CHAPTER 16

Milk Proteins

BY THOMAS L. McMEEKIN

Page
I. Biological Importance 389
II. Protein Distribution in Milk 391
1. Protein Distribution in Average Mixed Milk 391
2. Changes in the Protein Distribution of Milk During the Lactation Cycle 393
III. Separation and Properties of Milk Proteins 395
1. Casein 395
a. Heterogeneity of Casein 397
b. Methods for Separating the Components of Casein 397
c. Properties and Composition of the Caseins 400
d. Phosphopeptones from Casein 408
e. Clotting of Milk and Casein by Rennet 409
f. Casein from the Milk of Different Mammals 410
2. Proteins of Whey 411
a. Globulin Fraction 411
b. "Albumin" Fraction 416
c. Preparation and Properties of β-Lactoglobulin 419
IV. Amino Acid Composition of Milk Proteins 428
V. Enzymes in Milk 428
VI. Antibacterial Factor of Milk 432
VII. Relationship of Milk Proteins to Serum Proteins 433

I. Biological Importance

The importance of milk as a food for the newborn can hardly be overestimated. Milk is the last contact between the mother and the offspring, and the relationship between the composition of milk and the stage of development of the young at birth, as well as environmental influences, makes a fascinating study in adaptation. Thus at birth, cats, dogs, and rabbits are incapable of much movement, and need a milk containing large amounts of protein for rapid muscle development. In contrast, the young of cows, horses, and related animals have well-developed muscles and are capable of locomotion at birth, and they require higher concentrations of sugar for energy. Reindeer milk contains a high concentration of fat and protein to furnish calories for adaptation to low temperatures. The relationship of the composition of human milk to the development of the child does not fit into these apparent adaptive classes. The slow rate of development of the child in a protected environ-

ment perhaps gives a more complicated relation between food intake and the development of the offspring. The common practice of using diluted cow milk as food for infants is justified by the apparently satisfactory results obtained, though some studies indicate that human milk is of greater nutritional value for infants than cow milk.[1]

An illustration of the relationship between the composition of milk and the effect on the blood of the offspring is the finding that the concentration of the enzyme cholinesterase in the blood of young puppies is directly related to the cholinesterase content of the ingested milk.[2] Thus, the cholinesterase content of the blood serum of young puppies increases greatly when the colostrum from bitches, containing large amounts of cholinesterase, is ingested. When similar young puppies are fed cow's milk containing no cholinesterase, there is no increase in the cholinesterase content of the blood serum of puppies.

TABLE I
AVERAGE COMPOSITION OF THE MILK OF VARIOUS MAMMALS[3]
(Data in per cent)

Species	Casein	Whey protein	Sugar	Fat	Ash
Human	0.9	1.2	6.3	3.7	0.21
Horse	1.3	0.7	6.0	1.3	0.51
Cow	3.0	0.6	4.7	3.7	0.72
Cat	3.8	3.2	4.8	4.8	0.58
Dog	4.8	2.6	3.2	11.6	—
Reindeer	8.4	1.5	2.8	17.1	1.20
Guinea pig	4.7	0.6	2.3	7.0	—

Although the composition of the milk of a species is constant within limits, as implied in Table I,[3] numerous factors are responsible for wide variations in its composition. One of the most important of these factors is the stage of lactation. Variation in composition is particularly marked in the milk obtained at and immediately following parturition.[4] This first milk or colostrum of the cow contains large amounts of antibody protein, which serves a special function of increasing the resistance of the newborn to infectious diseases.

(1) O. Mellander, *Upsala Läkareförn. Förh.* **52**, 107 (1947).
(2) R. A. McCance, A. O. Hutchinson, R. F. A. Dean, and P. E. H. Jones, *Biochem. J.* **45**, 493 (1949).
(3) W. L. Davies, The Chemistry of Milk, Chapman and Hall Ltd., London, 1939, p. 7.
(4) I. G. Macy, H. Kelley, and R. Sloan, *Bull. Natl. Research Council* **No. 119** (Jan. 1950).

II. Protein Distribution in Milk

1. Protein Distribution in Average Mixed Milk

The proteins of milk, in common with proteins of other tissues, may be estimated by a variety of methods, though for adequate characterization, separation of the components is essential. The method of determining the protein distribution in milk,[5–7] based on the amount of protein

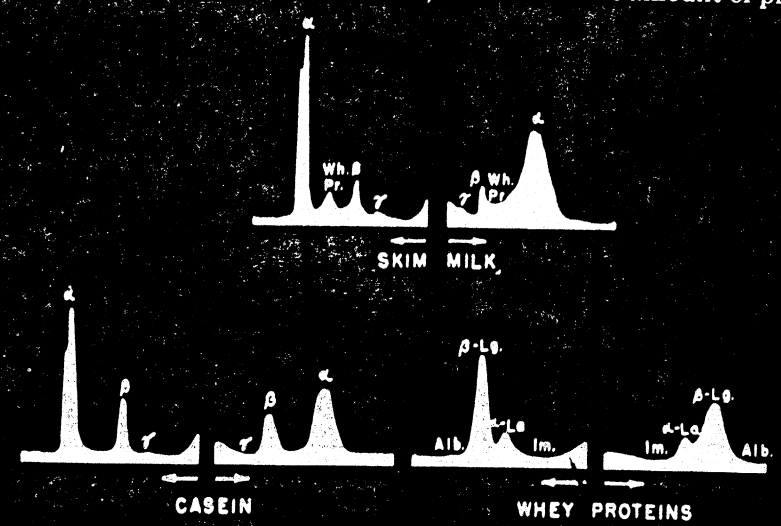


FIG. 1. Electrophoretic patterns of milk proteins in veronal buffer, pH 8.35; ionic strength 0.1. Wh. Pr., whey protein; β-Lg., β-lactoglobulin; α-La., α-lactalbumin; Alb., serum albumin; Im., immune globulin; α-, β-, and γ-components of casein. (After McMeekin.)[9]

nitrogen separated by isoelectric precipitation, salt or alcohol[8] fractionation, and heat coagulation, is relatively simple and gives useful comparative information. By these methods, the average protein composition of cow milk is reported[4] to be casein, 2.8 per cent; globulin, 0.2 per cent; and albumin, 0.4 per cent; as compared with casein, 0.4 per cent; globulin, 0.2 per cent; and albumin, 0.3 per cent; in human milk. Because of its availability, cow's milk has been investigated more frequently than any other milk. Unless otherwise stated in this review, the discussion refers to the proteins of cow's milk.

