregated at neutral pH and can be dissociated into monomers at pH 12.0 with a molecular weight of approximately 31,000. Its partial specific volume is being investigated.

McMeekin, Groves & Hipp (1957) have recently reported on the separation of a new component of casein. It was "homogeneous" on electrophoresis at pH 8.4, $\underline{I} = 0.1$, and has been termed α_2-casein since it moved at a slower rate than, but near that of α-casein. It was not precipitated by calcium salts and was rapidly split by rennet at pH 7.3 with the formation of an insoluble and a soluble fraction; it thus resembles κ-casein in these respects (see Section IV). However, it differs from the latter in being soluble at pH 4.7, with a minimum solubility at pH 5.8 to 6.0. Its behaviour on sedimentation has not been reported. It was obtained in approximately 1 per cent yield from acid casein, and is obviously related to κ-casein in some way. The connection between the two should not be difficult to determine.

SECTION IV

THE ACTION OF RENNIN ON CASEIN

INTRODUCTION

Milk will clot under the influence of rennin and certain other enzymes. The enzymic activity of rennin is not to clot milk directly but to alter it in some way so that it clots subsequently. The component of milk which is altered is the casein.

The mechanism of milk clotting can be resolved therefore into two stages (a) the enzymic conversion of casein into paracasein and (b) the subsequent coagulation in the presence of calcium ions.

$$\text{Casein} \xrightarrow{\text{rennin}} \text{Paracasein} \xrightarrow{Ca^{++}} \text{Clot}$$

The present work is concerned mainly with the first half of this process.

Linderstrøm-Lang (1929) suggested that the activity of rennin is due to its ability to destroy or inactivate a "protective colloid" component whose function it is to keep the other insoluble calcium caseinates in "solution". In the absence of calcium casein can be converted into paracasein without any clotting taking place.

There has been much controversy as to which of the components of casein functions as the protective colloid. Until very recently most of the evidence pointed to " α-casein"

as acting in this capacity, and as being the primary site of rennin attack.

Nitschmann & Lehmann (1947a) demonstrated the splitting of the electrophoretic " α-peak" of calcium free whole casein into two, α_1 and α_2, under the action of rennin. In addition, Cherbuliez & Baudet (1950b), on fractionating solutions of paracaseinate by procedures which had been developed for native casein, found that β- and γ-casein had not changed, and that " α-casein" had been modified into two proteins, para-α_1, and para-α_2, so that it gave a double peak on electrophoresis. The finding that β-casein is not attacked very readily by rennin has been supported by the work of Nitschmann & Keller (1955) on the liberation of non-protein nitrogen from casein fractions by the action of this enzyme. Cherbuliez & Wolf (1953) have fractionated γ-casein from both paracasein and native casein and the two products appear to be the same.

Cherbuliez & Baudet (1950a) claimed that the calcium salt of " α-casein" was soluble at 40° in the pH range 6 - 7.5 while those of β- and γ-casein were insoluble. This fact alone supported the evidence suggesting that " α-casein" acted as the protective colloid. It should be mentioned, however, that Hipp et al. (1950) claimed that both " α-" and " β-" casein were clotted by rennin and that only γ-casein was precipitated with calcium ions.

McMeekin (1954) concluded that "any theory of clotting based on the action of rennin on one of the components of casein is inadequate." Also, Malaney, Weiser & Van Winkle (1954), from a study of the physico-chemical alterations produced by Streptococcus agalactiae on casein in milk, concluded that β-casein must act as the protective colloid.

Nitschmann and his collaborators (Alais, Mocquot, Nitschmann & Zahler, 1953; Nitschmann & Keller, 1955) showed that during the rennet curdling of milk, the amount of nitrogen (NPN) which was not precipitated by 12 per cent trichloracetic acid (TCA) increased markedly before visible clotting occurred. Crystalline rennin acted in a similar manner on solutions of sodium caseinate at pH 6.8, the NPN values reaching a maximum within a relatively short time. The NPN originated from the " α-casein", a negligable amount being split from β-casein under the same conditions. It was concluded that the specific splitting reaction, which sets free a small amount of NPN very quickly, is directly responsible for the milk clotting.

More recently, Waugh & von Hippel (1956) reported the existence of κ-casein, present to the extent of approximately 15 per cent in whole casein. It was claimed that " κ-casein is the most important single factor responsible for micelle stabilization and is the protein on which rennin acts immediately". The existence of κ-casein and

its micelle stabilizing properties have since been confirmed by the candidate and, in addition, a method for the isolation of the pure protein has been described (Sections I and III).

κ-Casein is present in skim milk and whole casein as an α- κ-complex. The normally referred-to " α-peak" observed on electrophoresis of these two systems actually represents this complex. The alteration of the " α-component" of whole acid casein after rennin treatment 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 of " α-casein" and its behaviour under the action of rennin could be due to contamination with κ-casein. Because of the close association between α- and κ-casein, particularly on acid precipitation, it is most likely that many of the preparative procedures described for obtaining " α-casein" from acid casein do result in giving a mixture of the two.

In the first part of this Section it will be shown that the NPN which is spit from whole casein during rennin treatment stems from the κ-casein. It is concluded that the splitting reaction is directly responsible for milk clotting. An examination of the free α-amino end-groups of κ-casein before and after the action of rennin is also included, as well as some observations on the nature of the NPN.

Berridge (1945), De Baun, Connors & Sullivan (1953) and Simonart & Chow (1952) have all shown that commercial rennet contains pepsin. Berridge (1945) and Berridge & Woodward (1953) have described methods for the crystallization of pure rennin, and its physico-chemical properties have been examined by Schwander, Zahler & Nitschmann (1952). The crystallization procedures of Hankinson (1943) and De Baun et al. (1953) have been severely criticized by Berridge (1955) in that they result in "irregular particulate precipitates."

The procedure of Berridge & Woodward (1953) is probably the most satisfactory, certainly it is the simplest. Attempts by the candidate to use this method, however, have resulted in considerable difficulty. This complaint is not at all uncommon (Berridge, 1955). The difficulties which have arisen and the methods used to overcome them are therefore described under "Materials and Methods".

MATERIALS AND METHODS

Crystallization of Rennin

Several attempts to prepare crystalline rennin from Australian rennet (Producers' Co-operative Distributing Society Ltd., Sydney) by the method of Berridge & Woodward (1953), and various modifications of it, were unsuccessful. The final precipitates were always dark brown, even after

reprecipitating with NaCl several times. Electrophoresis of the freeze-dried material showed two components (Figure 19). Evidently the slower moving material was largely responsible for preventing crystallization of the rennin (Berridge 1955). However, after removal of this impurity by preparative electrophoresis the material still would not crystallize, even after several weeks.

