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INVESTIGATION OF THE CASEINATE-PHOSPHATE-CALCIUM COMPLEXES AS THEY EXIST NATURALLY IN MILK

by

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INTRODUCTION

The chemistry of casein has been studied extensively since the mid eighteen hundreds. The development of new techniques during the last thirty years, especially, ultracentrifugation, moving boundary electrophoresis, gel electrophoresis, and gel filtration, have added greatly to our knowledge, if not to our understanding of the calcium caseinate-phosphate complex. These newer techniques have allowed scientists to advance in a number of directions. However, only a small portion of the more recent literature deals with the intact or moderately treated complex.

The objectives in this investigation were to study the calcium caseinate-phosphate complex in a condition as closely approximating its native state as possible and to attempt to reconcile some of the discrepancies in the literature concerning the dependence or independence of casein micelle composition (α- and β-casein concentrations) on micelle size.

The dependence or independence of the amounts of calcium-sensitive (α_s-casein), calcium-insensitive (κ-casein), and β-casein on micelle size, was studied using urea- and urea-2-mercaptoethanol starch-gel electrophoresis. During the final phase of our investigations, Rose (1965) published material that had been presented at a symposium at the 59th Annual Meeting of the American Dairy Science Association, in June 1964. He reported a somewhat

similar approach using analytical and physical chemical methods rather than urea and urea-2-mercaptoethanol starch-gel electrophoresis of the calcium caseinate-phosphate complex.

REVIEW OF LITERATURE

The investigation of casein and "native" caseinates is important not only because of their nutritional importance but also because some problems such as gelation in some concentrated products cannot be solved until more information concerning the native milk proteins is available. The proteins of milk have been the object of extensive research. Casein, the most important of the milk proteins, and the calcium caseinate-phosphate complex have received most of the attention. Despite our increased knowledge of the complex and the improved research equipment and techniques, the nature of the colloidal phosphate and citrate salts and their association with calcium caseinate has defied elucidation and many conflicting data have been reported.

Casein has been the object of extensive research since the mid eighteen hundreds. The fact that the composition and properties of preparations of acid casein (Hammarsten, 1883) were surprisingly constant led to the assumption that casein was a single protein. The work of Osborne and Wakeman (1918) and later that of Linderstrom-Lang and Kodama (1925), employing extraction and precipitation methods, found that it was possible to fractionate casein into products having divergent compositions and properties. Since the work of Linderstrom-Lang, the heterogeneity of casein has been extensively investigated. The development of the Svedberg ultracentrifuge and the moving

boundary electrophoresis apparatus of Tiselieus added impetus to these investigations. Mellander (1939), in a classical paper, demonstrated the heterogeneity of casein by moving boundary electrophoresis; the three fractions obtained were designated α-, β-, and γ- in order of their decreasing mobilities. The outstanding chemical difference between these fractions is their phosphorus content. Phosphorus values, recently presented, are, in g/100g: α-casein, 0.99, β-casein, 0.61, (Gordon et al., 1949) and γ-casein, 0.11 (Gordon et al., 1953).

Following the work of Mellander (1939), these fractions (α-, β-, and γ-casein) and a few additional ones, have been characterized and numerous methods of isolation have been reported. These data have been reviewed by the Nomenclature of the Proteins of Bovine Milk Committee, (Brunner et al., 1960, Jenness et al., 1956, and Thompson et al., 1965).

In the remainder of this review, the molecular weights of the caseins, caseinate micelle size distribution and caseinate gel compositions are considered. In addition the relationship between the size of the caseinate micelles and their α-casein: β-casein ratios will be discussed.

Molecular Weight of Casein and Its Components

The molecular weight of casein was first estimated by Van Slyke and Bosworth (1913a, 1913b) from the sulfur and phosphorus contents. These workers found 0.72% sulfur and 0.71% phosphorus

in a sample of casein. They assumed one atom of each of these elements in the original protein and arrived at a molecular weight of 8888. Their method of preparation was questioned by Harden and Macallum (1914) who indicated that hydrolytic cleavage of the sulfur and phosphorus had taken place. Carpenter (1926), subsequently showed that there is considerable cleavage of nitrogen from the casein molecule using the Van Slyke and Bosworth procedure. Pauls and Matula (1919) calculated the valency of casein as 3 and concluded, from base-binding data, that the molecular weight was 3000. Yamakami (1920), using a method which compares solutions of equal vapor pressure, concluded that the minimum molecular weight was about 4000. Cohn et al. (1925) evaluated the molecular weight from the amino acid content. On the basis of one molecule of cystine per molecule of casein, the minimum molecular weight was calculated to be 96,000. However, because of the difficulty in reconciling the tryptophan content with the above figure, they decided on the value 192,000. Burk and Greenburg (1930), employing isoelectric casein in urea solutions, calculated from osmotic pressure data a molecular weight of 33,600 ± 250 for casein. More recently, D'yachenko and Vlodavets (1952), using a light scattering method, calculated the molecular weight of casein to be 30,000. However, the method of preparation of their casein resulted in complete removal of β-casein (Warner, 1944). In

a subsequent paper, D'yachenko and Vlodavets (1954), using a differential light scattering apparatus, reported that the mean particle weight of casein in skim milk to be from 266-780 million.

The introduction of the ultracentrifuge by Svedberg provided a new means of determining molecular weights of proteins. Svedberg et al. (1930a), using the sedimentation velocity method, found that casein was polydispersed which greatly increases its complexity. They found that the acid-alcohol soluble protein (Linderstrom-Lang and Kodama, 1925) extracted from casein prepared by the Hammersten method was homogeneous and had a molecular weight of 375,000 ± 11,000. In a subsequent paper, Svedberg et al. (1930b) found that casein prepared by the Van Slyke and Bosworth (1913a) method was also heterogeneous; a constant mixture of the various components could not be obtained. The bulk of the crude casein had a molecular weight between 75,000 and 100,000. Carpenter (1931) subsequently found 98,000 apparently to be the correct value. Subjecting the crude casein to a temperature of 40 C, during dispersion in the buffer solution, resulted in a casein having a molecular weight of 188,000 by the sedimentation equilibrium method. No molecules of molecular weight between 75,000 and 100,000 remained after this treatment. Pedersen (1936), using a salt concentration of 0.25 M vs 0.017 M used by Svedberg et al. (1930a, 1930b), obtained higher sedimentation

constants which he attributed to the higher salt concentration. Pedersen used milk ultrafiltrate as a buffer in one experiment with milk serum (obtained by subjecting skim milk to the action of a centrifugal force of 100,000 x g for "some minutes") and identified one major constituent (α- and β-casein); minor components were difficult to identify because of the refractive index gradient caused by sedimentation of the large amount of lactose. Attempts to take light absorption pictures failed.

Warner (1944) separated α- and β-casein. Subsequent investigators have dealt primarily with the separation and analysis of the various components of casein and the interaction among these components. Cherbuliez and Baudet (1950) estimated the minimum molecular weight of their α- and β-caseins, on the basis of tyrosine, tryptophan and phosphorus contents, to be 130,000 and 48,000, respectively. From the phosphorus content of α-casein, Perlmann (1954) suggested a minimum molecular weight of 31,000. Sullivan et al. (1955), using centrifugal methods, investigated the influence of temperature and electrolytes upon the apparent size and shape of α- and β-casein. They concluded that β-casein has a molecular weight of 24,100 below 15 C and has a marked tendency to form aggregates at room temperature. α-Casein was found to have a molecular weight of 121,800 and its aggregation was dependent upon ionic strength rather than temperature. Von Hippel and Waugh (1955) determined that at a temperature near 0 C and

pH 12, unfractionated soluble casein (soluble casein in equilibrium with micellar casein and readily soluble at pH 7.0) dissociates into monomeric units with an average molecular weight of 15,000. The α-casein monomer probably lies in the range of 13,000 to 15,000 and the β-casein monomer in the range of 15,000 to 25,000. Waugh (1958) subsequently reported a molecular weight of 23,300 for α_s-casein (calcium sensitive) and 16,300 for κ-casein (calcium insensitive). However, the α_s fraction was said to be 95% pure and the κ-fraction 90% pure. The above values for the molecular weight of the various casein components have been questioned by McKenzie and Wake (1959c). They state that the ease with which α- and β-casein aggregate have affected the results reported by various workers using "pure" fractions. In addition, the α-casein used by workers before 1956 could have been contaminated with the recently discovered κ-casein (Waugh and von Hippel, 1956). McKenzie and Wake (1959c) obtained a molecular weight for α-casein of 24,800 by sedimentation and diffusion measurements at pH 11, 25,000 by the Archibald (1947) method at pH 12, and 27,600 at pH 7 in 6 M urea. β-Casein was found to have a molecular weight of 17,300 by sedimentation and diffusion studies in glycine buffer at pH 11.0, and 19,800 in 6 M urea at pH 7.2. A preliminary value of 26,000 ± 3000 was obtained for κ-casein. Nielsen (1959) obtained the following molecular weights for caseins dissolved in 6.66 M urea solution: acid

precipitated casein 25,700, calcium precipitated casein 29,800, calcium precipitated α-casein 27,800, and calcium precipitated β-casein 23,100. The molecular weights for the whole casein are number averages while those for α- and β-casein are minimal, due to the strong disaggregating effect of urea on proteins. The discrepancies noted, prior to 1956 for α-casein, have been explained by McKenzie and Wake (1959c) as resulting from contaminated fractions. More recently, Swaisgood and Brunner (1962) reported a molecular weight of 29,000 using guanidine hydrochloride (5 M) at pH 5.0, urea (7.0 M) at pH 8.5, 33 and 66% acetic acid. They calculated the weight of the light component. Since previous molecular weight studies on κ-casein were conducted in phosphate buffer (pH 12) and Jolles et al. (1962) found cystine present, Swaisgood and Brunner (1963) suggest that the high pH may have catalyzed the cleavage of the disulfide bonds resulting in low molecular weight fragments. Evidence is given supporting this hypothesis and indicating that the basic monomer of κ-casein consists of two or more polypeptide chains which are cross-linked by disulfide bonds.

The total number of casein components present in milk is not known. According to Waugh et al. (1962), there are 8 components. Wake and Baldwin (1961) have observed as many as 20 components. Waugh et al. (1962) state that, at this time, it seems reasonable to assume that at least the four most abundant

caseins (α_{s1}-casein, α_{s2}-casein, β-casein and κ-casein) are primary caseins (γ-casein is not mentioned). The α_s-caseins are defined by Waugh et al. (1962) as the caseins which interact with κ-casein in appropriate weight ratios, to form complexes in the absence of divalent cations and, after rennin action, the micelles clot in the presence of divalent cations. The α_s-caseins can be resolved by starch gel electrophoresis in the presence of 6.5 M urea yielding two bands, α_{s1}- and α_{s2}-casein, at pH 3.2, 4.3 and 8.4. α_{s1}-Casein has the greater mobility at pH 8.4. The average molecular weight of α_{s1}- and α_{s2}-caseins, calculated from the tryptophan released by carboxypeptidase A, is 27,000 to 27,500; that by osmotic pressure in 6.5 M urea at pH 4.5 is 26,900 ± 2000. Dreizen et al. (1962) reported a molecular weight of 27,300 ± 1500 at pH 12 by a light scattering method.

The existance of γ-casein, obtained (and called γ) by Mellander (1939), was at first in doubt. It was thought to be a boundary abnormality. However, subsequent separation techniques have confirmed its existance. The percent of total casein reported as γ-casein has been from 3 to 7%, but very little has been reported on its chemical and physical properties (except for phosphorus content). Murthy and Whitney (1958) report that the γ-casein peak in electrophophoretic patterns of skim milk at pH 8.7 is made up of three proteins (γ-casein, pseudoglobulin, and euglobulin). The molecular weight of

γ-casein in veronal buffer (pH 8.7) was found to be 30,650 and in glycine HOl buffer at pH 2.3, 537,000.

Numerous sedimentation and diffusion coefficients have been reported for casein and its components as a result of molecular weight determinations. Since these coefficients depend upon experimental conditions such as pH, concentration and type of buffer used, they will not be covered.

Particle Size Distribution of the Caseinates

The particle size distribution of the caseinates in skim milk is of interest. Reports are not nearly as numerous as on other aspects of milk protein research. This probably results from the consistent results that have been obtained using a number of methods.

The determination of particle size distribution of caseinates in skim milk seems, at first glance, to be difficult because of the opacity of skim milk and because dilution of skim milk with water results in disaggregation of the caseinate particles (micelles). Particle size distribution of isoelectric casein is of minor interest because of artificial aggregation and will not be discussed. The calculation of particle size is possible from sedimentation data obtained in the determination of molecular weights; however, a majority of the molecular weight determinations have been performed on isoelectric casein and these calculations (for determining particle size) are of little value.

Nichols et al. (1931) minimized the disaggregating effect of dilution by employing skim milk ultrafiltrate as a diluent. They determined the particle size distribution by diluting skim milk (obtained by separating whole milk at 40 C) to 1/5 its original concentration and measuring the effect of centrifugation at 4000 rpm. (g force not given) by the light absorption technique. The estimated mean diameter was 90 mμ with most of the calcium caseinate particles being less than 200 mμ. A smaller amount of coarse material, probably colloidal calcium phosphate and larger aggregates of calcium caseinates, was also reported.

The increased use of differential ultracentrifugation (serially depleting the caseinates) to fractionate "Native" caseinates has led to newer methods for determining particle size distribution. De Kadt and van Minnen (1943), using "Tyandall light" on skim milk and caseinate gels diluted to a casein concentration of 0.03%, demonstrated that resuspended caseinates contained particles larger than those in the original milk. The development of light scattering techniques, primarily by Russian workers, and the use of the electron microscope, coupled with the demonstration by Nitschmann (1949) and Hostettler and Imhof (1952) that the addition of 0.3% formaldehyde to skim milk and then holding overnight at 4.0 C prevented the disintergration of the micelle when milk subsequently was diluted, gave impetus to additional work

on particle size.

Nitschmann (1949), using formalin and the electron microscope, obtained the following results:

diameter in mμ	% of Total	
	a[1]	b[2]
40-80	20.8	32
80-120	33.5	34
120-160	23.3	23.7
160-200	13.9	8.0
200-400	4.5	2.0
240-280	2.0	0.4

Hostettler and Imhof (1952), using a similar technique with a 1:300 dilution, obtained the following results:

diameter in mμ	% of Total
33	20.3
33-66	29.5
66-100	29.1
100-133	14.0
133-166	4.9
166-200	1.5
200-233	0.3

The largest particles found in the separator slime after separation of milk measured up to 800 mμ. D'yachenko (1953) estimated the average size of casein particles in milk as being

[1]Casein precipitated with 0.01 M $CaCl_2$, diluted 1:200.
[2]Skim milk, formalin treated, diluted 1:200.

100 to 120 mμ using ultrafiltration, ultracentrifugation and the electron microscope. In a subsequent paper, D'yachenko and Vlodavets (1954) reported a mean diameter range for casein particles in skim milk as 86 to 123 mμ. More recently, D'yachenko et al. (1955), using the method described by Nitschmann (1949) and the dilution (1:300) used by Hostettler and Imhof (1952) obtained the following results:

diameter mμ	% of Total
40	30
40-80	30
80-120	30
120-140	10

Yusa (1956) reported that no change in the shape of the casein particles was observed with temperature to 100 C for 30 min, but above that temperature the casein particles tended to aggregate. This is in agreement with Nichols et al. (1931) who found that preheating to 95 C had little effect on particle size distribution.

From the above data, it appears that the particle size distribution is somewhat restricted. The agreement among results especially between D'yachenko et al. (1954) and Hostettler and Imhof (1952), is quite good. However, the slight difference obtained when a 1:200 dilution vs. a 1:300 dilution is used, suggests a dilution effect. D'yachenko et al. (1954) pointed out that wet casein binds 0.69g of water per g of

casein and formalin treated casein only 0.55 g. This suggests that the use of formalin, to prevent micelle size changes on dilution, may slightly shrink the natural micelles, and make them appear smaller than they actually are in milk. If this were true, the data of Nichols et al. (1931) may more closely represent the conditions of casein micelles in the skim milk.

Ford and Ramsdell (1949) studied the molecular weights and micelle-size distribution of the calcium-caseinate-phosphate complexes. They found, by a differential centrifugation technique, that the smallest particle (basic "unit") had a sedimentation coefficient, S, of 179×10^{-3} and was 64 mμ in diameter, with an apparent molecular weight of 33 million. The dominant particle, about 30% of the total colloidal protein, had an S value of 1150×10^{-13}. Assuming that all the unit particles are spherical, they concluded that the larger particles must be made up of 16 "units"; the calculated diameter of this particular aggregate was 168 mμ with an apparent molecular weight of 530 million. The largest particles observed, $S = 4950 \times 10^{-13}$, had a calculated diameter of 348 mμ and an apparent molecular weight of 4.3 billion. They concluded that the colloidal particles in skim milk have a limited number of sizes and the size distribution varied as a result of concentration changes of caseinates left in the supernatant during centrifugation.

Composition of Caseinate Ultracentrifugal Gel

While the mineral composition of milk has been known for many years, the exact mode of combination of the individual acid and base constituents still requires elucidation. This discussion will be restricted to colloidal calcium phosphate and the calcium and phosphate associated within the caseinate molecule. The above are commonly referred to as the calcium caseinate-calcium phosphate complex or caseinate complex. The early work concerning the complex dealt chiefly with two separate areas, namely:

1. Is the colloidal calcium phosphate a di or tri calcium phosphate?
2. Is the colloidal calcium phosphate absorbed or chemically linked to the calcium caseinate?

Both questions have been studied extensively. Conflicting data and interpretations have been advanced for both sides of each question. The early work of Pyne (1934) and Ling (1936) refuted the contention of Van Slyke and Bosworth (1915) that the colloidal calcium phosphate in milk was dicalcium phosphate. Pyne (1934) found that addition of potassium oxalate lowered the titratable acidity of milk and reasoned that neutral colloidal tricalcium phosphate was converted into dissolved tri potassium phosphate having an alkaline reaction. The lowering corresponded to about 0.76g of calcium per liter of milk which was in agreement with Porcher and Chevallier

(1923). Analysis (Pyne, 1934) of synthetic calcium caseinate-calcium phosphate systems and their ultrafiltrates indicated that dicalcium phosphate would occur among the soluble salts and tricalcium phosphate among the colloidal constituents. Eilers et al. (1945) reached the same conclusions from potentiometric titrations. Ling (1936) developed a method of estimating tricalcium phosphate in milk using titrations of oxalated and non-oxalated milk and whey. He concluded that tricalcium phosphate was the major colloidal calcium phosphate. Pyne and Ryan (1950) modified Ling's procedure and concluded that tricalcium phosphate comprised about 88% of the colloidal phosphate. Davies and White (1960) obtained a value of 70% and indicated that the composition of the phosphate varied greatly in different milks. They reported that Evenhuis, in a personal communication, suggested that the colloidal calcium phosphate may be entirely in the tricalcic form. Pyne and McGann (1960a), employing a new technique (colloidal phosphate-free milk, obtained by a controlled acidification to pH 4.8 to 5.0, followed by dialysis against the original milk), suggest that the so-called colloidal phosphate of milk should be more properly termed a colloidal phosphate-citrate complex. This colloidal calcium phosphate-citrate component behaves as though it possessed the approximate imperical formula of the hypothetical citrate-apatite $3\ Ca_3(PO_4)_2\ CaH\ Cit^-$. From the overwhelming amount of circumstantial evidence, i.e., calcium:

phosphate ratios and oxalate titrations, it must be concluded, until other evidence is forthcoming, that the colloidal calcium phosphate is made up primarily of tricalcium phosphate.

The second question, nature of the association between calcium caseinate and the colloidal phosphate, however, is a much more difficult problem to solve. The evidence at this time, seems to favor a chemical union. However, no conclusive evidence has been presented. Pyne (1934) was one of the early proponents of a double-salt union in the complex. Ling (1936) favored a physical "protection" of colloidal phosphate by the caseinates. Van der Burg (1947), employing yeasts as an adsorbing agent, and ter Horst (1947) in a review tend to favor Ling's interpretation. Evenhuis and De Vries (1955, 1956a, 1956b, 1956c, 1957) concluded that the titration of oxalated milk could not be used to support the chemical union concept and that neither the use of yeast as a transferring agent nor the Ling titration could be used to support a physical adsorption concept. McGann and Pyne (1960) and Pyne and McGann (1960b), employing their colloidal phosphate-free milk to overcome the objections to the oxalate titration, concurred in Pyne's earlier concept of a chemical union. Schipper (1961), working with a sythetic complex, obtained no definite solution to the question, but considered that he proved that the presence of a double salt, as well as the independent binding of calcium and phosphate ions to the casein cannot be

of major importance, As can be seen above, the nature of the bond between colloidal calcium phosphate and calcium caseinate has not been resolved.

The development of preparative ultracentrifugal equipment has resulted in numerous papers on the composition of the calcium caseinate complex in milk, because it affords a means of obtaining the complex in practically its native state. De Kadt and van Minnen (1943), using the Sharples super-centrifuge, obtained several fractions of calcium caseinate by differential centrifugation and performed a number of analysis on the unwashed sediment and supernatants. They found that 0.55 g of water per gram of casein was bound water. They obtained slightly more calcium and phosphate with the first than with subsequent fractions and reported that the calcium: phosphate ratio (after subtracting the ester bound phosphate in casein) was 3:2. They concluded that the colloidal calcium phosphate is chemically linked to the casein and suggest the following as possible structures:

$$\text{Casein} - PO_4 = Ca \ldots\ldots\ldots\ldots\ldots\ldots Ca_3(PO_4)_2$$

or

$$\text{Casein} \begin{matrix} - Ca - PO_4 = Ca \\ - Ca - PO_4 = Ca \end{matrix}$$

Ramsdell and Whittier (1944) analyzed the water-washed, casein-containing colloids, obtained by ultracentrifugation. They reported that the complex contained 4.80% $Ca_3(PO_4)_2$ and 95.20% calcium caseinate. They concluded that their analyses

compare suprisingly well with analyses of isoelectric casein although their sulphur and phosphorus values were somewhat higher than values reported in the literature for casein (no specific references were given) repeatedly peptized and reprecipitated. Hostettler et al. (1949) using an angle centrifuged, separated 2/3 to 3/4 of the casein colloid of milk into 5 fractions. Their analysis of sedimented fractions indicate a decrease in the calcium and total phosphorus from the largest particles to the smallest. However, they started with whole milk which would contain all of the very large particles. Ford et al. (1955) fractionated the complex by differential ultracentrifugation and removed 75-90% of the casein. They obtained an organic phosphorus (OP): casein nitrogen (CN) ratio of 0.0563 and concluded that inorganic phosphorus (IP) decreased with decreasing particle size except that, for the smallest particles removed, a definite minimum proportion of inorganic phosphorus is indicated such that the molecular proportion of calcium: organic phosphorus: inorganic phosphorus is about 5:2:2. This ratio suggests a combination of two moles of calcium caseinate with one mole of tricalcium phosphate which is the inverse of that suggested by de Kadt and van Minnen (1943). Ford et al. (1955) also concluded that the complex, in fresh untreated skim milk, appears to exist as a single phosphoprotein, which is probably a definite mixture with fixed ratios of α-, β- and γ-caseins. The organic phosphorus: casein nitrogen ratio is in close

agreement with that of a mixture of these caseins in the proportion 16:4:1. In a subsequent paper, Ford and Martinez-Mateo (1958) investigated the compositions of the non-sedimented (whey-soluble) caseinate and found the same organic phosphorus: casein nitrogen (OP:CN) ratio for these smallest particles as for the remaining caseinate particles. More recently, Bohren and Wenner (1961) reported a OP:CN ratio of 0.045 and 0.044 for whey soluble casein obtained by centrifugation at 20 and 4 C respectively *vs.* an OP:CN ratio of 0.054 for the sedimented caseinate fraction. Tsugo *et al.* (1962), employing differential centrifugation, fractionated the complex and studied the effect of temperature, pH, heating (95 C, 30 min) and $CaCl_2$ precipitation on the composition of the sedimented complex. They report a decrease, in the OP:CN ratio, in the sedimented caseinate when the centrifugation temperature was reduced from 26 to 4-7 C.

The studies presented indicate that the data and interpretations are conflicting concerning the natural caseinate-complex in milk obtained by differential centrifugation. The problem of determining the composition of the sedimented complex is not a difficult one from the standpoint of analytical techniques. However, the composition of the adsorbed and occluded liquid (whey) is not known. This means that assumptions have to be made concerning this liquid, and, as a result, reported analytical data are only close estimates of the true

composition and will vary depending upon the assumptions made and the amount of liquid involved. Since inferences have been made from ratios of various constituents in the sediment, these assumptions may be critical.

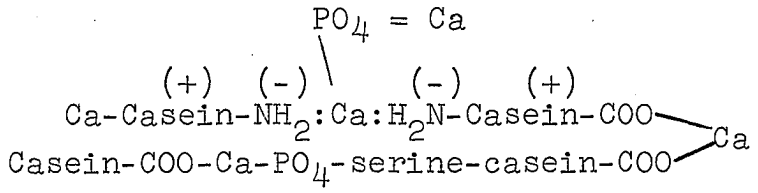
The data concerning the amino-acid contents of the various caseins is scanty. Gordon et al. (1949, 1953) have reported the amino acid compositions of α-, β- and γ-casein but the sequential arrangement of these amino acids in casein is unknown. Swaisgood and Brunner (1963) reported the amino composition of κ-casein. Gordon and Basch (1963) reported the amino acid composition of α_s-casein. In a review, Ling (1956) indicates that from casein hydrolysates, serine, threonine and arginine have been found to be attached to phosphorus, indicating that phosphorus is combined to these amino acids as an integral part of the casein molecule. Proteolytic enzymes have released a number of phosphopetones from casein. They show considerable differences in amino acid composition but seem to have phosphoric acid esterified to serine or threonine with the adjacent amino acid being dicarboxylic, generally glutamic acid frequently followed by leucine or isoleucine (Ling, 1956). Perlmann in a series of papers (reviewed by Perlmann, 1955) using specific phosphatases, reported that the phosphorus in α-casein was linked 40% as the monoester, $-O-PO(OH)_2$, 40% as the "diester" (20% as -O-PO(OH)-O- and 20% -O-PO(OH)(NH-), Perlmann, 1954) and 20% as pyrophosphate,

-O-PO(OH)-O-PO(OH)-. β-Casein was found to contain diesters of the -O-P-O- type as contrasted to the -O-P-O- and -NH-P-O- (Perlmann, 1954) diester types of α-casein. Unfortunately $\varkappa$-casein was unknown when this work was completed. Osterberg (1961) reported that the phosphorus linkages in α-casein were monoesters with six phosphate groups linked to serine residues and one to a threonine residue which is not in agreement with Perlmann (1954). Belec and Jenness (1962), working on the "dephosphorization" by heat treatment in caseinate solutions concluded that the phosphodiester of the type -N-P-O- as proposed by Perlmann (1954), may be considered absent in casein. If Osterberg (1961) and Belec and Jenness (1962) are correct, arginine would not be considered to be linked to phosphorus in caseins.

Recently, Aiyar and Wallace (1964) propose that casein contains -N-P-N- type bonds which are hydrolyzed by the action of rennet. They suggest that the phosphorus is attached to the guanido groups of arginine.

The nature of the association of calcium within the caseinate micelles has received little attention. Manson (1962) concluded from his work that there are two types of calcium linkages. The stronger is postulated as linking α_s-, β-, and $\varkappa$-caseins in constant proportions, which, according to Manson is consistent with the data of Waugh (1958) but not with those of Sullivan et al.

(1959) who reported different amounts of κ-casein in caseinate fractions obtained by differential centrifugation. The weak and more readily accessible calcium bonds Manson considers to be responsible for the loose combination of primary units into micelles of varying sizes, but of constant composition. Kirchmeier (1960), proposed the following structure for casein:



Reisfeld (1957) concluded, from his work on the calcium-binding properties of casein, that free carboxyl groups, Zwitterion forms of imidazole and guanidinium and phosphoric acid esters were the primary calcium-binding sites of casein.

The internal structure of casein is essentially unknown. The above reports show that, while much information has been obtained, our present knowledge is insufficient to allow the structure to be elucidated.

Relationship Between the Sizes of the Caseinate Micelles and Their α-Casein: β-Casein Ratios

The composition of whole and fractionated casein, obtained by ultracentrifugal sedimentation has been studied by de Kadt and van Minnen (1943), Ramsdell and Whittier (1944), Chanutin et al. (1942), Hostettler et al. (1949), ter Horst (1947, 1950) and Ford et al. (1955) to name a few. On

the basis of composition alone Eilers et al. (1947) found that casein preparations derived from coarse and fine "micellae" from the same milk had practically the same α- and β-casein composition and concluded that it is probable that these fractions, if they occur in milk in a preformed condition, are already mixed in the "micellae". Ford et al. (1955) concluded that on the basis of the OP:CN ratio (organic phosphorus: casein nitrogen) the native caseinates (obtained at 20 C) cannot be α-, β- or γ-casein alone. These casein fractions have the OP:CN ratio of 0.0637, 0.0398 and 0.0032. Ford et al. (1955) concluded that the OP:CN ratio, 0.056, found in a sedimented gel could result from the presence of all three caseins in a definite proportion of 16:4:1 yielding an OP:CN ratio of 0.0563. They do not rule out the possibility that α-, β-, and γ-casein could exist in separate particles and that the α-particles, β-particles, and γ-particles have comparable or identical size distributions.

In the work of Ford et al. (1955), 5-10 percent of the casein was not removed during centrifugation (which is true for all reported attempts to sediment casein without adding calcium or resorting to rather high centrifuging temperatures). In a subsequent paper, Ford and Martinez-Mateo (1958) investigated this non-sedimenting fraction, which contained the smallest casein particles, by isoelectric precipitation. They found that the OP:CN ratios were the same as in the

sedimented portions.

On the basis of the composition of casein fractions, obtained with the ultracentrifuge, there is evidence that the α-casein: β-casein ratio (by moving-boundary electrophoresis) is independent of casein particle size. However, the data reported on the electrophoretic patterns of ultracentrifugally separated fractions are conflicting. Hostettler et al. (1949) sedimented (1000-17,000 x g) the caseinates, at 2-3 C into five fractions. Using equal protein concentrations, moving boundary electrophoretic patterns were run and the α-: β-casein ratios were found to be independent of micelle size. Heyndrickx and De Vleeschauwer (1952) sedimented the caseinates (no temperature given) at 7,000, 25,000 and 38,000 x g, and determined the moving boundary electrophoretic patterns of the supernatants. They found that the α-: β-casein ratios in the supernatant decreased as the caseinate concentration was depleted. Heckman et al. (1958), in an effort to resolve the conflicting reports above (Hostettler et al., 1949 and Heyndrickx and De Vleeschauwer, 1952), repeated the work and reach the same conclusion reported in both papers. Heckman et al. (1958) then conclude that the presence of serum (whey) proteins in the dialized supernatants preparations cause changes in the interaction between α- and β-caseins and states that the constancy of compositions of the centrifugally separated complex is considered strong

evidence that the particles of various sizes in milk have essentially constant compositions in agreement with Ford et al. (1955) and Hostettler et al. (1949). Heckman et al.'s (1958) work is available only as an abstract of a paper presented at a meeting. Hence, a critical evaluation cannot be made.

More recent work on the α-casein: β-casein ratio vs. particle size relationship has indicated that the α-casein: β-casein ratio is dependent on particle size, adding further to the confusion caused by the conflicting data above. Annibaldi (1960) separated the fat from whole milk (30 C) and sedimented 90 percent of the caseinate at 4.0 C and 150,400 x g for 60 min. The sediment consisted of a lower opaque layer (large particles) and a clear upper layer (smaller particles). He separated these layers and determined their moving boundary electrophoretic patterns. Annibaldi reported that the transparent layer had more α-casein and less β-casein than the opaque layer, which contained the largest micelles. Annibaldi (1961) confirmed his previous results, with caseinates obtained at successive speeds of 10,000, 20,000 and 40,000 rpm (13,100, 53,600 and 144,700 x g respectively).

Recent work, reporting specifically on the α-casein: β-casein ratios in relation to particle size (Yamanchi and Tsugo, 1962) is in general agreement with Annibaldi (1960, 1961). Yamauchi and Tsugo (1962) likewise included the effect

of heating (85 C for 30 min) and cooling (4 C) the skim milk. They fractionated the caseinates into three fractions by differential centrifugation (42,000 and 65,620 x g). They resuspended and isoelectrically precipitated each fraction and obtained a fourth fraction by isoelectric (I.E.) precipitation of the final supernatant (fraction IV-a non-sedimented fraction). The fractions, all I.E. preparations, were dispersed in 1% concentrations in dilute NaOH at pH 7.0. The solutions were dialyzed against phosphate buffer (pH 7.0 $\Gamma/2 = 0.1$) at 4 C for 2 days and the moving boundary electrophoretic analyses were carried out. The I.E. (non-sedimented) caseins corresponding to fraction IV above, were turbid when resuspended and were centrifuged until clear. A loss of less than 2 percent of the total nitrogen was encountered. It should be pointed out that Yamauchi and Tsugo (1962) did not include any of the non-sedimented fractions (obtained in a number of experiments) when the α-casein: β-casein ratios were determined, because all these fractions exhibited a component designated X between the α- and β-casein peaks in the electrophoretic patterns. The authors concluded that the X component caused the turbidity. Yamauchi and Tsugo (1962) proposed that it might be the unidentified component obtained by McKenzie and Wake (1959a) during paper electrophoresis, or the peak observed by Kenyon and Jenness (1958). Larson (1958) also reports an X component. Yamauchi and Tsugo (1962) observed no difference,

in the α-casein: β-casein ratio, between fresh and heated milk. However, the ratio of α-casein: β-casein slightly increased from fraction I (the largest sedimented micelles) to fraction III (the smallest sedimented micelles). The organic phosphorus: casein nitrogen (OP:CN) ratios did not relate to the size of the micelle in agreement with Ford et al. (1955). However, the (OP:CN) ratios were low in fraction IV (non-sedimented fraction). Yamauchi and Tsugo (1962) also investigated the effect of low temperature on the α- and β-casein composition in the non-sedimented fractions. They reported an increase in β-casein composition in the non-sedimented fractions when the centrifugation was done at low temperature (4-7 C). They also reported a decreased sedimentability of the caseinates at low temperature and concluded that this is caused partly by the increased solubility of β-casein at low temperature. They further concluded that since the β-casein that is solubilized from the whole casein may be a very small portion of whole casein, no effect on the α-casein: β-casein ratios in the sedimented fractions may be discernable but would be discernable in the non-sedimented fraction since this fraction contains only 5-15 percent of the total casein. The authors (Yamauchi and Tsugo, 1962) consider their data as direct evidence for the solubilization of β-casein at low temperature vs. the indirect (analytical ultracentrifuge data) evidence of Sullivan et al. (1955). They concluded that

most of the β-casein was sedimented even at low temperatures, and suggested that the probably coexistence of calcium and calcium phosphate with native caseinates may keep β-casein with the other casein components in the micelle at low temperature and restrict the loss of β-casein to the monomeric form.