(5) E. F. Beach, S. S. Bernstein, O. D. Hoffman, D. M. Teague, and I. G. Macy, *J. Biol. Chem.* **139**, 57 (1941).
(6) S. G. Menefee, O. R. Overman, and P. H. Tracy, *J. Dairy Sci.* **24**, 953 (1941).
(7) S. J. Rowland, *J. Dairy Research* **8**, 1, 6 (1937).
(8) K. M. Shahani and H. H. Sommer, *J. Dairy Sci.* **34**, 1003 (1951).
(9) T. L. McMeekin, *J. Milk and Food Technol.* **15**, 57 (1952).

identical in molecular weight with the α-component of whey described by Pedersen, it could be classified as a globulin instead of an albumin which would be expected to be soluble in water at the isoelectric point. This protein, however, is insoluble in salt, which property distinguishes it from a globulin. The fact that the protein in whey, soluble in saturated magnesium sulfate, coagulated by heat, and described extensively in the literature as lactalbumin, has no relation to the protein with a molecular weight of 15,000, suggests that the crystalline protein should be given a different designation. Consequently, the component of whey with a sedimentation constant of $S_{20} = 1.75$ is given the name α-lactalbumin as has been suggested by Svedberg.[94]

(1) *Preparation of α-Lactalbumin.* Gordon and Semmett have perfected their method for the separation of α-lactalbumin so that a pure product is obtained without difficulty. In their method, casein is precipitated from skim milk by acidification in the usual manner. The fraction of whey insoluble in 2.3 *M* ammonium sulfate at pH 6 is removed by filtration. The remainder of the total protein of whey is precipitated from the filtrate by the addition of solid ammonium sulfate to 3.3 *M*. After filtration, the insoluble protein is dissolved in water and dialyzed free of salt at pH 5.2. Under these conditions β-lactoglobulin crystallizes and is removed by centrifugation. The protein in the supernatant liquor is precipitated by adding solid ammonium sulfate to 3.3 *M*. The precipitated protein is removed by filtration and dissolved in water and again dialyzed at pH 5.2. A precipitate appears after exhaustive dialysis. This precipitate is composed of crude α-lactalbumin and some additional β-lactoglobulin. This insoluble material is extracted with small amounts of 0.1 *M* sodium chloride, which dissolves the β-lactoglobulin, leaving α-lactalbumin as an insoluble precipitate. The precipitate is removed by centrifugation and dissolved in 0.03 *N* ammonium hydroxide, and the solution is filtered. The filtrate is acidified by the addition of 0.1 *N* sulfuric acid to pH 4.0. The α-lactalbumin, which is precipitated, is removed by centrifugation and again dissolved in ammonium hydroxide. The pH is adjusted to 6.6, and to the clear solution an equal volume of saturated ammonium sulfate solution (adjusted to pH 6.6) is added dropwise with stirring. The precipitate, which is formed at half-saturation with ammonium sulfate, is amorphous and is removed by centrifugation. To the supernatant liquor, saturated ammonium sulfate solution is added as before. When the concentration of salt reaches 60 per cent saturation, crystallization of α-lactalbumin begins and is complete at about two-thirds saturation.

α-Lactalbumin crystallized at pH 6.6 is easily soluble in water, but when the solution is adjusted to pH 4.6, the protein is precipitated in an amorphous condition. It may be recrystallized, however, by dissolving it in dilute ammonia and again salting it out with saturated ammonium sulfate solution at pH 6.6. As yet, no method has been found to crystallize the α-lactalbumin from water at pH 4.6. α-Lactalbumin, as prepared by this method, contains 15.86 per cent nitrogen, 1.91 per cent sulfur, and only a trace of phosphorus. The tryptophan content of 7.2 per cent is unusually high for a milk protein. At pH 6.8 the optical rotation $[\alpha]_D^{25}$ is −61°.

c. *Preparation and Properties of β-Lactoglobulin*

In attempting to prepare crystalline lactalbumin by the described methods, Palmer,[15] by dialysis from aqueous salt solution, obtained the principal protein of whey in the form of large crystals. The method used for separating β-lactoglobulin from milk is indicated under the section on the preparation of α-lactalbumin. Its sedimentation constant of $S_{20} = 3.1$ is identical with the β-component of whey described by Pedersen,[13] who also determined its isoelectric point to be 5.2.[97]

Palmer[15] determined the solubility of β-lactoglobulin in water, dilute sodium chloride, and concentrated sodium sulfate solutions. He observed that a fourfold increase in the amount of solid phase did not change the solubility, indicating the purity of this protein. In further study of its solubility, Sørensen and Palmer[98] found that a threefold variation in the amount of solid phase did not change the solubility more than 1 per cent. These results on its solubility, the ease of obtaining large crystals, and the other unique properties of β-lactoglobulin have made it a favorite protein for investigation. Crowfoot[99] determined its crystal structure and unit cell by means of x-ray measurements. Assuming eight molecules per unit cell, she calculated a molecular weight of 40,000 for air-dried β-lactoglobulin,[100] which was close to Pedersen's[97] value of 39,000 for its molecular weight. A value of 35,800 for the anhydrous molecular weight was deduced from Crowfoot's data on the assumption that air-dried crystals contain 9.78 per cent moisture.[101] Senti and Warner[102] obtained a value of 35,400 for the molecular weight of β-lactoglobulin from x-ray measurements, which is also in agreement with the value obtained by Bull and Currie[103] from osmotic-pressure measurements

(97) K. O. Pedersen, *Biochem. J.* **30**, 961 (1936).
(98) M. Sørensen and A. H. Palmer, *Compt. rend. trav. lab. Carlsberg. Sér. chim.* **21**, No. 21 (1938).
(99) D. Crowfoot, *Chem. Revs.* **28**, 215 (1941).
(100) I. Fankuchen, *in* E. J. Cohn and J. T. Edsall, eds., Proteins, Amino Acids and Peptides, Reinhold Pub. Corp., New York, 1943, p. 328.
(101) T. L. McMeekin and R. C. Warner, *J. Am. Chem. Soc.* **64**, 2303 (1942).
(102) F. R. Senti and R. C. Warner, *J. Am. Chem. Soc.* **70**, 3318 (1948).
(103) H. B. Bull and B. T. Currie, *J. Am. Chem. Soc.* **68**, 742 (1946).