At the suggestion of Dr. Berridge (personal communication), who also supplied seeding crystals, Sterren Cheese Making Rennet Powder (Benger's Ltd., Holmes Chapel, Cheshire, England) was used as starting material. The first attempt, using essentially the procedure of Berridge & Woodward and without seeding, was unsuccessful. In the second attempt the rennin solution was clarified by high-speed centrifugation. The final solution, light straw in colour, failed to yield crystals after two 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 one of the freeze-dried preparations from Australian rennet but only after two weeks.

No difficulties have since arisen in preparing crystalline rennin from Sterren Powder using the modified procedure as long as the final solutions were seeded with the crystalline material. Details of the procedure are given below.

FIGURE 19. Examination of rennin preparations by electrophoresis and sedimentation. Runs were carried out in sodium phosphate buffer at pH 6.0, $\underline{I}$ = 0.1 (0.08 M-NaCl).
(a) Electrophoresis of purified rennin precipitate obtained from Australian rennet; approx. 0.2 per cent protein and 4.0 V/cm., 50 min., 20°. (b) Electrophoresis of crystalline rennin; 0.7 per cent protein and 3.88 V/cm., 226 min., 40°.
(c) Sedimentation of crystalline rennin at 2° with 0.7 per cent protein, 109 min., 60°. Sedimentation is from left to right.

(over)

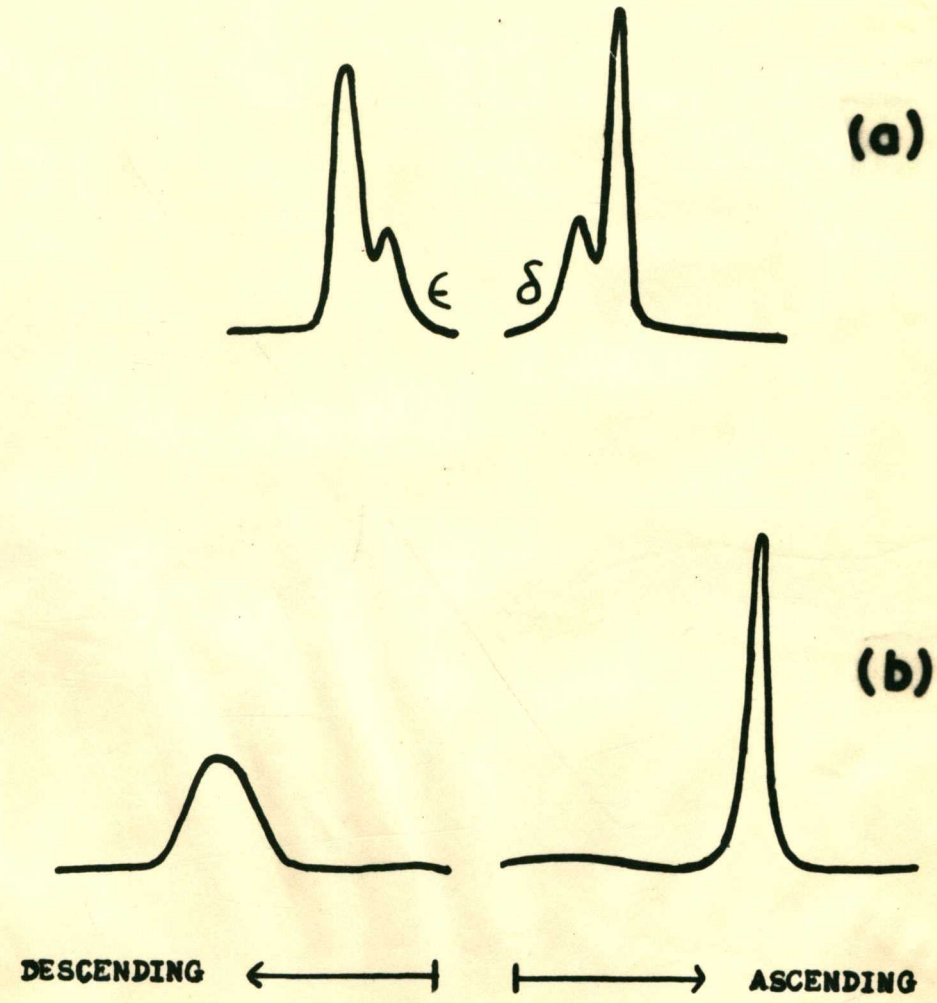


FIGURE 19

500 g. Benger's Sterren Powder were added to 4 l. water at 2° and stirred to dissolve as much of the material as possible. On warming to room temperature the mixture was adjusted to pH 5.4 with 0.1 N-NaOH and thymol added. After saturating with NaCl (C.P.) the precipitate was removed on a large Whatman No. 3 paper as suggested by Berridge & Woodward. The paper was extracted with 1 l. water and most of the insoluble material removed by centrifugation at 3,900 xg for 40 min. in an International Refrigerated Centrifuge. The supernatant was readjusted to pH 5.4 with 0.1 N-NaOH, and NaCl (A.R.) added through a rotating cellulose bag until the solution was saturated. The precipitate was removed by centrifuging at 3,900 xg for 20 min. and then dissolved in 150 ml. water. The brown, cloudy solution was clarified by centrifuging for 90 min. at 5° and 90,000 xg in a Spinco Preparative Ultracentrifuge, Model L. The clear, straw-coloured solution was readjusted to pH 5.4 and saturated with NaCl (A.R.) as before. The precipitate was removed by centrifugation and dissolved in a minimum quantity of water. A small amount of insoluble material was removed by centrifugation at 13,000 rev./min. and 5° in a M.S.E. type 92110 High-Speed Refrigerated Centrifuge using a type S775 rotor. The solution was seeded and allowed to stand at 2°. Crystallization occurred overnight and was allowed to proceed for two weeks. The crystals were obtained by centrifugation and washed twice with small volumes of 10 per cent

NaCl at 2°. Approximately 1 g. wet crystals were obtained from 500 g. Benger's Sterren Powder.

The rennin could be recrystallized by dissolution in a minimum quantity of 0.1 M-phosphate (0.1 M-Na_2HPO_4 + 0.1 M-KH_2PO_4) at pH 6.8 and 2° and readjustment to pH 5.4 with 0.1 N-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 purified by dissolving in 0.1 M-phosphate at pH 6.8 and 2°, adjusting to pH 5.4, and reprecipitating at room temperature by saturation with NaCl. The precipitated material was removed by centrifuging at 13,000 rev./min. in a M.S.W. High-Speed Refrigerated Centrifuge for 40 min., and stored in the moist condition at 2° in the presence of thymol. On dissolving this material in a minimum quantity of iced water crystallization proceeds very readily.