The caseinate fraction (5-15 percent) not sedimented in 180 min at 50,000 x g and 4.0 C, termed whey-soluble caseinate or non-micellar casein, has been investigated by moving boundary electrophoresis by Bohren and Wenner (1961). They reported that whey-soluble casein (WSC) and whole casein differ as regard organic phosphorus: casein nitrogen (OP:CN) ratios. The moving boundary electrophoretic patterns show a lower α-casein: β-casein ratio in WSC, obtained at 20 C, than in whole casein, when the quantity of WSC is less than 15 percent of the total casein. The α-casein: β-casein ratio of WSC is further reduced if the centrifugation is performed at 4 C. In fact, under these conditions there appears to be more β-casein than α-casein in the fraction. Bohren and Wenner (1961) concluded that ultracentrifugation of caseinates in the temperature range, 4 to 20 C, yields a sediment that is difficient in β- and γ-casein; they consider that $\varkappa$-casein (calcium-insensitive fraction of α-casein) might be an important factor in solubilizing β- and γ-caseins. These results may suggest a cause of the conflicting results that have been reported. It has been only recently (since 1955)

that there has been an appreciation of the increased solubility of some of the casein fractions, at low temperature. A few of the papers considered here do not indicate the temperature at which the casein was fractionated likewise there have been differences in the manner of handling the fractions, prior to electrophoretic analysis. The work of Bohren and Wenner indicates that some of the reported dependence of the α-casein: β-casein ratios on micelle size may result from a non-uniform solubilization of some of the β-casein, which could occur if constant temperatures were not used.

Additional work on the non-micellular milk proteins (whey-soluble casein and whey proteins) has been reported by Hansen et al. (1962). The significance of this work in relation to WSC is somewhat puzzling, since the authors do not mention α-casein in their fractions. They investigated the supernatant of milk centrifuged at 55,150 x g for 10 hours at 0-2 C. The "natural protein free milk system" (dialysate) was used as a buffer in the moving boundary electrophoresis. They compared these electrophoretic patterns with those obtained using a veronal buffer. Tentitative identification of components was accomplished using a boundary elimination technique. They concluded that in the native state at about 0 C, the major non-micellar proteins are not associated with each other and the mobilities are less in the native state than they are in veronal buffer. WSC was precipitated

by the Rowland (1938) technique and was subjected to moving boundary electrophoresis in veronal buffer. Hansen et al. (1962) indicated that the preparation contained 82.57% of β-casein and 2.90% of γ-casein, together with a component, consisting of 14.52% of the WSC, with a mobility of -4.73×10^{-5} cm^2 $volts^{-1}$ sec^{-1}. This component is possibly the X component reported by Larson (1958). The high percentage of β-casein is in general agreement with Bohren and Wenner (1961).

Morr (1965) reported results using a differential centrifugation procedure to fractionate the caseinates. The data are puzzling because the electrophoretic components (in phosphate buffer) of each fraction separated were not identified. The reported mobilities of these components do not agree with mobilities reported for α- and β-casein in phosphate buffer by McKenzie and Wake (1959b). Morr (1965) fractionated raw and heated (88 C for 10 min) milk to obtain particles of 55,83 and 135 m_μ in diameter. Only a slight dependence of chemical composition of the sedimented fractions to particle size was exhibited. Aliquots of each of the above fractions were solubilized (potassium oxalate) and moving boundary electrophoretic analysis were performed in phosphate buffer (pH 6.8, $\Gamma/2 = 0.1$). Sedimented proteins (135 m_μ and above), from raw and heated skim milks contained 72.5 and 69.5% of the protein in the fastest moving component (I),

(descending mobility -5.5×10^{-5} cm^2 $volts^{-1}$ sec^{-1}), whereas the fraction from 55 mμ and above contained 78.2 and 81.6% of their proteins in component (I) respectively. Assuming that component I is α-casein, Morr's (1965) results would support the conclusions of Annibaldi (1960, 1961), Yamauchi and Tsugo (1962) and Heyndrickx and De Vleeschauwer (1962), that the α-casein: β-casein ratio is micelle size dependent.

This review points out the diversity of results concerning the dependence or independence of the α-casein: β-casein ratios with micelle size. There appears to be an almost equal amount of evidence on both sides. As mentioned previously, the variety of methods and temperatures employed in the above studies could be the reason for their conflicting reports. Temperature has been indicated to be a critical factor in the results obtained (Sullivan et al., 1955) and undoubtedly the method of preparing samples for electrophoresis plays a part. Washing sediments with water may also affect the results, since preliminary work at this station has shown that sedimentation of all the caseinate from resuspended caseinate solutions is extremely difficult; however, this must be balanced against the problems of working with unwashed sediments which have been shown by Ford et al. (1955) to contain adsorbed whey proteins and non-protein nitrogen.

Considering the above difficulties it is small wonder that conflicting results have been reported. It is entirely

within the realm of possibility that all the work actually is in agreement when differences in technique are accounted for, barring missinterpretation of data.

Waugh and von Hippel (1956) demonstrated that α-casein is made up of a calcium-sensitive (α_s-casein) and calcium insensitive (κ-casein) components. These facts have extended the investigation of the α- and β-casein composition of the different caseinate micelle sizes to include the α_s- and κ-casein compositions as well. Sullivan et al. (1959) and Ribadeau-Dumas and Veaux (1964) report an increase in κ-casein with decreasing caseinate micelle size, using the sialic acid composition as an estimate of the amount of κ-casein. However, Sullivan et al. (1959) could show no difference by moving boundary electrophoretic analysis, between the original casein and the "subfractions". Rose (1965), using turbidimetric analysis to estimate β- plus κ-casein, and the sialic acid contend for κ-casein, reports a decrease in β-casein content with decreasing caseinate micelle size and a corresponding increase in κ-casein content.

The results of Rose (1965) and Sullivan et al. (1959) are in agreement as regards κ-casein contents; however, the fact that Sullivan et al. could show no difference between the original casein and its "subfractions" by moving boundary, implies that a corresponding decrease in α_s-casein should be obtained rather than the decrease in β-casein as reported by

Rose (1965).

Most of the literature cited is fairly recent. Because of the enormous amount of literature dealing with casein, it is impossible to review or even mention some of the excellent early work. These papers have been covered extensively by Sutermeister and Browne (1939), Cohn and Edsall (1943), and more recently but less extensively by Jenness and Patton (1959).

EXPERIMENTAL

Materials

Water

Distilled water Tap water softened by ion exchange treatment was distilled in a Barnstead, hard-water model, laboratory still and stored in a 100 gal. aluminum tank.

Redistilled water Redistilled water was prepared by the procedure described by Bird *et al.* (1961).

Milk

The fresh raw milk, composite of the evening milking from at least 75 cows, was obtained from the University dairy farm and separation procedures were initiated within 1 hr.

Reagents

All reagents were reagent grade unless otherwise indicated. Technical grade sodium hydroxide was used in the nitrogen determinations.

Starch The starch employed in urea starch gel electrophoresis was Connaught hydrolyzed starch, lot 153 (Connaught Medical Research Laboratory, Toronto, Canada).

Polyacrylamide gel The spacer gel was premixed upper gel solution (Canalco, Bethesda 14, Md.). The lower and sample gel solutions were made from dry ingredients (Distillation Products Industries, Rochester 3, N.Y.).

Apparatus

pH meter

A Beckman Model G portable pH meter with a glass electrode and calomel reference electrode assembly (Beckman Instruments, Inc., Pasadena, Calif.) was employed.

Centrifuges

A Model PR-2, refrigerated centrifuge with an automatic timer and temperature control (International Equipment Co., Boston, Mass.) was employed to obtain the skim milk.

A size 2 centrifuge (International Equipment Co., Boston, Mass.) was employed to sediment the precipitate obtained in the calcium and magnesium determination.

An Adams Safety Head centrifuge (Clay-Adams, Inc., New York, N.Y.) was employed for low speed centrifugation of dispersed (oxalate) calcium caseinates to remove the precipitated oxalates.

A Spinco Model L, refrigerated preparative ultracentrifuge (Beckman Instruments Inc., Pasadena, Calif.) was employed in obtaining the "native" calcium caseinate sediments.

Spectrophotometer

A Beckman Model DU Spectrophotometer (Beckman Instruments, Inc., Pasadena, Calif.) was employed in the phosphorus determinations.

Freeze-drying apparatus

The freeze-drying apparatus employed was described by Decelles (1963, p. 21).

Conductivity apparatus

A number 4960, line-operated, portable resistance indicator, with a 1-ml conductivity cell (Leeds and Northrup, Philadelphia, Penna.), was employed.

Moving boundary electrophoresis apparatus

A Model 38-A electrophoresis apparatus (Perkin-Elmer, Corp., Norwalk, Conn.), equipped with a modified Philpot-Svensson cylindrical lens system, was employed. Electrophoretic cells, 2-ml and 6-ml, utilizing a closed system were used.

Compensator

A Model 038-0071 compensator (Perkin-Elmer, Corp., Norwalk, Conn.), equipped with a 2 ml syringe, was used to adjust the initial boundaries in the moving boundary electrophoresis experiments.

Urea starch-gel electrophoresis apparatus

Power supply A Model IP-32 Heathkit (Heath Co., Benton Harbor, Mich.) was employed.

Gel trays The trays were made from 0.6 cm Plexiglas sheets with outside dimensions of 26.3 x 18.0 x 1.2 and inside dimensions of 22.3 x 14.0 x 0.6 cm.

Buffer vessels Each vessel consisted of two compartments with outside dimensions of 23.0 x 5.7 x 5.4 cm. The over-all dimensions were 23.0 x 11.0 x 5.4 cm. The vessels were made from 0.6 cm Plexiglas sheets.

Sample insertion knife The knife was made of 1.5 mm sheet stainless steel 14 cm wide by 11.3 cm long. One end of the sheet was sharpened, forming a blade 14 cm wide; it was bent at an angle of 90°, 1.3 cm from the sharpened end. The opposite end was bent in a like manner, 2.0 cm from the end. The knife was constructed to allow the gel to be cut, 6.0 cm from the anodic end of the gel, perpendicular to the direction of sample migration. The knife was pulled back, approximately 1.5 cm and held by a plastic block between the end of the tray and the non-sharpened portion of the knife, leaving the hands free for inserting the sample wicks. Thus, unnecessary handling of the gels is eliminated.

Buffer wicks The wicks for connecting the buffer vessels to the gels, and likewise the two buffer compartments were re-usable Nylonge-sponge Cloths (Nylonge Corp., Cleveland, Ohio).

Disc electrophoresis apparatus

A Model 12 Disc Electrophoresis apparatus, equipped with a Model 1400 power supply (Canalco, Bethesda, 14, Md.), was employed.

Densitometers

Densitometer for urea and urea-2-mercaptoethanol starch-gel patterns A Model 525 Photovolt Densitometer and a Model 42 A Photovolt Varicord Recorder (Photovolt Corp., New York, N.Y.) were employed.

Densitometer for polyacrylamide disc gel patterns

A Model E Microdensitometer (Canalco, Bethesda 14, Md.) was employed.

Methods

Analytical methods

Determination of total solids A modified Mojonnier procedure, designed to eliminate charring, was employed. Mojonnier total solids dishes, vacuum oven, hot plate and weighing pipettes were used. The dishes were cleaned, rinsed with distilled water and dried in a 100 C oven for 1 hr, then placed in a Mojonnier vacuum oven, under 25 inches of vacuum, at 80 C for 1 hr. The dishes were cooled in an aluminum desiccator oven P_2O_5 for 1 hr. Each dish was weighed, a 2-g sample of product was placed into it from a Mojonnier pipette and the dish reweighed. The samples were evaporated on a hot plate (155 C) until a slight browning occurred. They were then placed in the 80 C vacuum oven, under 25 inches of vacuum, for 1 hr, removed, cooled as above and weighed. The total solids were calculated in the usual manner.

Determination of moisture Mojonnier total solids dishes and a Mojonnier vacuum oven were employed. The dishes were cleaned as in the total solids determination, then placed in a Mojonnier vacuum oven, under 25 inches of vacuum, at 100 C for 1 hr. The dishes were cooled as above. Each dish was weighed and about a 0.2-g sample was distributed in the

dish and weighed. The samples were dried at 100 C in a vacuum oven, under 25 inches of vacuum, for 4 hr, removed, cooled as above and weighed. The percent moisture was calculated in the usual manner.

Determination of total, non-casein, and non-protein nitrogen Nitrogen determinations were conducted by the semi-micro Kjeldahl method of Rowland (1938), except that the ammonia was caught in 2.5% boric acid and titrated directly with 0.025 N HCl (Menefee and Overmann, 1940).

Determination of calcium and magnesium Approximately 50 mg of freeze-dried native calcium caseinate were weighed in platinium dishes, 5 ml of 0.06% (W/V) sodium oxalate (to distribute the light material) and 15 drops of concentrated HNO_3 were added, and the samples evaporated to dryness on a sand bath (80-85 C). The dishes were placed in a cold muffle furnace set at 450 C, the furnace was turned on and the samples were ashed for 24 hr. When a white ash was not obtained, a few drops of concentrated HNO_3 were added and the process was repeated. The ash in each dish was dissolved in 2 ml of 10% HCL (v/v), transferred quantitatively to a 25-ml g.s. volumetric flask and made to volume with redistilled water.

Calcium and magnesium were determined by the method of Bird et al. (1961) except that the bulk of the metastannate precipitate was removed by centrifuging in 250-ml Pyrex bottles in a size 2 International centrifuge, at 1000 x g for

10 min; the supernatant was filtered through Whatman, No. 40 filter paper.

Determination of total phosphorus Fontaine's (1942) spectrophotometric method was employed with a 1- or 2-ml aliquot of the solution resulting from the ashing procedure above. The total phosphorus was calculated from the following regression:

$$\text{mg phosphorus/25 ml} = \frac{\text{O.D.} - 0.00533}{29.2804}$$

Preparation of skim milk

Skim milk was obtained by centrifuging whole milk in 1-liter polyethylene bottles with a model PR-2 International refrigerated centrifuge at 2.5 ± 1 C at 2100 rev/min (1109 x g) for 90 min and the skim milk removed by a siphon.

Preparation of α_s-casein and κ-casein

α_s-Casein and κ-casein were prepared, from skim milk, for use as a standardizing guide in gel electrophoresis. The sulphuric acid procedure of Zittle and Custer (1963) was employed for preparing κ-casein, using four alcohol purifications. The α_s-casein was obtained from the by-product of the above preparation using the method of Zittle et al. (1959), after raising the pH to 4.6 with 2 N HCl. The α_s-casein was purified twice with alcohol according to the method of Zittle and Custer (1963).

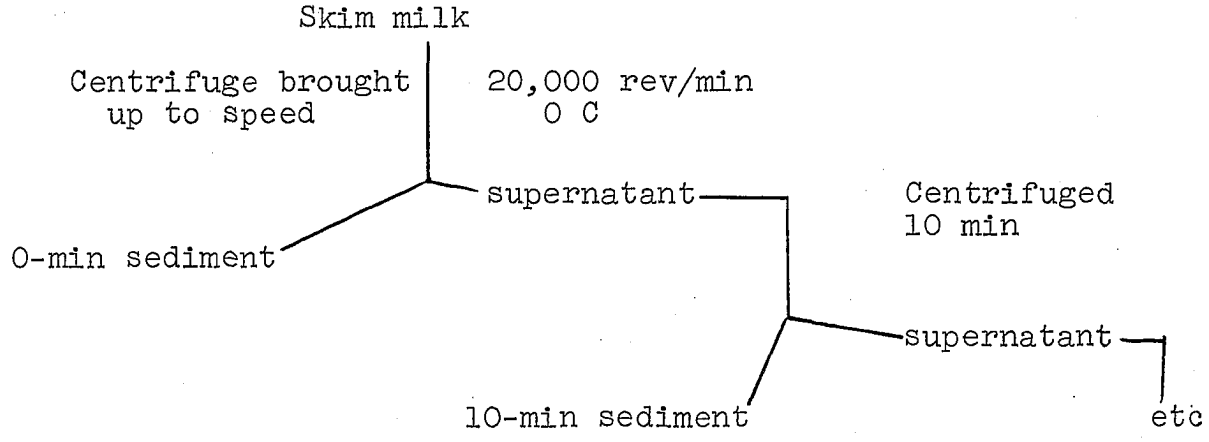
Preparation of isoelectric casein

Preparation 1 The casein was prepared by precipitation at pH 4.6 with 1 N HCl and peptized with NaOH at a pH $\leq$ 6.7; it was reprecipitated three times at pH 4.6 with 1 N HCl. The final casein preparation was washed three times with redistilled water and freeze-dried.

Preparation 2 The milk was from the same source as preparation 1, but obtained at a different period during the year. The casein was prepared by precipitation at pH 4.6 with 1 N HCl, peptized twice at pH $\leq$ 7.0 with NH_4OH and reprecipitated twice with 1 N HCl. The casein was washed three times with redistilled water, air dried between sheets of filter paper and soxhlet-extracted with ethyl ether overnight.

Ultracentrifugal fractionation of "native" calcium caseinates

Differential ultracentrifugation of the caseinate micelles Raw skim milk was obtained, June 6, 1963, as described above. The "native" calcium caseinates were differentially fractionated (serially depleted) by centrifuging in a Model L Spinco (No. 21 angle head) ultracentrifuge at 20,000 rev/min (maximum g force, 53,620) and 0 C. The filled centrifuge tubes were chilled in ice water, placed in the previously chilled rotor, the rotor was placed into the previously chilled centrifuge, and centrifuged. The supernatants, poured from the preceeding time increment, were used for the next time increment, as follows:



After pouring off each supernatant, the upper portion of the centrifuge tubes were wiped to remove any lipid material and a small amount of redistilled water added to the tubes. All sediments were redispersed, in the centrifuge tubes, in the redistilled water by gently rubbing the surface of the sediments with a glass rod. The centrifuge tubes, filled with the redispersed caseinates, the head, and centrifuge were chilled as above and the caseinates resedimented during 12 hr at 53,620 x g and 0 C. The supernatants were discarded and the resulting "washed" caseinates redispersed in redistilled water and freeze-dried for storage and later study. The dried caseinates were stored at room temperature in a vacuum desiccator over P_2O_5.

A problem of leaking tubes during centrifugation was encountered. This resulted in a reduced amount of supernatant so that only six time increments (0-, 10-, 20-, 30-, 40-, and 55-min) were obtained. Additional fractions, which

were thought to be analogous to those that would have been obtained at 70 and 80 min were obtained by centrifuging the same skim milk for 60 min, followed by fractionation of the supernatants at 10 min centrifuging intervals. These additional two fractions were washed and freeze-dried as previously described.

A composite of the above fractions were also obtained, from the same milk, by subjecting the skim milk to the same centrifugation conditions for 20 hours. The 20-hour sediment was washed as described above except that a 20-hour centrifugation was employed in sedimenting the caseinate after dispersion in water. The supernatant from the 20-hour centrifugation contained the "whey-soluble" caseinate, which was precipitated with 1 N HCl at pH 4.6 (not repeptized), washed three times by dispersing the precipitate in redistilled water and sedimenting at room temperature and 1000 x g. The whey-soluble casein was freeze-dried and stored as above. The total solids, and total nitrogen contents of the supernatants were determined. The freeze-dried caseinate fractions were analyzed for moisture, total nibrogen, total phosphorus, calcium and magnesium.

The caseinate fractions are designated according to the total centrifuging time at 53,620 x g (i.e., 0-min, 10-min, 20-min, 30-min, 40-min, 55-min, 60+10(70)-min, 60+10+10(80)-min, and 20-hour); the whey-soluble casein is designated

isoelectric "whey-soluble" casein.

Preparation of "total native" calcium caseinate An attempt was made to sediment all of the calcium caseinate from skim milk. Raw skim milk was obtained, October 26, 1963, as described above. The "native" calcium caseinate was sedimented in a Model L Spinco (No. 40 rotor) during 100 hours at 0 C and 40,000 x g (maximum g force, 144,700). The sediment from several tubes was dispersed as above and freeze-dried as an "unwashed" control. The sediment in the remaining tubes was washed and freeze-dried as previously described except that 100 hours at 144,700 x g and 0 C was employed in sedimenting the caseinate after dispersion in water. The supernatants and freeze-dried caseinates were analyzed for the same constituents as above. In addition, the supernatants and wash water were analyzed for casein nitrogen and non-protein nitrogen. The "total native" calcium caseinates are designated as 100-hour W (washed fraction) and 100-hour UW (unwashed fraction).

Moving boundary electrophoresis

Preparation of buffers

Veronal buffer A standard sodium veronal (barbital) buffer, pH 8.6 (at 2 C) and 0.1 ionic strength, prepared as described by Alexander and Block (1960, p. 202), was employed for isoelectric casein preparations. The pH was about 8.3 at 23 C.

Veronal-urea-buffer The standard veronal buffer containing 5 M urea was employed. No effect was made to adjust the resistance of this buffer to correspond to the resistance of the standard veronal buffer.

Veronal-oxalate buffer Sodium oxalate was chosen as a means of dispersing calcium caseinate. Since a non-standard veronal buffer was to be employed, it was necessary to use some uniform criterion for preparing the oxalate buffers because of the variation in calcium (Ca) and magnesium (Mg) contents of the caseinate fractions to be examined. A constant conductivity of sample free buffer was chosen. Sufficient sodium oxalate was added to a series of single strength veronal buffers, without NaCl, ($\Gamma/2 = 0.02$) to just react with the Ca and Mg in a 1.0% protein solution of the 20-hour fraction (7.01 mM/liter) and varying quantities of 5.0 M NaCl were added and their resistance determined. The veronal-oxalate buffer (7.01 mM oxalate/liter) having the same resistance as the standard veronal buffer above ($\Gamma/2 = 0.1$) was found to require 13.48ml of 5.0M NaCl/liter resulting in an ionic strength of 0.1084. The oxalate buffers for the other caseinate fractions were prepared by adding the prescribed amount of sodium oxalate, based on the Ca and Mg content of the individual fractions, and adjusting the ionic strength to 0.1084 with 5.0 M NaCl. The resulting veronal-oxalate buffers had resistances, within experimental error, equivalent to the

standard buffer above. The fact that the oxalate buffers could be prepared in this manner is probably due to the narrow range of Ca and Mg contents encounted in the caseinate fractions. No further adjustment of the pH was necessary when sodium oxalate was added to veronal buffer.

In some cases, veronal-oxalate buffers containing an excess of sodium oxalate were used. This was accomplished by replacing all of the NaCl with sodium oxalate. To obtain a buffer with a resistance equivalent to a standard veronal buffer (220 ohms); 0.0534 M/liter of sodium oxalate was required with a resulting ionic strength of 0.1802 in the sample free buffer.

Veronal-disodium ethylenediaminetetracetate (EDTA) buffer In order to obtain a comparison with the data of Annibaldi (1960, 1961), veronal buffer containing disodium dihydrogen ethylenediaminetetracetate dihydrate (EDTA) was prepared. Since EDTA is acidic, the veronal buffer was prepared as described by Alexander and Block (1960, p. 202) except that the HCl, employed to adjust the pH, was replaced with EDTA. Approximately 4.0 mM/liter of the dihydrate sodium salt of EDTA was required to adjust the veronal buffer to the desired pH of 8.6 at 2.0 C.

Preparation of "native" calcium caseinates for moving boundary electrophoresis The veronal-oxalate buffers, previously described, containing the appropriate amounts of

sodium oxalate were employed with all caseinate fractions. The calcium caseinates, 0.1g protein on a dry basis, samples were placed in 10 ml volumetric flasks and approximately 9.0 ml of the appropriate buffers added. The samples were allowed to hydrate (overnight at 4.4 C), warmed to room temperature and made to volume with the appropriate buffer. The Ca and Mg oxalates were removed by centrifugation at 1000 x g for 10 min at room temperature. The samples were placed in Visking seamless cellulose tubing, 26/32 inches inflated diameter (Visking Co., Chicago, Ill.) and dialysis carried out at 4.4 C, against the same buffer employed in dispersing the sample. Dialysis was carried out in 1-liter dialysis units (American Instrument Co., Silver Spring, Md.) consisting of a stirring motor, stirrer and an attachment for holding the cellulose tubing. The stirrer was designed to stir the contents within the cellulose tubing. The buffer outside the cellulose tubing was stirred by a magnetic stirrer. The samples were dialized for 24 hr against 2 changes (500 ml each) of buffer. After dialysis, the samples were removed from the cellulose tubing and centrifuged again at 1000 x g for 10-min at room temperature to remove the oxalates previously unsedimented due to their almost colloidal character. The resulting samples were in most cases, reasonably clear. When veronal oxalate buffers, containing excess sodium oxalate, were employed, the same procedure employed above was used.

When veronal-EDTA buffer was used, the above procedure was employed to disperse the caseinate samples except that centrifugation was not necessary. The sample plus buffer was dialized as above against standard veronal buffer, to simulate Annibaldi's conditions.

Moving boundary electrophoresis The procedure described in the Perkin-Elmer instruction manual for the closed system was employed with one exception, when 5.0 M urea was used in the veronal buffer a 3N NaCl solution, instead of the usual 1/3 saturated NaCl soltuion, was used to surround the electrodes because of the high density of the urea buffer. The electrophoretic cells were filled in a 4.4 C cold room and the buffer vessels charged with the second buffer dialysate. The cell was placed in the ice water bath of the apparatus (1.5-2.0 C) and allowed to equilibrate for 30-min. During this time, the hose and 2-ml syringe connecting the compensator to the closed side of the cell were filled with buffer. Care was used to eliminate bubbles.

The hydrostatic head created during this operation was released by opening the closed side of the cell for a few minutes. After the cell had equiliberated, the center section of the cell was pushed into alignment and the boundary brought into view on the ground glass screen with the compensator (using the small pully). Approximately 30-min was required to move the boundary 3-4 mm into the field of view. The

compensator was turned off and a current of 2 watts applied across the cell (about 14 mA). Pictures of the descending patterns, using Type 146-Polaroid transparent film and a Land Camera, were taken after approximately 1 hr of running time had elapsed (current on, bath stirrer off). Additional pictures were taken, after 1.5 hr, of the ascending pattern (current on) and the descending pattern (current off). One to two minutes exposure times were used depending upon the clarity of the patterns.

Conductivity measurements of the sample-free buffer and sample plus buffer were made, using the same bath as the cell, while the electrophoresis was in progress. The pH of the above solutions were determined at room temperature.

Analysis of the moving boundary electrophoretic patterns The patterns were enlarged (3-4 times) with an Omega D-3 Enlarger (Simmon Brothers, Inc., Long Island City, N.Y.) and the patterns traced on graph paper. The magnification factor was calculated by measuring the length of a 2.54-cm line enlarged the same amount as the pattern as follows:

$$\frac{(\text{enlarged line in cm})(1.06)}{2.54 \text{ cm}} = \text{magnification factor}$$

Where the 1.06 is the camera magnification factor (from the instruction manual).

Calculation of the relative percents of components in the pattern The Gaussian curves, as transparencies, of Weidemann (1947) were used in the enlarger to extend the

enveloping curve (outside curve) of the peaks in the pattern to baseline. The baseline was taken as the bottom of the entire pattern. In cases where incomplete resolution of the peaks was obtained, the method by Svedberg and Pedersen (1940, p. 296), as suggested by Longsworth and McInnes (1940), was employed; this method consists of drawing the Gaussian curves for each peak such that the sum of the areas of the peaks involved is equal to the total area under the enveloping curve. The relative areas under all peaks were determined by planimetry.

Calculation of electrophoretic mobilities The position of the bisecting line for each peak, used to determine the distance of migration, was determined with the aid of the Gaussian transparencies. In cases where the peak was asymetrical, the position of the bisecting line was determined by trial and error.

The electrophoretic mobilities of the casein components were calculated from the descending pattern as outlined by Alberty (1948) using the false boundary (ε peak) as the initial boundary as suggested by Tobias et al. (1952a). The actual distance that the peaks migrated was determined by measuring the distance on the enlarged tracing and dividing this distance by the magnification factor.

Gel electrophoresis

Horizontal urea starch-gel electrophoresis (USG) The

method employed was basically the one described by Smithies (1955) and Wake and Baldwin (1961) with a few minor modifications. Pieces of Plexiglas, 3 mm thick, were fastened to the top of the 6.0 mm gel trays with silicone grease and held in place with rubber bands forming a tray with a depth of 9.0 mm. The gels were prepared as described by Wake and Baldwin (1961), except that degassing was held to a minimum. When 2-mercaptoethanol (MCE) was used it was added after degassing according to the method of Neelin (1964). The gels were poured into the trays, covered with Parafilm and allowed to gel at 4.4 C for 16 to 20 hr. After gelling, the added pieces of Plexiglas were removed and the top 3.0 mm of gel, which contained the air bubbles was sliced from the gel with a 26 gauge Nichrome wire. The knife, previously described, was used to slice the gel perpendicular to the direction of current flow, 6.0 cm from the anodic end of the gel. The samples were applied by dipping 9.0 x 5.5 mm filter paper wicks (No. 319329, Beckman Instruments, Inc., Palo Alto, Calif.) into the sample and laying the impregnated filter paper against the cut surface of the gel. Care was exercised to eliminate air bubbles. The filter papers were placed about 6 mm apart and 5 mm from each edge enabling 8 samples to be run on a 14 cm gel. After placing the samples in the gel, the knife, restraining the gel, was removed and the cut surfaces of the gel were pushed together. The gel was wrapped

with Saran film except for 2.0 cm at each end. Both compartments of the buffer vessels were filled with borate buffer (pH 8.6), Poulik (1957). This compartment was bridged to the nearer one in each vessel with the Nylonge-sponge cloth. The buffer vessels were placed in a refrigerator at 3.0 C, 26.0 cm apart, with the electrode compartments the ones farthest from the gel. The poured gel tray, was supported by the buffer vessels. The buffer vessel containing the anode supported the end of the gel containing the samples so that the protein which in these experiments are negatively charged, migrate toward the cathode. The buffer wicks were placed on the gel so that they covered the exposed 2.0 cm on each end of the gel; they were held in place by Saran wrapped brass bars (12.0 x 1 x 0.5 cm). A potential of 180 volts, resulting in a current density of 40 mA, is applied across the system during 16 hr, or until the brown borate boundary has migrated a distance of 12.0 cm from the sample wicks. The current density at the end of the run is about 13 mA.

After the desired migration had occurred, the gel was unwrapped, the tray inverted onto a sheet of filter paper and the tray slowly removed. The sample wicks were removed and the gel sliced into two gels 3.0 mm thick. The desired thickness was obtained by placing strips of plastic, 3.0 mm

thick, along side the gel. A piece of filter paper was placed on the top of the gel and the top slice removed for staining. The filter paper adheres to the gel and facilitates handling the gel which is weak. One slice of the gel was stained with Nigrosine and the other with Amido Black 10B as described by Smithies (1955). The stained gels were washed in methanol-water-acetic acid (10:10:1) separately until no more background stain was removed. The gels were then stored by wrapping the wet gels in Saran film.

Dispersion of the "native" caseinates for urea starch-gel electrophoresis Two methods are available for redispersing the natural calcium caseinates for USG electrophoresis. The calcium caseinates were readily dispersed by the urea (6.6 M) and citric acid present in the gel buffer (urea-tris-citrate) and with urea-tris-citrate-oxalate buffers (0.01M $Na_2C_2O_4$). In the latter buffer, calcium was removed as the oxalate by centrifugation (1000 x g). The caseinate samples were dispersed in the buffers by allowing them to hydrate overnight at 4.4 C and were used within three days. All samples were dispersed in non-oxalated buffer for USG and urea-2-mercaptoethanol starch-gel (USG-MCE) electrophoresis. In addition, three of the caseinate samples were dispersed with the oxalate containing buffer for USG-MCE electrophoresis. In USG-MCE electrophoresis, the κ-casein of the samples were depolymerized (reduced) by adding 0.01 ml

MCE to 1.0 ml of the sample plus buffer (Woychik, 1964), and were allowed to stand at least 3 hr prior to electrophoresis.

Protein concentrations of samples for urea starch-gel electrophoresis

Urea starch-gels (USG) Preliminary experiments indicated that good resolution of the caseinates was obtained in USG at protein concentrations between 1 and 2 percent with 1.5 percent appearing to be optimal. Subsequent gel electrophoresis of caseinates were performed at 1.5 percent protein except the 60+10+10(80)-min fraction, which was electrophoresized at a protein concentration of 0.75 percent because of the limited supply of this fraction. The isoelectric whey-soluble (at 0.0 C) portion of the caseinates (precipitated at pH 4.6 with 1 N HCl from the supernatant of skim milk that had been centrifuged for 20 hours at 53,620 x g and 0 C) was employed at a protein concentration of 0.75 percent because of its high β-casein content, to eliminate excessive lateral diffusion. The α_s-casein protein concentration was 0.5 percent to eliminate excessive lateral diffusion. κ-Casein was electrophoresed at 1.0 percent protein so that the extensive smearing would show up readily on photographs. Isoelectric casein preparation 1 was electrophoresised at 1 and 2.0 percent protein to insure the visibility of minor components on photographs and to determine the effect of protein

concentration on the relative amounts of α_s- and β-casein.