reted, or colostrum, and in milk secreted during the end of the lacta-
n period when, as in the cow, gestation is usually superimposed on the
tating cycle. The proteins of milk appear to be qualitatively the same
ring the entire lactating cycle, though the total protein, as well as
ative proportions of each protein, may vary markedly. Thus Smith
found colostrum globulin to be essentially the same as the immune
bulin of normal milk.[17] Variations in the protein distribution of cow

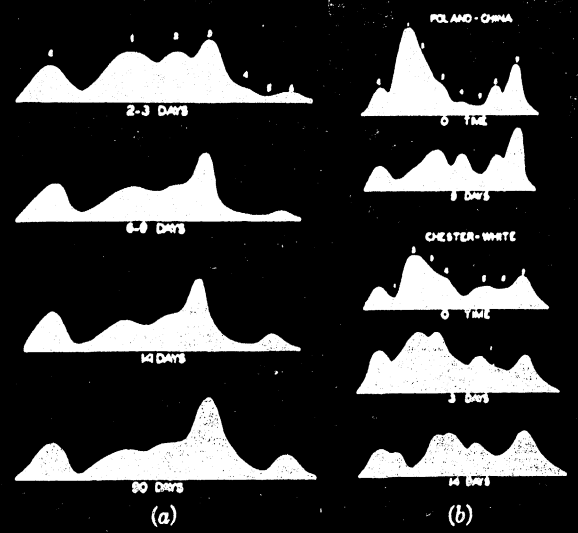


FIG. 3. (a) Descending electrophoretic boundaries of human whey at various times
t partum. (b) Descending electrophoretic boundaries of whey of Chester white
d Poland-China pigs at various times post partum. (After Deutsch.)[19]

lk during the lactating cycle have been determined by electrophore-
.[9,18] The electrophoretic components of acid casein from mixed milk,
well as milk from most cows, were found to be remarkably constant;
wever, the casein from an occasional cow differed in that the α-casein
mponent was split into two components as shown in Fig. 1. The rela-
ve proportions of α- and β-casein were constant during the entire
tating cycle. Casein from the "regression" milk, the fluid that can
obtained from the udder during the end of the gestation cycle, and
lostrum casein, however, contained less β-casein than normal milk
sein. This difference in the composition of casein from normal milk
d from colostrum can be seen by comparing the relative areas of α-

(17) E. L. Smith, *J. Biol. Chem.* **164**, 345 (1946).
(18) A. Grönwall, *Nature* **159**, 376 (1947).

and β-casein in normal milk (Fig. 1) with that of casein from colostrum (Fig. 9). The protein components of milk whey varied more during the lactating cycle than the casein components. The electrophoretic pattern shown in Fig. 1 was obtained on normal milk 14 days after parturition and remained the same until 70 days before parturition or 30 days before discontinuing milking. At this time the immune or colostrum globulin and the albumin components began to increase progressively. At parturition the colostrum globulin exceeds by far all the other proteins in colostrum, dominating the electrophoretic pattern.

Deutsch[19] has compared the whey proteins of bovine, goat, pig, human, sheep, and horse milk by means of electrophoretic and ultracentrifugal analyses. Marked differences in the composition of the whey proteins of these species were found. For a given species, however, the composition at the same stage of lactation is essentially constant. Large differences in the electrophoretic composition of the whey proteins were found immediately following parturition, as illustrated for human and pig whey in Fig. 3. With the exception of the similarity of the colostrum globulin of milk whey and the γ-globulin of blood serum, little relation was noted between the electrophoretic composition of a given milk whey and the corresponding blood serum.

III. Separation and Properties of Milk Proteins

Analytical methods are useful for determining the approximate type of protein in milk. For the identification and characterization of milk proteins, isolation is essential. Because of the availability of milk and its nutritional importance, separation and characterization have been the subject of many investigations.

1. Casein

Casein was one of the first proteins obtained in a relatively pure form. In 1838, Mulder[20] devised the method of separating casein from milk by acidification. For a long time, casein prepared by Hammarsten's method[21] was extensively used. Casein was prepared by this method as follows: skim milk is diluted with 4 vol. of water, and the mixture is treated with acetic acid to 0.75–1 part per million. After separating by filtration, the casein is purified by repeated solution in water with the aid of the smallest quantity of alkali possible, and reprecipitated with acetic acid. After dissolving and reprecipitating the casein several times in this manner, it is

(19) H. F. Deutsch, *J. Biol. Chem.* **169**, 437 (1947).
(20) G. J. Mulder, *Ann. der Pharm.* **28**, 73 (1838).
(21) O. Hammarsten, Textbook of Physiological Chemistry (translated by J. A. Mandel), John Wiley and Sons, New York, 1900.

The relationship between the composition and density of β-lactoglobulin crystals and that of sucrose solutions used as a suspending medium has been determined.[110] The solubility of β-lactoglobulin in sucrose solutions is small and does not affect these measurements. For comparative purposes the densities of β-lactoglobulin crystals equilibrated in serum albumin solutions were also determined. The density of the protein

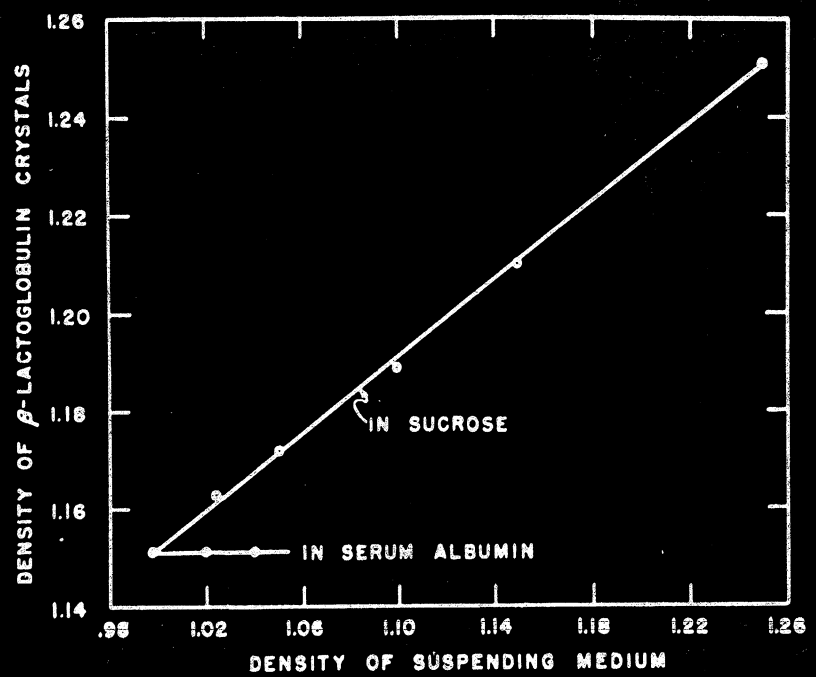


FIG. 12. Densities of β-lactoglobulin crystals equilibrated in sucrose and serum albumin solutions. (After McMeekin *et al.*)[110]