The crystalline material has the same form as that described by Berridge (1955). It gives a single peak on electrophoresis in phosphate buffer at pH 6.0, $\underline{I}$ = 0.1, 0.7 per cent protein (Figure 19), and has a mobility of - 2.95. In agreement with Schwander et al. (1952) the material shows heterogeneity on sedimentation in phosphate buffer under the same conditions (Figure 19). S_{20} is approximately 4.4, which is near the value reported by Schwander et al.

NPN Liberation Experiments

Total Whey Protein. The supernatant, obtained after removal of casein micelles from skim milk by the method of von Hippel & Waugh (1955), was used as a source of the total whey protein. It was filtered through Whatman No. 3 paper at 2° to remove most of the fat, centrifuged at 90,000 xg and 5° for 90 min. in a Spinco Preparative Ultracentrifuge, and dialysed exhaustively against 0.1 M-NaCl at 2°. It was then freeze-dried.

Experimental Procedure. Stock solutions of milk protein fractions were prepared by dissolving the freeze-dried material in 0.1 M-NaCl and dialysing against a large volume of the same solvent at 2° overnight. After nitrogen estimations, solutions of required concentrations were made up by first adjusting to pH 6.7 with 0.05 N-HCl or 0.05 N-NaOH, and then diluting in the correct proportion with 0.1 M-NaCl. Stock rennin solutions were prepared by dissolving the once crystallized and reprecipitated material in ice-cold 0.1 M-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° for 20 min. Approximately 0.1 ml. rennin solution (final concentration 0.1% rennin N/ml.) were pipetted into the protein solution with mixing. The solution was mixed thoroughly and 2 ml.

samples removed at suitable intervals and pipetted quickly into 4 ml. 18 per cent TCA at room temperature. After mixing, the latter 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 a sample taken just prior to the addition of the rennin.

Nitrogen Estimations. These were obtained by direct Nesslerization of a sample, after digestion under suitable conditions. The procedure described below afforded results reproducible to within 5 per cent over the range 40 - 150 γ N. Tryptophan, the most refractory of the amino acids, gave 98 per cent recovery (100 γ N) even in the presence of 12 per cent TCA.

The sample, approximately 2 ml., was mixed with 0.3 g. K_2SO_4 and 0.3 ml. 36N - H_2SO_4 in a 1" boiling tube marked at the 25 ml. level. Water was boiled off on a brass block, containing holes $\frac{1}{2}$" deep to take the tubes in an upright position, heated by a gas burner. Heating was continued until the mixture began to fume (where TCA is present this treatment, which provides even and gentle heating, is essential in order to prevent bumping and consequent loss of digestion mixture). The tube was then transferred to an electric rack of the type described by McKenzie & Wallace (1954) - new asbestos inserts were made to contain holes $\frac{1}{2}$" in diameter

on which the smaller tubes rested. The digestion mixture was refluxed gently for 20 min., cooled, and diluted with a small volume of water. The contents of the tube were neutralized by firstly adding 40 per cent NaOH drop by drop until blue to washed litmus paper and then 5 per cent H_2SO_4 till just red. The volume was made up to 25 ml. with water and 2.5 ml. Nessler's Reagent, prepared by the method of Bock & Benedict (Hawk, Oser & Summerson, 1954) were blown in. The solution was mixed thoroughly, allowed to stand for 10 min., and the optical density determined, against a suitable blank, using an Evelyn Photoelectric Colorimeter (Rubicon Co., Philadelphia, Pa., U.S.A.) with a filter at 490 mμ. The nitrogen content of the unknown was obtained by reference to a standard curve.

α-Amino End-Group Analysis

Preparation of Para- κ-Casein. Approximately 50 ml. 1 per cent κ-casein in 0.1 M-NaCl at pH 6.7 were allowed to react with rennin (0.1δ rennin N/ml. final concentration) at 30° for 30 min. The reaction mixture was cooled in an ice-bath and centrifuged at 13,000 rev./min. for 50 min. in a M.S.E. High-Speed Refrigerated Centrifuge. The clear supernatant was poured off and the sediment treated with water at 80° 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 0.05 N-HCl gave only a slight cloudiness. It was heated to 80° for 10 min., cooled,

and readjusted to pH 7 with 0.05 N-NaOH. It was then freeze-dried. Approximately 400 mg. para- κ-casein and 100 mg. freeze-dried "soluble nitrogen" (after correcting for NaCl) were obtained.

End-Group Analysis Procedure. FDNB (L. Light & Co.) was used throughout. The DNP-derivatives of all the commonly occurring amino acids were prepared by the methods indicated in Table 11 - other information is also included. Most of the derivatives had melting points close to those reported in the literature, and all moved as well-defined spots at 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 & Levy (1955). All the DNP-amino acids except DNP-leucine and DNP-isoleucine could be separated.

Ethylene chlorhydrin and toluene (BDH laboratory reagents) and tert.-amyl alcohol (L. Light & Co.) were distilled before use. Solvents (laboratory grade) used for washing and extracting the DNP-derivatives were also distilled. Peroxide-free ether was used throughout. All other solvents and chemicals were A.R. grade.

The DNP-proteins were prepared as follows: 0.6 g. κ-casein were dissolved in 40 ml. water and adjusted to pH 9.2 with 0.5 N-NaOH. 0.6 ml. FDNB in 5 ml. ether were

TABLE 11

Details of DNP-Amino Acid Preparations

DNP-Amino Acid	Amino Acid Source*	Times Crystallized	M.P.
DNP-D-Alanine[2]	L	2	174-5
DNP-L-arginine[1]	GBI	1	254-9(d)
DNP-DL-aspartic acid[1]	BDH	2	179-94(d)
Di-DNP-L-cystine[1]	L	1	121(d)
DNP-L-glutamic acid	NBC	oil	
DNP-glycine[2]	HW	2	200-1
DNP-L-hydroxyproline[1]	L	oil	
DNP-DL-isoleucine[2]	L	2	168-70
DNP-L-leucine[1]	BDH	1	
Di-DNP-L-lysine[1]	GBI	1	240
-DNP-lysine[3]	GBI	1	168-74(HCl)
DNP-D-methionine[1]	L	oil	
DNP-DL-phenylalanine[1]	GBI	1	205-7
DNP-L-proline[2]	L	1	131-4
DNP-DL-serine[1]	-	1	186-94(d)
DNP-L-threonine[1]	L	1	142-5
Di-DNP-L-tyrosine[1]	BDH	1	140-90(d)
DNP-L-tryptophan[2]	GBI	2	173-5
DNP-DL-valine[2]	BDH	2	180-2

The methods used are indicated in the first column:

1, Fraenkel-Conrat *et al.* (1955); 2, Sanger (1945);

3, Porter & Sanger (1948)

* L, L. Light; BDH, British Drug Houses' laboratory reagent; HW, Hopkin & Williams; GBI, General Biochemicals Inc.; NBC, Nutritional Biochemicals Corp.

added with vigorous stirring. The pH was maintained at 9.2 for 2½ hours 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. N-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 and this is the reason for using such a 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° in a sealed tube, usually for 16 hours. After cooling it was diluted with 4.7 ml. water and centrifuged to remove the brown, insoluble humin, which was most marked in the case of DNP- κ-casein. The solution was extracted four times with 5 ml. ether, only a faint yellow colour being transferred to the ether layer. The combined extracts were evaporated in a vacuum at room temperature. The water layer was evaporated to dryness in a vacuum at 50°. They 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 et al. (1955). After chromatography the spots were cut out, eluted with 4 ml. 1 per cent $NaHCO_3$, and the optical densities at 360 mμ measured against suitable blanks in a Hilger Uvispek Spectrophotometer.

The simplified procedure for the identification and estimation of DNP-arginine described by Wissmann & Nitschmann (1957) was applied to the water layer in each case. This procedure involved the removal of free amino acids by adsorption of the DNP-derivatives on a column of acid talc, elution of the latter with ethanol, and subsequent estimation of DNP-arginine by means of the colour reaction with Sakaguchi's reagent. The procedure was carried out exactly as described by Wissmann & Nitschmann.

RESULTS

The Liberation of NPN from Casein 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 20. The 1st and 2nd c.s. casein, fraction S, and total whey protein were all prepared from the one milk sample. With 1st c.s. casein 1.0 per cent NPN was released within 20 min. while none was split from the total whey protein. There was no significant release of NPN with casein from which the κ-component had been removed (2nd c.s. casein), while a marked increase to 3.4 per cent was evident with fraction S. With pure κ-casein approximately 6.7 per cent NPN was released rapidly. No significant splitting occurred with pure α-casein.

These results clearly support the conclusion that κ-casein is the site of primary attack when rennin acts on whole casein (Waugh & von Hippel, 1956). They are also in accordance with the assumption that the specific splitting reaction, which sets free a small amount of NPN very quickly, is directly responsible for the milk clotting (Alais et al., 1953; Nitschmann & 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 1st c.s. 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. This value agrees well with the estimate

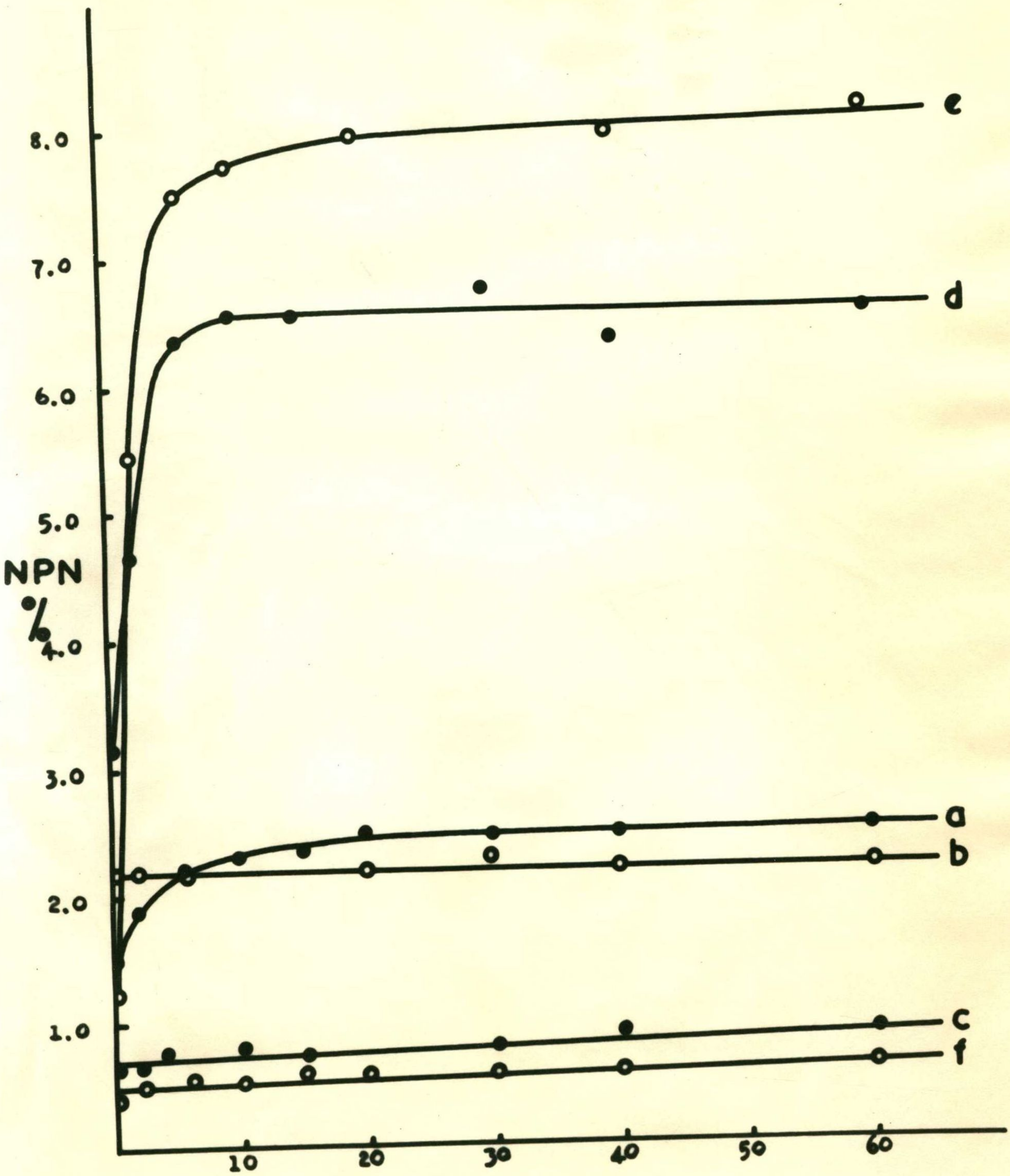


FIGURE 20. Liberation of NPN (soluble in 12 per cent TCA) from milk protein fractions by rennin. All studies were made at pH 6.7 and 30° using 0.1 γ rennin N/ml. (a) 2 per cent 1st c.s. casein; (b) 1 per cent total whey protein; (c) 2 per cent 2nd c.s. casein; (d) 1 per cent fraction S; (e) 1 per cent κ-casein; (f) 2 per cent α-casein.

made by Waugh & von Hippel. Also, in agreement with these workers, κ-casein during treatment with rennin formed aggregates. On addition of rennin to a clear solution of κ-casein the latter began to appear cloudy. This cloudiness increased in intensity, and the reaction mixture had the appearance of skim milk by the time the maximum value for NPN was reached. The aggregates could be centrifuged out to leave a clear supernatant which gave no significant precipitate on adjustment to pH 4.6. This supernatant contained approximately 23 per cent of the total nitrogen and will be considered further later on. The aggregated material, which will be referred to as para- κ-casein was found to move as a single component and more slowly than κ-casein on paper electrophoresis in veronal buffer at pH 9.0, $\underline{I}$ = 0.1 (Figure 21). Extensive adsorption in the case of para- κ-casein is evident.