Urea mercaptoethanol starch-gels (USG-MCE) All caseinate samples contained 1.5 percent protein except the 60+10+10(80)-min fraction which contained 0.75 percent protein due to the limited supply of the fraction. The α_s-casein and isoelectric whey-soluble casein samples contained 0.5 percent protein so that the major band would not show excessive lateral diffusion. The κ-casein protein concentrations was 0.21 percent, which is approximately the κ-casein concentration in the caseinate fractions based on the assumption that the caseinates contain 14 percent κ-casein (0.14 x 1.5 = 0.21 percent). This was done in order to establish which κ-casein bands would be visible at the caseinate concentrations employed so that the traces could be analyzed on a sound basis. The isoelectric casein preparation 2 was electrophoresised at 0.75 and 1.5 percent protein to determine the effect of protein concentration on the relative amounts of α_s- and κ- and β-casein.

Preparation of transparencies for analysis of urea starch-gels (USG) The major objection to urea starch-gel electrophoresis, for this study, is the problem of clearing the gels for densitometry. A number of methods for clearing starch-gels (made opaque because methanol is used in the washing solvent for destaining) for their preservation and/or densitometry are described in the literature. Most of these

methods employ a basic plastisizing treatment of the gels with glycerol, with variations in technique, from method to method. Wake and Baldwin (1961), using glycerol, describe a method for plastisizing and drying urea starch-gels, but they do not discuss densitometry. An attempt was made to clear gels for densitometry using the Wake and Baldwin procedure, but satisfactory results could not be obtained. Baur (1964) states that previous methods, for clearing starch-gels, causes their contraction and distortion; he suggested using glycerol with drying of the gels on a glass plate. Baur (1964) covered the gels with water permeable cellulose sheets and dried the gels in a warm air stream. The method of Baur (1964) was employed with some of the urea starch-gels in this study. Reasonably clear gels were obtained. However, smooth gel surfaces were difficult to obtain due to formation of air bubbles between the gels and the cellulose sheets during drying. This resulted in rough spots on the surface of the gel, which had an adverse affect on the densitometer traces. To eliminate the problems, transparent prints were employed to analyze the gels.

After the analyses of the gels (transparent prints) were completed, it was discovered that Goldberg (1958) had suggested and used transparent prints to analyze starch-gel electrophoretic patterns of hemoglobulin. Gratzer and Beaven (1960) stated that "this method cannot give valid results since

emulsion blackening is not directly proportional to the gel zone optical density (i.e., pigment concentration)". Their argument is an obvious one, but would not seem to apply in this study, since comparison among patterns rather than among bands in individual patterns is the primary aim in the analysis of the urea starch-gels (with and without mercaptoethanol).

Electrophoretic patterns Pictures of the gels were taken with Kodalith Ortho type 3 film (Estar base), Eastman Kodak Co., Rochester, N.Y. A 4 x 5 in. speed Graphic camera, Graflex Inc., Rochester, N.Y., without a filter, was employed. The gels were submerged in the washing solvent (methanol, water, acetic acid, 10:10:1) and illuminated with two 500 watt photo flood lamps with a shield on the camera to prevent reflections.

Positive transparencies were printed with a contact printer. In both the negatives and prints, extreme care was taken while agitating them during developing to insure even development.

Analysis of urea starch-gel electrophoretic patterns

Identification of bands in urea starch-gel (USG) patterns The method of Wake and Baldwin (1961) for numbering bands was used. The method consists of arbitrarily choosing a reference band (second sharp band behind the major α_s-band) and relating its migration to the migration of the

other bands. This method is analogous to using a reference compound, glucose for example, in paper chromatography of sugars and dividing the distance each sugar migrates by the distance that the glucose migrates; values are reported as R_g.

A schematic diagram of a pattern of isoelectric casein, preparation 1 (IE p-1), is shown in Figure 1. The relative band positions agree quite well with those reported by Wake and Baldwin (1961), except that these workers indicate the occurrence of several minor bands between the No. 13 and 14 bands in Figure 1. The relative positions of the various genetic variants of α_s- and β-casein are also in excellent agreement with the data of Schmidt (1964). The data of Neelin et al. (1962), who used a number of different buffers, are difficult to compare with our data, because the buffer used will have considerable effect on relative band positions and the authors merely numbered the bands. An exact identification of the minor bands is considered impossible because of a limited knowledge concerning the effect of the methods of isolating whole casein and its fractions; therefore, only the major α_s- (Figure 1, band 5, 6, and 7) and β- (Figure 1, bands 12 and 13) casein bands will be considered in the analysis of transparent prints of the patterns. The minor bands (Figure 1, 8, 9, 10 and 11), however, are also included in the traces of the transparencies because they are involved in

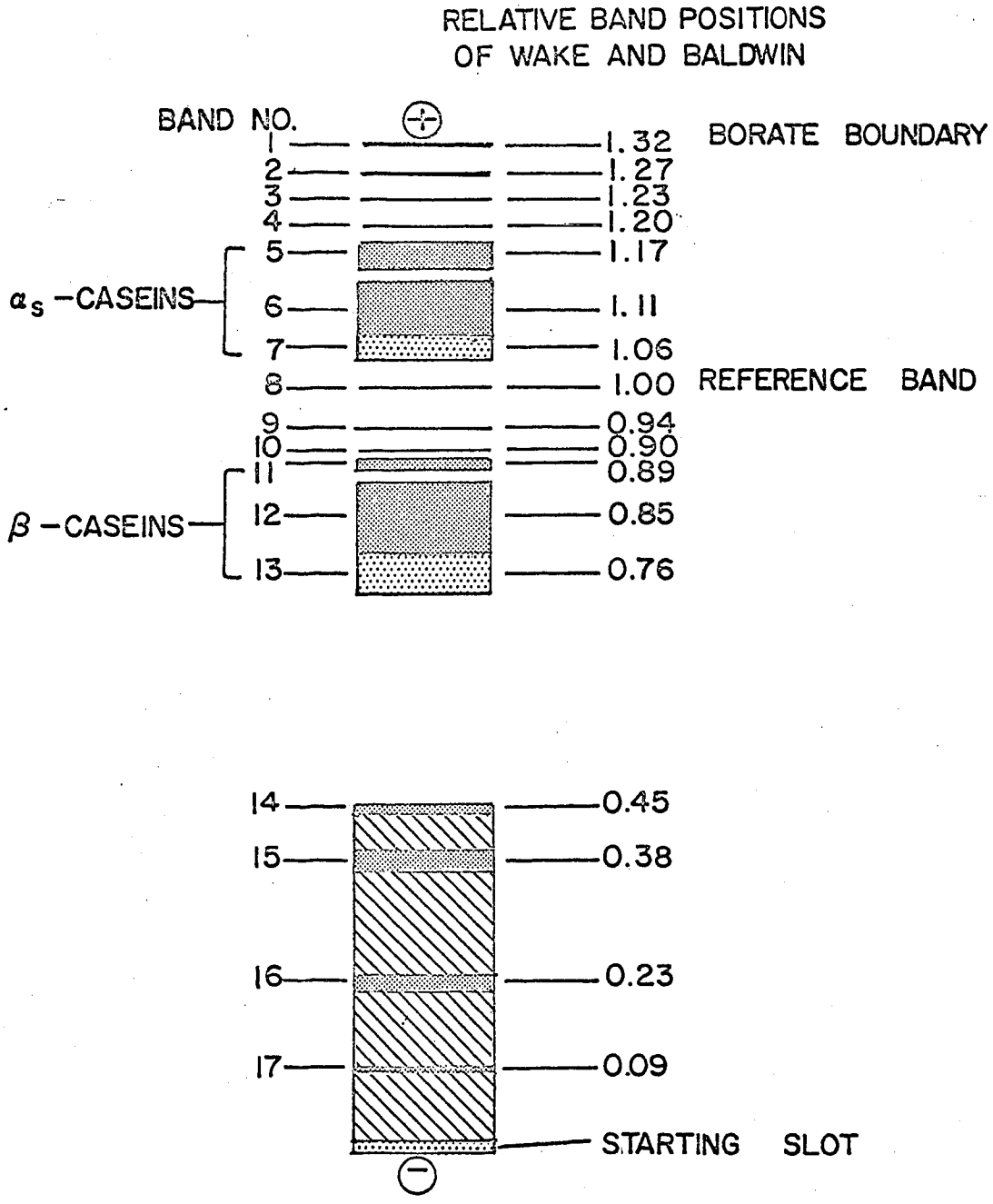


Figure 1. Schematic diagram of a horizontal urea starch-gel electrophoretic pattern, of an isoelectric casein, obtained in this study.

establishing the baseline of the densitometer traces.

It is interesting to note, that Wake and Baldwin (1961) are of the opinion that bands 14, 15, 16 and 17, which appear in urea starch-gels, are related to γ-casein. They show that these bands are not attacked by rennin whereas κ-casein, which migrates as a smear in this region, disappears. Likewise these four bands are greatly intensified in γ-rich fractions. However, the inability to obtain highly purified γ-fractions makes further inferences concerning these bands questionable. Since γ-casein migrates as a smear in the region of bands 14, 15, 16 and 17 (Figure 1), without MCE this region is ignored in this section.

Identification of bands in urea starch-gel patterns with 2-mercaptoethanol (MCE) A schematic diagram of isoelectric casein and κ-casein is presented in Figure 2. The two major bands, 16 and 17, Figure 2, of κ-casein agree closely in band position with the data of Schmidt (1964). However, Schmidt (1964) observed an increase in relative migration of the β-casein bands, which was not observed during this study. Neelin (1964) reported casein USG patterns using MCE. His data are difficult to compare with ours. Estimates of relative mobilities from Neelin's (1964) photographs show a lower relative mobility for the κ- and β-casein components than was found in this study. The system of whole numbers (starting at the cathode end) shown in Figure 2, yields a

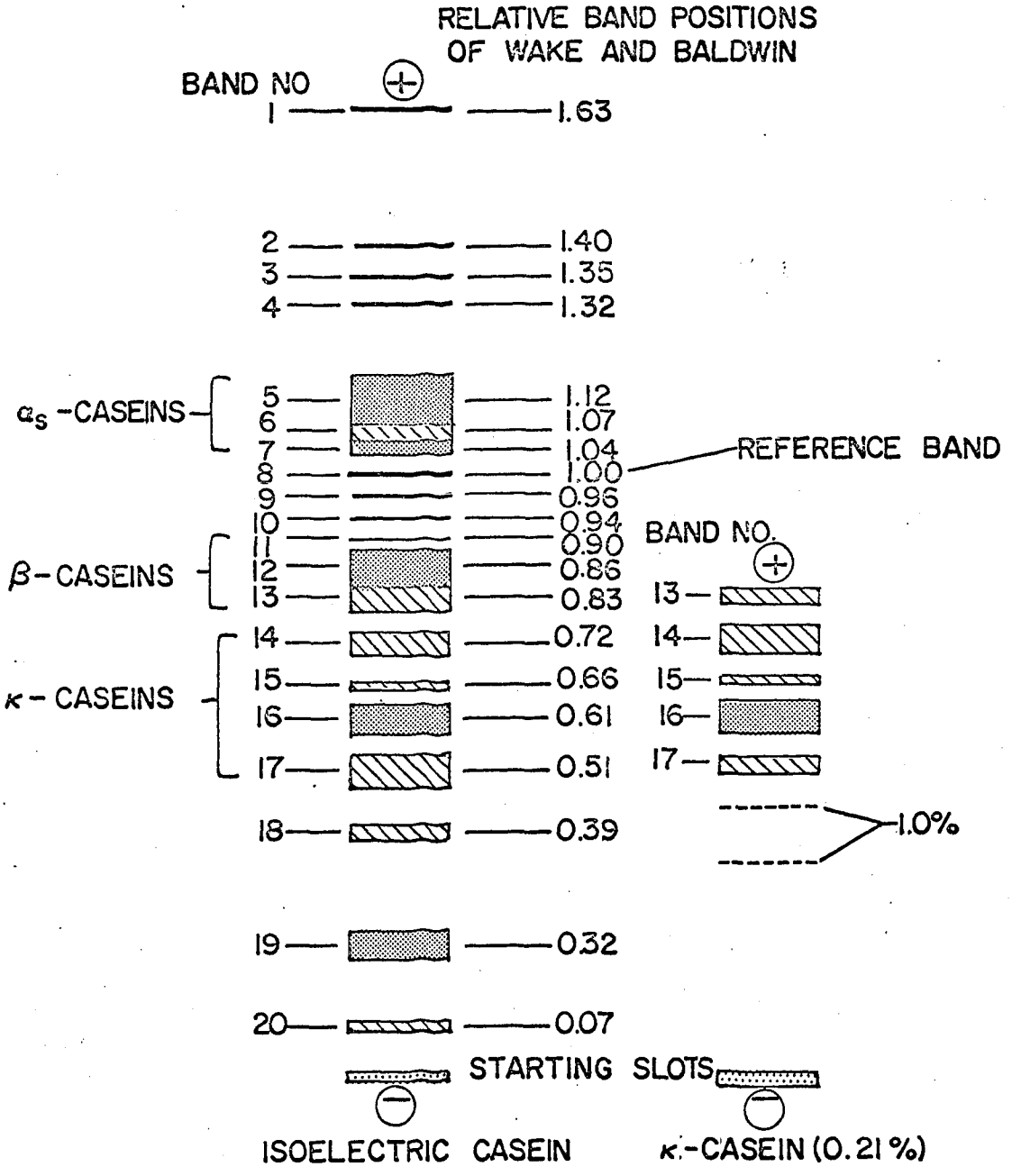


Figure 2. Schematic diagrams of horizontal urea-2-mercapto-ethanol starch-gel patterns of isoelectric casein and κ-casein obtained in this study.

band sequence in general agreement with Neelin (1964).

The characteristics of the bands of USG are changed with MCE is used. The most striking difference is that, although the borate boundary line was allowed to migrate the full 12.0 cm, the migration distances of the components from band 5 through 20 (Figure 2) were shorter. Since the reference band (9, Figure 2) is included, the relative mobilities of these bands (5-20) agree quite well with gel patterns without MCE, the decrease in migration appears to be uniform. The above difference may be responsible for the very faint pre-α_s-casein (bands 1 through 4, Figure 2) bands when MCE is used, compared with the better resolution of these bands when it is not.

Isoelectric casein USG-MCE patterns gave the desired resolution of κ-casein. κ-Casein, Figure 2, resolved into two major bands 16 and 17, and several minor bands in agreement with Neelin (1964), Schmidt (1964), and Mackinlay and Wake (1964). Band 16 (Figure 2) was the most pronounced of all the κ-casein bands.

Densitometry of urea starch-gel transparent prints

Transparent prints were taken of the gels as previously described. The prints were cut into strips each representing a pattern of a single preparation and traces were obtained with a Photovolt Densitometer model 525 using a Photovolt Varicord Recorder model 42A (Photovolt Corp., New York, N.Y.). The

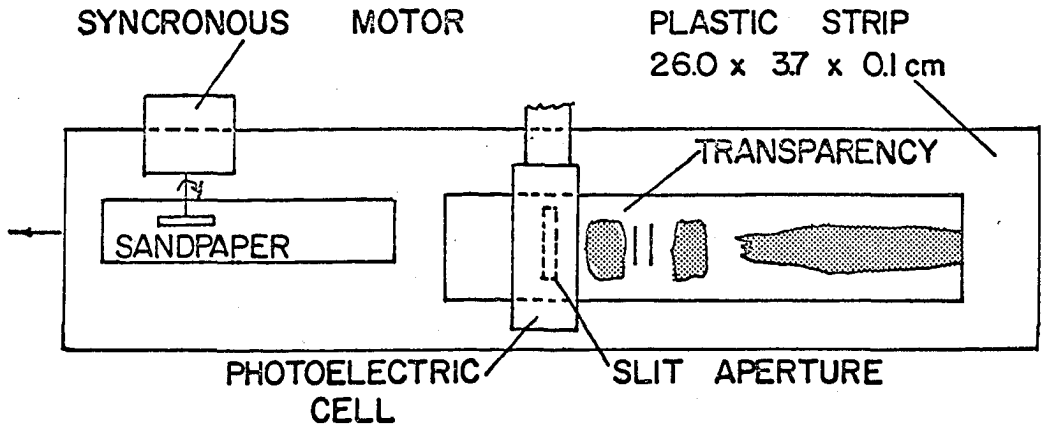


Figure 3. Diagram of the method employed to scan transparencies of urea starch gel and urea 2-mercaptoethanol starch gel electrophoretic patterns with the densitometer.

synchronous motor assembly on the transmission density unit was displaced 3.0 cm to the left (using holes already present in the cover) in order that the drive wheel would not slip on the transparencies during the last half of the trace. The transparent strip and a piece of sandpaper (Figure 3) were taped to a strip of plastic (26.0 x 3.7 x 0.1 cm). The sandpaper was employed to eliminate slipping.

With USG or USG-MCE gels, the bands representing the components are not all the same length. Therefore, the slit aperture on the densitometer was masked with black electrical Scotch tape to yield a slit length equal to the longest band in the pattern to be scanned. Although this method decreases the accuracy with which the shorter bands can be traced because of extraneous light, the longer bands, α_s- and

β-caseins, were considered more important in this analysis. Likewise it was thought that the length of the bands was a function of the protein concentration presented by the bands.

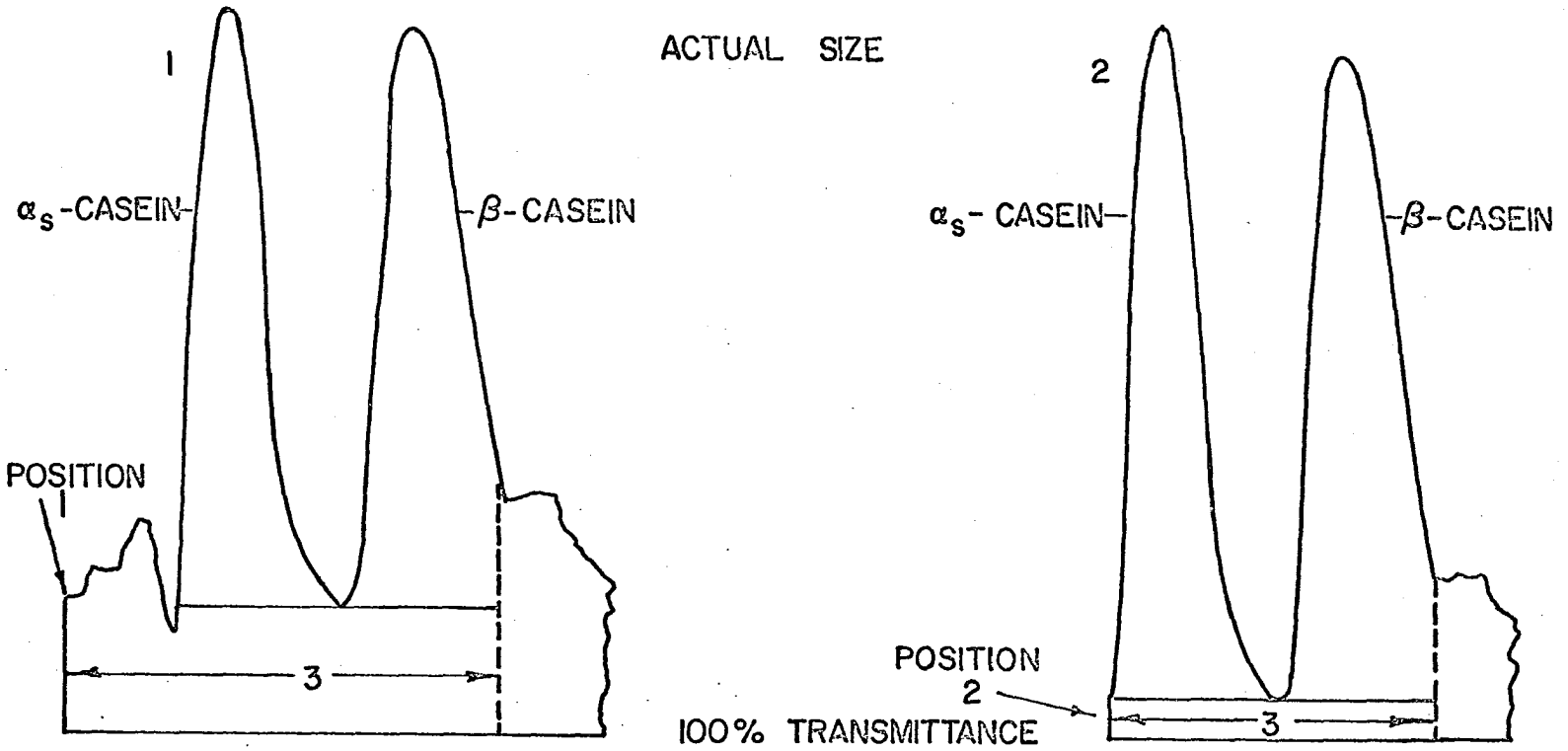
The No. 1 response on the recorder was used to obtain a linear response for all the traces. The range setting on the Electronic Photometer was set on the lowest range, usually No. 1 or 2, that gave 100 percent transmittance (zero optical density) on the photometer and still allowed the dark zero on the photometer and recorder to be set. The recorder chart speed was 7.7 cm/min and the syncronous motor drove the plastic strip 4.2 cm/min resulting in an enlargement of the pattern on the transparent strip of 1.83 in the traces.

Analysis of the densitometer traces The major problem in the analysis of the traces was the difference in background depth among and within the transparencies and the fact that the slit aperture length was not always the same. To eliminate errors in this regard, a consistant but arbitrary method for determining the baseline was used, because comparison of patterns was desired.

Urea starch-gel (USG) patterns Since κ-casein does not resolve into discreet bands with this system, only the α_s- and β-casein components were considered. The base line was arbitrarily taken at the minimum between these two peaks. Two positions on the transparencies, the first just ahead of the borate boundary line, 1, and the second just

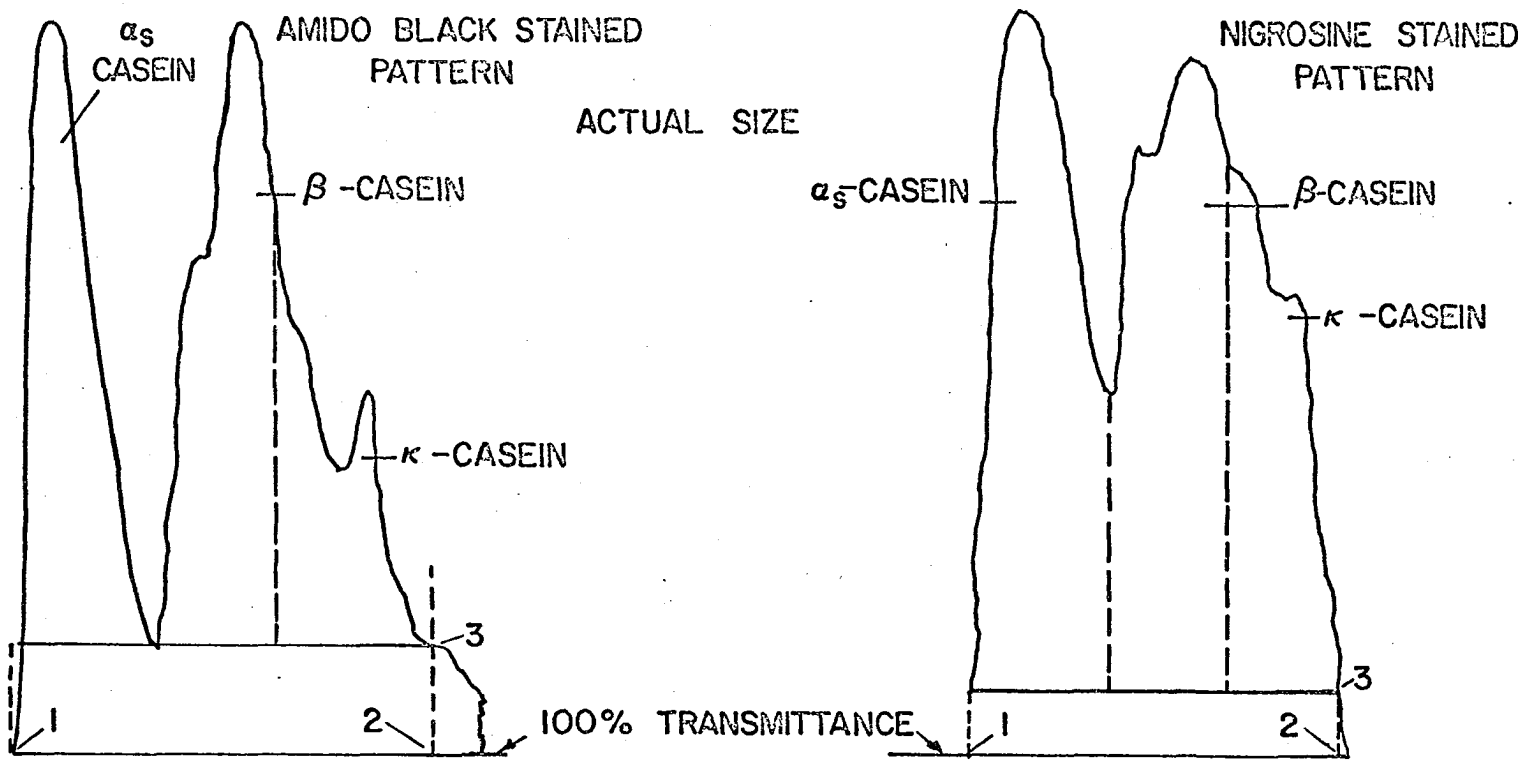
ahead of the major α_s-band, 2, (band 5, Figure 1) were used to set the zero on the densitometer (Figure 4). The distance from the zero positions (band 2), to the point that just included the β-casein peak were measured on the transparency, multiplied by the magnification factor (1.83) and these distances were laid-off on the traces. The patterns were traced, the traces cut out and the peaks were weighed.

Urea starch-gel with 2-mercaptoethanol (USG-MCE) patterns With this treatment, κ-casein forms descreet bonds, at least for isoelectric preparations. In order to obtain estimates of the κ-casein contents as well as the α_s- and β-casein in the samples, a different method of determining the base line was used. Since there appeared to be excellent agreement between the two methods of setting the densitometer zero in the USG patterns, only the zero position before the major α_s-band was employed. This was necessary due to the very faint borate line which was obtained with the USG-MCE patterns. The distance from the zero position on the transparency (the point just ahead of the major α_s-casein band, No. 5, Figure 2) to and including κ-casein (band 17, Figure 2), was measured on the transparency and was multiplied by the magnification factor (1.83) to convert it to the distance of the equivalent portion of the trace. This distance was laid-off on the trace (Figure 5) from the same zero position. At the end of this distance, the point farthest from the zero



1 Densitometer zero set just ahead of the borate boundary, position 1.
2 Densitometer zero set just ahead of the major α_s-casein band, position 2.
3 Distance determined from the picture or transparency, multiplied by the magnification factor (1.83) and laid-off on the traces.

Figure 4. Method of determining the baseline on the densitometer traces of urea starch-gel electrophoretic patterns.



1 Densitometer zero set just ahead of the major α_s-casein band.

2 Distance to band 17, Figure 2, as measured from the picture or transparency.

3 Intersection of a verticle line from point 2 to the densitometer trace. The calculated baseline is a horizontal line from point 3.

Figure 5. Method of determining the position of the baseline on the densitometer traces of urea, 2-mercaptoethanol, starch-gel electrophoretic patterns.

position, a line is drawn perpendicular to the bottom line of the chart, on which the trace is drawn. The point at which this perpendicular crosses the trace is taken as the baseline position. In Figure 5, (1) is the zero position, (2) the point at which the perpendicular is drawn and (3) the point of intersection of the perpendicular and the trace, i.e., the point at which the baseline is established, parallel to the bottom boundary of the chart. The disecting lines for the separation of the major components, on the traces, were checked with distances on the original pattern. No problems were encountered using this technique for the Nigrosine stained patterns. However, two caseinate fractions (10 min and 20 min) stained with amido black had minima between the α_s- and β-casein peaks below the calculated baseline. In these cases, the baseline was drawn at the calculated point and the low minima were ignored.

Disc electrophoresis The basic standard gel method of Ornstein and Davis (1961) as described in the Canalco Manual (revised March, 1962), together with subsequent modifications described in the Canalco Disc Electrophoresis Newsletters, was used with two modifications. To prevent back diffusion of unpolymerized sample gel, which would cause a partial loss of sample, the gels were covered with about 0.04 ml of spacer gel solution, which was covered with buffer (tris-glycine pH 8.3) and the gel then was photopolymerized.

The second modification was in the preparation of the sample gel solutions, from dry ingredients, with which HCl (Ornstein and Davis, 1961) rather than H_3PO_4 (indicated in the Canalco manual) was employed to adjust the pH of the buffer used with the upper gel. An attempt was made to use sucrose rather than sample gel in the disc electrophoresis experiments as suggested by Narayan et al. (1964). However, the data implied that diffusion of some of the sample into the upper buffer compartment took place so the method was abandoned.

Electrophoresis was carried out at room temperature with a current density of 5 mA per tube (initial voltage 750-790, final voltage 600-650 when 12 tubes were used). The electrophoresis was completed in about 50 min. The procedure, as described in the Canalco Manual, uses no inhibitor in the lower gel thus speeding up the polymerization and eliminating wavy bands. Six tubes could easily be prepared at one time under these conditions when non-urea gels were used. Urea appears to speed up the polymerization; only four tubes could be prepared at one time.

Dispersion of calcium caseinates for disc electrophoresis

Disc electrophoresis without urea Three reagents for dispersing the caseinates were compared, namely, sodium oxalate, citric acid and disodium ethylenediaminetetra-acetate (EDTA). The buffers used in the sample gel were changed to incorporate an excess of the above reagents as

follows:

Tris-EDTA Buffer

5.98 g Tris (2-Amino-2(hydroxymethyl) 1-3 propanediol

0.2 g EDTA

2.0 N HCl, to adjust the pH to 6.90 (about 22.4 ml)

Made to 100 ml

Tris-Citrate Buffer

5.98 g Tris

1.0 M Citric acid, to adjust the pH to 6.90 (about 15 ml)

Made to 100 ml

Tris-oxalate buffer

5.98 g Tris

10.0 ml, 0.1 M sodium oxalate

2.0 N HCl, to adjust the pH to 6.90 (about 24.0 ml)

Made to 100 ml

The sample gel solutions were prepared, using the above buffers, as described in the Canalco Manual.

The caseinates were dispersed in the sample gel solutions (1.0 per cent protein), hydrated overnight at 4.4 C and diluted with spacer gel to the desired concentration (usually 100 μg of protein 0.1 ml of sample gel solution). With the oxalate containing buffered gel solution, the calcium and magnesium oxalates were removed by centrifuging (1000 x g) at room temperature for 10 min prior to diluting with spacer gel

solution. The electrophoresis was accomplished using the standard gel procedure in the Canalco Manual.

Disc electrophoresis with urea Two sample gel solutions were employed: 1) Sample gel solution containing 6.6 M urea and 2) sample gel solution 1) containing oxalate. A number of variations in technique were also studied. In all cases, the sample gel solutions contained 6.6 M urea; these were used in conjunction with the spacer and lower gel combinations tabulated below. These combinations were used with both the oxalate-urea and urea sample gel solutions.

Spacer gel 5.0 M urea, Lower gel 5.0 M urea
Spacer gel 5.0 M urea, Lower gel 6.6 M urea
Spacer gel 6.6 M urea, Lower gel 6.6 M urea

Because the procedure for preparing the working gel solutions includes a final 1:1 dilution of the upper gel with water, or a catalyst solution in the case of the lower gel, some problems were encountered in attaining these high urea concentrations. The 5.0 M urea concentration in the spacer gel was obtained by diluting the premixed gel solution (see above) with an equal volume of aqueous 10 M urea solution. The lower gel containing 5.0 M urea was obtained by diluting the lower gel solution (solution 1) with a catalyst solution (solution 2) that was 10 M in urea.

The 6.6 M urea concentrations were prepared using a slightly different method, because urea, equivalent to a

concentration of 13.2 M is completely soluble only with heating. The lower gel solution 1 was made 3.2 M in urea so that the completed lower gel would be 6.6 M urea when mixed with an equal volume of solution 2 (catalyst) that was 10 M in urea. The premixed spacer gel (Canalco) was made 6.6 M in urea by adding 39.6 g of urea to 50 ml of undiluted spacer gel solution and diluting to 100 ml. The sample gel, prepared from dry ingredients, was made 6.6 M in urea by adding the urea when the stock solutions were mixed in the amounts stated in the Canalco Manual. The pH values of all the urea gel solutions, which increase slightly because of the urea, were adjusted with 1.0 N HCl, to values given in the procedure e.g. pH 6.7-6.9 for the upper gel, pH 8.7-9.0 for lower gel. In the case of the urea-oxalate dispersion, the same buffer was employed without urea. The caseinates were dispersed as described in the non-urea gel section.