crystals was determined in bromobenzene–xylene mixtures after equilibrating in the suspending medium. It was found that the density of the protein crystal is proportional to the density of the sucrose suspending medium and that the serum albumin solutions have no effect, as shown in Fig. 12. The composition of the protein crystal suspended in sucrose solutions is graphically illustrated in Fig. 13 as a function of sucrose concentration. It can be seen that there is a reciprocal relationship between the amount of sucrose and water in the protein crystal. The total volume of the crystal, however, decreases with increasing concentrations of sucrose owing to loss of water not replaced by the volume of sucrose.

(110) T. L. McMeekin, M. L. Groves and N. J. Hipp, *J. Am. Chem. Soc.* **72**, 3662 (1950).

The similarity between the density of β-lactoglobulin crystals in sucrose suspensions and the sedimentation rates of tobacco mosaic virus in sucrose solutions, as reported by Schachmann and Lauffer,[111] has been taken to indicate that the environment affects proteins in solution in a similar manner to its effect on the protein crystal.[110]

The combination of β-lactoglobulin with acids and bases has been described in a comprehensive manner as that of a dissociating curve.[58,112] This curve expresses the relation between the pH of the protein solution

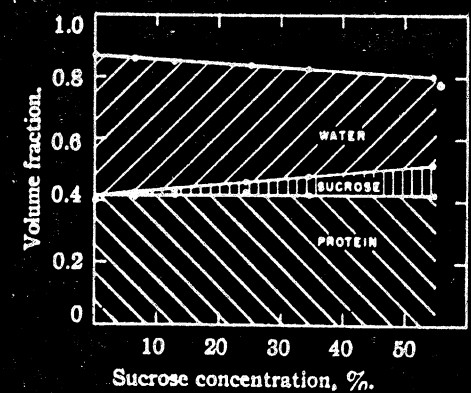


FIG. 13. Volume fractions of sucrose, protein, and water in crystals of β-lactoglobulin suspended in varying concentrations of sucrose solutions. (After McMeekin *et al.*)[110]

and the amount of acid or base bound to the protein, as shown in Fig. 14. The temperature and the salt and protein concentrations are important factors in determining the dissociation of a protein. An analysis of the curve for β-lactoglobulin[58] indicated the presence of 58 carboxyl, 34 amino, 6 imidazole, and 6 guanidino groups in 1 mole (40,000 g.) of β-lactoglobulin. By amino acid analyses,[113] 48 cationic and 64 anionic groups were found, which is in excellent agreement with the titration data.

Grönwall[114] found that the solubility of β-lactoglobulin in dilute salt solution varies with the method used for its preparation. In agreement with Palmer's results, the solubility did not increase more than 1 per cent when the solid phase was increased several times. As a test of purity, these solubility measurements were deficient in that a large excess of solid phase was used and there was no indication that the solubility values represent equilibrium values. The fact that different preparations of

(111) H. K. Schachmann and M. A. Lauffer, *J. Am. Chem. Soc.* **71**, 536 (1949).
(112) R. K. Cannan, *Chem. Revs.* **30**, 395 (1942).
(113) E. Brand, L. J. Saidel, W. H. Goldwater, B. Kassell, and F. J. Ryan, *J. Am. Chem. Soc.* **67**, 1524 (1945).
(114) A. Grönwall, *Compt. rend. trav. lab. Carlsberg. Sér. chim.* **24**, Nos. 8–11 (1942).

method is based on precipitation of the casein components from a solution in 6.0 *M* urea by adding water. In each of these methods, α-casein is the least soluble and is removed first.

(*1*) *Water-Solubility Method.* In Warner's method,[16] α-casein is prepared by dissolving undried, purified, acid-precipitated casein in sodium hydroxide to give a 1 per cent solution with a pH of 6.5. The solution is then adjusted to pH 3.5 by adding dilute hydrochloric acid. After the solution is chilled to 2°, it is diluted to a protein concentration of 0.2–0.3 per cent, and 0.01 *N* sodium hydroxide is added until pH 4.2 is reached. At this point a precipitate is formed. Addition of sodium hydroxide is continued until a clear supernatant is given on centrifugation, usually at pH 4.4. The precipitate is dissolved, and the entire procedure is repeated at least six times. The protein isolated in this manner gives the electrophoretic pattern of α-casein free of β-casein. Mellander[1] was able to separate α-casein from cow milk casein by this method, but he was unable to separate the α-component of human milk casein by this procedure.

(*2*) *Alcohol Method.* In separating the components of casein by fractionation in alcohol,[12] purified, acid-precipitated casein is dissolved in dilute alkali and made to contain 50 per cent alcohol and 0.2 *M* ammonium acetate. On adjusting the alcoholic casein to pH 6.5 by the addition of 1 *N* acetic acid in 50 per cent alcohol, approximately one-third of the casein is precipitated (fraction *A*). This fraction contains 92 per cent α-casein and 8 per cent β-casein. It also contains the proteolytic enzyme of milk.[37] The filtrate from fraction *A* is adjusted to pH 5.7 by adding acetic acid in 50 per cent alcohol. The precipitate formed at this point (fraction *B*) amounts to two-fifths of the total casein, and is composed of 80 per cent α-casein and 20 per cent β-casein. The protein in the filtrate from fraction *B* is precipitated by cooling to 2° or by diluting with an equal volume of water at room temperature (fraction *C*). This fraction contains an equal amount of α- and β-casein and about 10 per cent of γ-casein.

α-Casein may be prepared from either fraction *A* or *B*. It may be prepared from fraction *A* by reprecipitating at pH 7.2 in the presence of ammonium acetate in 50 per cent alcohol. For the preparation of α-casein from fraction *B*, this fraction is dissolved in borax solution and precipitated in 50 per cent alcohol in the presence of 0.15 *M* NaCl by adding hydrochloric acid until the pH of the solution reaches 5.7. It is necessary to repeat this precipitation twice to obtain electrophoretically pure α-casein. A yield of approximately one-third of the α-casein in whole casein can be obtained by reworking fractions *A* and *B*.