The NPN values obtained at zero time might be of some significance in indicating a small amount of TCA soluble nitrogen in 1st c.s. casein and fraction S. The zero values for the various casein fractions were as follows: 1st c.s. casein - 1.4 per cent, 2nd c.s. casein - 0.6 per cent, fraction S - 3.1 per cent, κ-casein - 1.2 per cent. A certain zero 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

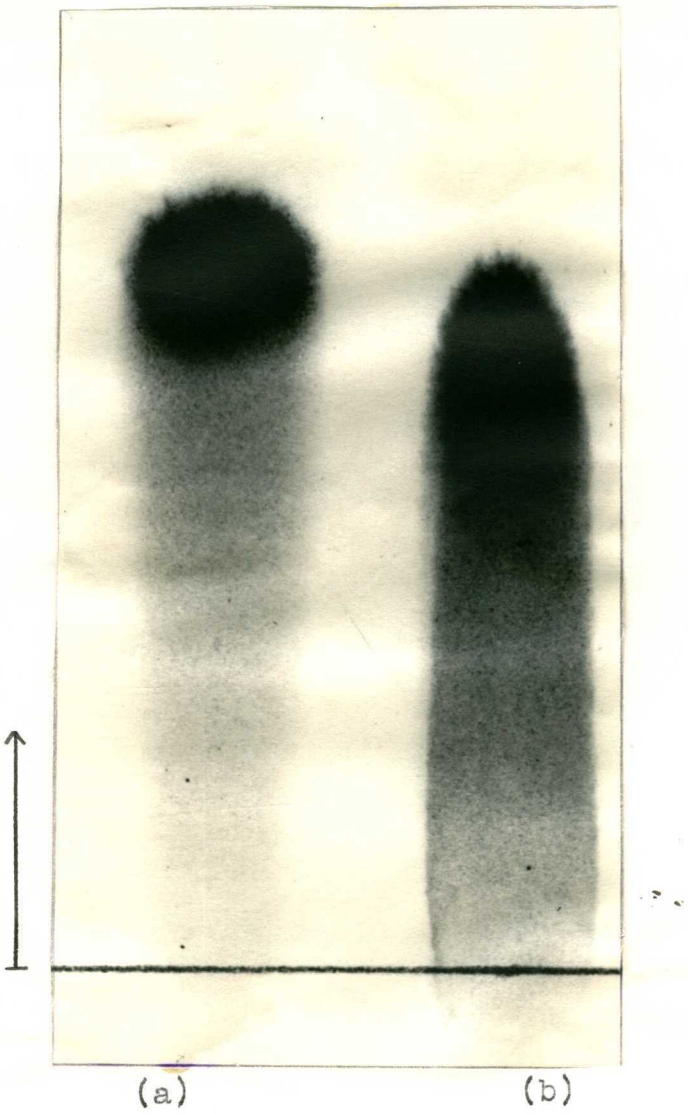


FIGURE 21. Paper electrophoretic patterns of (a) κ-casein and (b) para- κ-casein. The run was carried out in veronal (1) at pH 9.0, $\underline{I}$ = 0.1, and 2° on Whatman No. 1 paper for 14 hr. at 140V.

0° before addition of the protein sample. It is possible that we have approximately 0.6 per cent TCA soluble nitrogen in 1st c.s. casein and this remains in the supernatant (fraction S) on treatment with 0.25 M-$CaCl_2$ to give the precipitated 2nd c.s. casein. All, or most, of it is removed during the isolation of pure κ-casein. It would certainly not correspond to any of the whey proteins which give a zero value of only 2.2 per cent.

α-Amino End-Group Analysis of κ-Casein and Para- κ-Casein

The rapid release of over 20 per cent nitrogen from κ-casein by rennin to give insoluble para- κ-casein suggests that the specific action of this enzyme is to break some bond (or bonds) by which this material is combined in the parent molecule. Does the enzyme rupture some specific peptide bond and thus set free a part of a polypeptide chain in the κ-casein? As a step towards answering this question the free α-amino end-groups of κ-casein and para- κ-casein have been examined.

Examination of the ether layer, obtained from the hydrolysis of DNP- κ-casein by the two-dimensional toluene-phosphate, and the 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 dintrophenol it was decolourized 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 & Anfinsen (1956) in 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 hours. It is unlikely that DNP-proline, DNP-glycine, and di-DNP-cystine would have been completely destroyed under these conditions. Certainly the two breakdown derivatives of DNP-proline (Fraenkel-Conrat et al. 1955) were absent. The absence of cysteine from whole casein (Gordon, Semmett, Cable & Morris, 1949) made it unnecessary to check for the presence of this amino acid as an end-group.

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

In both cases only DNP-aspartic and DNP-glutamic acids were present in estimable 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 shown in Table 12. Taking 23 per cent nitrogen being split from κ-casein during the action of rennin a monomer molecular weight of 24,000 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 layers were ε-DNP-lysine, DNP-arginine, and di-DNP-histidine. ε-DNP-lysine is always present in large excess and this, unfortunately renders the identification and estimation of the other two very difficult. No trace of di-DNP-histidine could be detected in either of the ether layers, even after exhaustive extraction with ether. It was therefore unlikely to appear in significant quantities in the water layers and could not be detected in any of the chromatographic systems used. The phosphate system proved most useful in this respect as di-DNP-histidine moved very slowly in comparison with the others. It was necessary,

TABLE 12

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	
	Mol./10^5g.	Mol./Mol.	Mol./10^5g.	Mol./Mol.
Asp	0.13	approx.0.04	0.16	approx.0.04
Glu	0.12	approx.0.04	0.13	approx.0.03

however, to apply only a portion (approx. 25 per cent from a 20 mg. hydrolysate) of the water layer as a spot to the paper because of the large excess of ϵ-DNP-lysine. DNP-arginine moved rather close to ϵ-DNP-lysine in the chromatographic systems used and could not be separated satisfactorily from the large excess of the latter - an attempt to use the benzene system of Mellon, Korn & 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 et al., 1953; Wissmann & Nitschmann, 1957) and the difficulty in detecting and estimating it by the procedures described above, the simplified procedure of Wissmann & Nitschmann (1957) was applied once to each of the water layers in an attempt to clarify the position. The results indicated the presence of 0.6 and 0.5 arginine α-amino end-groups per 10^5g. 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 using this method and it cannot be said with certainty that κ-casein and para- κ-casein contain 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 & Hannan (1950)

and Schwartz & 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 et al. (1953) and Wissmann & Nitschmann (1957). Seno, Murai & Shimura (1955), during a study of the lysylpeptides in α-casein by the FDNB technique, could not detect any terminal arginine residues in this component.