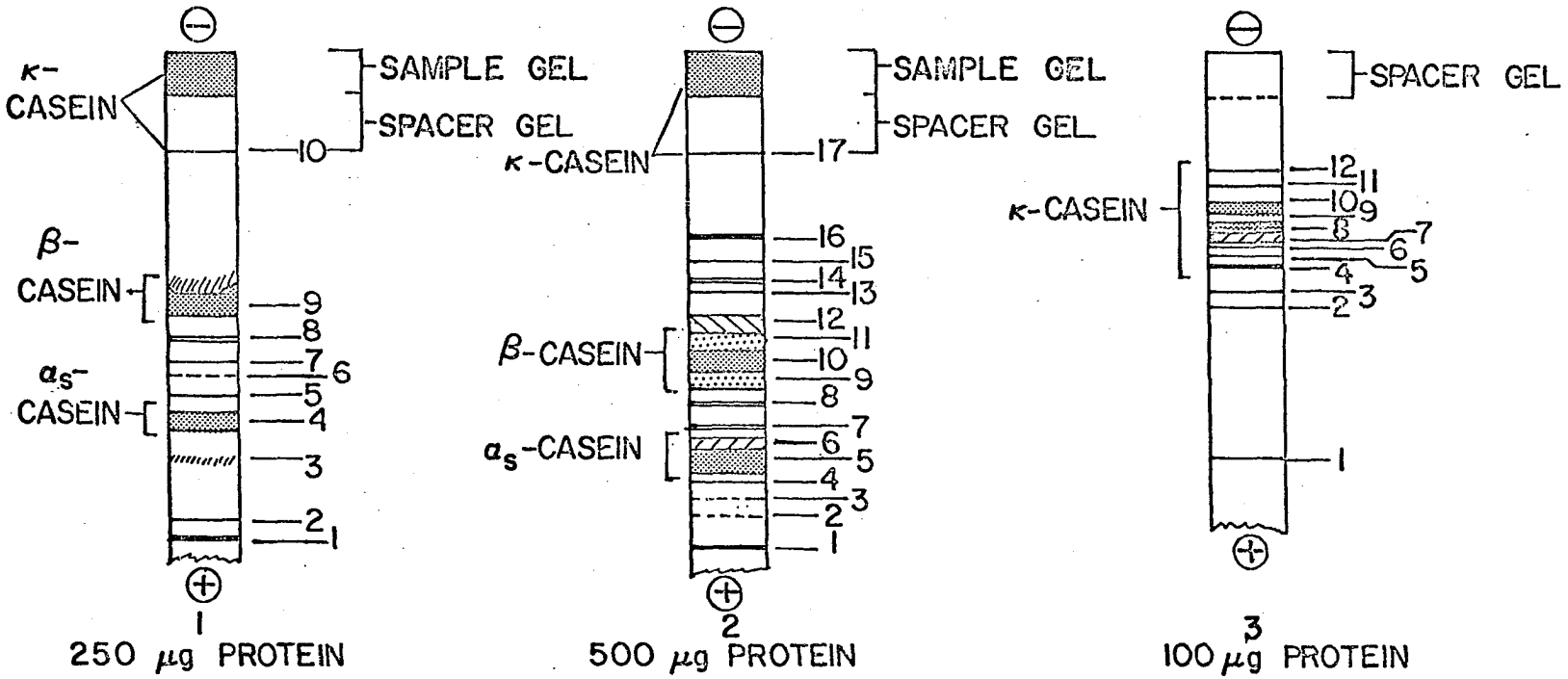
Disc electrophoresis with urea and 2-mercaptoethanol (MCE) The use of MCE did not alter the basic procedure. MCE was added to the sample gel solution. (0.01 ml/ml of solution) at least 3 hr before electrophoresis (Woychik, 1964). Preliminary experiments indicated that MCE could not be added to the spacer gel because it completely inhibited photopolymerization. One of the fundamental requirements of disc electrophoresis is that the spacer gel must gel and have a perfectly flat minescus, therefore, MCE could not be used in

the spacer gel. Since Woychik (1964) reported that it was not necessary to add MCE to acrylamide gels (Cyanogum), MCE was added only to the sample gel solution.

Identification of bands in disc electrophoretic patterns

Polyacrylamide gel (PAG) electrophoresis has not been used as extensively for casein as had urea starch-gel (USG) electrophoresis. The numbering system of Wake and Baldwin (1961) for USG electrophoresis has been employed for the bed type PAG electrophoresis (Thompson and Pepper, 1964, Thompson et al., 1965), but little or no work has been done using the disc technique. The gel patterns obtained using 7.5 percent PAG are not identical with USG patterns (Woychik, 1965). The major objection to disc electrophoresis is the variability of the rate of tracking dye migration which results in reproducible patterns but not reproducible positions on the gel. This makes it impossible to use the Wake and Baldwin (1961) method of designating the bands on the basis of relative band positions. A simple numbering system, based on an isoelectric preparation will be presented and used.

Non-urea disc electrophoretic patterns A schematic diagram of a non-urea pattern of isoelectric preparation is shown in Figure 6 (Pattern 1). Ten bands, seven clearly defined, were obtained without urea. Band 1, Figure 6, Pattern 1, appeared on all gels and is in about the same position as the tracking dye or front. Since the tracking dye



1) Non-urea disc electrophoretic pattern of an isoelectric casein.
2) Urea disc electrophoretic pattern of an isoelectric casein.
3) Urea 2-mercaptoethanol disc electrophoretic pattern of a κ-casein preparation.

Figure 6. Schematic diagrams of polyacrylamide disc electrophoretic patterns obtained in this study.

was not fixed to protein and is ionic, it is electrophoresed from the gel during the destaining process.

Neelin, et al. (1962) suggests that the component moving with the front in USG could be the λ-casein component of Long et al. (1958). Good resolution between α_s- and β-casein is obtained and κ-casein does not migrate into the lower gel. Band 10, at the origin of the lower gel, may be the simpler polymers (or smaller aggregates) of κ-casein that are able to migrate through the more porous upper gel but too large to penetrate the smaller pores of the lower gel. The major portion of the κ-casein and possibly other components (Neelin et al., 1962) are trapped in the sample gel.

Urea disc electrophoretic patterns A schematic diagram of a urea pattern of an isoelectric casein preparation (No. 1) is shown in Figure 6 (Pattern 2). Seventeen bands, 15 quite distinct, were obtained with excellent resolution although κ-casein does not appear to migrate into the lower gels even in the presence of urea. In some cases a visible smear was obtained when high concentrations of the κ-casein preparation were employed (> 100 μg of protein tube). The disc pattern of isoelectric casein correlates reasonably well with the USG pattern, Figure 1, especially as regards the major α_s- and β-casein bands, except that a sizable pre-β-casein component (bands 9, Figure 6, Pattern 2) was observed. Similar variation in the minor banding was also observed.

Urea-mercaptoethanol (MCE) disc electrophoretic patterns A schematic diagram of a urea-MCE pattern of κ-casein is shown in Figure 6 (Pattern 3). Twelve bands were observed, the first 3 bands (1 through 3, Figure 6, Pattern 3) may be contaminants in this κ-casein preparation. Band 1 (Figure 6, Pattern 3) is very faint compared to the corresponding bands in Figure 6 (Patterns 1 and 2). Bands 2 and 3 probably correspond to bands 7 and 8 in Pattern 2 and bands 6 and 7 in Pattern 1, Figure 6. The general pattern agrees quite well with the ones separated by Woychik (1964, 1965) for vertical bed type PAG. Further results obtained with disc electrophoresis using MCE were of little value, hence a schematic diagram of whole isoelectric casein is not included. Apparently MCE, which inhibits photopolymerization of the sample gel has some affect on the surface of the already polymerized spacer gel for the α_S-casein and β-casein bands were quite irregular, nonreproducible and unsuited for densitometry. No reason can be given for the fact that generally the κ-bands were not affected.

Densitometry of disc electrophoretic gels Densitometry, using the model E microdensitometer (Canalco, Bethesda 14, Maryland) was relatively simple. The B-filter position, for Amido Black stained gels, with the gain control set on the number 5 position (for a linear response of the optical density) and a slow chart speed (setting 9) was used. The

intergrator, usually set to give a blip for every 20 mm^2 was employed. In most cases, both the 2.0 and 0.2 optical density settings were used, because resolution of all bands could not be obtained at one sensitivity. However, the great differences in concentration between the major and minor bands made it impossible to measure them both at one sample concentration. Because the major bands only have been identified, sample concentrations that afforded good densitometer measurements of these bands were used (usually 100 µg protein tube).

The magnification of the gels by the densitometer was 3.6. The gels traveled across the slit apperature at the rate of 8.64 cm/min and the chart speed was 16.1 cm/min. These conditions gave a magnification for the tracing of 6.71. Efforts to correlate actual distances on the gel and traces, as was done with the USG-MCE patterns, proved unsatisfactory due to the difficulty in measuring distances on the disc gels. Therefore, an arbitrary method of dividing the traces was employed.

Non-urea disc electrophoretic patterns A typical densitometer trace of citric acid dispersed "native" calcium caseinate without urea is shown in Figure 7. The banding of the caseinates was not as sharp as for isoelectric casein, especially for β-casein. The bands between the α_s- and β-casein bands were variable and some problems were encountered in background as well as in tailing of the major bands. A

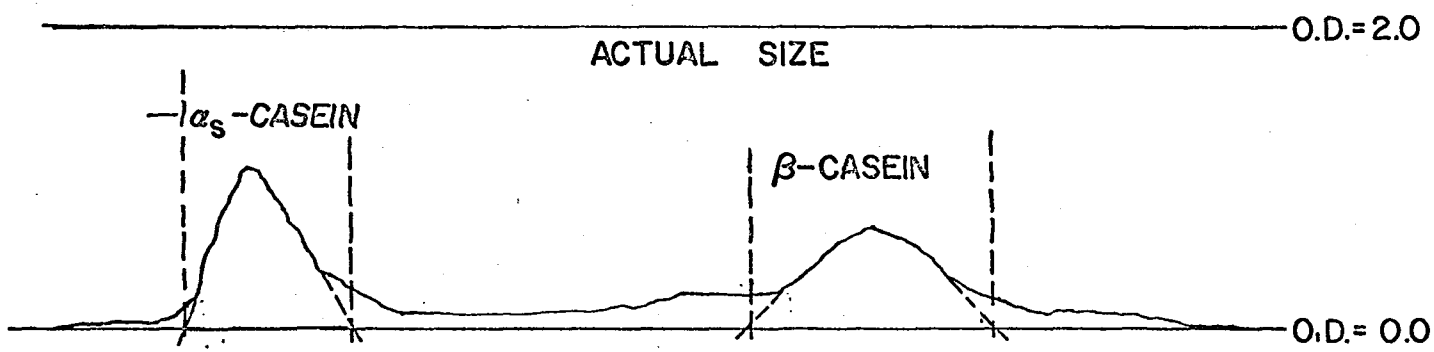


Figure 7. Method of dividing a typical polyacrylamide disc electrophoretic densitometer trace of citric acid dispersed native calcium caseinate without urea; optical density setting = 2.0.

number of arbitrary methods for dividing the tracing were tried and the method diagramed in Figure 7 was chosen.

The method consists of extrapolating the slopes of the major peaks to baseline, the area under the tracing between the lines perpendicular to the chart baseline at the points at which the extrapolations cross the baseline was considered α_s- or β-casein. Relative areas were calculated using the intergrator.

Urea disc electrophoretic patterns A typical densitometer tracing of native calcium caseinate in urea gels is shown in Figure 8. Both the low (a) and high (b) sensitivities are shown. The banding of the α_s- and β-casein components was much sharper than when urea was not used, especially as regards β-casein. No bands corresponding to bands 6, 7, 8 and 9, Figure 6 (Pattern 2), were observed between α_s- and β-casein, hence the tracing reached the baseline between the two bands. The division between α_s- and β-casein was taken at one-half the distance between the two maxima (Figure 8). Faint but discreet banding, obscured by slight smearing was obtained behind the β-casein band, corresponding to bands 13 through 16 in Figure 6 (Pattern 2). A consistent but arbitrary method for determining the division between the end of the β-casein peak and the remainder of the trace was used. The usually straight slope on the trailing edge of the β-casein peak was extrapolated until it bisected the baseline (Figure 8). A vertical line through this point was taken as the end

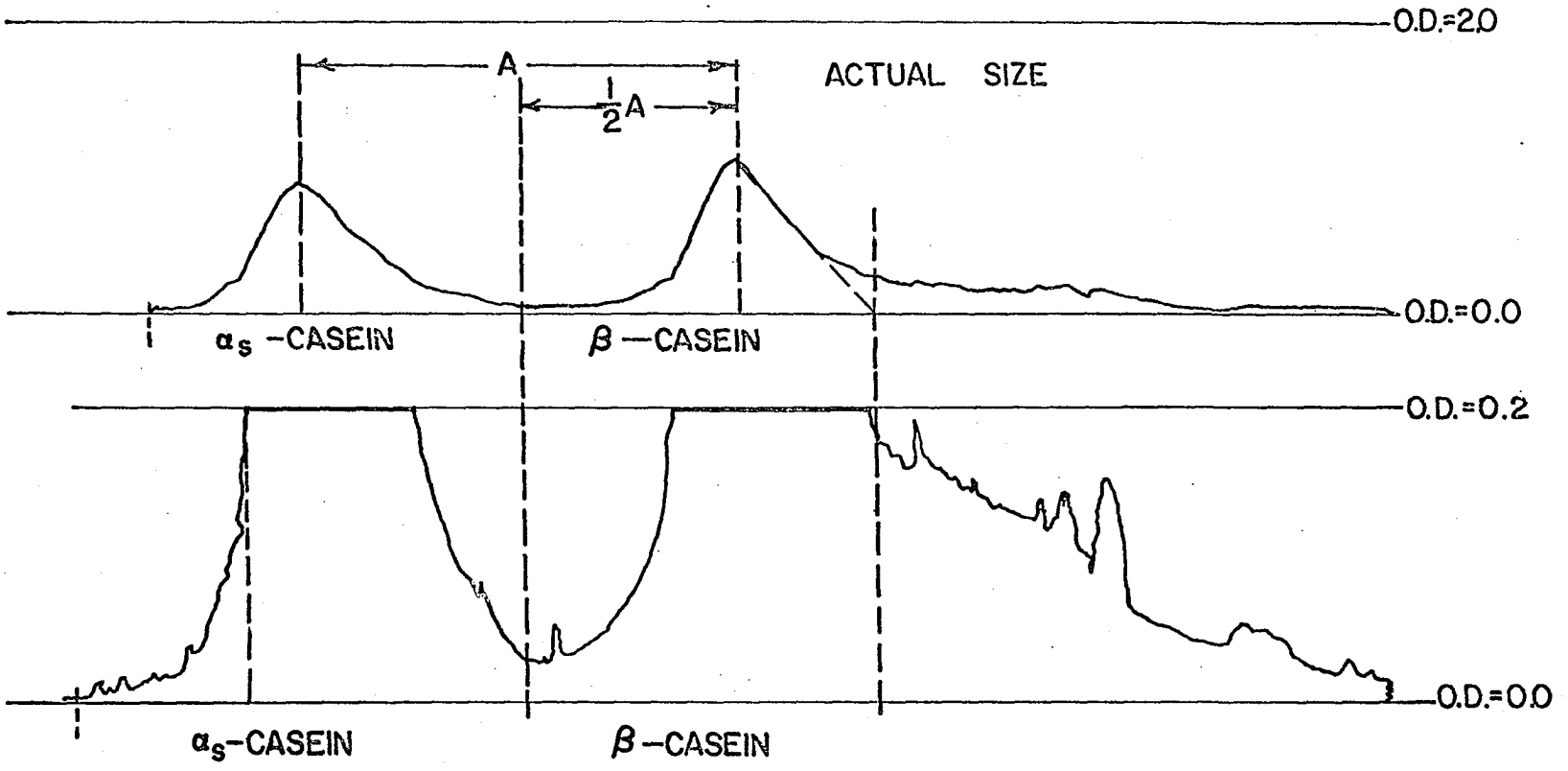


Figure 8. Method of dividing a typical polyacrylamide disc electrophoretic densitometer trace of native calcium caseinate in urea gels; optical density settings = 2.0 and 0.2.

of the β-casein band. The high sensitivity trace (b-Figure 8) could not be used to estimate the end of the β-casein band because in most cases this point had an optical density greater than 0.2. Relative areas under the traces were determined with the intergrator.

RESULTS AND DISCUSSION

Elemental Analysis of the Supernatants and Sediments of Ultracentrifuged Skim Milk

Total nitrogen and total solids contents of the calcium caseinate depleted supernatants

The results of the total solids (TS) and total nitrogen (TN) analysis of the supernatants are given in Table 1. Non-casein nitrogen of the original skim milk was considered to be the TN of the whey resulting from centrifugation of skim milk for 20 hours, at 53,620 x g and 0 C, and subsequent precipitation of the whey-soluble casein at pH 4.6; the value corrected for the dilution caused by the acid precipitation of the casein. The data in Table 1 are plotted in Figures 9, 10, and 11.

The depletion of casein (the "native" calcium caseinate), Figure 9 appears smooth. However, it should be noted that more caseinate was sedimented in 30 min using the differential centrifugation technique (serial depletion) than could be sedimented in 60 min of continuous centrifugation, Table 1, Figure 9. This probably results from a more efficient sedimentation due to the decreasing calcium caseinate concentration in the serially depleted supernatants. No attempt was made to estimate the diameters of the caseinate micelles sedimented in a given fraction. The resulting micelle distribution, Figure 10, shows that the fraction sedimented

Table 1. Analysis of the supernatants obtained by differential ultracentrifugation of skim milk at 53,620 x g and 0 C

Centrifuging time in min	Total[a] solids	Total nitrogen[b] mg/100 g skim	% Casein removed	% of total casein in fraction
Original skim	9.18	527.5	0.0	
0[c]	8.98	489.0	9.4	9.4
10[c]	8.42	403.2	30.3	20.9
20[c]	8.07	346.0	44.3	14.0
30[c]	7.62	720.2	62.8	18.5
40[c]	7.28	227.8	73.1	10.3
55[c]	7.05	205.9	78.5	5.4
continuous[d]				
60[d]	7.94	304.9	45.5	
70[d]	7.16	208.5	77.8	32.3
80[d]	6.92	182.0	84.3	7.5
20 hours[e]	7.05	174.6	86.1	86.1
I.E. Whey[f]	6.38	117.6	100.0	13.9

[a]Grams/100g of sample.

[b]Corrected using the factor of Davies and White (1960). $\frac{100 - \text{total solids in skim milk}}{100 - \text{total solids in supernatant}}$ x(mg nitrogen/100g supernatant).

[c]Differentially centrifuged skim milk supernatants, preceeding supernatant used for the next time increment.

[d]Skim milk centrifuged for 60 min. The supernatant was centrifuged for 10 min (70) and the resulting supernatant centrifuged for 10 min (80).

[e]Ultracentrifugal whey obtained by centrifuging skim milk for 20 hr.

[f]Whey-soluble casein remaining in the 20 hour supernatant was precipitated at pH 4.6 with 1 N HCl. The total solids and total nitrogen values were corrected for the dilution of the acid and the total nitrogen value taken as the non-casein nitrogen content of the skim milk.

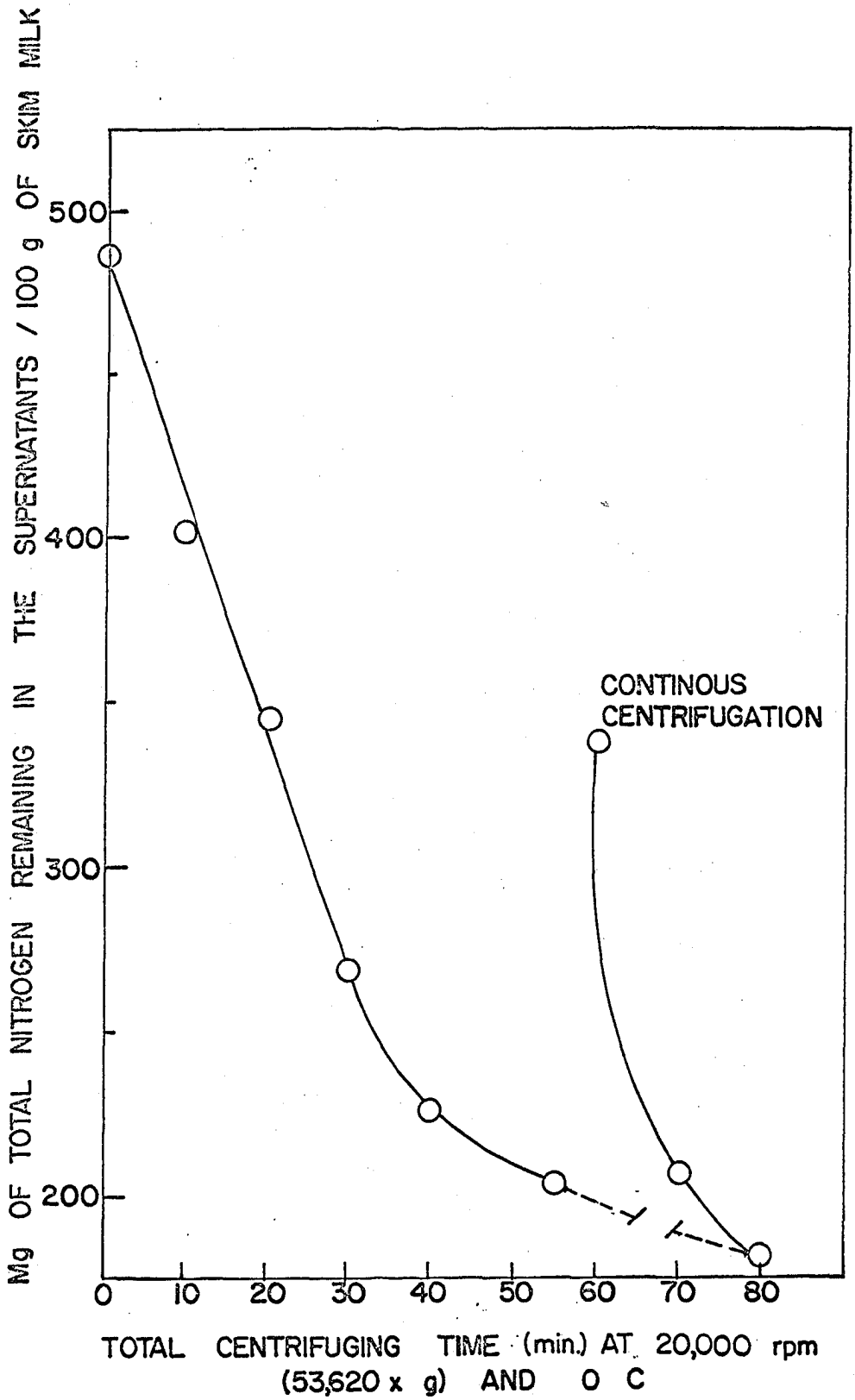


Figure 9. Depletion of total nitrogen in differentially ultracentrifuged supernatants.

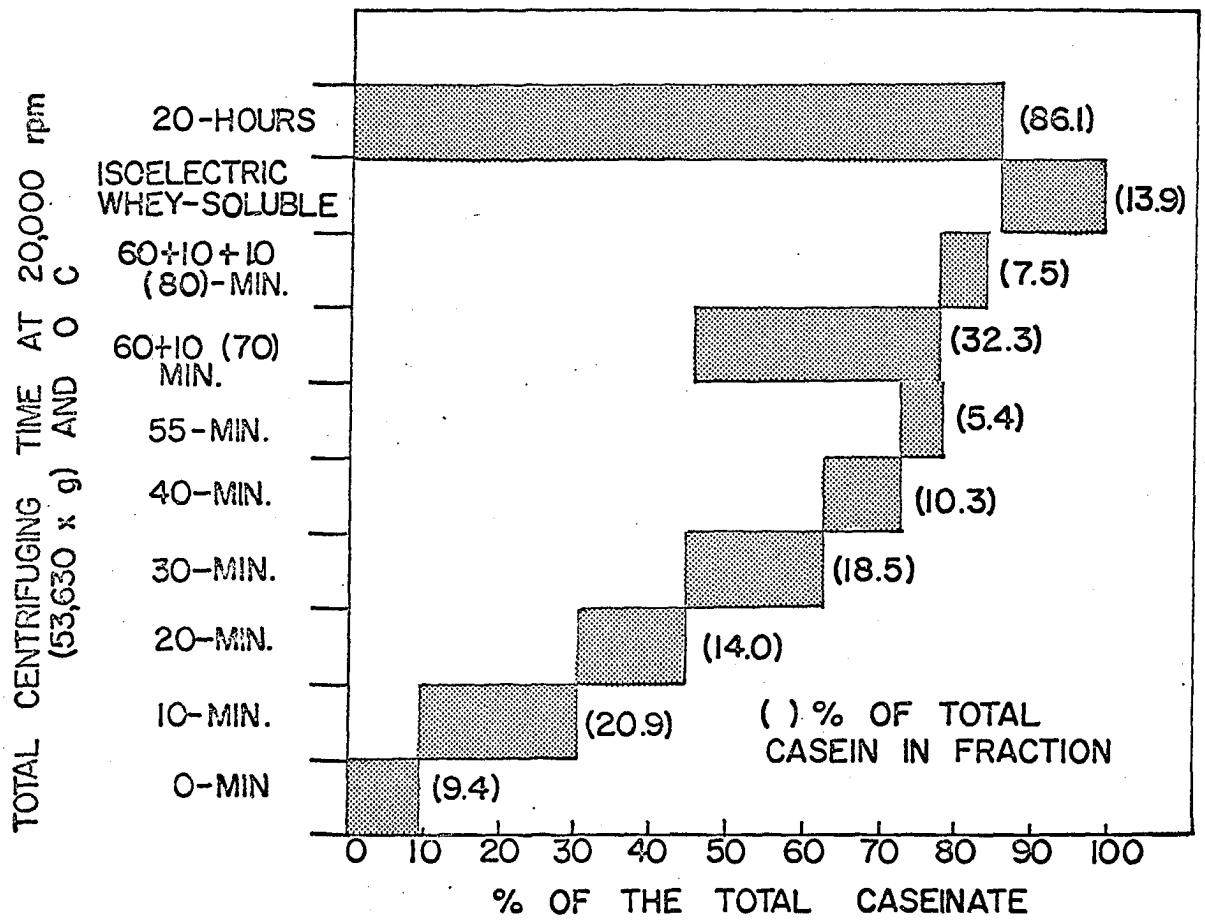


Figure 10. Caseinate quantities obtained by the differential (serial) sedimentations indicated; quantity not sedimented during 20 hours at 20,000 rpm (whey-soluble).

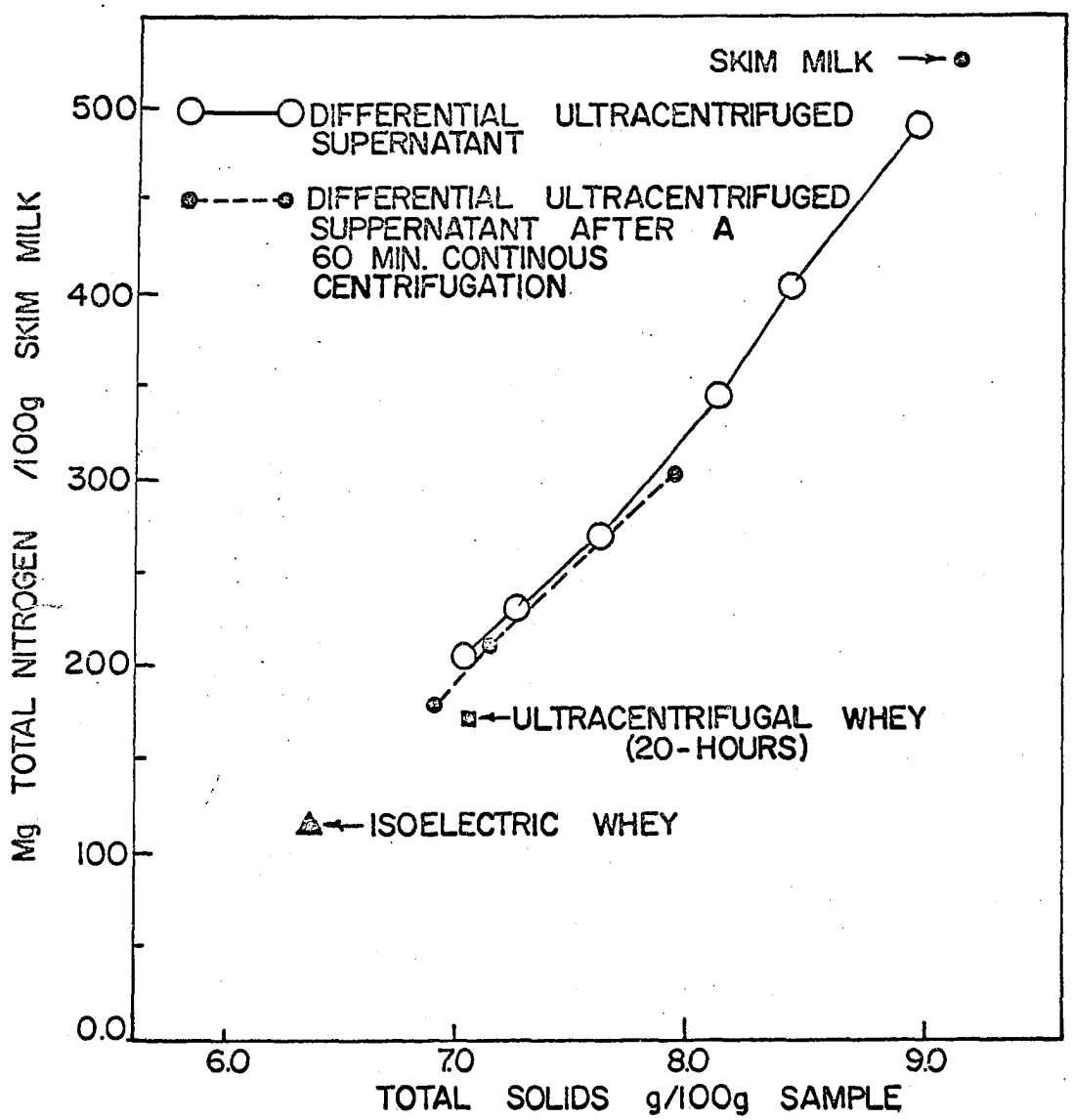


Figure 11. Relationship between total solids and total nitrogen in caseinate depleted supernatants.

during 10 min from the supernatant obtained during 60 min of continuous centrifugation (60+10 = 70-min fraction) of skim milk is apparently a composite of the 30, 40, and 55-min fractions. The results in Figure 10 show that 7 sequential caseinate fractions, were obtained; these did not include the 70-min fraction and the non-micellar (whey-soluble) fraction. The fraction sedimented during 20 hours of centrifugation and containing 86.1% of the total caseinate is a composite of these 7 sedimented fractions.

It is interesting to note that the relationship between the TN and TS in the supernatants is almost linear (Figure 11) for the range of micelles sedimented and the conditions employed.

Elemental analysis of the sedimented and non-sedimented caseinate and casein fractions

The analyses of the ultracentrifugal "native" calcium caseinate sediments and the isoelectrically precipitated whey soluble casein are shown in Table 2. The moisture values were employed to determine the analytical results on a dry basis. The variations in moisture from fraction to fraction are probably due to the fact that the internal surface area of the freeze-drying flasks and the amount of caseinate material in the flasks (variation in the thickness of the shell of frozen fractions) was not constant. Calcium and total phosphorus decreased and total nitrogen increased as micelle size decreased (Table 2); these results agree with those of

Table 2. Analysis of the freeze-dried differentially centrifuged, at 53,620 x g and 0 C, native calcium caseinate fractions and isoelectric whey soluble casein

Fractions[a]	Dry basis								
	% Moisture	% Total nitrogen	Total calcium mg/g	Ca/N[b]	Total magnesium mg/g	Ca/Mg[c]	Total phosphorus mg/g	Ca/TP[b]	TP/N[b]
0-min	0.95	13.81	25.9	0.188	1.79	8.78	20.4	1.27	0.148
10-min	1.36	13.86	26.0	0.187	1.93	8.15	20.2	1.28	0.148
20-min	2.86	13.79	25.5	0.185	1.91	8.10	19.9	1.28	0.148
30-min	3.97	14.07	24.9	0.177	1.69	8.96	18.6	1.34	0.132
40-min	1.94	14.10	24.6	0.175	1.36	11.00	18.0	1.37	0.127
55-min	3.70	14.18	23.8	0.168	1.20	12.05	17.4	1.37	0.123
60+10(70)-min	1.04	14.07	24.4	0.174	1.45	10.20	18.3	1.33	0.132
60+10+10(80)-min	1.32	14.19	22.0	0.155	1.09	12.25	13.8	1.59	0.098
20-hours	1.16	13.99	25.5	0.174	1.44	10.60	20.0	1.28	0.143
I.E. whey sol. casein	0.70	15.01					6.6		0.0438

[a]For the method of isolating each fraction see methods section (pp. 42-46).

[b]Weight ratios: (gA/100g)/(gB/100g).

[c]Moles Ca/Moles Mg.

de Kadt and van Minnen (1943), Hostettler *et al.* (1949), and Ford *et al.* (1955). The calcium:total nitrogen, calcium: total phosphorus, total phosphorus:total nitrogen, and total phosphorus:total nitrogen weight ratios generally decreased with micelle size. The magnesium contents, in general, decreased with micelle size while the molar Ca:Mg ratios increased with decreasing micelle size. The molar Ca:Mg ratio in the 20-hour fraction was 10.6, which is lower than the value reported by Alexander and Ford (1957) for twice washed (distilled water) caseinate sedimented at 18-20 C. They report a mean molar Ca:Mg ratio of 14.6 with a range of from 11.7 to 17.0. The low molar Ca:Mg ratio obtained in this study may reflect the differences in washing, twice by Alexander and Ford (1957) *vs.* once in this study, and the temperature of which the centrifuging was done, 18-20 C *vs.* 0 C in this study.

Perhaps the most important information in Table 2 is the phosphorus content of the isoelectric whey soluble casein fraction. The total phosphorus (TP) in this fraction should be equivalent to the organic (ester-bound) phosphorus (OP) concentration, since this fraction was isoelectrically precipitated. The TN value is equivalent to the casein nitrogen (CN) concentration for the same reason. Ford *et al.* (1955) report a constant OP:CN ratio of 0.055 for all their differentially sedimented fractions obtained at 18-20 C.

Later Ford and Martinez-Mateo (1958) obtained the same ratio for the non-sedimented (18-20 C) whey-soluble casein recovered by isoelectric precipitation. The OP:CN ratio for the whey soluble casein fraction in this study was 0.0438, which is in good agreement with the data of Bohren and Wenner (1961), who report OP:CN ratios for the whey soluble fraction obtained at 4 and 20 C of 0.044 and 0.048, respectively. Yamauchi and Tsugo (1962) likewise obtained an OP:CN ratio of 0.048 for the whey-soluble casein fraction obtained at 20 C. Since β-casein has a lower organic phosphorus content than α-casein, 0.6% and 1.0%, respectively, and β-casein is reportedly more soluble at temperatures below 30 C (Sullivan et al., 1955), the low OP:CN ratio for the whey-soluble casein fraction is undoubtedly a reflection of the increased β-casein content in this fraction. This fact is substantiated by moving boundary and gel electrophoresis to be discussed in later sections. No explanation can be given for the discrepancy in the data of Ford and Martinez-Mateo (1958) and the data obtained in this study.