β-Casein is prepared from fraction *C*, which contains about 45 per cent of β-casein. It is separated from the other components by utilizing the difference in solubility of the three components in water at 2 and 32°C.[16] α-Casein is precipitated from an aqueous solution of this fraction at 2° and pH 4.5. Crude β-casein is precipitated by warming the filtrate to 32°, leaving most of the γ-casein in solution. After two extractions at 2° and pH 4.5, the insoluble fraction is free of γ-casein, and β-casein is obtained by further extraction and precipitation with these conditions. A yield of about 15 per cent β-casein is obtained.

γ-Casein can be prepared from the aqueous filtrates obtained in preparing β-casein, or it can be prepared from unfractionated casein by extraction with 50 per cent alcohol[38] in a manner similar to that used by Osborne and Wakeman[39] for preparing their alcohol-soluble protein. In both methods it is necessary to concentrate the γ-casein by removing α-casein at 2° and pH 4.4, and most of the β-casein by warming the filtrates from these precipitates to 32°. After the removal of the protein insoluble at 32°, by adjusting to 0.5 *M* sodium chloride, a fraction is precipitated from the filtrate that contains 44 per cent γ-casein. The precipitate is dissolved in alkali, and sodium chloride is removed by dialysis at 2°. The crude γ-casein solution containing about 15 per cent protein is adjusted to pH 6.4 by addition of dilute hydrochloric acid at room temperature. The protein is precipitated as a sirup, which is extracted three times with 50 per cent alcohol at 35° and then cooled at room temperature. γ-Casein is precipitated by cooling the 50 per cent alcohol-soluble portion to 2°. A yield of about 20 per cent of the total γ-casein present is obtained.

(*3*) *Urea Method.* Freshly prepared wet casein or dried casein is easily soluble in 6.6 *M* urea, and the electrophoretic components can be separated by merely adding water.[12] When the concentration of the urea of a casein solution in urea is reduced to 4.6 *M* by adding water, α-casein is largely precipitated. Crude β-casein is precipitated from the filtrate of α-casein by addition of enough water to make the urea concentration 1.7 *M*. γ-Casein, which is concentrated in the filtrate from the β-casein, is precipitated by adjusting to 1.6 *M* ammonium sulfate. Essentially pure α- and β-casein are obtained when the crude products are reprecipitated at the same urea concentration as originally used. Final purification of the γ-casein fraction is accomplished by precipitation in aqueous solution by a variation of pH and temperature as described under the section on γ-casein.

The principles of these methods for separating the components of

(37) R. C. Warner and E. Polis, *J. Am. Chem. Soc.* 67, 529 (1945).

(38) N. J. Hipp, M. L. Groves, J. H. Custer, and T. L. McMeekin, *J. Am. Chem. Soc.* 72, 4928 (1950).

(39) T. B. Osborne and A. J. Wakeman, *J. Biol. Chem.* 33, 243 (1918).

of the protein structure. Further hydrolysis of the protein gave a contraction of 20 cc. per peptide bond split, which is the value given by synthetic peptides. The influence of temperature on the denaturation of β-lactoglobulin in urea was also measured by changes in optical rotation.[125] In an analysis of denaturation in urea, a distinction was made between a reversible denaturation with a negative temperature coefficient

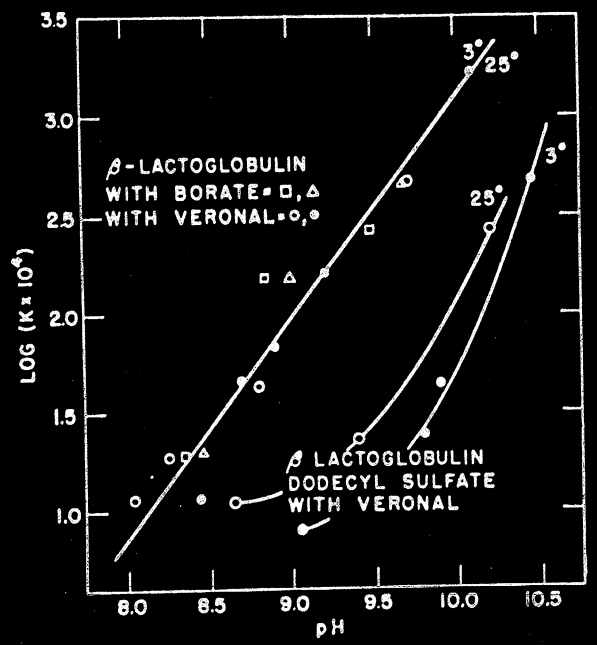


FIG. 15. Comparison of the effects of pH and temperature on the velocity constant of denaturation of β-lactoglobulin and β-lactoglobulin dodecyl sulfate. □, borate at 25°; △, borate at 3°; ○, veronal at 25°; ●, veronal at 3°. (After Groves *et al.*)[126]

and an irreversible denaturation with a positive temperature coefficient. The irreversible portion of the reaction is associated with the oxidation of sulfhydryl groups unmasked in reversible denaturation.[125] It was also shown that trypsin caused a rapid denaturation of β-lactoglobulin at pH 9.3 in the absence of urea. The rate of denaturation of β-lactoglobulin has been found to be the same at 3° and at 25° from pH 8.0 to 10.0, as shown in Fig. 15.[126] Under these conditions the rate of denaturation is inversely proportional to the 1.1 power of the hydrogen-ion concentration. β-lactoglobulin containing two molecules of dodecyl sulfate, however, was much more slowly denatured at these pH values than was β-lactoglobulin, and the rate of denaturation was greater at 25° than at 3°.

(125) L. K. Christensen, *Compt. rend. trav. lab. Carlsberg. Sér. chim.* **28**, 37 (1952).
(126) M. L. Groves, N. J. Hipp, and T. L. McMeekin, *J. Am. Chem. Soc.* **73**, 2790 (1951).