The soluble nitrogen (23 per cent) obtained from the action of rennin on κ-casein was not examined for free α-amino end-groups. It was found to give a deep red colour when boiled with Bial's reagent and this is probably due to the presence of neuraminic acid. The large amount of humin formed on hydrolysing DNP- κ -casein with HCl is also probably a result of appreciable quantities of neuraminic acid which is known to decompose on acid treatment, with the production of humin matter (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, was split from κ -casein by rennin along with the soluble nitrogen. The latter also contained phosphorus (molybdate test) and gave a positive sulphuric acid-cysteine test (Dische, 1953, 1954) for galactose - the candidate is indebted to Dr. K. Knox of this Department for this result.

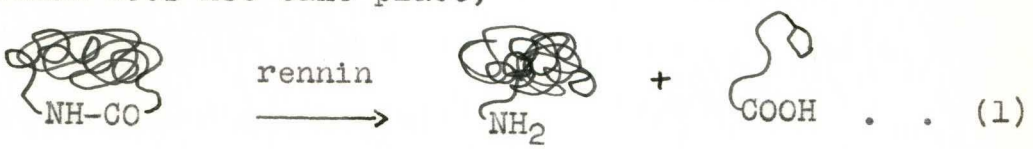
GENERAL DISCUSSION

The primary action of rennin on casein is to destroy the stabilizing properties of a "protective colloid" component whose function it is to keep the other insoluble calcium caseinates in suspension as micelles. The protective colloid is κ-casein, present to the extent of approximately 15 per cent in whole casein. Under the action of rennin, κ-casein is rapidly converted to an insoluble fraction, para- κ-casein, and a soluble fraction which accounts for approximately 23 per cent of the total nitrogen. The specific action of rennin rests in its ability to rupture some particular bond (or bonds) by which the soluble nitrogen is linked in the parent molecule. The course of this reaction with whole casein and various casein fractions has been followed by measuring the NPN (soluble in 12 per cent TCA) which is released by the enzyme after various intervals of time.

Previous evidence which has indicated " α-casein" to be the "protective colloid" and the primary site of rennin attack can be explained by the presence of κ-casein which occurs in milk and whole casein as an α- κ-complex.

No free α-amino end-groups could be detected in significant quantities in κ-casein, even after treatment with rennin. If arginine is present as such there is no appreciable difference as to its content in κ-casein and para- κ-casein. It is obvious that the specific action of rennin on

κ-casein does not involve the rupture of a peptide bond which would result in the formation of a free α-amino end-group in para- κ-casein. In other words the following reaction does not take place,

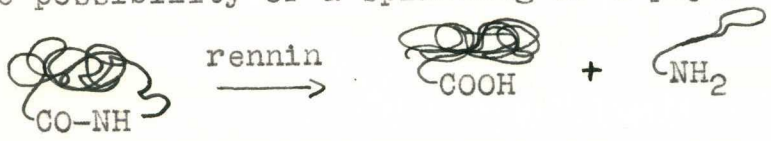


κ-casein para- κ-casein soluble nitrogen . . (1)

This result was unexpected in view of the observations of Wissmann & Nitschmann (1957) who reported the appearance of phenylalanine 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. This result suggested that "the primary reaction in the rennet curdling of milk is a specific limited proteolysis." In a recent report by Fish (1957) it has been shown that rennin has a proteolytic activity similar to that of trypsin. In the B chain of insulin it splits the following five bonds: Leu. Val, Leu. Tyr, Tyr. Leu, Phe. Phe, and Phe. Tyr (abbreviations according to Brand & Easall, 1947). The optimum effect is at pH 4. It is possible that the appearance of the phenylalanine α-amino end-group reported by Wissmann & 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. Alternatively it could have been due to some specific attack on the α-casein molecule which is distinct from this type of proteolytic activity. It will be

recalled that Payens (1958) reported a split in the α-casein peak of 2nd c.s. casein, which is free from κ-casein, on treatment with commercial rennet. The appearance of traces of phenylalanine, leucine, and lysine as α-amino end-groups in para- κ-casein is most likely due to very slight proteolysis.

The results presented so far have not ruled out the possibility of a splitting of a peptide bond as follows,



. . . (2)

κ-casein para- κ-casein soluble nitrogen

In this case any new α-amino end-groups would appear in the soluble nitrogen.

It is appropriate at this juncture to consider the two important contributions made recently by Alais (1956) and Nitschmann, Wissmann & Henzi (1957). These workers 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°. The NPN - 12 per cent was "almost homogeneous, a large molecule which is not dialysable." The greater part of NPN - 2 per cent behaved like NPN - 12 per cent; in addition there were some smaller components probably produced by non-specific proteolysis. The major component

in NPN - 12 per cent proved to be a "glyco-macropeptide" with a molecular weight of 6,000 - 8,000. It is obvious that 12 per cent TCA caused precipitation of most of the macropeptide, only a portion of it remaining in solution. On precipitating the protein in the reaction mixture by adjustment to pH 4.7, instead of with TCA, between 4 and 5 per cent NPN remained in solution. This was found on paper chromatography to consist of the glyco-macropeptide as well as a smaller peptide which formed comparatively slowly. The macropeptide had a very unusual composition (Table 13).