Elemental Analysis of the Supernatant, "Wash" Water and Sediments of Skim Milk Centrifuged for 100 Hours at 144,700 x g and 0 C

Total solids and nitrogen distribution of the supernatants and "wash" water

The total solids and nitrogen distribution (Rowland, 1938) values for the original skim milk, ultracentrifugal whey (UCW)

obtained during 100 hours at 144,700 x g and 0 C, and the water with which the sedimented caseinate was washed, are summarized in Table 3. The data show that only 95.8% of the total

Table 3. Analysis of the original skim milk, supernatant and sediment wash water of skim milk centrifuged for 100 hours at 144,700 x g and 0 C

Fraction	% Total solids	Total[a] nitrogen	Non-casein[a] nitrogen	Non-protein[a] nitrogen	Casein[a] nitrogen
Skim milk	9.15	529.9	120.1	31.2	409.8
UCW[b]	6.22	82.6	65.2	29.1	17.4
Wash water[c]	0.43	26.0	16.8	3.6	9.2

[a]Reported as mg/100g skim milk using the correction factor of Davies and White (1960):

$$\frac{100 - \text{total solids in skim milk}}{100 - \text{total solids in fraction}} \text{ (mg constituent/100g fraction).}$$

[b]Ultracentrifugal whey from which 95.8% of the total caseinate had been removed.

[c]Redistilled water in which the sediment, caseinate, was redispersed and resedimented using the same conditions.

caseinates were removed with the conditions employed. The TN content of the supernatant (UCW) appears to be low, for the total nitrogen content of UCW should be greater (by the amount of nitrogen in the whey-soluble) than the non-casein in the original skim milk. The low TN in the UCW probably results from the fact that serum (whey) proteins are adsorbed on and sediment with the caseinate micelles (Ford et al., 1955). The NCN in the wash water is in agreement with this fact. It should be noted that some of the caseinates are lost during the washing

procedure so that the washed caseinate constitutes about 93.5% of the total caseinate compared with 95.8% for the unwashed caseinate. The sum of the non-protein nitrogen (NPN), in the supernatant and wash water, agrees well with the NPN in the original skim milk (32.3 mg NPN/100g skim milk *vs.* 31.2 mg NPN/100g skim milk, respectively), indicating that the washing procedure has probably removed all of the NPN from the sediment. However, the sum of the NCN in the supernatant and wash water is only 68% of the NCN in the original milk. indicating that the washing procedure did not remove all of the adsorbed whey proteins. These data are in agreement with those of Ford *et al.* (1955). Assuming that all of the NPN has been removed, as suggested previously, by the washing procedure, the washed sediment still contains approximately 7.6% non-casein protein (NCP) in reasonable agreement with a value of 5.3% calculated from the TN and NCN data reported by Ford *et al.* (1955), for their sediments. Their data also show that more NCP is associated with the smallest micelles sedimented which probably results from the increased surface area that can adsorb NCP.

Analysis of the sedimented 100-hour caseinate

The analyses of the washed and unwashed 100-hour sediments are summarized in Table 4. The washed sediments contained greater amounts of Ca, TP, and TN than the unwashed ones, which is in agreement with the results of Ford *et al.* (1955). Ratios calculated from the data of Ford *et al.* (1955) are: Ca:TP 1.62

Table 4. Analysis of the freeze-dried caseinates, washed and unwashed, sedimented during 100 hours at 144,700 x g and 0 C

Fraction[a]	% Moisture	Dry basis: % Total Nitrogen	Total Calcium[b]	Total Magnesium[b]	Total Phosphorus[b]
W 100 hr	1.42	14.71	25.4	1.41	18.0
UW 100 hr	4.89	14.40	24.8	1.34	17.5

	Ratios: Calcium[c] / nitrogen	Calcium[c] / Phosphorus	Phosphorus[c] / Nitrogen	Calcium[d] / Magnesium
W 100 hr	0.173	1.41	0.122	10.8
UW 100 hr	0.172	1.42	0.122	11.1

[a]For the method of isolating each fraction see methods section (pp. 42-46).

[b]Reported as mg/g of sample.

[c]Weight ratios.

[d]Molar ratios.

and 1.61, Ca:TN 0.184 and 0.203, and TP:TN 0.117 and 0.125 for washed and unwashed sediments, respectively in comparison with Ca:TP 1.41 and 1.42, TP:TN 0.122 and 0.122, and Ca:TN 0.173 and 0.172 for the washed and unwashed sediments obtained in this study. The molar Ca:Mg ratios are somewhat lower than the values reported by Alexander and Ford (1957), but are in agreement with the value obtained for the 20 hour fraction (Table 2). While there appears to be some difference in composition of the wediment from the 20-hour and 100-hour

washed caseinate fractions, Tables 2 and 4, (especially as regards TN) there is excellent agreement among the various ratios with the exception of the TP:TN ratio. It is difficult to make further comparisons among the data in Tables 2 and 4, because organic phosphorus and casein nitrogen values of the sediments were not determined.

Moving Boundary Electrophoresis

Comparison of various electrophoretic conditions employed

A number of moving boundary electrophoretic conditions were employed with the 20-hour fraction and the results were compared with isoelectric casein preparation 1 (Table 5). Typical casein patterns were obtained for the 20-hour caseinate fraction regardless of the electrophoretic conditions employed. A low rather broad peak was observed ahead of the α-casein peak. This peak, designated as unknown, had a high mobility (about -11 to -12 x 10^{-5} $cm^2 volt^{-1} sec^{-1}$) and this is thought to be a salt complex rather than a protein component, since its mobility is so much greater than any known protein component in milk. In general, the mobilities of α- and β-casein, obtained using a number of different conditions, are in reasonable agreement (Table 5) especially sample Nos. 3(6-ml cell, 0.5% protein) and 4 (2-ml cell, 1.0% protein). The α-casein mobilities obtained with the veronal-E.D.T.A. buffer, sample No. 1, Table 5, (similar to the buffer employed by Annibaldi, 1960, 1961) were slightly higher and in better agreement with the mobility obtained for α-casein in isoelectric

Table 5. Moving boundary electrophoretic analysis of freeze-dried native calcium caseinat

Sample[b] No.	Buffer	pH at 23 C sample	pH at 23 C buffer	Res[c] in Ohms sample	Res[c] in Ohms buffer	Cell size	Volts/cm	Time (sec)	Mo ur
1	veronal-E.D.T.A.[f]	8.10	8.20	228	220	2-ml	8.87	3360	
								5160	
2	veronal-oxalate[g]	8.17	8.25	228	222	6-ml	4.94	9900	
3	veronal-oxalate[g]	8.20	8.23	228	220	6-ml	5.17	6120	
								10170	
4	veronal-oxalate[g]	8.17	8.20	226	220	2-ml	8.91	3675	
								5400	
5	veronal-oxalate[g,h]	8.51	8.49	233	218	2-ml	8.99	5914	
6	veronal-oxalate[g,i]	8.21	8.22	221	216	2-ml	8.59	3780	
								5640	
7	veronal[f,j]	8.14	8.17	228		2-ml	8.75	3852	

[a]Areas, determined by planimetry (average of 4 values), include the outer perimeter representing the electrophoresis cell.

[b]Sample concentrations were 1.0% protein (sample No. 2, 0.5% protein).

[c]Conductivity cell constant was 1.201.

[d]Descending mobilities x 10^{-5} cm^2 $volt^{-1}$ sec^{-1}, calculated from the false (ϵ) bounda

[e]Ascending pictures taken at about 5400 sec.

[f]Dialized for 24 hr against two changes of standard veronal buffer ($\Gamma/2 = 0.1$).

[g]Dialized for 24 hr against two changes of the same veronal-oxalate buffer ($\Gamma/2 = 0.$

[h]Not dialized.

[i]Veronal-excess oxalate buffer ($\Gamma/2 = 0.1802$).

[j]Isoelectric casein, preparation 1.

re calcium caseinate sedimented during 20 hours at 53,620 x g and 0 C

Volts/cm	Time (sec)	Mobilities[d] of components unknown	α-casein	β-casein	Relative areas %[a] descending α-casein	descending β-casein	ascending[e] α-casein	ascending[e] β-casein
8.87	3360	-12.45	-6.63	-2.91	77.0	23.0		
	5160		-6.68	-3.05	73.0	27.0	66.5	33.5
4.94	9900		-6.82	-3.31	76.2	23.8		
5.17	6120	-11.90	-6.38	-2.95	82.2	17.8		
	10170		-6.38	-3.07	80.9	19.1		
8.91	3675	-11.94	-6.33	-3.04	77.6	22.7		
	5400		-6.41	-2.99	75.1	24.9	64.3	35.7
8.99	5914		-6.38	-3.19				
8.59	3780	-10.99	-6.81	-3.42	75.8	24.2		
	5640		-6.68	-3.26	75.1	24.9	64.4	35.6
8.75	3852		-6.82	-3.20	71.1	28.9		

e outer perimeter of the peaks (Gaussian distribution) to the bottom of the rectangle

in).

e false (ϵ) boundary.

(Γ/2 = 0.1).

e buffer (Γ/2 = 0.1084) that was used to disperse the sample.

casein (standard veronal buffer), sample No. 7, Table 5, than when veronal-oxalate buffers were used (samples 3, 4, and 5). However, there was more variation in relative areas of α- and β-casein with electrophoresing time when veronal-E.D.T.A. buffer was used. The mobilities obtained, when veronal-excess oxalate buffer (sample No. 6, Table 5) was employed, were generally higher and more variable with electrophoresing time than when the "normal" veronal-oxalate buffers were used.

The relative areas of α- and β-casein are in good agreement among samples, despite the variations in electrophoretic conditions, except for sample No. 3, Table 5, in which a 6-ml cell was used with a sample concentration of 1.0% protein. In general, γ-casein peaks were not observed in the descending patterns although a small γ-casein peak appeared in most of the ascending patterns. These γ-casein peaks were ignored when they appeared only in the ascending patterns. Variations in relative areas of α- and β-casein were observed between the ascending and descending patterns (Table 5), in agreement with Warner (1944), Krejci (1942) and Krejci *et al*. (1941).

The mobilities obtained for α- and β-casein in the caseinate samples are in good agreement with Tobias *et al*. (1952b) who report mobilities of -6.35 and -3.34 x 10^{-5} cm^2 $volt^{-1}$ sec^{-1} for α- and β-casein respectively in unheated skim milk diluted 1:4, dialized against veronal buffer pH 8.6, and electrophoresed using the same buffer. The mobilities are somewhat lower than the values reported by Hipp *et al*. (1952)

who reported mobilities of -6.75 and -3.05 x 10^{-5} cm^2 $volt^{-1}$ sec^{-1} for α- and β-casein, respectively. It should be pointed out that these mobilities were obtained for the individual caseins by isolating them and determining their mobilities separately.

On the basis of better agreement for the mobilities and relative areas of α- and β-casein between 1 and 1.5 hr electrophoresing time, veronal-oxalate buffer (previously described) with the 2-ml cell, 24 hr dialysis, and sample concentrations of 1.0% protein were employed in the remaining moving boundary electrophoretic analyses of caseinate fractions.

Moving boundary electrophoretic analysis of sedimented caseinates and non-sedimented whey-soluble casein

The electrophoretic analysis of the fractions are summarized in Table 6, and typical and atypical patterns are shown in Figure 12. In general, the results for the 0-, 10-, 20-, 30-, 40-, 55-, and 60+10(70)-min fractions are in agreement with the data in Table 5 for the 20-hour fraction. The data, Table 6, show no change in relative areas of α- and β-casein, for the above mentioned fractions, as the micelle size changes. These data are in agreement with Hostettler et al. (1949) and Heckman et al. (1958). However, the smallest micelle sedimented, 60+10+10(80)-min fraction, shows an atypical electrophoretic pattern (Figure 12, II). A rather large peak is observed between the α- and β-casein

Table 6. Moving boundary electrophoretic analysis, 2-ml cell, of freeze-dried differentia fractions and isoelectric whey soluble casein

Fractions[c] 1% protein	pH at 23 C sample	pH at 23 C buffer[f]	Res[d] (ohms) sample	Res[d] (ohms) buffer	Volts/cm	Time (sec)	Mobilities of casein[e] unknown	α-	X-	co
0 min	8.22	8.20	227	220	8.80	3600	-12.02	-6.29		-3
						5220		-6.34		-3
10 min	8.16	8.20	228	222	8.92	3480	-12.51	-6.48		-3
						5400		-6.54		-3
20 min	7.95	8.02	229	221	8.77	3800	-12.00	-6.50		-3
						5400		-6.54		-3
30 min	8.20	8.20	223	220	8.85	3600	-11.97	-6.53		-3
						5400		-6.50		-3
40 min	8.28	8.38	223	219	8.54	3600	-11.77	-6.22		-2
						5400		-6.39		-2
55 min	8.22	8.32	226	220	8.66	3600	-12.69	-6.77		-3
						5400		-6.52		-3
60 + 10 (70) min	8.23	8.20	225	221	8.62	3800	-11.76	-6.30		-3
						5340		-6.30		-2
60 + 10 + 10 (80) min	8.22	8.30	222	219	8.69	3000	-12.24	-6.64	-4.96	-3
60 + 10 + 10 (80) min	7.81	7.88	221	213	8.77	4080		-6.72	-4.56	-3
						5400		-6.73	-4.78	-3
I.E. whey sol casein	8.19	8.20	223	219	8.63	3765	-11.30		-4.92	-3
						6540			-5.24	-3
I.E. whey sol casein	8.22	8.27	224	220	8.67	3840	-11.47		-4.72	-3
						6660			-4.95	-3

[a] Areas, determined by planimetry (average of 4 values), include the outer perimeter representing the electrophoretic cell.

[b] Ascending pictures taken at about 5400 sec.

[c] For the method of isolating each fraction see methods section (pp. 42-46).

[d] Conductivity cell constant was 1.201.

[e] Descending mobilities x 10^{-5} cm^2 $volt^{-1}$ sec^{-1}, calculated from the false (ε) bounda

[f] All buffers were veronal-oxalate buffers (see methods section), (Γ/2 = 0.1084), exc standard veronal buffer, (Γ/2 = 0.1), was used. Samples were dialized for 24 hr against

[g] ( ) Relative areas (%) α- plus β-casein = 100%.

, of freeze-dried differentially centrifuged, at 53,620 x g and 0 C, native calcium caseinate

me ec)	Mobilities of casein[e] components					Relative areas (%) of casein components[a,b] descending				ascending			
	unknown	α-	X-	β-	γ-	α-	X-	β-	γ-	α-	X-	β-	γ-
00	-12.02	-6.29		-3.13		75.9		24.1					
20		-6.34		-3.11		75.7		24.3		63.10		36.9	
80	-12.51	-6.48		-3.19		75.4		24.6					
00		-6.54		-3.14		75.3		24.3		64.9		35.1	
00	-12.00	-6.50		-3.08		77.3		22.7					
00		-6.54		-3.08		75.4		24.6		64.9		35.1	
00	-11.97	-6.53		-3.17		77.7		22.3					
00		-6.50		-3.08		75.9		24.1		65.5		34.5	
00	-11.77	-6.22		-2.82		76.6		23.4					
00		-6.39		-2.80		74.2		25.8		70.2		29.8	
00	-12.69	-6.77		-3.15		76.8		23.2					
00		-6.52		-3.00		74.2		25.8		68.3		31.7	
00	-11.76	-6.30		-3.05		76.5		23.5					
40		-6.30		-2.99		74.8		25.2		64.8		35.2	
00	-12.24	-6.64	-4.96	-3.27		56.3 (75.4)[g]	25.3	18.4 (24.6)		56.3 (79.4)	29.1	14.6 (20.6)	
80		-6.72	-4.56	-3.04		56.6 (72.6)	22.0	21.4 (27.4)		54.9 (77.4)	29.0	16.1 (22.6)	
00		-6.73	-4.78	-3.04		53.5 (73.6)	27.4	19.1 (26.4)					
65	-11.30		-4.92	-2.86	-1.51		35.9	57.1	7.0				
40			-5.24	-3.03	-1.65		38.7	53.3	8.0				
40	-11.47		-4.72	-2.91	-1.30		32.1	59.0	8.9				
60			-4.95	-2.90	-1.45		37.7	52.6	9.7		29.5	58.4	12.1

include the outer perimeter of the peaks (Gaussian distribution) to the bottom of the rectangle

ection (pp. 42-46).

ed from the false (ε) boundary.

ection), (Γ/2 = 0.1084), except for the isoelectric whey soluble casein fraction with which a dialized for 24 hr against two changes of the buffer used to disperse the sample.

Figure 12. Moving boundary electrophoresis patterns, 2-ml cell, of freeze-dried differentially centrifuged, at 53,620 x g and 0 C, of native calcium caseinate fractions and isoelectric whey-soluble casein

I)[1] A typical electrophoretic pattern of oxalate dispersed calcium caseinate, protein concentration 1.0%. The 20-min caseinate pattern is shown. Similar patterns were obtained with the 0, 10, 30, 40, 55, and 60+10(70)-min fractions as well as the 20-hour fraction.

II)[1] The atypical electrophoretic pattern obtained with the 60+10+10(80)-min caseinate fraction, protein concentration 1.0%.

III)[1] The atypical electrophoretic pattern obtained with the isoelectric whey-soluble casein fraction, protein concentration 1.0%.

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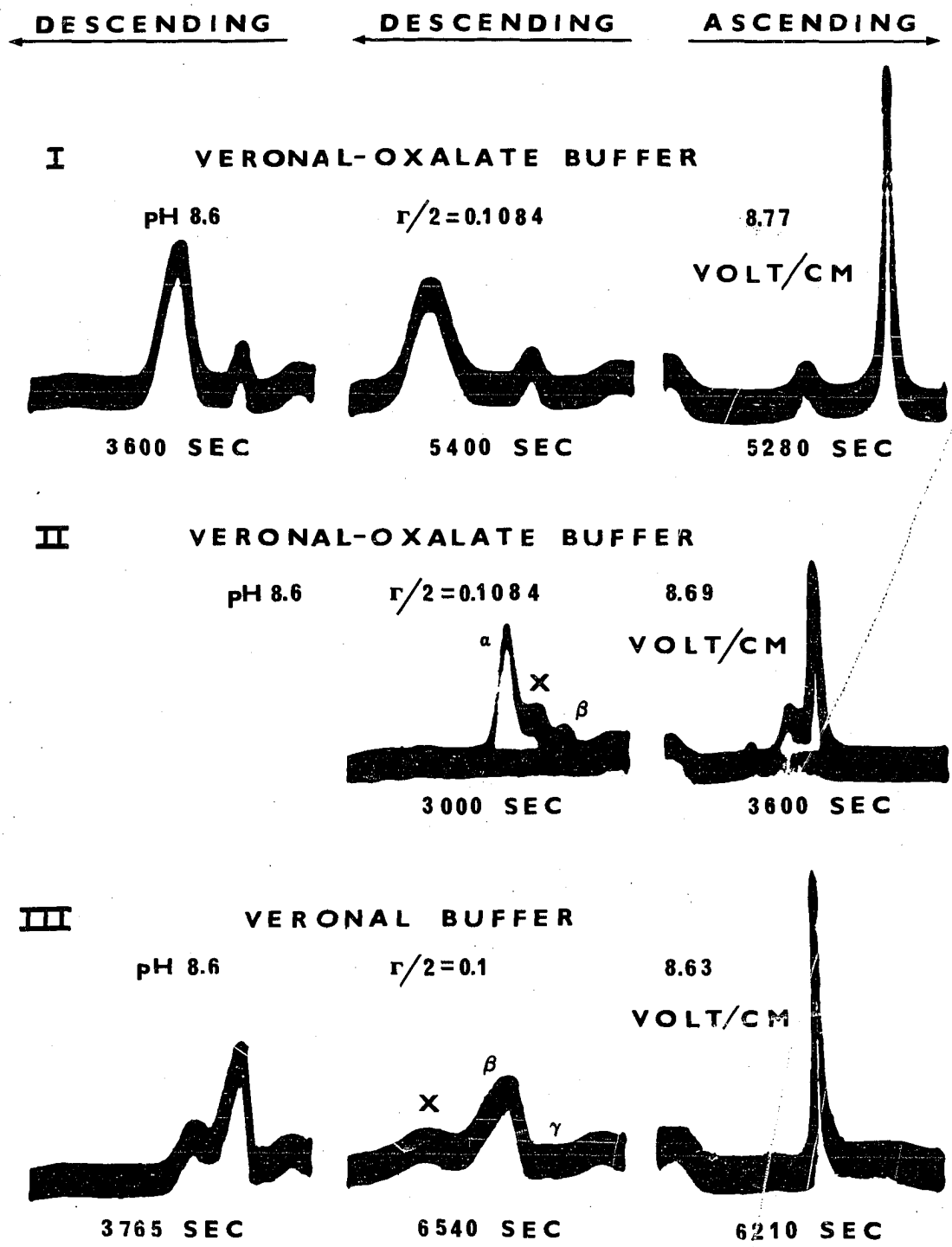
[1]For the method of isolating each fraction see methods section (pp. 42-46).


DESCENDING
DESCENDING
ASCENDING
I
VERONAL-OXALATE BUFFER
pH 8.6
r/2=0.1084
8.77
VOLT/CM
3600 SEC
5400 SEC
5280 SEC
II
VERONAL-OXALATE BUFFER
pH 8.6
r/2=0.1084
8.69
VOLT/CM
α
X
β
3000 SEC
3600 SEC
III
VERONAL BUFFER
pH 8.6
r/2=0.1
8.63
VOLT/CM
β
X
γ
3765 SEC
6540 SEC
6210 SEC

peaks which is not completely resolved from the α-casein peak. This intermediate peak is designated "X"-casein, since a number of workers (Larson, 1958, Kenyon and Jenness, 1958, and McKenzie and Wake, 1959a) have reported a similar unidentified component in this region and Larson (1958) has used the term "X"-casein. The mobility of this X-casein component (Table 6) is in reasonable agreement with the mobility of β-lactoglobulin (-5.09×10^{-5} cm^2 $volt^{-1}$ sec^{-1}) reported by Tobias et al. (1952b) in skim milk diluted 1:4. The adsorption of whey proteins on the small micelles which cannot be completely removed with the washing procedure employed (Table 3: Ford et al., 1955), suggests that the X-casein component may be, wholly or partly, β-lactoglobulin. However, the large relative area of this component in the 80-min fraction suggests that the X-casein component cannot all be β-lactoglobulin.

The whey-soluble casein fraction also exhibits an atypical electrophoretic pattern (Figure 12, III). No peak with a mobility comparable to α-casein is observed. The mobility of the first peak suggests that it is the X-casein component. A large β-casein peak is observed which accounts for more than 50% of the total pattern (compared with 25% in a typical casein pattern). The ascending relative areas of α-, β-, and γ-casein (Table 6) are in excellent agreement with the values reported by Bohren and Wenner (1961) for a whey-soluble casein precipitated with $CaCl_2$ from ultracentrifugal whey

obtained during 300 min at 50,000 x g and 4 C. A definite γ-casein peak is observed in the descending, as well as in the ascending, whey-soluble casein pattern (Figure 12, III): γ-casein is not observed in the descending patterns of any of the other fractions. Hipp et al. (1952) report that β- and γ-casein are more soluble at low than at high temperatures, thus the increase in γ-casein as well as β-casein in the whey-soluble casein fraction may result from their greater solubilities at low temperature (Sullivan et al., 1955, Hipp et al., 1952, and Bohren and Wenner, 1961). Hansen et al. (1962) report descending, moving-boundary, electrophoretic patterns for "non-micellar" casein obtained by a Rowland (1938) fractionation of ultracentrifugal whey obtained during a 10 hr centrifugation at 55,150 x g and 0-2 C. The mobilities reported by Hansen et al. (1962) are in agreement with the mobilities obtained for whey soluble casein in this study (Table 6), except for the mobility of γ-casein. They report a mobility of -2.05×10^{-5} cm^2 $volt^{-1}$ sec^{-1} for γ-casein vs. about -1.50×10^{-5} cm^2 $volt^{-1}$ sec^{-1} in this study. Hansen et al. (1962) report 14.5, 82.5 and 2.9% for X-, β-, and γ-casein respectively in contrast with 35, 55, and 10% respectively obtained in this study. However, Hansen et al. (1962) were not specific as to the method of analyzing their patterns.

In general, the electrophoretic results obtained for the

whey-soluble casein fraction in this study are in agreement with the results of Bohren and Wenner (1961) with one exception, they do not report mobilities of their casein components as such. They presented patterns of whey-soluble casein to which a known amount of whole isoelectric casein has been added. They conclude from the first peak in the pattern of the combined caseins and the whey-soluble casein that this component had the same mobility as α-casein. This is not in agreement with the mobilities, calculated directly, for this component in this study nor the mobilities reported by Hansen et al. (1962) for the first component in "non-micellar" casein. It is interesting to note that Hansen et al. (1962) list this first peak in their "non-micellar" casein as β-lactoglobulin but suggests that it could be the X-casein component observed by Larson (1958). There is a strong possibility that the X-casein component in the 80-min fraction (Figure 12) is the same component as the X-casein component in the whey soluble casein fraction, since their mobilities are in good agreement (Table 6). Because the whey-soluble casein fraction is an isoelectric preparation, it would appear that only a small part, if any, of the X-casein component, can be β-lactoglobulin. Gel electrophoresis data, to be presented, indicate that whey-soluble casein is almost devoid of α_s-casein. It is possible that the X-casein component could be κ-casein. Swaisgood and Brunner (1962) and Zittle and Custer

(1963) report the mobility of κ-casein to be comparable to that of α-casein. The mobility of the X-fraction is lower than that of α-casein. This suggests the possibility that the lower mobility of the X-fraction could result from an interaction between κ- and β-casein. McMeekin et al. (1957) reported separating a new α-casein fraction which they termed α_2-casein. This fraction had a mobility of -5.0×10^{-5} cm^2 $volt^{-1}$ sec^{-1} which is approximately the mobility obtained for the X-casein component in this study. The α_2-casein is reported to have properties (calcium-insensitive; split by rennin) similar to κ-casein isolated by Waugh and von Hippel (1956). The nomenclature committee on milk proteins (Brunner et al., 1960) lists α_2-casein with κ-casein.

Gel electrophoresis has shown that the α_s-casein: κ-casein ratio has been altered in whey-soluble casein. This supports the contention of a κ-casein-β-casein interaction. If this is true, the addition of whole isoelectric casein to whey-soluble casein to compare mobilities (Bohren and Wenner, 1961) could reestablish a normal α_s-casein: κ-casein ratio and yield a mobility for the X-casein + α-casein that might appear to be the same as the mobility of a normal α-casein.

Moving boundary electrophoresis of calcium caseinate in the presence of urea

Little if any work has been reported on the use of high urea concentrations in moving boundary electrophoresis of

caseins or caseinates. The 20-hour caseinate fraction was electrophoresed in veronal buffer containing 5.0 M urea and the patterns are shown in Figure 13. The calcium caseinate was easily dispersed in the veronal-urea buffer and the sample was not dialized. The sample concentration was 1.0% protein. The rather large false boundaries (ε peak) that were obtained probably reflect the high urea concentrations used and the fact that the sample was not dialized. The patterns (Figure 13) show the disaggrating effect of the urea on the caseinate and show as many as 12-13 peaks in the descending pattern.

The patterns were not analyzed because of the excessive number of peaks. Mobilities were not determined because of the extremely slow migrations rates obtained which may have resulted from the high viscosity of the buffer. No further experiments, using urea buffers, were performed by moving boundary.

Moving boundary electrophoretic analysis of "total" washed and unwashed calcium caseinate

The results of moving boundary electrophoresis of the 100-hour washed and unwashed calcium caseinates (sedimented at 144,700 x g and 0 C) are summarized in Table 7. The results are in reasonable agreement with the data in Tables 5 and 6 with two exceptions; 1) γ-casein peaks were obtained in the descending patterns of the washed 100-hour fraction (in veronal-oxalate and veronal-excess oxalate, buffers) samples

Figure 13. Moving boundary electrophoretic pattern, 2-ml cell, of the 20-hour native calcium caseinate fraction in veronal buffer containing 50 M urea, protein concentration 1.0%.

VERONAL UREA BUFFER
pH 8.6 $\Gamma/2 = 0.1$

9.86 VOLT/CM

11340 SEC

DESCENDING

8700 SEC

ASCENDING

Table 7. Moving boundary electrophoretic analysis, 2-ml cell, of freeze-dried, washed and during 100 hours at 144,700 x $\underline{g}$ and 0 C

Fractions	pH at 23 C of sample	pH at 23 C of buffer	Res (ohms)[b] sample	Res (ohms)[b] buffer	Volts/cm	Time (sec)	Mobilities[c] of casein unknown	α-	X-
100-hour W[e]	8.31	8.34	224	220	8.83	3930	-11.96	-6.29	
						5522		-6.44	
100-hour W[g]	8.22	8.23	221	215	8.65	3780	-10.49	-6.27	-4.41
						5460		-6.46	-4.38
100-hour UW[e]	8.40	8.38	228	223	8.67	3600	-11.95	-6.38	
						5520		-6.39	

[a]Areas, determined by planimetry (average of 4 values), include the outer perimeter rectangle representing the electrophoresis cell.

[b]Conductivity cell constant = 1.201.

[c]Descending mobilities x 10^{-5} cm^2 $volt^{-1}$ sec^{-1}, calculated from the false (ϵ) bounda

[d]Ascending pictures taken at about 5400 sec.

[e]Dialized for 24 hours against two changes of the same veronal-oxalate buffer ($\Gamma/2$ =

[f]( ) Relative areas (%) α- plus β-casein = 100%.

[g]Dialized for 24 hours against two changes of veronal-excess oxalate buffer ($\Gamma/2$ = 0

1 and 2, Figure 14 and, 2) an X-casein component is barely visible in the descending 100-hour washed caseinate pattern, sample 2, Figure 14, when excess oxalate is used in the buffer.

As regards the γ-casein peak in the descending pattern of the washed caseinate sample, the presence of γ-casein probably reflects the fact that this fraction contains about 93.5% of the total caseinate *vs.* 86.1% of the total caseinate in the 20-hour fraction. However, the fact that a γ-casein peak is not visible in the unwashed 100-hour fraction suggests that γ-casein is not lost during the washing procedure and the presence of more adsorbed serum (whey) proteins in the unwashed caseinates may mask the γ-casein peak in the descending pattern. It should be pointed out that γ-casein peaks are observed in all ascending patterns. The presence of an X-casein component in the descending patterns of the washed caseinate, electrophoresed in the presence of excess oxalate is puzzling. The mobility of this X-casein is lower (about -4.4 *vs.* -5.0×10^{-5} cm^2 $volt^{-1}$ sec^{-1}) than had been obtained in the 80-min and isoelectric whey-soluble fractions (Table 6). The X-casein peak is extremely small and could be a salt abnormality due to the excess oxalate. However, its magnitude is about the same as for γ-casein and no X-casein peak was observed in the 20-hour fraction when it was electrophoresed in the presence of excess oxalate. If this peak is indeed a protein component, it is apparent that it exists in the micelle

Figure 14. Moving boundary electrophoretic patterns, 2-ml cell, of freeze-dried, washed (W) and unwashed (UW), native calcium caseinate fractions sedimented during 100-hours at 144,700 x g and 0 C

I)[1] 100-hour W calcium caseinate, protein concentration 1.0%.

II)[1] 100-hour W calcium caseinate, protein concentration 1.0%.

III)[1] 100-hour UW calcium caseinate, protein concentration 1.0%.

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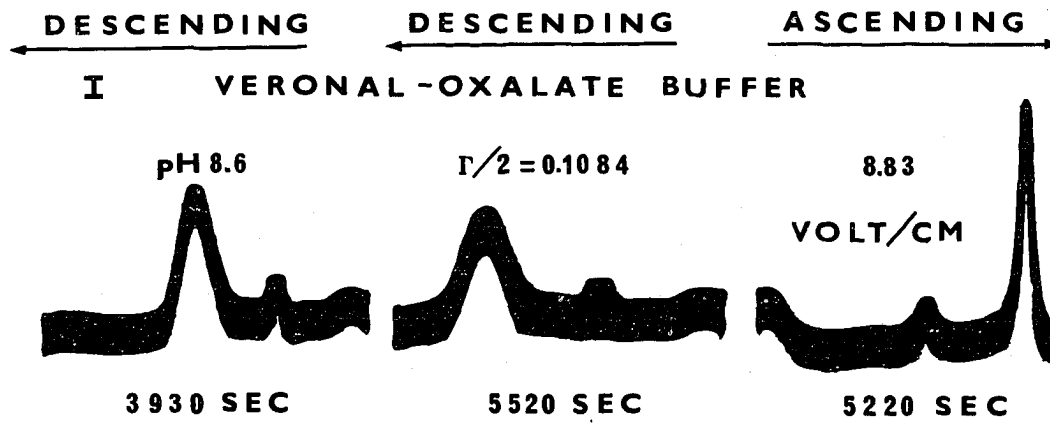
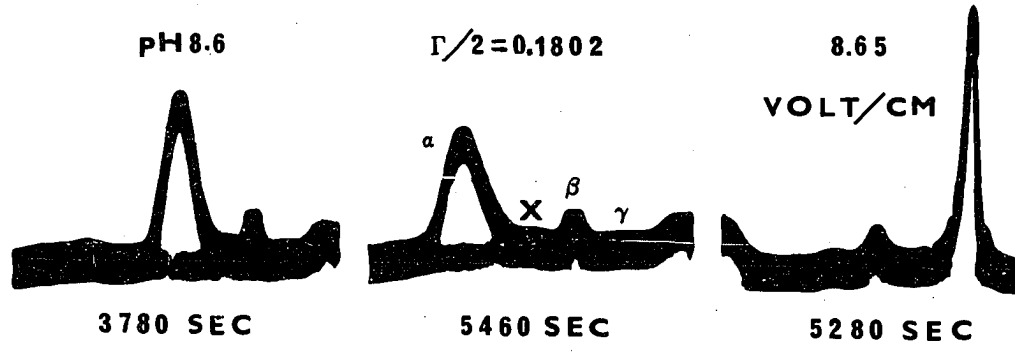
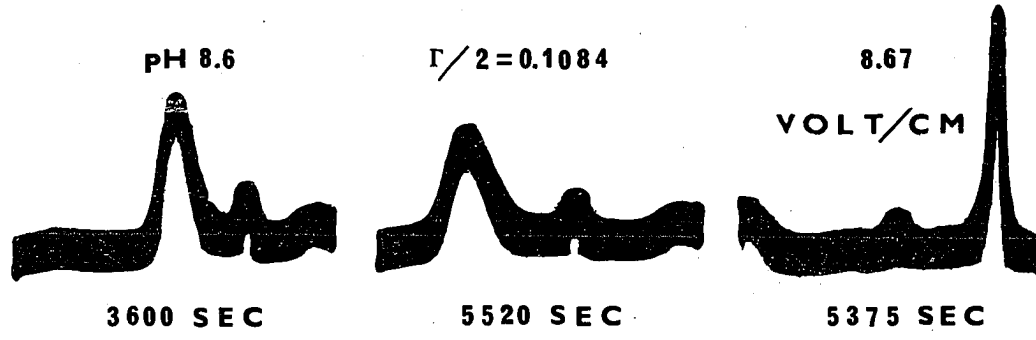
[1]For the method of isolating each fraction see methods section (pp. 42-46).