TABLE VI
AMINO ACID COMPOSITION OF MILK PROTEINS[a]

Constituent	Human casein[d]	Cow[b] casein[49]	Cow β-lactoglobulin	Cow colostrum globulin	Immune globulin of cow milk
Total N	14.89	15.63	15.60[118]	15.63[17]	15.67[c11]
Total S	0.88	0.76[1]	1.60[118]	1.1[128]	1.00[c87]
Total P	0.57	0.86			
Amino N		0.93	1.24[118]		
Amide N		1.6	1.07[118]		
Glycine	0.0	2.0	1.4[118]		
Alanine	2.0	3.2	7.4[127]		
Valine	5.0	7.2	5.8[118]	10.2[17]	9.6[87]
Leucine	12.2	9.2	15.6[118]	8.9[17]	9.6[87]
Isoleucine	6.3	6.1	6.1[87]	2.77[87]	3.03[c87]
Proline	8.9	10.6	4.1[118]	10.0[128]	
Phenylalanine	5.8	5.0	3.5[118]	3.6[17]	3.88[87]
Cysteine			1.11[118]		
Cystine	0.64	0.34	2.29[118]	3.26[87]	3.15[c87]
Methionine	2.4	2.8	3.22[118]	0.89[87]	0.90[c87]
Tryptophan	1.4	1.7[d]	1.94[118]	2.74[17]	2.70[87]
Arginine	3.4	4.1	2.88[118]	4.36[87]	4.05[c87]
Histidine	1.8	3.1	1.58[118]	1.90[87]	2.05[c87]
Lysine	5.4	8.2	11.4[118]	6.3[87]	6.8[c87]
Aspartic acid	4.6	7.1	11.4[118]	9.4[128]	
Glutamic acid	20.9	22.4	19.5[118]	12.3[128]	
Serine	5.4	6.3	5.0[118]		
Threonine	4.5	4.9	5.85[118]	9.4[87]	10.5[c87]
Tyrosine	5.7	6.3	3.78[118]	6.7[128]	
Totals	96.3	110.5	113.85	92.81	56.26

[a] Grams per 100 g. protein.
[b] Except for sulfur.
[c] Average of milk euglobulin and milk pseudoglobulin.
[d] Spies method.

(127) A. S. Keston, S. Udenfriend, and R. K. Cannan, *J. Am. Chem. Soc.* **68**, 1390 (1946).
(128) R. G. Hansen, R. L. Potter, and P. H. Phillips, *J. Biol. Chem.* **171**, 229 (1947).

value of about $S_{20} = 12$ as compared to a value of $S_{20} = 23$ for a 2.6 per cent solution. Svedberg, Carpenter, and Carpenter[41] reported that casein preparations are heterogeneous and that the sedimentation constants of the components vary with the method of preparation. The main component of casein prepared by the Van Slyke and Baker methods

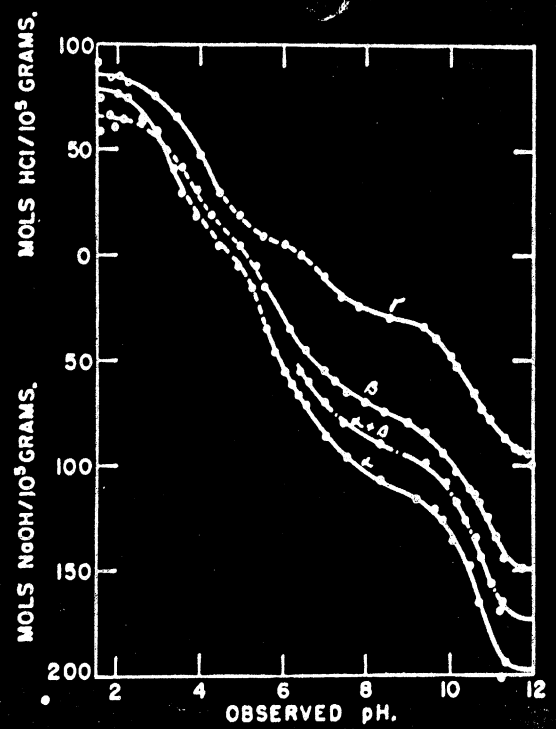


FIG. 4. Acid–base titration curves for α-, β-, and γ-casein and an equal mixture of α- and β-casein at protein concentration of 1 per cent, 0.05 ionic strength and 25°. Ordinates are moles bound/10^5 g. The dotted line is the pH region where, under the conditions of the experiment, all the protein was not soluble. For clarity, only a portion of the curve for the mixture of α- and β-casein is given. (After Hipp *et al.*)[44]

had a molecular weight of 75,000–100,000. The apparent conflict in the sedimentation constants for casein as reported by Pedersen and by Svedberg, Carpenter, and Carpenter was ascribed to the dissociation tendency of casein and also to the difference in buffer solutions used.[18] Another factor of possible significance in the reported sedimentation constants for casein is the finding of Philpot (unpublished experiments quoted by Svedberg and Pedersen)[47] that the addition of small amounts

(47) T. Svedberg and K. O. Pedersen, The Ultracentrifuge, Oxford Univ. Press, London, 1940, p. 408.

of calcium to casein solutions causes the sedimentation constant to increase from $S_{20} = 6$ to $S_{20} = 10$. No studies have been reported on the molecular weights of the separated electrophoretic casein components.

The combination of casein with acid and base has been used to estimate its dissociating groups.[48] The acid- and base-combining capacity of the components of casein has also been found to vary[44] as would be expected from their amino acid compositions. The marked difference in the titration curves of α-, β-, and γ-casein (Fig. 4) is consistent with the analytical data for ionic groups in these proteins as reported by Gordon *et al.*[49]

In addition to the usual ionizing groups found in proteins arising from amino acid residues, the casein components also contain varying amounts

TABLE IV
MAXIMUM COMBINING CAPACITY OF α-, β- AND γ-CASEIN

	α	β	γ
Moles acid/10^5 g. casein	78	66	85
Moles base/10^5 g. casein	198	150	96
Grams casein/equivalent of acid	1282	1500	1176
Grams casein/equivalent of base	505	666	1042

of phosphoric acid which must be considered in the analysis for ionic groups. By selecting the pH values where the ionic groups of the amino acid residues would be expected to dissociate, the amount of the ionizing groups present can be estimated from the titration curve. Thus at pH 6.35, carboxyl groups plus one equivalent of phosphoric acid are considered to be dissociated. At this point calculations based on the titration data indicate that α-casein contains 144 moles and β-casein contains 108 moles of carboxyl groups including one equivalent of phosphoric acid per 10^5 g., which is in agreement with the results obtained for these groups when calculated from the amino acid analysis. Similar comparisons at other pH values give reasonable agreement between the values estimated from the titration curve and those determined by amino acid analysis. The maximum acid- and base-combining capacities of the casein components are given in Table IV as well as the number of grams of casein combining with one equivalent of acid or base.[44] The values given in Table IV for the base-combining capacity are higher than the

(48) E. J. Cohn and R. E. L. Berggren, *J. Gen. Physiol.* 7, 45 (1924).
(49) W. G. Gordon, W. F. Semmett, R. S. Cable, and M. Morris, *J. Am. Chem. Soc.* 71, 3293 (1949); W. G. Gordon, W. F. Semmett, and M. Bender, *ibid.* 72, 4282 (1950); W. G. Gordon, W. F. Semmett, and M. Bender, *ibid.* 75, 1678 (1953).

on content to be 0.069 per cent. This lactoperoxidase contained two ectrophoretic components which were separated. Some evidence indi- tes that there is an equilibrium between these two forms of ctoperoxidase.