In view of this previous work it is almost certain that the soluble fraction (23 per cent) obtained here by the action of rennin on κ-casein is made up mainly, if not entirely, of this glyco-macropeptide or a product similar to it. Correcting for non-specific proteolysis, the amount of NPN - 2 per cent split from whole casein under conditions similar to those used here is between 3 and 4 per cent (Nitschmann & Bohren, 1955), which would be near the maximum amount of macropeptide produced. Using 23 per cent as the amount of soluble nitrogen produced from κ-casein, present to the extent of 15 per cent in whole casein, we would expect approximately 3.5 per cent to be released from whole casein under similar conditions. This is of the same order as that estimated. In addition, the molecular weight of the soluble material would be roughly 23 per cent of 31,000 (the monomer

TABLE 13

Composition of Macropeptide Split from Whole Casein by Rennin (Nitschmann, Wissmann & Henzi, 1957)

Component	Percentage
Nitrogen	11.4
Amide-N	1.83
Peptide	60
Galactose	15.2
Glucosamine	4.3
Neuraminic acid	11.4
Phosphorus	0.57

Amino acids per 10^5g. peptide were as follows: Asp, 49; Glu, 160; Ser, 110; Thr, 252; Pro, 104; Val, 82; Leu/Ileu, 85; Ala, 96; Gly, 14; Lys, 10.

molecular weight of κ-casein) or, in other words, 7,000. This is near the value of 6,000 - 8,000 proposed by Nitschmann and his collaborators. Furthermore the rather unexpected presence of galactose and neuraminic acid, as well as phosphorus, has been found here in the soluble material split from κ-casein. (The galactose content is possibly much less than 15.2 per cent estimated in the glyco-macropeptide obtained from whole casein). Finally, para- κ-casein moves at a slower rate on 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 in Table 13.

It is therefore proposed that the specific action of rennin on κ-casein at pH 7 is to release a glyco-macropeptide of rather unusual composition with the consequent formation of insoluble para- κ-casein. Nitschmann et al. (1957) found no free α-amino end-groups in the purified macropeptide, and this rules out the mechanism for rennin action considered in (2) above. Some bond (or bonds) other than peptide must be involved in linking the macropeptide to the larger molecule. The presence of such a high proportion of aspartic and glutamic acids (probably as the amides), serine and threonine, and the structurally unique proline, as well as the unusual occurrence of galactose and neuraminic acid in the macropeptide would suggest a number of factors contributing to

the stabilizing properties of κ-casein and its behaviour under the action of rennin.

Over the years it had been generally agreed that the action of rennin on casein did not lie in its ability to split peptide bonds. Marini & Levey (1955) have recently shown that sulphate inhibits the proteolytic activity of pepsin but augments its milk clotting activity. Many workers have considered the problem regarding the nature of the bonds split by rennin but their results have been rendered extremely difficult to interpret because of the use of whole casein as substrate in their experiments. Nitschmann & Varin (1951) observed that fewer basic than acidic groups were liberated on treatment of casein with rennin. This indicated that splitting of amide bonds of glutamic and aspartic acids could at the most account for only a portion of the groups liberated, while the splitting of ester bonds such as those between phosphoric acid and serine would lead to no increase in basic groups . Berridge (1954) suggested that possibly both kinds of bonds could be hydrolysed by rennin, giving some liberation of basic groups and a greater liberation of acidic groups. Rimington (1927) observed that dephosphorylated casein was not coagulated by rennin, and suggested that the ability to be so coagulated might be dependent on the presence of phosphorus. Cherbuliez & Baudet (1950b) also considered the possibility of an increase in the number of

phosphoric acid residues during rennin action. D'yachenko (1950) has made a study of the specific chemical nature of the linkages attacked by rennin. His potentiometric titrations on casein, paracasein, and their formaldehyde derivatives indicated the most important difference between casein and paracasein to be in the ability of the latter to bind hydroxyl ions in the pH range 10.2 - 12.2. The groups liberated were not all capable of binding formaldehyde. This behaviour was ascribed to the rupture of phosphoric acid ester bonds with the liberation of the third basicity of phosphoric acid. This, according to Higgins (personal communication) would implicate the presence of a triester of orthophosphoric acid in casein, and it is well known that only mono- and di-esters are hydrolysed by phosphatases (Roche, 1950). Undoubtedly, many workers who have suggested an increase in the number of available phosphoric acid residues during rennin treatment of casein, have been influenced by the possibility that calcium acts as a bridge between phosphoric acid groups on different paracasein molecules and that this is responsible for the clotting process.

In contrast to Nitschmann & Varin, Higgins & Fraser (1954) found a greater liberation of basic than acidic groups when casein was treated with <u>commercial rennet</u>. They concluded that the ϵ-amino groups of lysine were not responsible for the increased liberation of basic groups and have suggested

that the imidazole group of histidine might be involved. The differences in the ultraviolet absorption of casein and paracasein suggested that rennin might also effect the absorbing capacity of the tyrosine groups, perhaps by rupturing intramolecular hydrogen bond structures involving the phenolic hydroxyls. The absorption spectra of the azo-derivatives of casein and paracasein supported these suggestions.

No doubt much valuable information will be obtained when the techniques used by previous workers in attempting to establish the nature of the linkages attacked by rennin are applied to κ-casein. The exact chemical structure, including the amino acid sequence, of the glyco-macropeptide will be of paramount importance in solving this problem, as well as a knowledge of the organization of the polypeptide chain(s) in κ-casein to give its tertiary structure. Unfortunately the aggregation of para- κ -casein could hinder the use of certain optical methods, such as ultraviolet absorption and optical rotation, in following the enzymic process. It might be possible to prevent this aggregation reaction while still allowing the enzymic step to proceed, by the addition of M-sodium bromide or similar compounds as in studies on the conversion of fibrinogen to fibrin monomer under the action of thrombin (Ehrenpreis, Laskowski, Donnelly & Scheraga, 1957).

Mention should be made of the suggestion by Holter (1932) that the primary reaction brought about by rennin might be due to a phosphoamidase activity. More recently, Holter & Li (1950) have demonstrated the remarkable equality between coagulating and phosphoamidase activities of crystalline rennin, pepsin and chymotrypsin, and this supported the view of a connection between the splitting of P-N bonds and the coagulation of casein. Mattenheimer, Nitschmann & Zahler (1952) investigated this possibility and found that rennin did not split ammonia from casein and that it was inactive as a phosphatase against casein, phosphopeptone and glycerophosphate. However, when the results of Perlmann (1954a, 1954b) on the nature of the phosphorus linkages in " α -" and β-casein were taken into consideration the picture became somewhat different. The only bond, containing phosphorus and nitrogen was a-N-P-O-, present only in " α-casein". Also, it was shown that the complete removal of phosphorus from " α-casein" was accompanied by a disintegration of the protein into smaller units which were soluble in 10 per cent trichloracetic acid. Possibly the " α-casein" was contaminated with κ-casein and the -N-P-O- crosslink could have been present in the latter. The rupture of this bond by rennin might set free a polypeptide corresponding to the glyco-macropeptide. No free ammonia or phosphorus would be liberated in this process, but to rupture this bond rennin

would have to exhibit a diesterase rather than a monoesterase (phosphoamidase) activity as measured by Holter & Li on N - (p - chlorophenyl) - amidophosphoric acid. However, the present author found that under a variety of conditions rennin had no effect on the synthetic substrate N - monophenylphosphoroglycine (Wake, unpublished). The conclusions of Perlmann concerning the nature of the phosphorus linkages in casein have been criticized by Hofmann (1955), who found also that the snake venom diesterase from Crotalus adamanteus, which Perlmann used in her experiments, did not hydrolyse N - monophenylphosphoroglycine (personal communication). Perlmann's conclusions have also been criticized recently by Peterson, Nauman & McMeekin (1958) and Kalan & Telka (1958). In addition, the possibility of the specific action of rennin involving an attack on a P-N bond has been ruled out by Fish (1957), who showed that the phosphoamidase activity of rennin was due to a contaminant not present in the crystals prepared by Berridge's latest method (Berridge & Woodward, 1953).