DESCENDING
DESCENDING
ASCENDING
I VERONAL-OXALATE BUFFER
pH 8.6
Γ/2 = 0.1084
8.83
VOLT/CM
3930 SEC
5520 SEC
5220 SEC


II VERONAL-EXCESS OXALATE BUFFER
pH 8.6
Γ/2 = 0.1802
8.65
VOLT/CM
α
β
γ
3780 SEC
5460 SEC
5280 SEC


III VERONAL-OXALATE BUFFER
pH 8.6
Γ/2 = 0.1084
8.67
VOLT/CM
3600 SEC
5520 SEC
5375 SEC

range between 85% (amount of caseinate in the 20-hour fraction) and 93.5% (amount of caseinate in the 100-hour fraction) of the total caseinate.

The mobilities (Table 7) of α-, β-, and γ-caseins are in general agreement with the mobilities obtained, for these caseins, with other caseinate fractions (Tables 5 and 6). The relative percentages of these caseins are also in agreement with the exception of the 100-hour W (5460 sec) pattern, using veronal-excess oxalate buffer, which appears to contain slightly more α-casein, when α- plus β-casein is taken as 100%. The 100-hour W ascending patterns, with veronal-oxalate and veronal-excess oxalate buffers, appear to contain more α-casein than the 100-hour UW ascending pattern when the α- plus β-casein contents only are considered.

Starch-Gel Electrophoresis

There are a number of empirical observations that can be made from the photographs of the gels or the gels themselves. The banding seems to be much more distinct when the gels are stained with Nigrosine rather than with Amido Black; this is particularly true for the pre-α_s-casein bands. However, for densitometry, Nigrosine does not appear to be as satisfactory as Amido Black because of the much darker background. Further observations, with regard to urea starch-gel patterns, are discussed in the sections that deal with urea starch-gels, with and without the addition of 2-mercaptoethanol.

Urea starch-gel (USG) electrophoretic patterns

The minor components (Figures 15 and 16) pre-α_s-casein (bands 1 through 4), pre-β-casein (bands 9 and 10) and bands 14 through 17, are quite distinct for the more rigorously treated, isoelectric casein preparations, but are either absent or tend to smear in the "native" caseinate preparations. This is especially true of bands 14 through 16 (Figures 15 and 16). However, bands 14, 15, and 16 are observed in the 100-hour washed sample (Figure 16, slot No. 7), indicating that possibly these components are concentrated in the micelle sizes located between 86 and 93 percent of the total caseinate, since the 20-hour fraction contains about 86 percent of the total native caseinate, while the 100-hour fraction contains 93 percent of the total native caseinate. Because different pooled milks are involved here, band 17 (Figures 1 and 16) may result from variations between milks. Band 17 is not sharp on the isoelectric whole casein (Figure 16, slot 1 and 2) and it could be masked by the darker smearing in this region on the native caseinate patterns (Figures 15 and 16). The whey-soluble portion of the caseinates (precipitated at pH 4.6 with 1 N HCl from the supernatant of skim milk centrifuged 20 hours, 53, 620 x g, 0 C), which constitutes about 14 percent of the total native caseinates (Figure 16, slot No. 4), shows virtually no major α_s-casein component. It does show discernible banding in the 0.47 and 0.54 relative band region (between bands 13 and 14, Figures 1 and 15). This apparent

Figure 15. Horizontal urea-starch gel electrophoretic patterns of caseins and native calcium caseinate fractions

Electrophoretic conditions: The basic procedure of Wake and Baldwin (1961) was employed. Gel size 14 x 22.3 x 0.6 cm, potential 180 volts (40 mA), borate line migrated 12 cm beyond the sample slot. All fractions were dispersed in 6.6 M urea-tris-citrate buffer, pH 8.6.

Plate 1 A Nigrosine stained.
Plate 1 B Amido Black stained.

Samples[1] (slot No. and sample protein concentration):

1) Isoelectric casein, preparation 1, 2.0%; 2) isoelectric casein, preparation 1, 1.0%; 3) α_s-casein, 0.5%; 4) isoelectric whey soluble casein, 0.75%; 5) κ-casein, 1.0%; 6) 20-hour caseinate fraction, 1.5%; 7) 100-hour W caseinate fraction, 1.5%; 8) 100-hour UW caseinate fraction, 1.5%.

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[1]For the method of isolating each fraction, see methods section (pp. 42-46).

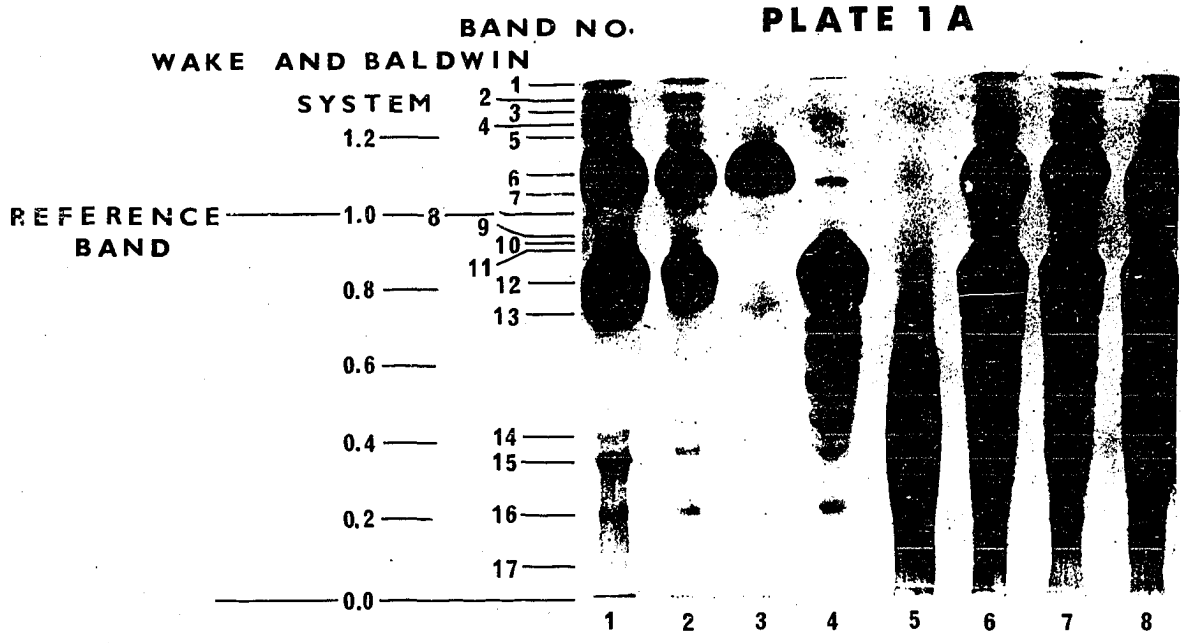
PLATE 1A
BAND NO.
WAKE AND BALDWIN
SYSTEM
REFERENCE BAND
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17

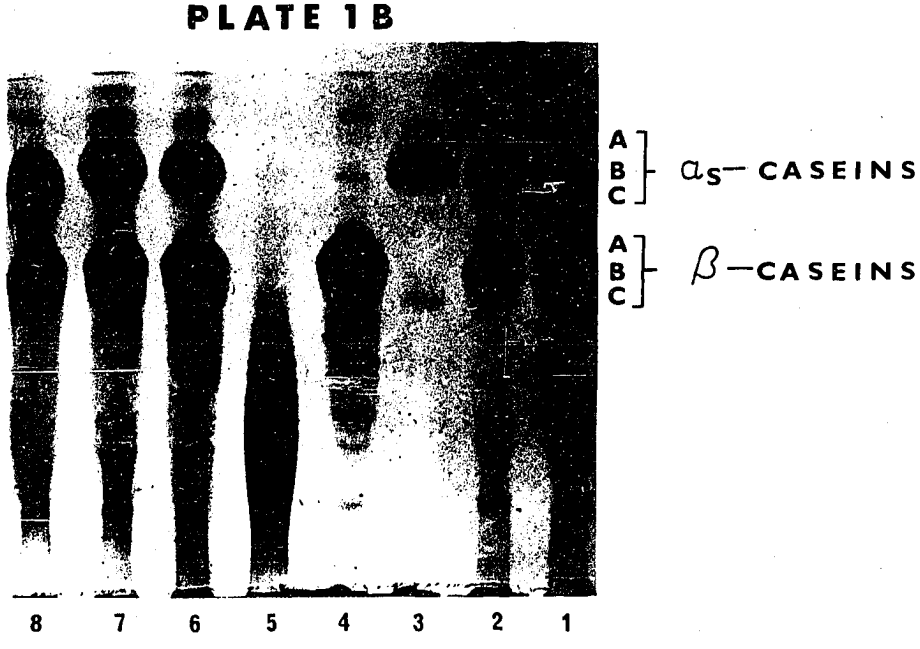
PLATE 1B
A
B
C
α_s— CASEINS
A
B
C
β—CASEINS
8
7
6
5
4
3
2
1

Figure 16. Horizontal urea starch gel electrophoretic patterns of differentially centrifuged, at 53,620 x g and 0 C, native calcium caseinate fractions

Electrophoretic conditions: The basic procedure of Wake and Baldwin (1961) was employed. Gel size 14 x 22.3 x 0.6 cm, potential 180 volts (40 mA), borate line migrated 12 cm beyond the sample slot. All fractions were dispersed in 6.6 M urea-tris-citrate buffer, pH 8.6.

Plate 2 A Nigrosine stained.
Plate 2 B Amido Black stained.

Samples[1] (slot No. and sample protein concentration):

1) 0-min fraction, 1.5%; 2) 10-min fraction, 1.5%; 3) 20-min fraction, 1.5%; 4) 30-min fraction, 1.5%; 5) 40-min fraction, 1.5%; 6) 55-min fraction, 1.5%; 7) 60+10(70)-min fraction, 1.5%; 8) 60+10+10(80)-min fraction, 0.75%.

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[1]For the method of isolating each fraction see methods section (pp. 42-46).

PLATE 2A

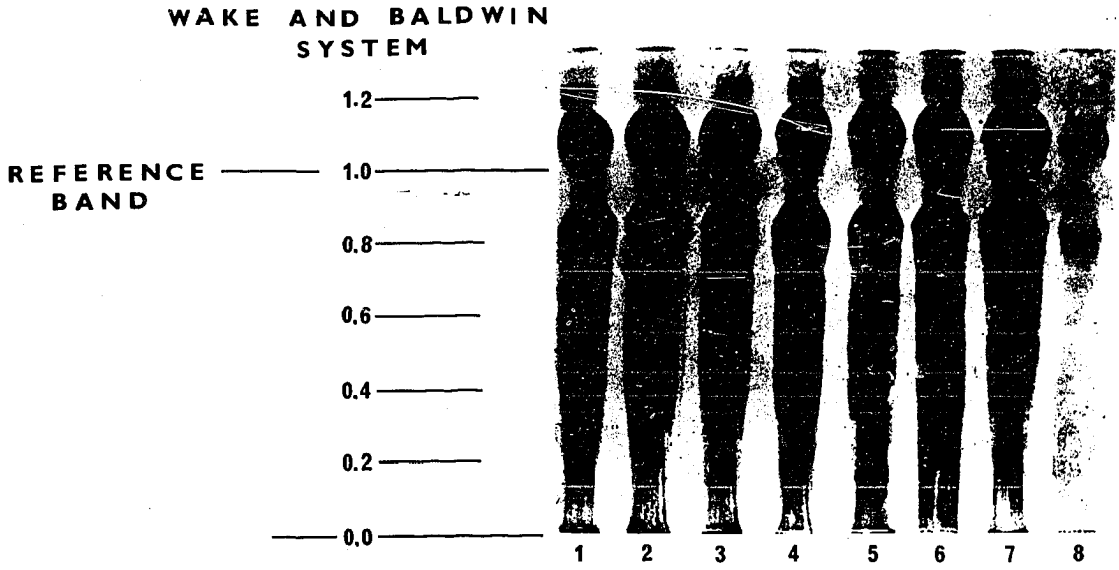
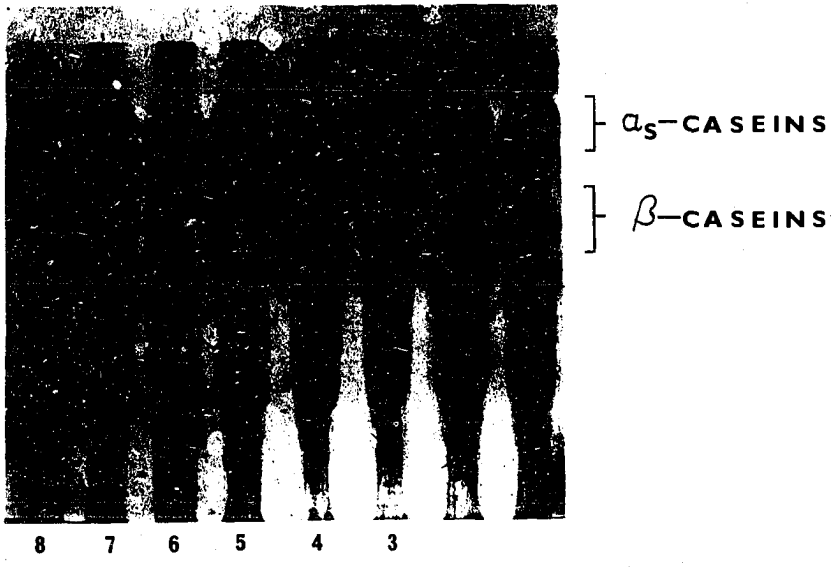


PLATE 2B



decrease in the quantity of α_s-casein agrees with the data presented in the section concerning moving boundary electrophoresis and likewise with the moving boundary electrophoretic results of Bohren and Wenner (1961) for the caseinate fraction not sedimented at low temperature. Sullivan et al. (1955) had previously reported that β-casein is depolymerized (more soluble) at low (4.4 C) than at high (25-30 C) temperatures a fact supported by Waugh (1962) who found that the minimum conditions for stable micelle formation are α_s-, κ-casein, and divalent cations and that β-casein is incorporated into the micelle as the temperature is increased. It is interesting to note that the bands in the 0.47 and 0.54 relative band region may correspond to the major κ-casein bands in the USG-MCE patterns (Figure 2, p. 63) despite the fact that the relative band positions do not agree because of the effect of MCE on the relative band positions in this region (to be discussed in the following section). USG patterns, of the whey-soluble casein fraction, also show very intensive banding in the region of bands 14, 15 and 16 (Figures 1 and 15), especially as regards band 16. If the suggestion of Wake and Baldwin (1961), that these bands are related to γ-casein, is correct, bands 14, 15 and 16 (Figure 15, slot No. 4) suggest that γ-casein concentration may be dependent on the micelle size. However, Hipp et al. (1952) indicated a close similarity between β-casein and γ-casein based on their solubilities at

2.5 and 25 C. These data confirm the results with moving boundary electrophoresis for this fraction (whey-soluble casein) in which a well defined γ-casein peak was obtained (Table 6).

The densitometric data (Table 8, Appendix, Table 17) show

Table 8. The α_s- and β-casein contents of isoelectric casein, α_s-casein and native calcium caseinate fractions based on densitometry of transparencies of urea starch gel electrophoretic patterns[a] (α_s- plus β-casein = 100%)

Fractions[c]	% Protein in sample	Amido black stain relative %[b]		Nigrosine stain relative %[b]	
		α_s-casein	β-casein	α_s-casein	β-casein
0-min	1.5	45.0	55.0	46.4	53.6
10-min	1.5	44.4	55.6	45.1	54.9
20-min	1.5	47.7	52.3	46.9	53.1
30-min	1.5	44.4	55.6	43.2	56.8
40-min	1.5	44.4	55.6	48.7	51.3
55-min	1.5	47.5	52.5	47.0	53.0
60+10(70)-min	1.5	46.7	53.3	44.8	55.2
60+10+10(80)-min	0.75	46.9	53.1	45.5	54.5
20-hour	1.5	46.0	54.0	43.3	56.7
I.E., whey-sol. casein	0.75	3.6	96.4	5.0	95.0
100-hour W	1.5	46.1	53.9	44.2	55.8
100-hour UW	1.5	49.6	50.4	49.0	51.0
α_s-casein	0.5	95.8	4.2	95.8	4.2
I.E. casein[d]	2.0	47.0	53.0	56.3	53.7
I.E. casein[d]	1.0	49.1	50.9	49.0	51.0

All samples were dispersed in 6.6 M urea-tris-citrate buffer (pH 8.6).

[a]Transparencies correspond to the pictures in Figures 17 and 18.

[b]Average of two values, densitometer zero set just ahead of the borate boundary and the α_s-casein band. (See Appendix, Table 17.)

[c]For the method of isolating each fraction see methods section (pp. 42-46).

[d]Isoelectric casein preparation 1.

reasonable agreement between Amido Black and Nigrosine stained patterns with the exception of the 40-min fraction.

The data (Table 8) show some variation, especially for the Nigrosine stained patterns, with varying micelle size. However, no trend is evident. The 100-hour unwashed fraction, Table 8, appears to contain more α_s-casein (or less β-casein) than the 100-hour washed fraction. This may be due to inclusion of a whey protein contaminant in the α_s-casein bands or to the loss of a component in the wash water. Some variation in α_s-casein and/or β-casein content is noted for the isoelectric casein preparation (Table 8) possibly due to casein concentration (1.0 percent vs. 2.0 percent) differences. However, the variation in α_s-casein and/or β-casein, noted above, is not sufficient to suggest that α_s- and β-casein contents of the 60+10+10(80)-min fraction are in error because of concentration differences (0.75% protein vs. 1.5% protein for the other caseinate fractions, Table 8).

Urea-mercaptoethanol starch-gel (USG-MCE) electrophoretic patterns

A few problems were encountered in maintaining a straight borate boundary during electrophoresis when MCE was used, especially if all the samples were caseinates. For this reason, the borate boundary was allowed to migrate only 10 cm from the sample slot, instead of the usual 12.0 cm, on gels containing eight caseinate samples (Figure 17). The resulting

Figure 17. Horizontal urea-2-mercaptoethanol starch-gel electrophoretic patterns of differentially centrifuged, at 53,620 x g and 0 C, native calcium caseinate fractions

Electrophoretic conditions: The basic procedure of Wake and Baldwin (1961) as modified by Neelin (1964) was used. Gel size 14 x 22.3 x 0.6 cm, potential 180 volts (40 mA), borate boundary migrated 10 cm beyond sample slot. All samples were dispersed in 6.6 M urea-tris-citrate buffer, pH 8.6, and 0.01 ml of 2 mercaptoethanol added/ml of sample at least 3 hr before electrophoresing (Woychik, 1964).

Plate 3 A Nigrosine stained.
Plate 3 B Amido Black stained.

Samples[1] (slot No. and sample protein concentration):

1) 0-min fraction, 1.5%; 2) 10-min fraction, 1.5%; 3) 20-min fraction, 1.5%; 4) 30-min fraction, 1.5%; 5) 40-min fraction, 1.5%; 6) 55-min fraction, 1.5%; 7) 60+10(70)-min fraction, 1.5%; 8) 60+10+10(80)-min fraction, 0.75%.

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[1]For the method of isolating each fraction see methods section (p. 42-46).

BAND NO.

PLATE 3A

WAKE AND BALDWIN SYSTEM

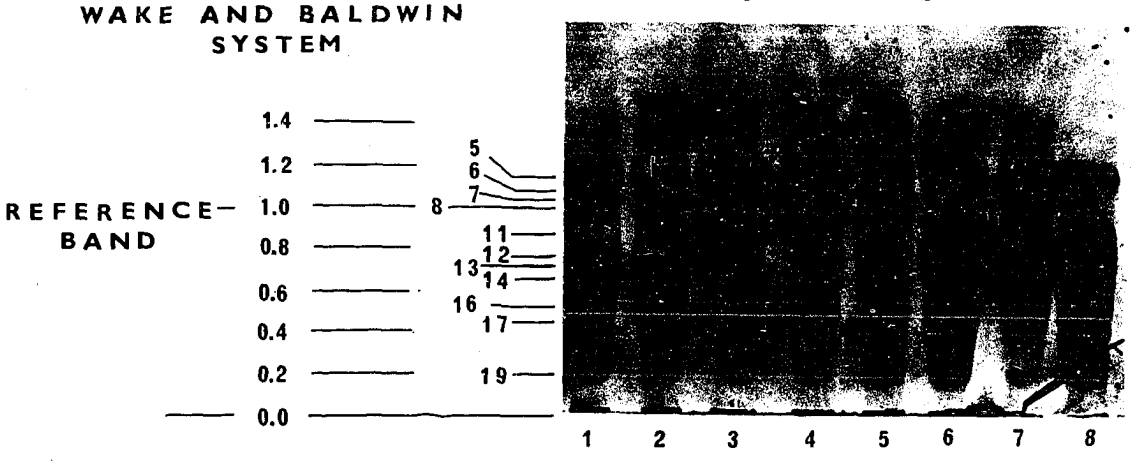
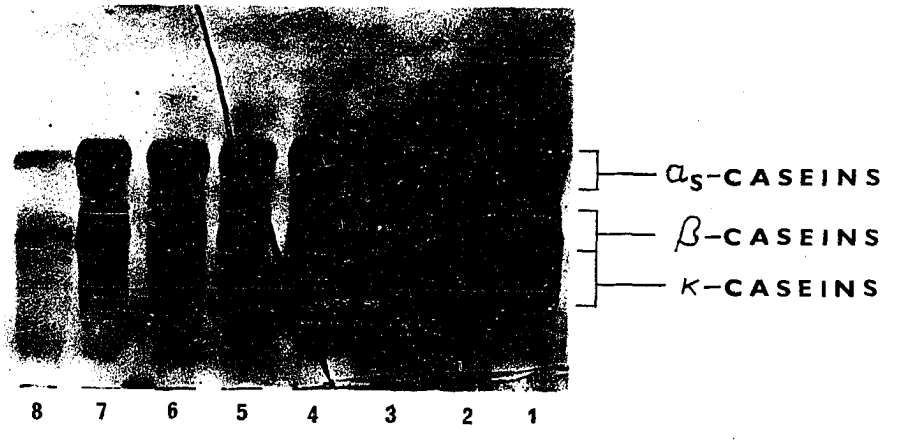


PLATE 3B



patterns are in general agreement with caseinate samples on gels in which the borate boundary migrated 12.0 cm (Figures 18 and 19). The only observed difference was a slight decrease in relative band positions of the β- and κ-casein components (for κ-casein from 0.39 to 0.64 vs. 0.48 to 0.77).

All caseinate samples did show typical κ-casein patterns, (Figures 17, 18, and 19). However, some smearing was still observed. In general, all USG-MCE patterns (Figures 17, 18, and 19) were not as clear as the USG patterns (Figures 15 and 16) due to darker backgrounds. All caseinate fractions show a large pre-β-casein band, 0.94 relative band position, for a 12-cm borate boundary migration (Figures 18 and 19) and 0.86 for a 10-cm migration (Figure 17) at about the No. 10 and 11 band position of Figure 2 (p. 63). This band probably is β-A casein and despite some uncertainty, it is included in the β-casein fraction in the densitometer analysis of the patterns. Both isoelectric casein preparations, 1 and 2, Figure 18, contained band 18 of Figure 2(p. 63), 0.39 and 0.65 relative band positions respectively. Caseinates obtained in 100 hours at 144,700 x g and 0 C did show band 18 (Figures 18 and 19), the washed sample showing it more clearly. These results suggest the possible micelle-size dependence of band 18. However, since three different milks are involved and the whey-soluble casein fraction (Figures 18 and 19, slot No. 4) contained no band 18, there is a possibility that band 18 is

Figure 18. Horizontal urea-2-mercaptoethanol starch-gel electrophoretic patterns of isoelectric casein, α_s-casein, κ-casein, and native calcium caseinate fractions

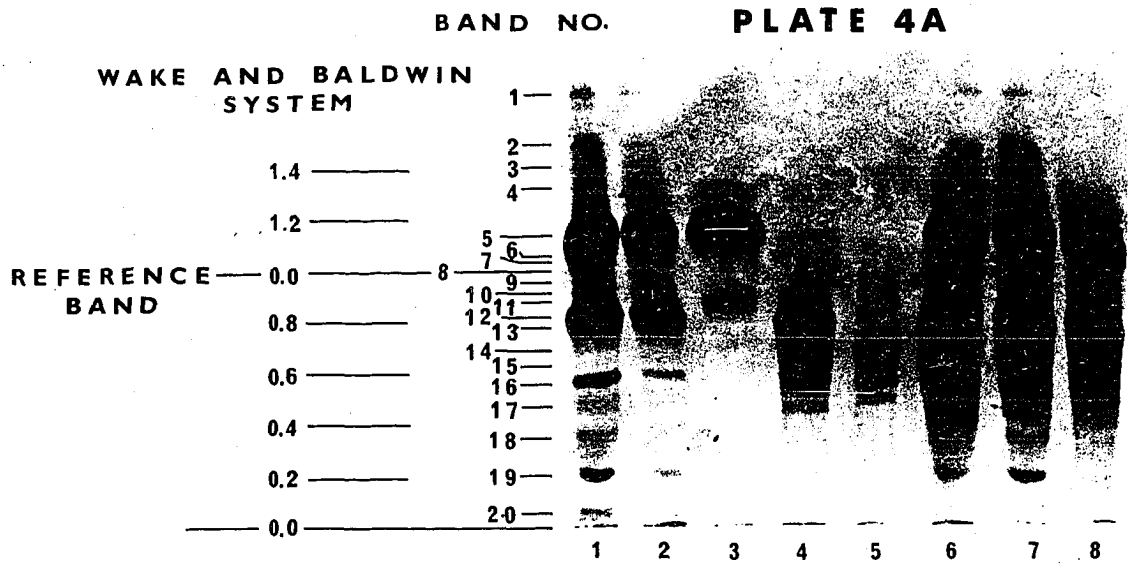
Electrophoretic conditions: The basic procedure of Wake and Baldwin (1961) as modified by Neelin (1964) was used. Gel size 14 x 22.3 x 0.6 cm, potential 180 volts (40 mA), borate boundary migrated 12 cm beyond sample slot. All samples were dispersed in 6.6 M urea-tris-citrate buffer (pH 8.6), except the caseinate fractions which were dispersed in 6.6 M urea-tris-citrate-oxalate buffer (pH 8.6). All Samples contained 0.01 ml of 2-mercaptoethanol/ml of sample added at least 3 hr before electrophoresing (Woychik, 1964).

Plate 4 A Nigrosine stained.
Plate 4 B Amido black stained.
Plate 4 C Nigrosine stained.

Samples[1] (slot No. and sample protein concentration):

1) Isoelectric casein preparation 2, 1.5%; 2) isoelectric casein preparation 2, 0.75%; 3) α_s-casein, 0.5%; 4) isoelectric whey soluble casein, 0.5%; 5) κ-casein, 0.21%; 6) 20-hour fraction, 1.5%; 7) 100-hour W fraction, 1.5%; 8) 100-hour UW fraction, 1.5%; 1A) isoelectric casein preparation 1, 2.0%; 5A) κ-casein, 1.0%.

[1]For the method of isolating each fraction see methods section (pp. 42-46).


BAND NO.
PLATE 4A
WAKE AND BALDWIN SYSTEM
REFERENCE BAND
1.4
1.2
0.0
0.8
0.6
0.4
0.2
0.0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
1 2 3 4 5 6 7 8

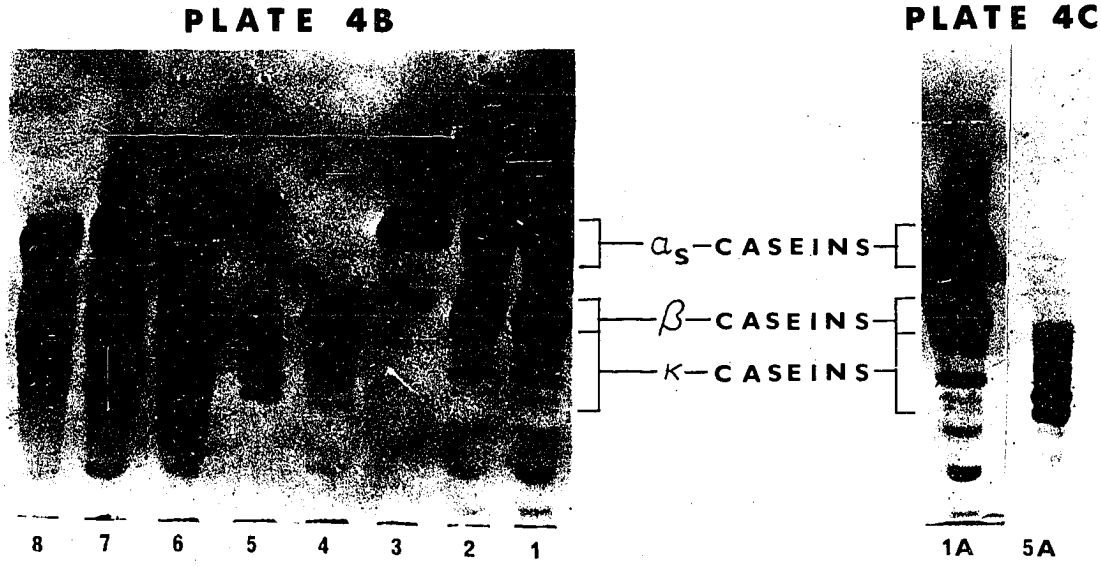


PLATE 4B
PLATE 4C
α_s-CASEINS
β-CASEINS
κ-CASEINS
8 7 6 5 4 3 2 1
1A 5A

Figure 19. Horizontal urea-2-mercaptoethanol starch-gel electrophoretic patterns of isoelectric casein, α_s-casein, κ-casein and native calcium caseinate fractions

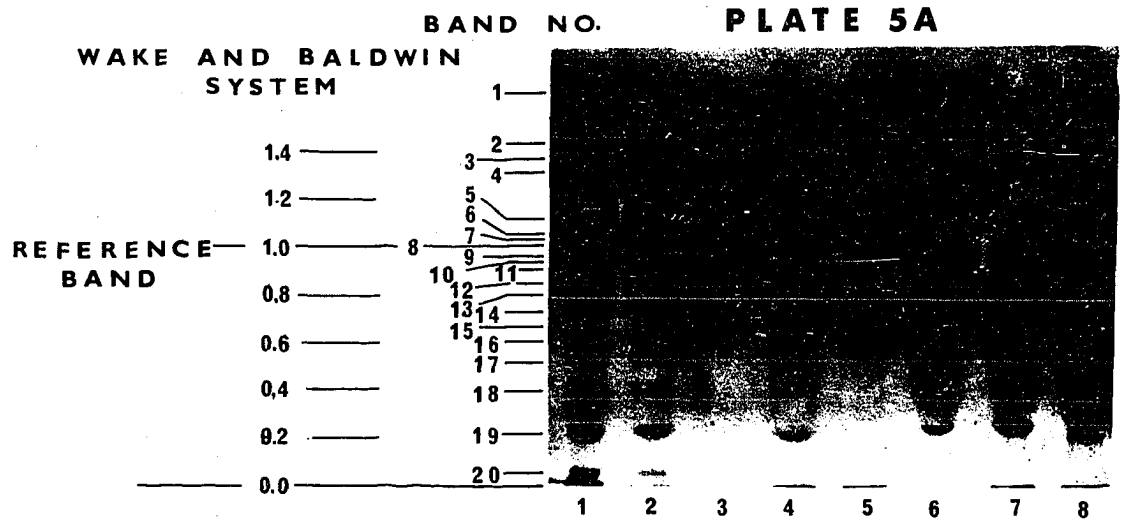
Electrophoretic conditions: The basic procedure of Wake and Baldwin (1961) as modified by Neelin (1964) was used. Gel size 14 x 22.3 x 0.6 cm, potential 180 volts (40 mA), borate boundary migrated 12 cm beyond the sample slot. All samples were dispersed in 6.6 M urea-tris-citrate buffer (pH 8.6) and 0.01 ml of 2-mercaptoethanol/ml of sample added at least 3 hr before electrophoresing (Woychik, 1964).

Plate 5 A Nigrosine stained.
Plate 5 B Amido Black stained.

Samples[1] (slot No. and sample protein concentration):

1) Isoelectric casein preparation 2, 1.5%; 2) isoelectric casein preparation 2, 0.75%; 3) α_s-casein, 0.5%; 4) isoelectric whey soluble casein, 0.5%; 5) κ-casein, 0.21%; 6) 20-hour fraction, 1.5%; 7) 100-hour W fraction, 1.5%; 8) 100-hour UW fraction, 1.5%.

[1]For the method of isolating each fraction see methods section (pp. 42-46).