Amylase. The concentration of this starch-splitting enzyme in milk is onstant. It appears to be associated principally with cream, although kim milk has some amylase activity.[138]

Lactase. This enzyme acts on lactose, splitting the molecule into ucose and galactose, and has been reported to be a constituent of normal ilk.[139]

Lipase. The presence of a true lipase resembling pancreatic lipase as demonstrated to be present in cow's milk.[140] The amount of this nzyme in milk usually increases with advanced lactation.

Xanthine Oxidase. Morgan, Stewart, and Hopkins[141] showed that ilk is a good source of an enzyme capable of converting xanthine to uric cid. It was shown by Ball[142] that the xanthine oxidase of milk is largely ssociated with the cream fraction. A method was devised for its puri- ication which resulted in a preparation 500 times more active per unit of ry weight than whole milk. Cream was extracted with an equal volume f 0.2 *M* disodium acid phosphate at 38°, and the aqueous layer was emoved after centrifugation at a low temperature. After incubating the olution with pancreatic lipase for 3½ hr. at 38°, a solution of calcium hloride was added which produced a precipitate of calcium phosphate. The yellow solution obtained after the removal of calcium phosphate was made to 60 per cent saturation with ammonium sulfate and allowed to tand at 0°. The precipitate was removed at 0° and dissolved in water. The solution was made to 33 per cent saturation with ammonium sulfate, and the precipitate was removed at 0° and discarded. The xanthine oxidase in the supernatant was precipitated by making to 42 per cent saturation with ammonium sulfate and then removed by centrifugation at 0°. Xanthine oxidase was found to have an isoelectric point at pH 6.2 as determined by cataphoresis.

On the basis that each molecule of protein combines with one molecule of flavin, and purity considerations lead to an estimate of 74,000 for the maximum value for the molecular weight of xanthine oxidase. The flavin group was split off from the protein reversibly by prolonged dialysis

(138) C. J. Koning, *Milchw. Zentr.* 4, 156 (1908); (*C. A.* 2, 1986).
(139) A. J. J. Vandevelde, *Biochem. Z.* 11, 61 (1908).
(140) F. E. Rice and A. L. Markley, *J. Dairy Sci.* 5, 84 (1922).
(141) E. J. Morgan, C. P. Stewart, and F. G. Hopkins, *Proc. Roy. Soc.* (London) *Ser.* B94, 109 (1922).
(142) E. G. Ball, *J. Biol. Chem.* 133, 51 (1939).

against water at 0°. Corran *et al.*[143,144] obtained a preparation of xanthine oxidase 1000 times more active than milk. Their method involves fractionation of milk with ammonium sulfate, precipitation of the active fraction with alcohol, absorption and elution of the enzyme from alumina gel, and further fractionation with ammonium sulfate. The flavin group of xanthine oxidase prepared in this manner was not split off by dialysis. This preparation of xanthine oxidase contained three components as determined in the ultracentrifuge. Most of the material with an absorption of 475 mμ had a sedimentation constant of $S_{20} = 12.6$.[145]

Phosphatase. Raw milk contains a phosphatase that has the characteristics of mammalian phosphatase in its activation by magnesium ions and pH activity relations.[146] Zittle and DellaMonica,[147] by comparative tests with inhibitors, have concluded that milk phosphatase bears a closer relationship to bone and kidney phosphatase than to intestinal phosphatase. They also determined the effects of anions, cations, and amino acids on milk phosphatase and devised methods for its purification. The greater heat resistance of bacterial phosphatase has been used as a means of its differentiation from milk phosphatase.[148]

Milk phosphatase is destroyed more slowly by heat than many bacteria, so that the phosphatase test is widely used as a test for pasteurization. Milk has been shown to contain small amounts of acid phosphatase which is more heat-resistant than the alkaline phosphatase.[149] From a study of the phosphatase activity of raw milk and its distribution in various milk fractions, it has been concluded that phosphatase is present to a large extent but not entirely at the fat–serum interphase.[150] It was found that cream contains about eight times as much phosphatase as skim milk and that buttermilk derived from making butter is particularly high in phosphatase.

Aldolase. The muscle enzyme aldolase, which reversibly splits fructose 1,6-diphosphate into dihydroxyacetone phosphate and phosphoglyceric aldehyde, was reported by Polis and Shmukler to be present in milk.[151]

(143) H. S. Corran and D. E. Green, *Biochem. J.* 32, 2231 (1938).
(144) H. S. Corran, J. W. Dewan, A. H. Gordon, and D. E. Green, *Biochem. J.* 33, 1694 (1939).
(145) J. St. L. Philpot, *Biochem. J.* 33, 1707 (1939).
(146) W. R. Graham, Jr., and H. D. Kay, *J. Dairy Research* 5, 54, 63 (1933).
(147) C. A. Zittle and E. S. DellaMonica, *Arch. Biochem.* 26, 112 (1950); *ibid.* 26, 135 (1950); *ibid.* 35, 321 (1952); *Proc. Soc. Exptl. Biol. Med.* 76, 193 (1951).
(148) R. P. Tittsler, D. S. Sager, and G. P. Sanders, *J. Dairy Sci.* 31, 705 (1948).
(149) J. E. C. Mullen, *J. Dairy Research* 17, 288 (1950).
(150) J. H. Hetrick and P. H. Tracy, *J. Dairy Sci.* 31, 367 (1948).
(151) B. D. Polis and H. W. Shmukler, *J. Dairy Sci.* 33, 619 (1950).