The possibility of a crosslinkage mechanism involving neuraminic acid being responsible for binding the glycomacropeptide in the κ-casein molecule is at present being investigated by the candidate in collaboration with Dr. K. Knox of this Department. The action of the enzyme neuraminidase, obtained from the culture filtrate of Vibrio

cholerae, on κ-casein before and after rennin treatment is being examined.

Although Lundsteen (1938) claimed to be able to bring about the coagulation of calcium free casein solutions by the action of chymotrypsin, it has been generally agreed that the presence of calcium or some other alkaline earth is necessary for the clotting of paracasein. Nitschmann & Lehmann (1947b) calculated that one calcium ion per 200 amino acid residues was all that was required for clotting.

Two theories have been put forward to explain the function of calcium in coagulation. The first is that calcium acts as a bridge between different paracasein molecules. D'yachenko (1950) is of the opinion that the bridge is formed between free hydroxyl groups of phosphoric acid. Hostettler & Ruegger (1950) suggest that the calcium ions attach themselves to the electron pairs of amino groups. Higgins & Fraser (1954) studied the variation with ionic strength of the amount of calcium required for coagulation of paracasein in the presence of sodium chloride. It was found that a certain electrolyte balance was necessary for clotting as in the case of blood plasma (Lovelock & Porterfield, 1952). The latter authors suggest that the function of calcium is to maintain the surface charge on one or more of the colloidal components at a value suitable for interaction with the others. The same could apply in the case of

paracasein. However, it is quite possible that the calcium is involved in a crosslinkage mechanism between two groups in adjacent paracasein molecules. The sodium ions would compete with the calcium for these sites and consequently a higher concentration of calcium would be required to bring about clotting in the presence of sodium chloride. From a study of the stability of the clot Higgins & Fraser conclude that the intermolecular bonds involved are of a predominantly ionic character.

The fact that para- κ -casein itself aggregates readily, introduces a strong alternative as to the mechanism of milk clotting which will have to be investigated. It is not at all unlikely that the mutual attraction between para- κ -casein molecules would bring about a primary cross-linking between the casein micelles. This process would not necessarily involve the direct participation of calcium ions, although they would be needed as an integral part of the micelle. Perhaps calcium influences the manner and extent of aggregation of para- κ -casein.

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SUPPORTING PAPER

All the experimental work described in this paper was carried out by Mr. A. N. Glazer under the joint supervision of Dr. H. A. McKenzie and the candidate.

(submitted in accordance with section 13 of the By-Laws)

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LIBERATION OF TYROSINE HYDROXYL GROUPS IN UREA SOLUTIONS OF BOVINE SERUM ALBUMIN AND OVALBUMIN

The process of denaturation of proteins is being investigated in this laboratory by following changes in various properties which depend on the structure and the state of aggregation of the protein molecule[1]. In view of the possible importance of hydrogen-bonded tyrosine hydroxyl groups in maintaining the native configuration of the protein molecule[2] the liberation of tyrosine hydroxyl groups has been studied by examining the changes with time in the ultra-violet absorption spectra of bovine serum albumin and ovalbumin under different conditions of pH, both in the presence and absence of urea.

The spectrum (above 255 mμ) of aqueous solutions of bovine serum albumin was found to be essentially independent of pH over the range 4.4 - 8.0 in the absence of urea. However, when the pH was adjusted to less than 4.4, the absorption peak at 279 mμ shifted instantaneously to lower wave-lengths and the spectrum more closely resembled that of free tyrosine. At pH 3.2 the peak had shifted to 277 mμ. No further shift in the peak occurred on decreasing the pH below 3. Differential absorption spectra were obtained by comparing the spectrum of the protein at pH values below 4.3 with that of the protein in neutral solution. At any given pH below 4.3 the difference in

optical density was found to be greatest at 287-288 mμ. The difference at 287 mμ reached a maximum near pH 2.6. No further change occurred on decreasing the pH below 2.6 until pH 1.0 was reached, when there was some decrease in this difference in optical density. These changes in the spectrum are almost entirely due to changes in the environment of the tyrosine hydroxyl groups[2,3]. The change in optical density at 287 mμ closely parallels the increase in viscosity of bovine serum albumin at low pH reported by Tanford et al.[4].

On exposure of native bovine serum albumin to urea at concentrations above 2M, over the pH range 4 - 9, an instantaneous shift of the absorption maximum towards 277 mμ occurred. The spectrum then resembled that of the protein in acid solutions below pH 3. Likewise differential spectra obtained by comparing the spectrum of the protein in urea solution with that of the native protein at neutral pH showed a maximum difference at 287 - 288 mμ.

Unlike bovine serum albumin, ovalbumin showed no change in the 280 mμ band over the pH-range 2.0 - 9.0 in the absence of urea. On exposure of ovalbumin to urea at concentrations greater than 4M, a hypsochromic effect was observed. The extent of this effect varied with the pH, urea concentration and time of exposure to urea. The greatest shift observed was obtained by exposing the protein to 9M urea at pH 3.1. The absorption peak under these conditions

was shifted from 280 mμ for the native protein to 277 mμ for the denatured protein. Examination of differential spectra showed that the maximum difference in optical density occurred at 288 mμ. These spectral changes were very strongly dependent on the urea concentration.

The rate of liberation of the tyrosine hydroxyl groups in urea solutions of ovalbumin at pH 5.8 and 7.8 was studied by examining the rate of change of optical density at 288 mμ. At both pH values the rate has apparent first-order dependence. This observation may be compared with Harrington's studies[5] on the acid-base titration curve of ovalbumin in the presence of guanidine hydrochloride.

The rate of liberation of tyrosine hydroxyl groups in urea solutions of ovalbumin was a minimum near pH 8. This observation agrees with the finding of Simpson and Kauzmann[6] that ovalbumin has its maximum stability towards urea denaturation in the pH-range 7 - 9.

A comparison has also been made of the rate of liberation of tyrosine hydroxyl groups with the rate of change of other properties such as optical rotation. This and the present work will be published in detail elsewhere.

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