BAND NO.
PLATE 5A
WAKE AND BALDWIN SYSTEM
REFERENCE BAND
1.4
1.2
1.0
0.8
0.6
0,4
0.2
0.0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
1 2 3 4 5 6 7 8

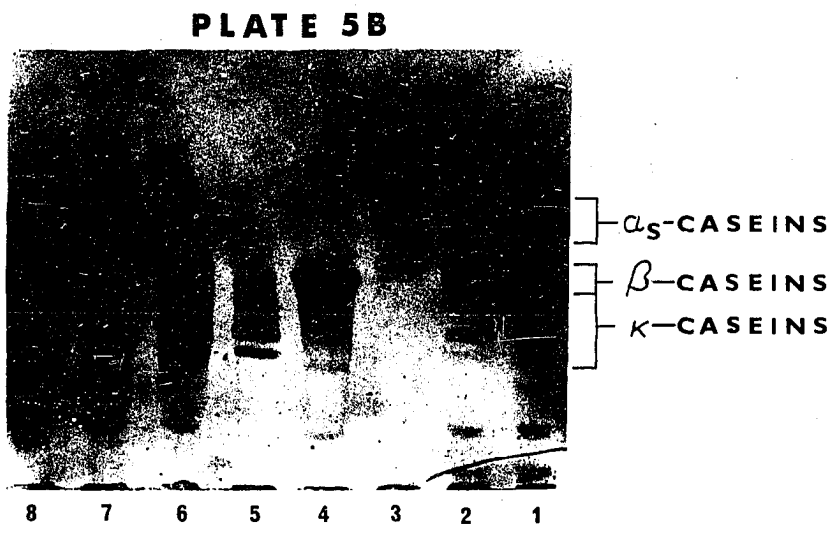


PLATE 5B
α_s-CASEINS
β-CASEINS
κ-CASEINS
8 7 6 5 4 3 2 1

a genetic variant of κ-casein. The three milks are all pooled samples from the same herd so it would seem fortuitous that differences in genetic variants could be detected. Band 18 appears in the κ-casein fraction at 1.0% concentration (Figure 18, slot No. 5A) but not at the 0.21% concentration (Figures 18 and 19, slot No. 5). Band 18, in this κ-casein fraction, may be a contaminant which would support the contention that this band is micelle size dependent. Band 20 of Figure 2 (p. 63) appeared only on patterns of whole isoelectric casein (preparations 1 and 2, Figures 18 and 19), suggesting that it may be an artifact of the method of isolation or result from the effect of MCE. The band appears much darker than the corresponding one reported by Wake and Baldwin (1961) for a few of their isoelectric preparations. They also report this band in alcohol fraction C of Hipp's et al. (1952). Wake and Baldwin (1961) did not use MCE. Bands 18 and 20 of Figure 2 (Figures 17, 18, and 19) appear to correspond to bands 15 and 17 of Figure 1(p. 61) in the patterns without MCE.

Band 19 of Figure 2 (No. 16 without MCE, Figure 1) appears in every sample except α_s- and κ-casein (Figures 17, 18, and 19) and is probably, as suggested in the previous section, related to γ-casein. Wake and Baldwin (1961) report a greatly intensified band in this position, without MCE, in the alcohol fraction C of Hipp's et al. (1952). By a visual observation, the intensity of band 19 in the 100-hour washed

caseinate fraction and the whey-soluble fraction (Figures 18 and 19) appears to be high. These results are in agreement with the data obtained by moving boundary electrophoresis (Table 6) suggesting either that γ-casein occurs in restricted micelle sizes or it is more soluble at low temperature.

A comparison can be made between two methods of dispersing caseinates fractions for USG-MCE electrophoresis. The caseinate fractions, 20-hour and 100-hour washed and unwashed fractions, (Figure 18) were dispersed in urea-tris-citrate-oxalate buffer and the calcium oxalate removed by centrifugation; the same caseinates (Figure 19) were dispersed in urea-tris-citrate buffer. The two sets of patterns (Figures 18 and 19) appear similar, especially as regards κ-casein resolution. However, the washed caseinates dispersed without oxalate appear to have more intense but less diffuse α_s- and β-casein bands. The unwashed (100-hour) fraction gel pattern in both methods (Figures 18 and 19) show poorly defined κ-casein bands.

The α_s-, β- and κ-casein values obtained for isoelectric casein, preparations 1 and 2, Table 9, are in general agreement with the values reported in the literature (Table 10). The data of Tessier et al. and Rose (Table 10) show a wide range of values. However, Rose and Marier (1963) have questioned the uniformity of κ-casein and its sialic acid content. Since their methods (Tessier et al., 1965 and Rose, 1965), for estimating κ-casein are dependent upon the sialic acid

Table 9. The α_s-, $\varkappa$-, and β-casein contents of isoelectric casein, α_s-casein, and native calcium caseinate fractions based on densitometry of transparencies of urea-2-mercaptoethanol starch-gel (Amido black stained) electrophoretic patterns[a]

Fractions[c]	% Protein in sample	Relative % of casein components[b] Plate 4 B (Fig. 18) α_s-	β-	$\varkappa$-	Plate 5 B (Fig. 19) α_s-	β-	$\varkappa$-
20-hour	1.5	37.3	36.9	25.8	32.9	38.3	28.8
I.E. whey sol. casein	0.5		65.0	35.0		68.9	31.1
100-hour W	1.5	34.9	40.5	24.6	35.9	39.7	24.4
100-hour UW	1.5	32.8	45.2	22.0	35.4	38.8	25.8
α_s-casein	0.5	91.3	8.7		90.0	10.0	
I.E. casein[d]	0.75	50.7	34.2	15.1	49.5	34.2	16.3
I.E. casein[d]	1.5	48.9	33.8	17.3	51.9	33.3	14.8
I.E. casein[e]	1.0	48.4	31.4	17.2			
I.E. casein[e]	2.0	48.9	34.4	16.7			

All samples were dispersed in 6.6 M urea-tris-citrate buffer except the 20-hour, 100-hour W, and 100-hour UW caseinate fractions in plate 5 B which were dispersed in 6.6 M urea-tris-citrate-oxalate buffer (pH 8.6).

All samples contained 0.01 ml of 2-mercaptoethanol/ml of solution which was added at least 3 hr before electrophoresing.

[a]Transparencies correspond to the pictures in Figures 18 and 19.

[b]Single values, densitometer zero set just ahead of the α_s-casein band.

[c]For the method of isolating each fraction see methods section (pp. 42-46).

[d]Isoelectric casein preparation 2.

[e]Isoelectric casein preparation 1, a Nigrosine stained pattern plate 4 C Figure 18.

Table 10. A comparison of the distribution of α_s-, β-, and $\varkappa$-casein in whole casein as reported in the literature

Reference	Percent		
	α_s-casein	$\varkappa$-casein	β-casein
Waugh and von Hippel (1956)[a]	55	15	30
Wake (1957)[b]		15	
Sullivan et al. (1959)[c]		16	
Payens (1961)[d]	51.52	7-13	36-41
Libbey and Ashworth (1961)[e]	39	11	43
Tessier et al. (1963)[f]	49-60 (54.)[g]	10-23 (16)	19-36 (30)
Rose (1965)[f]	50	16	34
This study[h]			
Preparation 1	50	16	34
Preparation 2	48.6	17	34.4

[a]Estimated by moving boundary data of fractionated casein.

[b]Estimated on the basis of the nonprotein nitrogen released by rennin from whole and $\varkappa$-casein.

[c]Estimated from the sialic acid content of skim milk.

[d]Estimated by spectrophotometric analysis of casein fractions separated by zone electrophoresis (cellulose column) with urea buffers.

[e]Estimated by paper electrophoresis in urea buffers (7% minor components not included).

[f]Estimated by tubidometric analysis of β- + $\varkappa$-casein, $\varkappa$-casein estimated by the sialic acid content.

[g]( ) Average of 10 determination on individual cow milks.

[h]Estimated by densitometric analysis of transparent prints of urea-mercaptoethanol starch-gel electrophoretic patterns.

content, it is not apparent whether a variation in sialic acid has caused the wide ranges (10-23% κ-casein) in their data or whether the κ-casein contents actually are varying. Tessier et al. (1963) and Rose (1965) examined individual cows milks which may account for some of the variation. The data (Table 10) of Libbey and Ashworth (1961) are not in agreement with the remaining data in the table which no doubt reflects the methods they employed. Under their experimental conditions (paper electrophoresis using urea buffers) κ-casein migrated with α_s-casein at urea concentrations of 0-2.5 M and with β-casein at urea concentrations of 6.0-7.0 M. The κ-casein contents were estimated by subtracting the β-casein contents from the β- + κ-casein contents or the α_s-casein contents from the α-casein (α_s- + κ-casein) contents of whole casein. Since the method, for estimating the α_s-, β-, and κ-casein contents of casein, used in this study is based on measurements on the separated fractions, the problems discussed above do not apply. However, an indirect method of measuring the separated fractions was used. It is reassuring that the values obtained for isoelectric casein are in agreement with most of the values reported in the literature (Table 10). The isoelectric caseins, preparation No. 1 and 2 (Table 9) contained relatively more α_s-casein and less κ-casein than did the caseinate fractions (Tables 9 and 11) which may be due to the smearing obtained in the κ-casein region (Figures 18

Table 11. The α_s-, κ-, and β-casein contents of differentially centrifuged, at 55,620 x g and 0 C, native calcium caseinate fractions based on densitometry of transparencies of urea-2 mercaptoethanol starch-gel electrophoretic patterns[a]

Fractions[c]	% Protein in sample	Relative % of casein components[b] Amido Black stained α_s-	β-	κ-	Nigrosine stained α_s-	β-	κ-
0-min	1.5	44.2	39.6	16.2	39.3	37.6	23.1
10-min	1.5	34.2	42.2	23.6	33.9	40.2	25.9
20-min	1.5	32.9	40.6	26.5	35.6	37.6	26.8
30-min	1.5	38.0	42.1	19.9	39.2	37.5	23.3
40-min	1.5	34.4	39.6	26.0	37.6	34.4	28.0
55-min	1.5	29.0	43.2	27.8	31.4	36.8	31.8
60+10(70)-min	1.5	37.0	41.3	21.7	37.7	36.2	26.1
60+10+10(80)-min	0.75	27.2	40.9	31.9	26.7	38.9	35.4

All samples were dispersed in 6.6 M urea-tris-citrate buffer (pH 8.6), and contained 0.01 ml of 2 mercaptoethanol/ml of sample which was added at least 3 hr before electrophoresing.

[a]Transparencies correspond to the pictures in Figure 17.

[b]Single values, densitometer zero set just ahead of the α_s-casein band.

[c]For the method of isolating each fraction see methods section (pp. 42-46).

and 19) of the USG-MCE electrophoretic patterns. This should not affect the results shown in Figure 20, since consistency in gel electrophoresis and analysis of the densitometer traces was maintained.

The data in Table 11, graphed in Figure 20, show some variability in micelle composition as micelle sizes decrease.

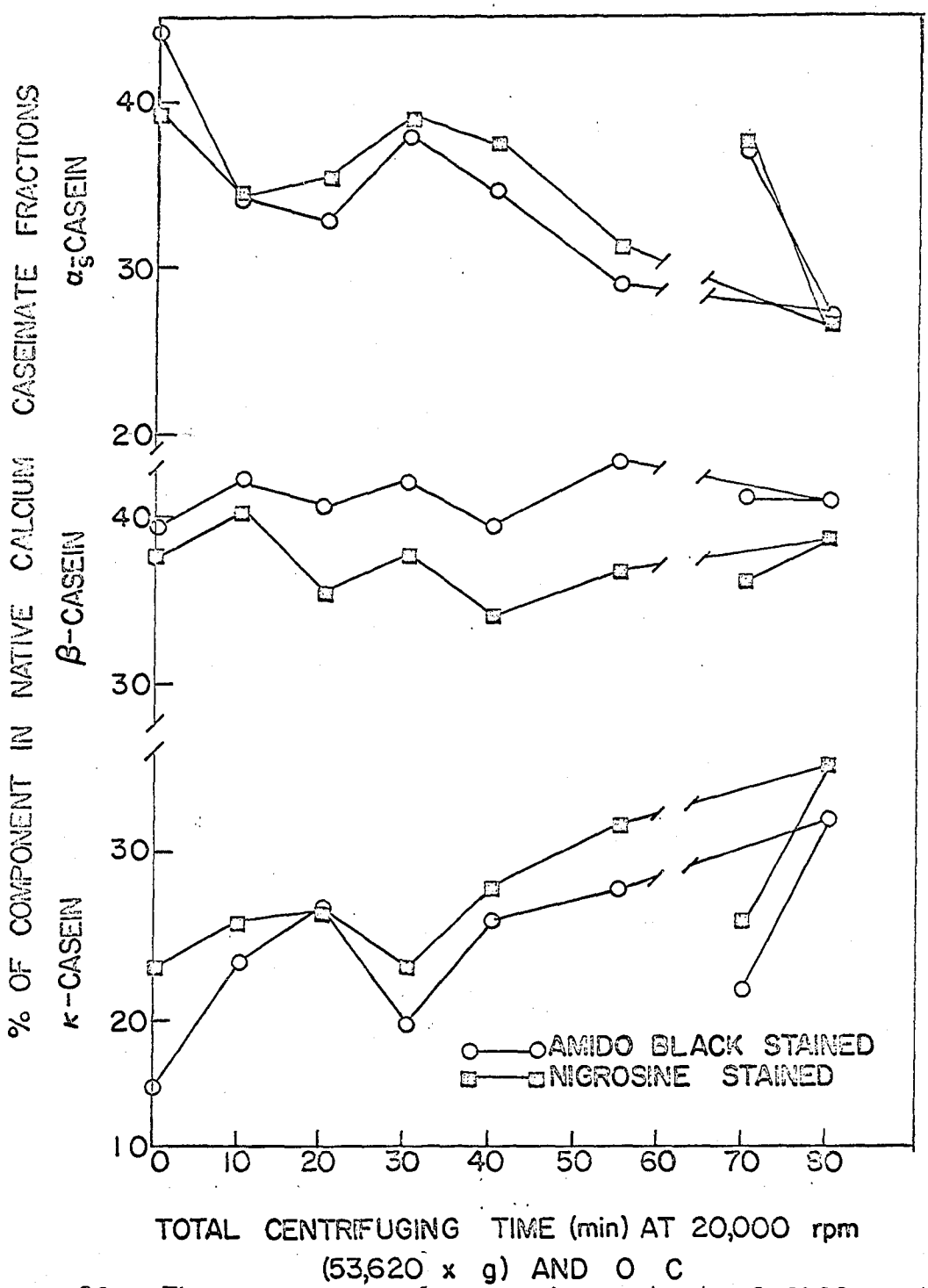


Figure 20. The α_S-, β-, and κ-casein content of differentially centrifuged native calcium caseinate fractions based on densitometry of transparencies of urea 2-mercaptoethanol starch gel electrophoretic patterns.

It is recognized that variability may result from fluctuations in the micelle composition or from experimental errors or both. The data show (Table 11) that generally the α_s- and κ-casein values are slightly higher and the β-casein values slightly lower in the Nigrosine stained patterns than in the Amido Black stained patterns. However, the values for both stains fluctuate in a similar manner (Figure 20) which is reassuring in view of the fact that the Nigrosine stained patterns appear to have darker backgrounds. The agreement between the two stains may reflect the fact that κ-casein, employed as a standard, was electrophoresed at a concentration approximating its concentration in the caseinate fractions (0.21% based on the assumption of 14% κ-casein in the caseinate fractions) in order to establish the region occupied by the κ-casein in the caseinate patterns and the method employed in establishing the baseline was selected in a manner that was considered to reduce background errors. It is considered that despite the variability in the data (Figure 20) they show a general trend toward an increase in κ-casein accompanied by a decrease in α_s-casein content as micelle size decreases.

The densitometric data (Table 9) for the isoelectric caseins, electrophoresed at protein concentrations of 0.75% and 1.5% (preparation 2) and 1.0% and 2.0% (preparation 1), show that the concentration employed had no adverse affect on the relative compositions of these samples. It seems probable

that this would hold for the caseinate samples and implies that the relative compositions obtained for the 60+10+10(80)-min fraction, electrophoresed at 0.75% compared with 1.5% protein for the remaining micelle fractions, is valid.

The electrophoretic mobility of κ-casein is changed markedly by the method of electrophoresis employed. In moving boundary electrophoresis, κ-casein migrates with α_s-casein as a complex designated α-casein. In USG the κ-casein migrates as a long smear just behind β-casein. This is caused by the sieving effect of the starch-gel on the aggregated κ-casein (Wake and Baldwin, 1961) and it has been postulated by Neelin (1964) that smearing is caused by random disulfide linkages resulting in a wide particle size distribution. The addition of MCE to the USG and to the sample, breaks these disulfide bonds resulting in a more restricted particle size distribution, yielding more descrete banding in the region where previously κ-casein appeared as a smear. The improved resolution of κ-casein upon disruption of these disulfide bonds makes it possible to estimate κ- as well as α_s- and β-casein by analysis of USG-MCE patterns. The sum of the α_s- and κ-casein values in the USG-MCE patterns of the micelle fractions (Table 11), comparable to α-casein in moving boundary, ranged from 57 to 60% for Amido Black stained patterns and 60 to 66% for Nigrosine stained patterns while the values obtained by moving boundary for α-casein (Table 6)

ranged from 74 to 77% in the descending patterns and 63 to 70% in the ascending patterns. It is apparent that moving boundary and USG-MCE (Table 11) can be shown to agree empirically with the moving boundary electrophoresis data (Table 6). The fact that no trend was observed in the relative % of α- and β-casein with most of the caseinate fractions (except the 80-min fraction) in moving boundary electrophoresis, suggests that there is no change in the β-casein content as micelle size decreases, under the conditions employed to obtain and electrophoresis the micelle fractions. Since α-casein consists of α_s- and κ-casein, the fact that no change appeared to occur in the β-casein content, as the micelle size changed, with moving boundary electrophoresis suggests that any change in micelle composition must be in the α_s- and κ-casein, which is in agreement with the USG-MCE data.

Waugh (1958) has postulated that the basic requirement for micelle formation is the presence of α_s- and κ-caseins and divalent cations. He suggests that rennin activity (the primary reaction being a splitting off of a glycomacropeptide from κ-casein) is a surface phenomenon, since all of the κ-casein is not attacked by rennin. If this is true, the increase in surface area of the smaller micelles would make it possible for more κ-casein to be associated with these micelles. The addition of β-casein complicates this picture and its role has yet to be elucidated. The increased solubility of

β-casein at low temperature (Sullivan et al., 1955) has been suggested by moving boundary electrophoresis in this study (I.E. whey soluble fraction) and by Bohren and Wenner (1961) and confirmed by USG and USG-MCE in this study. In addition, the preferential depletion of α_s-casein from the whey-soluble fraction has been demonstrated by USG and USG-MCE electrophoresis, but was only implied by Bohren and Wenner (1961). Since a constant temperature was employed in the differential sedimentation of the micelles, the data (Table 11) suggest that an equilibrium was obtained between solubilized (monomeric) and polymeric β-caseins and that this equilibrium was not changed during the differential sedimentation of the micelles, for the β-casein content of the micelles was shown to be essentially constant by both moving boundary and USG-MCE electrophoresis.

The USG-MCE data (Tables 9 and 11, Figure 20) are not in agreement with the USG data (Table 8) as regards α_s- and β-casein compositions; no relationship between micelle size and α_s-casein compositions was observed with USG. The different methods employed in establishing the baseline on the traces of USG (Figure 4, p. 68) and USG-MCE (Figure 5, p.69) patterns may be the reason that changes in α_s-casein contents of the caseinate micelle fractions could not be detected on USG. In addition, the extensive smearing of κ-casein when MCE is not used may trap either α_s- or β-casein or both resulting in

variable results that might mask existing trends. Since quantitative analysis of USG or USG-MCE casein patterns are not reported in the literature, insufficient information concerning the possibility of trapping the more mobile protein components by the less mobile protein components, i.e., κ-casein, is available. In this regard, Neelin et al. (1962) suggested that the more mobile contaminants (α_s- or β-casein) in several of the κ-casein preparations they studied may be trapped by κ-casein in non-urea gels.

The α_s-casein component in the whey-soluble casein fraction, which is shown to be equal to about 4.0 percent of the β-casein content, when the fraction is electrophoresed on USG (Table 8), appears to be absent in the USG-MCE gels stained with Amido black (Figures 18 and 19, plates 4B and 5B) and is barely visible when Nigrosine is used (Figures 18 and 19, plates 4A and 5A). The α_s-casein component in the whey-soluble casein fraction when electrophoresed on USG-MCE is so faint that it has been ignored in the densitometer analysis of the gel pattern (Table 9). These data suggest a slight effect on MCE on the α_s-casein which is illustrated by the apparent increase in the β-casein contaminant in the α_s-casein fraction with MCE (about 5% β-casein without MCE, Table 8 vs. 9% β-casein with MCE, Table 9). This β-casein contaminant in the α_s-casein fraction also exhibits an increase in its relative band position from about band position 13 to

band position 12 when MCE is used (Figures 15, 18 and 21).

In general, the densitometer data (Table 9) between the caseinates dispersed with and without oxalate (Plate 4B, Figure 18 and plate 5B, Figure 19, respectively) show some variation between dispersing methods. The washed 100-hour fraction showed good agreement between methods; the 20-hour and unwashed 100-hour fractions do not. This is due, in part, to the slightly skewed pattern obtained with the latter fractions in plate 5B, Figure 19.

The increase in κ-casein with decreasing micelle size (Table 11, Figure 20) observed in this study is in agreement with Sullivan et al. (1959), Ribadeau-Dumas and Veaux (1964), and Rose (1965). These workers results were based on the sialic acid content as an index of the κ-casein concentrations. McGann and Pyne (1960) obtained similar results based on the amount of NPN released by rennin in whole milk and "small particle" milk. Rose (1965), whose data are the only ones published to date which present α_s-, β-, and κ-casein contents of various micellar fractions, reported an increase in κ-casein and a decrease in β-casein with decreasing micelle size. This trend is not in agreement with the data obtained in this study; a decrease in α_s-casein was found to accompany an increase in κ-casein in this study. It is not clear why the discrepancy between the data in Table 11 (Figure 20) and those of Rose exists. The paper by Rose (1965) is a review

and the author did not explain in detail the conditions he used. A portion of his fractions were obtained at temperatures other than refrigerated temperature and his last sedimented fraction was obtained at refrigerated temperature. This change in temperature during fractionation of the caseinate micelles undoubtedly affected the composition of Rose's last sedimented fraction as well as his non-sedimented casein fraction, since β-casein is considered to be more soluble at low temperature than at room temperature (Sullivan et al., 1955). As regards the turbidimetric method employed by Rose (1965), it is possible that the results obtained with native caseinates may not fit a standard curve which has been established with acid casein, isolated α_s-, β-, and $\varkappa$-casein fractions, and mixtures of these fractions approximating whole casein. If this is true, the data of Rose (1965) for the α_s-casein compositions of fractionate micelles may be incorrect, since α_s-casein is estimated by difference (100- (β- + $\varkappa$-casein)). Rose also reports that a visual observation of his USG-MCE patterns (not included in the publication) show a decrease in β-casein content with decreasing micelle size, but only a slight increase in $\varkappa$-casein intensities was observed. Unfortunately Rose (1965) does not indicate his method of dispersing the caseinate for USG-MCE electrophoresis.

Polyacryalimide Disc Electrophoresis

As with the urea starch gel (USG) patterns, empirical results were obtained by simply viewing the gels. These results are considered under the appropriate headings.

Recoveries of α_s-casein in mixtures of α_s- and κ-casein

Since κ-casein does not migrate in $7\frac{1}{2}$ percent polyacrylamide gels (PAG) in the concentrations used, it seemed desirable to determine whether the κ-casein trapped in the sample gel held back part of the α_s-components. The data are summarized in Table 12.

Table 12. Amounts of α_s-casein that migrate in mixtures of α_s- and κ-casein using disc electrophoresis with and without urea

Concentration μg Protein/tube			Percent α_s Recovered[a,b]	
α_s-casein	κ-casein	α_s:κ	without urea	with urea
50	0.00	-	100.0	100.0
50	6.25	4	104.0	95.0
50	12.50	2	90.8	93.3
50	25.00	1	80.0	75.3
50	50.00	0.5	45.0	54.8

[a]Average of two tubes (gels)(See Figure 21).

[b]The percentages of α_s-casein are based on the amount that migrated in the absence of κ-casein as 100%.

The percentage of α_s-casein that migrated during electrophoresis decreased markedly as the κ-casein content of the mixture increased. It should be noted that the total protein concentrations placed on the gels were not constant, so that part of the reduction in the amount of α_s-casein that migrates

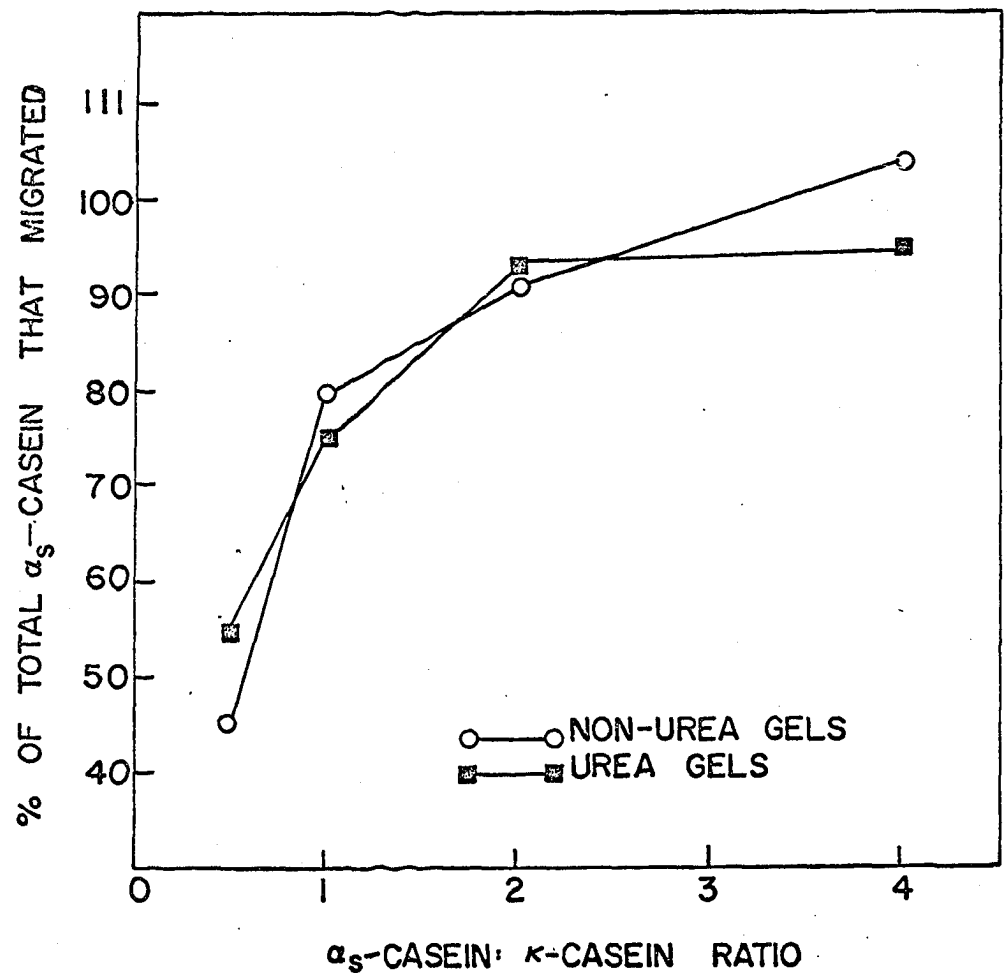


Figure 21. Relative amounts of α_s-casein that migrate in mixtures of α_s- and κ-casein using urea and non-urea polyacrylamide gels in disc electrophoresis.

probably results from this variation in total concentration. It must also be pointed out that the relative areas of individual peaks of samples electrophoresed in duplicate are quite variable. The above considerations not withstanding, a definite reduction in α_s-casein with increasing κ-casein concentration in the mixture is apparent (Figure 21). This decrease may result from the formation of a complex between α_s- and κ-casein or because κ-casein closes the pores of the gel before all the α_s-casein has entered the spacer gel. In the case of the latter, β-casein should also have been affected and reproducible trapping of the various components should not have been obtained. The possibility that κ-casein complexes with β-casein has been suggested by Swaisgood and Brunner (1962). Further evidence that would support either of the above hypothesis was not obtained in this study.

From USG-MCE data (Table 11), it would appear that all the micelle fractions have α_s-casein: κ-casein ratios greater than 1 except the 60+10+10(80)-min fraction. If this relationship holds for disc electrophoresis, and the data of Table 12 are applicable to caseinate samples, the sum of the percentages of α_s- and β-casein that migrate should exceed 80; the α_s-casein: β-casein ratio may or may not be affected.

Comparison of methods of dispersing native calcium caseinate (20-hour fraction) without urea

Three methods, oxalate, EDTA, and citric acid were used

to disperse the 20-hour fraction as previously described, and the results summarized in Table 13.

Table 13. Methods of dispersing calcium caseinates (20-hour fraction) without urea

Method of[a] dispersing	Percent of α_s-casein	Percent of β-casein	Ratio α_s:β
Sodium Oxalate	47.60	52.40	0.910
EDTA[b]	47.00	53.00	0.887
Citric Acid	47.70	52.30	0.913

[a]Sample concentration 100 μg protein/tube (gel), in duplicate.

[b]Disodium ethylenediaminetetraacetate.

The data by the three methods of dispersion are in good agreement. On the basis of sharper banding, citric acid was used as the dispersing agent for the "native" calcium caseinate in all subsequent disc electrophoresis without urea.

Comparison of methods of dispersing native calcium caseinate (20-hour fraction) in the presence of urea

Two methods, urea and urea-oxalate, were used to disperse the 20-hour fraction as previously described. Three variations in electrophoretic technique were also compared and the results summarized in Table 14.

The data in Table 14 show that the method of dispersing the caseinate had little affect on the respective quantities or ratios of α_s- and β-caseins. Some variation is observed

Table 14. Comparison of methods of dispersing the "native" calcium caseinates[a] (20-hour fraction) in the presence of urea

Urea concentrations in Gels[b]		Percent of α_s- and β-casein urea dispersed			urea-oxalate dispersed		
Spacer Gel	Lower Gel	α_s	β	α_s:β	α_s	β	α_s:β
5.0 M urea	5.0 M urea	45.20	54.80	0.825	45.90	54.10	0.848
5.0 M urea	6.6 M urea	46.87	53.13	0.882	46.45	53.55	0.867
6.6 M urea	6.6 M urea	45.70	54.30	0.842	45.30	54.70	0.828

[a]Sample concentration, 100 μg protein/tube (gel).

[b]All sample gels were 6.6 M urea.

among the urea concentrations in the gel; these do not appear to be excessive. All subsequent disc electrophoreses of caseinates were conducted using 5.0 M urea in the spacer and lower gels and 6.6 M urea in the sample gels.

Disc electrophoretic patterns without urea

The banding in the non-urea patterns of the caseinates (Figure 22) was almost identical to the banding obtained with isoelectric whole casein diagrammed in Figure 6 (p. 76). The bands between the major α_s- and β-casein bands (6 and 7) were present in most of the micelle fractions, but were quite low in intensity and do not show in the photographs. The number 8 band was well defined in all patterns. Bands 6, 7 and 8, were well defined in the 100-hour washed caseinate sample. In general, the α_s-casein band was quite sharp, compared with the slightly less intense, but broader β-casein

Figure 22. Non-urea polyacrylamide disc electrophoretic patterns of isoelectric casein, α_s-casein, κ-casein and native calcium caseinate fractions

Electrophoretic conditions:
Current, 5 mA per gel for approximately 50 minutes, stained with Amido Black for at least 1 hr and destained with a current of 10 mA per gel. All samples dispersed in sample gel solutions containing tris-citrate buffer (pH 6.9).

Samples[1] (gel No. and μg of protein per gel):

Plate 6 A 1) Isoelectric casein preparation 1, 100 μg; 2) α_s-casein, 50 μg; 3) isoelectric whey-soluble casein, 100 μg; 4) κ-casein, 100 μg; 5) κ-casein, 200 μg; 6) 20-hour fraction, 100 μg; 7) 100-hour W fraction, 100 μg; 8) 100-hour UW fraction, 100 μg.

Plate 6 B 1) 0-min fraction, 100 μg; 2) 10-min fraction, 100 μg; 3) 20-min fraction, 100 μg; 4) 30-min fraction, 100 μg; 5) 40-min fraction, 100 μg; 6) 55-min fraction, 100 μg; 7) 60+10 (70)-min fraction, 100 μg; 8) 60+10+10(80)-min fraction, 100 μg.

[1]For the method of isolating each fraction see methods section (pp. 42-46).

PLATE 6A

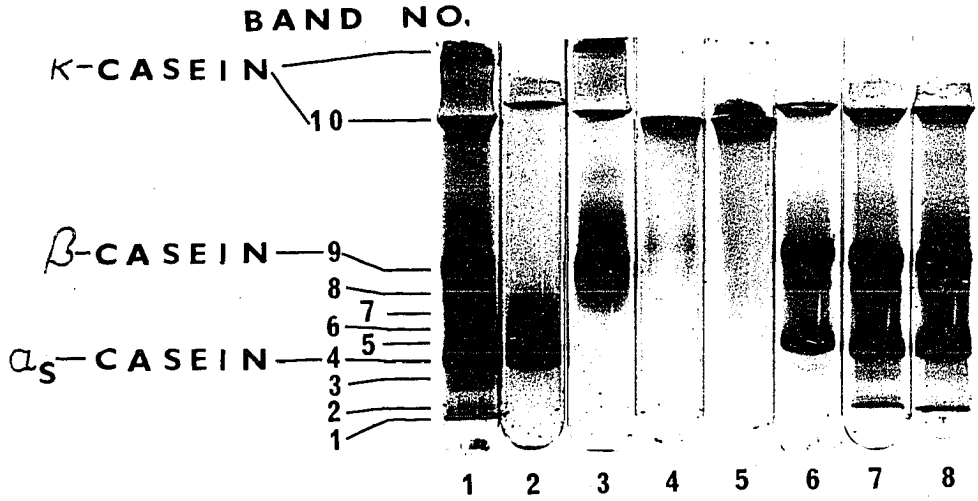
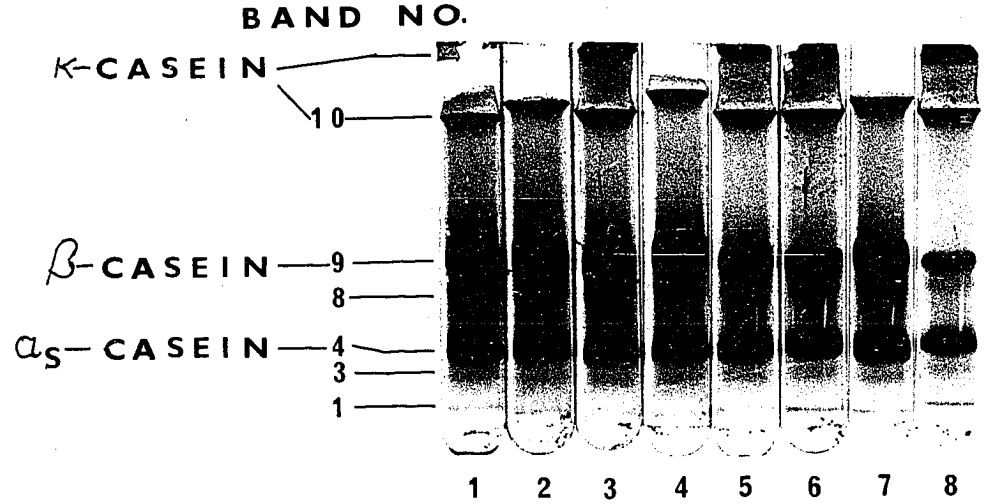


PLATE 6B



band which had a concave trailing edge. The whey-soluble casein fraction, precipitated at pH 4.6 from the supernatant of skim milk centrifuged for 20 hours at 53,620 x g and 0 C, Figure 22, showed essentially no α_s-casein component (less α_s-casein than was obtained with USG), in agreement with the USG data (Table 8). Smearing, noted to a much lesser extent on the caseinate fractions, was noted behind the β-casein band in the whey-soluble casein fraction; it probably is due to casein components other than β-casein.

The densitometer data, Table 15, show a somewhat random variation in α_s- and β-casein contents of the micelle fractions, regardless of the method used for analyzing the tracings. No definite trend is shown. The 60+10+10(80)-min fraction (smallest sedimented micelle) shows a high α_s-casein content which agrees with the data of Rose (1965) but is contradictory to the data in Tables 8, 9 and 11 for USG and USG-MCE. Since the other fractions do not exhibit a trend, the meaning of the 80-min sample values it is not clear. The 80-min fraction showed an atypical moving boundary electrophoretic pattern (Figure 12, p. 102)compared with all the other caseinate fractions. A broad peak between the α-casein and β-casein peaks, with an intermediate mobility of approximately -4.7×10^{-5} cm^2 $volts^{-1}$ sec^{-1}, was observed. The percentages of α-casein, X-casein and β-casein, in the descending moving boundary electrophoretic patterns were approximately 55.0,

Table 15. The α_s- and β-casein contents of isoelectric casein and native calcium caseinate based on densitometry of non-urea disc electrophoretic patterns (α_s- plus β-casein = 100%)

Fractions[a]	μg protein per gel	Relative % of casein components				ave	ave
		α_s-	α_s-	β-	β-	α_s-	β-
0-min	100	46.9	45.2	53.1	54.8	46.10	53.90
10-min	100	46.6	48.2	53.4	51.8	47.40	52.60
20-min	100	48.3	48.0	51.7	52.0	48.15	51.85
30-min	100	44.7	43.9	55.3	56.1	44.30	55.70
40-min	100	48.9	49.2	51.1	50.8	49.05	50.95
55-min	100	47.9	56.6	52.1	53.4	47.25	52.75
60+10(70)-min	100	47.5	56.2	52.5	53.8	46.85	53.15
60+10+10 (80)-min 20-hour	50	48.6		51.4			
20-hour	100	47.5	47.3	52.5	52.7	47.40	52.60
I.E. whey sol. casein	100	0.4	0.4	99.6	99.6	0.40	99.60
100-hour W	100	51.8	51.4	48.2	48.6	51.60	48.40
100-hour UW	100	51.8	50.9	48.2	49.1	51.35	48.65
I.E. casein[b]	250	47.7	47.8	52.3	52.2	47.75	52.25
I.E. casein[b]	200	46.0		54.0			
I.E. casein[b]	100	46.7		53.3			

All samples were dispersed in sample gel solutions containing a tris-citrate buffer (pH 6.9).

[a]For the method of isolating each fraction see methods section (pp. 42-46).

[b]Isoelectric casein preparation 1.

25.0 and 20.0, respectively. It was also noted that the band intensities of this fraction, in PAG, were not as great as in all the other caseinate fractions despite the fact that the same protein concentrations were used. The band intensities

for this fraction (Figure 22) were roughly $\frac{1}{2}$ of those for the other caseinate fractions. The low β-casein content, obtained with moving boundary electrophoresis, may indicate a β-casein-κ-casein interaction as suggested by Swaisgood and Brunner (1962), because the whey-soluble fraction has been shown, in this study and by others, to contain a high κ-casein concentration (Table 9) (Rose, 1965 and Sullivan et al., 1959). The decreased intensities in PAG, for the 80-min fraction, stronly suggests that components other than κ-casein did not migrate. Such a retardation of fractions may invalidate the analytical results of all gel electrophoreses of casein, including urea as well as non urea gels unless MCE is used, because κ-casein may hold back other components when it does not migrate.

Disc electrophoretic patterns in the presence of urea

In general, the bands in the urea PAG native calcium caseinate patterns, Figure 23 were sharper, especially that of β-casein, than in the non-urea PAG patterns. The distance between the α_s-casein band No. 6, Figure 23, and β-casein bands No. 9 and 10, Figure 23 in the urea PAG was less than in the non-urea PAG patterns (Figure 22), due in part, to the fact that both sets of bands migrated closer to the tracking dye or front in non-urea PAG. They thus migrated further into the lower gels. The minor bands (Figures 22 and 23) between α_s-casein and β-casein corresponding to bands 7 and 8

Figure 23. Urea polyacrylamide disc electrophoretic patterns of isoelectric casein, α_s-casein, $\varkappa$-casein, and native calcium caseinate fractions

Electrophoretic conditions:
Current, 5 mA per gel for approximately 50 minutes, stained with Amido Black for at least 1 hr and destained with a current of 10 mA per gel. All samples dispersed in sample gel solutions containing 6.6 M urea-tris-citrate buffer (pH 6.9).

Samples[1] (gel No. and µg protein per gel):

Plate 7 A 1) Isoelectric casein preparation 1, 500 µg; 2) α_s-casein, 50 µg; 3) Isoelectric whey soluble casein, 100 µg; 4) $\varkappa$-casein, 50 µg; 5) $\varkappa$-casein, 200 µg; 6) 20-hour fraction, 100 µg; 7) 100-hour W fraction, 100 µg; 8) 100-hour UW fraction, 100 µg.

Plate 7 B 1) 0-min fraction, 100 µg; 2) 10-min fraction, 100 µg; 3) 20-min fraction, 100 µg; 4) 30-min fraction, 100 µg; 5) 40-min fraction, 100 µg; 6) 55-min fraction, 100 µg; 7) 60+10 70)-min fraction, 100 µg; 8) 60+10+10 (80)-min fraction, 50 µg.

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[1]For the method of isolating each fraction see methods section (pp. 42-46).

PLATE 7A

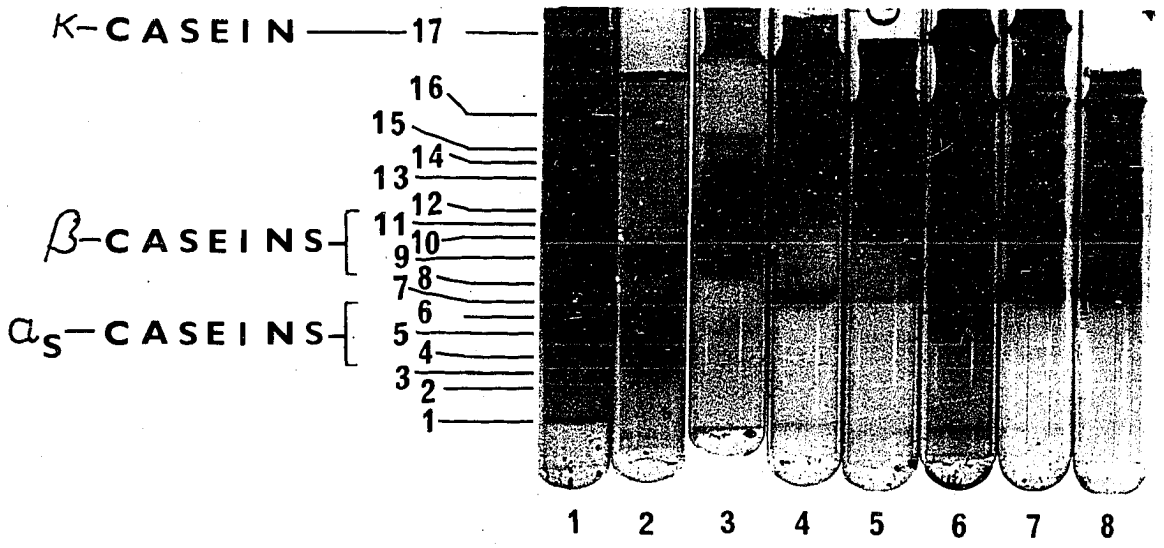
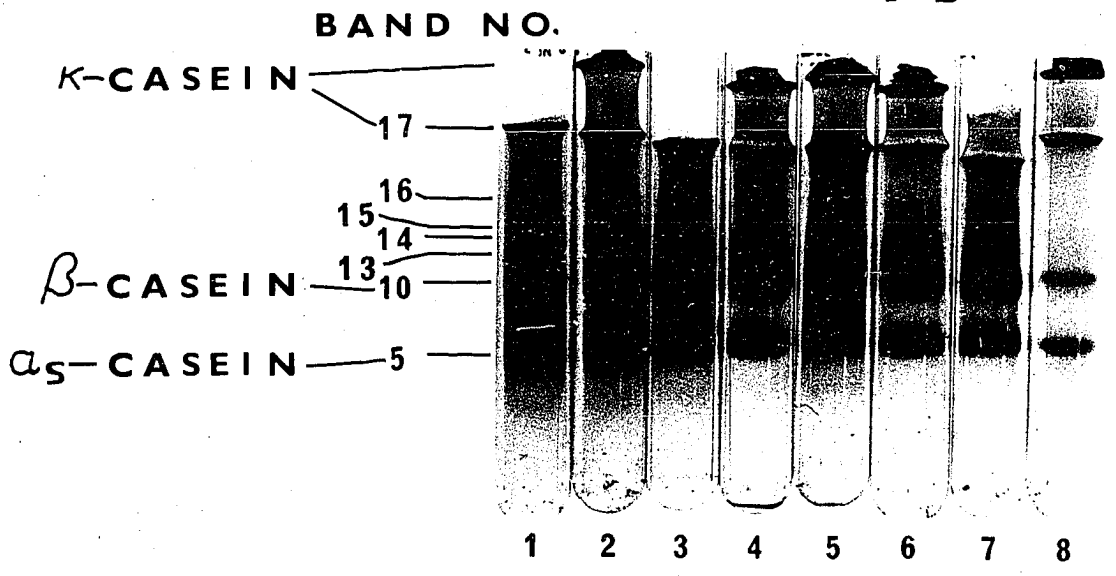


PLATE 7B



(Figure 6, p. 76) with urea-containing gels, were absent in the native calcium caseinate PAG patterns, due in part to the much lower sample concentrations (100 μg protein for caseinate vs. 500 μg protein for isoelectric casein). Higher protein concentrations of caseinates resulted in more diffuse bands in PAG. Bands are visible between the β-casein bands and the spacer gels; these correspond to bands 13 through 17 in Figure 6 (Pattern 2). These bands were not considered in the densitometric analysis of the urea-PAG patterns. The β-casein components (Figures 22 and 23) appear in most cases, as two bands, corresponding to bands 10 and 11 in Figure 6. The urea PAG pattern of the isoelectric whey-soluble fraction (Figure 23, gel No. 3) was similar to the non-urea PAG and the USG patterns. A barely discernible α_s-casein band (negligible in the densitometer trace) is obtained. Discrete bands also occur behind the β-casein components (Figure 23, gel No. 3).

The densitometer data, Table 16, show almost constant α_s-casein and β-casein contents in the micelle fractions, with the exception of the 80-min fraction in agreement with the USG data, Table 8. The relative proportion of α_s-casein in the 80-min fraction containing urea was slightly less (56.7 vs. 60.2 percent) than for the non-urea PAG (Tables 15 and 16). The total area under the densitometer trace of the 20-hour fraction, which had a protein concentration of

Table 16. The α_s- and β-casein contents of isoelectric casein and native calcium caseinate fractions based on densitometry of urea disc electrophoretic patterns (α_s- plus β-casein = 100%)

Fractions[a]	μg protein per gel	Relative % of casein components					
		α_s-	α_s-	β-	β-	ave α_s-	ave β-
0-min	100	43.7	42.3	53.6	47.7	43.00	57.00
10-min	100	42.3	42.3	56.3	57.7	42.55	57.45
20-min	100	45.2	56.2	57.7	57.2	45.75	54.25
30-min	100	44.7	42.9	54.8	53.8	43.80	56.20
40-min	100	43.4	44.1	56.3	57.1	43.75	56.25
55-min	100	44.9	42.7	56.6	55.9	43.80	56.20
60+10(70)-min	100	42.4	44.9	55.1	57.3	56.70	43.30
60+10+10 (80)-min	50	57.7	55.7	42.3	44.3		
20-hour	50	47.6		52.3			
20-hour	100	46.7	45.5	53.8	54.5	56.1-	53.90
I.E. whey sol. casein	100	0.0	0.0	100.0	100.0	0.00	100.00
100-hour W	100	44.0	42.0	56.0	58.0	43.00	57.00
100-hour UW	100	44.2	43.0	55.8	57.0	43.60	56.40
I.E. casein[b]	500	48.9	48.7	51.2	51.3	48.80	51.20

All samples were dispersed in sample gel solutions containing a 6.6 M urea-tris-citrate buffer, pH 6.9.

[a]For the method of isolating each fraction see methods section (pp. 42-46).

[b]Isoelectric casein preparation 1.

50 μg/tube, was approximately ½ of the total area obtained when 100 μg of protein/tube was used. However, in the 80-min fraction (50 μg protein/tube) the total area under the trace was approximately 3/4 of the total area obtained for the 20-hour fraction at the same concentration. This decrease in total area is more than would be expected upon considering

the difference in κ-casein contents (Table 9).

The discussion concerning the α_s-casein: κ-casein ratio of the 80-min fraction would seem to be similar to the discussion under the preceding (non-urea) section.

Disc electrophoretic patterns in the presence of urea and mercaptoethanol

As previously stated, PAG urea-MCE patterns were of little value due to the irregular and non-uniform appearance of the major α_s-casein and in some cases the major β-casein bands. A typical pattern, 20-hour fraction, obtained for caseinates is shown in Figure 24 (gel No. 2). The irregularity in the α_s-casein band is not apparent in the photograph.

In general, the overall band intensities of the major β-casein bands was greater than the intensities obtained in non-urea and urea PAG patterns for the same protein concentrations, implying that components other than κ-casein may not migrate when MCE is not used. Unfortunately, because the sample gel did not polymerize, it was impossible to determine whether or not protein remained in the sample gel.

Figure 24. Urea 2 mercaptoethanol polyacrylamide disc electrophoretic patterns of κ-casein and a typical native calcium caseinate fraction

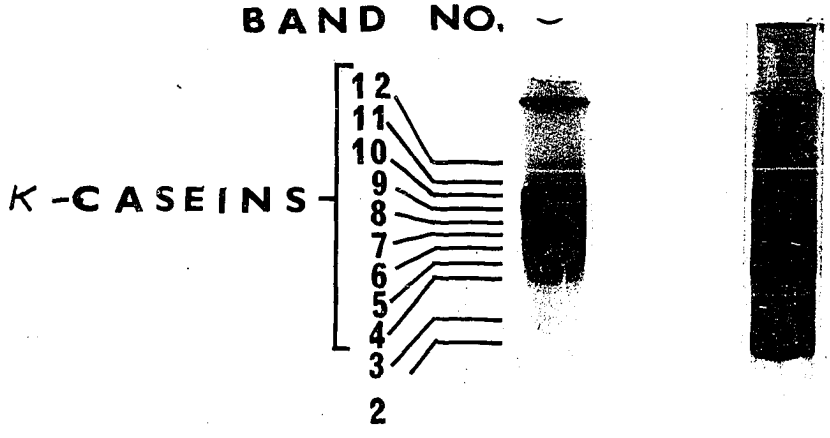
Electrophoretic conditions:
Current 5 mA per gel for approximately 50 minutes, stained with Amido Black for at least 1 hr and destained with 10 mA per gel. Samples were dispersed in a sample gel solution containing a 6.6 M urea-tris-citrate buffer (pH 6.9) and 0.01 ml of 2 mercaptoethanol added per ml of sample gel solution at least 3 hr before electrophoresing (Woychik, 1964).

Samples[1] (gel No. and μg protein per gel):

1) κ-casein, 100 μg; 2) 20-hour fraction, 100 μg.

[1]For the method of isolating each fraction see methods section (pp. 42-46).

PLATE 8



SUMMARY AND CONCLUSIONS

Investigations were carried out on the calcium caseinate-phosphate complex in raw skim milk. The protein portion of the complex (caseinate) was studied using conditions designed to maintain the protein as close to its native state as was feasible.

Raw skim milk was subjected to differential ultracentrifugation in a Model L Spinco ultracentrifuge (No. 21 rotor) at 20,000 rev/min (maximum g force of 53,620) and 0 C. Six native calcium caseinate fractions of decreasing micelle size, 0-, 10-, 20-, 30-, 40-, 55-min fractions, were sedimented in 10-min centrifuging increments (15 min for the last fraction) using the supernatants from the preceding increment. Two additional fractions were obtained, using 10-min increments, after an initial centrifugation of 60 min (60+10,70-min and 60+10+10,80-min fractions). A composite of the sedimented fractions above was obtained, containing 86.1% of the total caseinate, by centrifuging the same skim milk for 20 hours using the same conditions. The caseinate (13.9%) remaining in the supernatant was recovered by isoelectric precipitation at pH 4.6 resulting in a non-sedimented fraction designated as isoelectric whey-soluble casein. All of the sedimented native calcium caseinates were mechanically dispersed in redistilled water and were sedimented, using the initial conditions, for 12 hours (the 20-hour fraction was sedimented

for 20 hours). The fractions were again dispersed, freeze-dried and stored over P_2O_5 at room temperature in a vacuum dessicator.

The supernatants resulting from the fractionation above were analyzed (Table 1) for total nitrogen (TN) and total solids (TS); a smooth TN depletion curve (Figure 9) was obtained. The data show that more caseinate could be sedimented in 30 min using the differential technique than could be sedimented during 60 min of continuous centrifugation because of the decreasing calcium caseinate concentrations in the serially depleted supernatants. From these data, the casein nitrogen distribution of the sedimented and non-sedimented fractions was determined (Figure 10). Seven sequential caseinate fractions were obtained; these did not include the 60+10(70)-min fraction and the non-micellar (whey-soluble) fraction. The 60+10(70)-min fraction was found to represent a composite of the 30-, 40-, and 55-min fractions.

The analysis of the sediments (Table 2) show decreasing total phosphorus (TP) and calcium contents with decreasing micelle size accompanied by an increase in TN, in agreement with de Kadt and van Minnen (1943), Hostettler *et al.* (1949) and Ford *et al.* (1955). The TP:TN, Ca:TN, and TP:Ca weight ratios decreased with decreasing micelle size. The molar Ca:Mg ratios were lower than those reported by Alexander and Ford (1957). The isoelectric whey-soluble casein showed a

casein N(CN): organic phosphorus (OP) ratio of 0.0438, in agreement with Bohren and Wenner (1961) and Yamauchi and Tsugo (1962) for similar fractions. This reflects the increased solubility of β- and γ-casein at low temperature.

An attempt was made to remove essentially all of the native calcium caseinate from skim milk by low-temperature ultracentrifugation of skim milk in a Model L Spinco ultracentrifuge (No. 40 rotor) at 40,000 rev/min (maximum g force 144,700) for 100 hours and 0 C. A portion of the sediment was dispersed in redistilled water, as above, and freeze-dried as an unwashed control (100-hour UW fraction). The remaining sediment was washed and freeze-dried, as above, using the centrifuging conditions employed with the skim milk, for sedimenting the redispersed caseinates (100-hour W fraction). The total solids and nitrogen (TN, CN, non-casein nitrogen, and non-protein nitrogen) values were determined for the original skim milk, the supernatant, and wash water (Table 3). The results show that only 95.8% of the total caseinates were sedimented in 100 hours. Whey proteins, which are adsorbed to the caseinate, are sedimented and could not be completely removed by the washing procedure employed although the non-protein nitrogen was completely removed. A small amount of the caseinate was lost in the wash water with the result that the washed (100-hour W fraction) caseinate contained 93% of the total caseinate. Approximately 7.5% of the protein in the

washed caseinate was calculated (from analyses of the supernatant and wash water (Table 4) to be serum or non-casein protein. Analyses (Table 4) of the 100-hour W and UW sediments show that the washed fraction contained slightly higher values for TN, TP, Ca, and Mg than the unwashed fraction, in agreement with Ford *et al.* (1955).

All native calcium caseinates and the isoelectric whey-soluble casein, isolated during this investigation, were subjected to moving boundary electrophoresis. A number of methods (buffers) for dispersing the caseinates were compared: veronal buffer in which the pH was adjusted to 8.3 (23 C) with E.D.T.A. ($\Gamma/2 = 0.1$); veronal-oxalate buffer with enough sodium oxalate just to react with the Ca and Mg in a given fraction and sufficient NaCl to give a resistance comparable to a standard veronal buffer ($\Gamma/2 = 0.1084$); veronal-excess oxalate buffer (no NaCl) with enough sodium oxalate to give a resistance comparable to a standard veronal buffer ($\Gamma/2 = 0.1802$). These buffers were employed with the 20-hour fraction and the results (Table 5) compared with isoelectric casein. Mobilities and relative percentages of α- and β-casein were determined after 1 and 1.5 hr electrophoresis time. The data (Table 5) show that there was reasonable agreement among the data obtained using the several conditions described. The veronal-oxalate buffer ($\Gamma/2 = 0.1084$) yielded mobilities and relative areas that were in closer agreement at the two

electrophoresing times employed than did the other methods and was used in the remaining moving boundary analyses of the caseinate fractions.

The moving boundary electrophoretic data (Table 6, Figure 12) of the caseinate fractions show no difference among the relative percentages of α- and β-casein (approximately 75% and 25% respectively, descending patterns) for the 0-, 10-, 20-, 30-, 40-, 55-, and 60+10(70)-min fractions in agreement with Hostettler et al. (1949) and Heckman et al. (1958). However, the smallest micelles sedimented (60+10+10, 80-min fraction) exhibit an atypical electrophoretic pattern (Figure 12, II). A peak between the α- and β-casein peaks (designated as X-casein) with a mobility of approximately -4.7×10^{-5} cm^2 $volt^{-1}$ sec^{-1}, which is in agreement with the mobility of β-lactoglobulin reported by Tobias et al. (1952b), was observed. However, the magnitude of the peak (22-27%) suggests that the X-casein peak cannot all be β-lactoglobulin. The non-sedimented whey-soluble casein fraction also exhibits an atypical electrophoretic pattern (Figure 12, III). The first peak has a mobility comparable to the X-casein component above. Hansen et al. (1962), in a study of non-micellar (whey-soluble) casein report a similar X-casein component having a mobility of -4.73×10^{-5} cm^2 $volt^{-1}$ sec^{-1}. The β-casein peak shows an increase in relative area (approximately 50% of the pattern, compared with 25% in a typical

caseinate pattern) in agreement with Bohren and Wenner (1961) and Hansen et al. (1962). A γ-casein peak was also observed in the descending pattern which was not observed in any of the other fractions (γ-casein peaks were observed in all ascending patterns). The data suggest that the X-casein component in the whey-soluble casein and the 60+10+10(80)-min fraction may be the same. If this is true, it suggests that only a small portion of the X-casein component can be β-lactoglobulin, because the whey soluble fraction is an isoelectric preparation. Urea-2-mercaptoethanol starch-gel electrophoresis of the whey-soluble casein fraction has shown, in this study, that it is almost devoid of α_s-casein and contains high amounts of β- and κ-casein. It is concluded that the X-casein component is κ-casein which exhibits a low electrophoretic mobility because of a κ-casein: β-casein interaction.

The 20-hour fraction was electrophoresed (moving boundary) in the presence of 5 M urea. The patterns (Figure 13) show the disaggregating effect of the urea on the casein. Thirteen peaks were observed in the descending pattern. No further experiments using urea in moving boundary were attempted.

Moving boundary electrophoresis of the 100-hour W and 100-hour UW fractions (Table 7, Figure 14) show that a γ-casein peak is observed in the descending pattern in the 100-hour W fraction, but not in the 100-hour UW fraction

suggesting that washing has removed some NCN constituents that may mask the γ-casein in the unwashed fraction. The washed fraction was also electrophoresed in veronal-excess oxalate buffer ($\Gamma/2 = 0.1802$) and the descending pattern (Figure 14, II) shows a small peak between the α- and β-casein peaks. The mobility of this peak is slightly lower than the mobility of the X-casein peak observed in the 60+10+10(80)-min and whey-soluble casein fractions. No further data concerning this peak was obtained in this study, but it is concluded that if this peak is a protein component, it exists in the micelle range between 86.1% (amount of the total caseinate in the 20-hour fraction) and 93.5% (amount of the total caseinate in the 100-hour W fraction).

All caseinate and the whey soluble casein fractions as well as α_s-casein, κ-casein, and isoelectric caseins were subjected to urea and urea-mercaptoethanol horizontal starch-gel (USG and USG-MCE) electrophoresis at 2-3 C. The relative percent of the casein components were determined by densitometry of transparent pictures of gel patterns stained with Amido Black and Nigrosine. Seventeen bands were obtained for isoelectric casein USG patterns (Figure 15). The caseinates, however, exhibit more smearing and fewer bands, than the isoelectric casein preparations (Figures 15 and 16). This suggests that some of the bands observed in the isoelectric casein patterns are artifacts resulting from the

method of preparation. The 100-hour W caseinate fraction exhibited banding in the region where κ-casein migrates as a smear (Figure 15) indicating that these bands are present in the micelle size between 86.1 and 93.5% of the total caseinate. The 100-hour UW caseinate fraction did not show these bands. USG electrophoresis of the whey-soluble casein fraction confirms the suggestion obtained by moving boundary electrophoresis in this study and implied by Bohren and Wenner (1961), that this fraction is deficient in α_s-casein, since only 4% α_s-casein was observed in the pattern (Table 8). In contrast with moving boundary electrophoresis, κ-casein migrates as a smear behind the β-casein, rather than in combination with α_s-casein as α-casein, in USG electrophoresis. The densitometer data (Table 8), in which the relative percentages of α_s- and β-casein in the calcium caseinate micelle fractions were determined, show a somewhat random variation in composition and no trend could be detected with decreasing micelle size.

In the USG-MCE patterns, 20 bands were obtained with an isoelectric casein preparation and κ-casein formed descrete bands (about 4 bands) just behind the β-casein bands (Figures 18 and 19). The caseinates exhibit more discreet banding when MCE was employed, but some smearing was evident in the κ-casein region (Figures 17, 18, and 19). The relative percentages of α_s-, β-, and κ-casein (50, 34, and 16%

respectively) obtained for isoelectric casein (Table 9) are in good agreement with values reported in the literature (Table 10) which were obtained by other types of experimental methods. Higher κ-casein and lower α_s-casein values were obtained for caseinate samples (Tables 9 and 11) than for the isoelectric preparations. The densitometer data (Table 11) obtained with the calcium caseinate fractions show some variability in component quantities with changing micelle sizes, but in general they show a trend toward an increase in κ-casein and a decrease in α_s-casein as the micelle sizes decrease (Figure 20). The α_s- and κ-casein values were slightly higher and the β-casein values slightly lower in the Nigrosine stained patterns than in the Amido Black stained patterns. However, the relative quantities of the components of the caseinate fractions appear to fluctuate in the same directions with both staining procedure (Figure 20). These results confirm the results obtained by moving boundary (Table 6). Since α-casein consists of α_s- and κ-casein, the fact that no change appeared to occur in the β-casein content, as micelle size changed, with moving boundary suggested that any change in micelle composition must be in the α_s- and β-casein. The USG-MCE densitometry data (Table 9) for the whey-soluble casein fraction show a negligible amount of α_s-, 65-68.9% β-, and 31.1-35% κ-casein. This is in agreement with the contention that β-casein is more soluble at low than

at high (room) temperature. In addition, the preferential depletion of α_s-casein from skim milk by ultracentrifugation, which has been demonstrated in this study, was only implied by Bohren and Wenner (1961). Since a constant temperature was employed in the fractionation of the micelles in this study, the data (Table 11) suggests that an equilibrium was obtained between solubilized (monomeric) and polymerized β-caseins and that this equilibrium was not changed during the sedimentation of the micelles, for the β-casein content of the micelles was shown to be essentially constant by both moving boundary and USG-MCE electrophoreses. The fact that trends in micelle composition with decreasing micelle size was not shown by USG electrophoresis (Table 8) suggests that κ-casein, which migrates as a smear without MCE, may trap either α_s- or β-casein or both resulting in variable results that might mask existing trends.

The fractions isolated in this study were also subjected to disc electrophoresis (gels in glass tubes electrophoresed in a vertical position) in non-urea and urea polyacryalimide gels (PAG) at room temperature. Preliminary experiments indicated that κ-casein did not migrate in either non-urea or urea PAG. Comparison of various mixtures of α_s- and κ-casein (Table 12) indicate that when the α_s-: κ-casein ratio is less than 1, a sharp drop in the amount of α_s-casein that migrates occurred, suggesting that either an

α_s-, κ-casein interaction had occurred or that the non-migrating κ-casein blocked the pores of the gel before all the α_s-casein had migrated. Comparison of methods of dispersing calcium caseinate (sodium oxalate, E.D.T.A., and citric acid) for disc electrophoresis in non-urea PAG (Table 13) show that the three methods are in good agreement. On the basis of sharper banding, citric acid was used as the dispersing agent in non-urea disc electrophoresis. In urea PAG disc electrophoresis, a number of variations in urea gel concentrations were compared (Table 14) and good agreement among urea gel concentrations was obtained. All subsequent gel electrophoreses of caseinates were conducted using 5.0 M urea in the spacer and lower gels and 6.6 M urea in the sample gels.

Ten bands were obtained with isoelectric casein in non-urea PAG disc patterns (Figure 22). The caseinates showed similar patterns (6 bands) with some of the minor bands being absent. The urea PAG disc patterns (Figure 23) of isoelectric casein showed 17 bands. The caseinates showed only 7 bands which was caused, in part, by the difference in protein concentrations employed (500 μg protein per gel for the isoelectric casein compared with 100 μg protein per gell for the caseinates). The whey-soluble casein fraction in the urea and non-urea PAG disc patterns contained essentially no α_s-casein and almost discreet banding behind the β-casein band in agreement with the USG and USG-MCE patterns (Figures 15, 17, and 18).

The relative percentages of α_s- and β-casein (obtained by direct densitometry of the gels) of the non-urea (Table 15) and urea (Table 16) PAG disc electrophoretic patterns are in general agreement with the results obtained with the USG patterns in that no trend in micelle composition was obtained with decreasing micelle size. One exception was noted. The 60+10+10(80)-min fraction showed a definite increase in α_s-casein composition. Since this fraction was shown to contain the highest κ-casein concentration of any of the sedimented micelles by USG-MCE, exhibited an atypical moving boundary electrophoretic pattern, and decreased band intensities in comparison with the other caseinate fractions in PAG, it is concluded that wholly or in part, some components other than κ-casein may not have migrated. Such a retardation of fractions may invalidate the analytical results of all gel electrophoresis of caseins unless USG-MCE electrophoresis is used because κ-casein may hold back the other components.

Disc electrophoresis employing urea and MCE was attempted in this study. However, it was found that MCE inhibits the photopolymerization of the upper gels and caused distortion of the α_s-casein bands and to a lesser extent the β-casein bands making the patterns unsuitable for densitometry. The κ-casein bands were apparently unaffected and 12 bands were obtained for the κ-casein preparation employed in this study (Figure 24).

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APPENDIX

Table 17. The effect of the densitometer zero position on the α_s- and β-casein content of isoelectric casein, α_s-casein and native calcium caseinate fractions based on densitometry of transparencies of urea starch gel electrophoretic patterns[a] (α_s- plus β-casein = 100%)

Fractions[b]	% protein in sample	Relative % of casein components: Amido Black stained α_s-		β-		Nigrosine stained α_s-		β-	
0 min	1.5	45.0[c]	45.0[d]	55.0[c]	55.0[d]	45.9[c]	47.0[d]	54.1[c]	53.0[d]
10-min	1.5	45.4	45.4	54.6	54.6	44.5	45.6	55.9	54.4
20-min	1.5	47.9	47.4	52.1	52.6	45.2	46.7	52.8	53.3
30-min	1.5	44.2	44.5	55.7	55.5	43.0	43.3	57.0	56.7
40-min	1.5	44.7	44.1	55.3	55.9	48.8	48.6	51.2	51.4
55-min	1.5	47.5	47.5	52.5	52.5	47.1	46.9	52.9	53.1
60+10(70)-min	1.5	47.1	46.2	52.9	53.8	44.8	44.7	55.2	55.3
60+10+10 (80)-min	0.75	47.1	46.6	52.9	53.4	45.4	45.7	54.6	54.3
20-hour	1.5	46.1	46.0	53.9	54.0	45.0	42.6	55.0	57.4
I.E. whey sol. casein	0.75	4.3	2.8	95.7	97.2	4.8	5.2	95.2	94.8
100-hour W	1.5	46.2	46.1	53.8	53.9	43.8	44.5	56.2	55.5
100-hour UW	1.5	49.9	49.6	50.1	50.4	48.1	47.9	51.9	52.1
α_s-casein	0.5		95.8		4.2		95.8		4.2
I.E. casein[e]	1.0	49.2	49.1	50.8	50.9	49.3	48.9	50.7	51.1
I.E. casein[e]	2.0	47.1	47.0	52.9	53.0	47.0	45.6	53.0	54.4

[a]Transparencies correspond to the pictures in Figures 17 and 18 (see Table 8).

[b]For the method of isolating each fraction see methods section pp. 42-46.

[c]Densitometer zero just ahead of the borate boundary.

[d]Densitometer zero just ahead of the α_s-casein band.

[e]Isoelectric casein preparation 1.