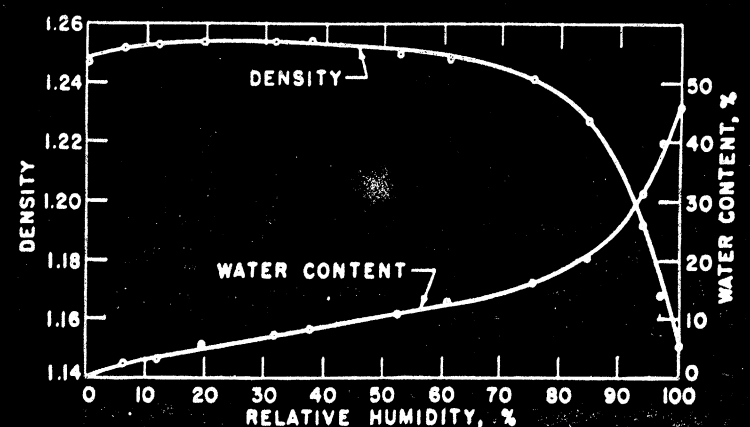
s component groups or atoms. Calculations of the solution volumes any proteins from their amino acid compositions give values that n good agreement with the observed solution volumes though there ome exceptions.[56]

TABLE V

CIFIC VOLUMES OF MILK PROTEINS IN THE SOLID STATE AND IN SOLUTION

Protein	Solid state: Density, g./ml.	Solid state: Sp. vol., ml./g.	In solution: Determined, ml./g.	In solution: Calcd. from amino acid compn., ml./g.	Volume change, ml./g.
n, unfractionated	1.292	0.774	0.731	0.731	−0.043
ein	1.293	0.773	0.728	0.725	−0.045
ein	1.271	0.787	0.741	0.743	−0.046
ein	1.250	0.800	0.750	0.756	−0.050
toglobulin	1.247	0.802	0.751	0.746	−0.051

The contraction in volume of proteins on going into solution is remark- constant, with an average value of 0.046 ml./g. or about 6 per cent



6. Effect of relative humidity on the water content and density of β-lactoglobulin crystals. (After McMeekin *et al.*)[57]

ble V). Careful measurements on the densities and water content of e β-lactoglobulin crystals as a function of relative humidity (Fig. 6) e shown that the volume of the protein in the crystal obtained by apolating the plot of water content against specific volume (Fig. 7) is 2 ml./g. when the water content is between 14 and 46 per cent, as

(56) T. L. McMeekin and K. Marshall, *Science* 116, 142 (1952).

compared to 0.751 ml. in dilute solution and 0.802 ml. for the anhydrous protein (Table V).[57] By assigning 0.030 ml. (0.802 minus 0.772) of the total contraction of 0.051 ml./g. of protein to the apparent contraction of the protein, a value of 0.021 ml. (0.772 minus 0.751) is obtained for the electrostriction of water, which is the approximate electrostriction

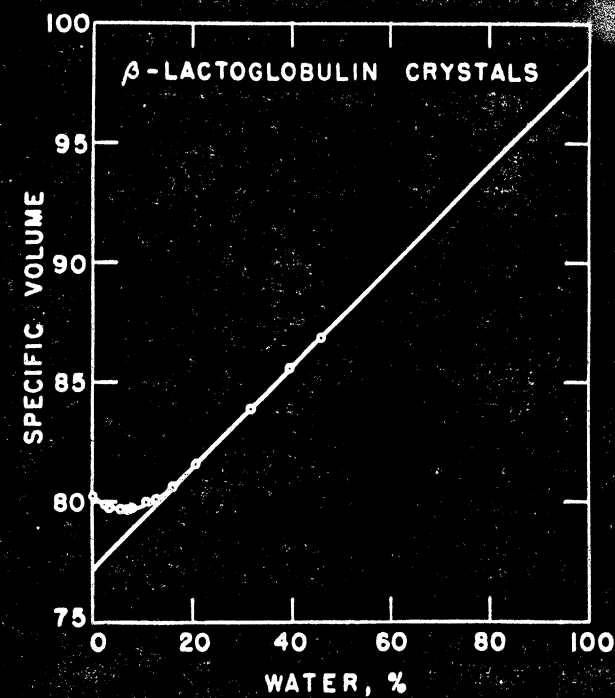


FIG. 7. Relationship between the specific volume and the water content of β-lactoglobulin crystals. (After McMeekin *et al.*)[57]

expected per gram of protein on the basis of the ionic groups in the protein. The apparent contraction of the protein is ascribed to the presence of molecular voids in the protein that were not penetrated by the organic solvent used in measuring the density or to a packing effect on the protein caused by the electrostriction of water when small amounts of water are added to the anhydrous protein. β-Lactoglobulin contains 104 ionic groups per mole of 40,000 g.[58] and it has been estimated that each pair of ionic groups causes a contraction of 20 ml. in volume;[54] consequently the value of 0.026 ml./g. of protein is obtained for electrostriction on the basis

(57) T. L. McMeekin, M. Groves, and N. J. Hipp, *J. Polymer Sci.*, January (1954).
(58) R. K. Cannan, A. H. Palmer, and A. C. Kibrick, *J. Biol. Chem.* 142, 803 (1942).

identical in molecular weight with the α-component of whey described by Pedersen, it could be classified as a globulin instead of an albumin which would be expected to be soluble in water at the isoelectric point. This protein, however, is insoluble in salt, which property distinguishes it from a globulin. The fact that the protein in whey, soluble in saturated magnesium sulfate, coagulated by heat, and described extensively in the literature as lactalbumin, has no relation to the protein with a molecular weight of 15,000, suggests that the crystalline protein should be given a different designation. Consequently, the component of whey with a sedimentation constant of $S_{20} = 1.75$ is given the name α-lactalbumin as has been suggested by Svedberg.[94]

(*1*) *Preparation of α-Lactalbumin.* Gordon and Semmett have perfected their method for the separation of α-lactalbumin so that a pure product is obtained without difficulty. In their method, casein is precipitated from skim milk by acidification in the usual manner. The fraction of whey insoluble in 2.3 *M* ammonium sulfate at pH 6 is removed by filtration. The remainder of the total protein of whey is precipitated from the filtrate by the addition of solid ammonium sulfate to 3.3 *M*. After filtration, the insoluble protein is dissolved in water and dialyzed free of salt at pH 5.2. Under these conditions β-lactoglobulin crystallizes and is removed by centrifugation. The protein in the supernatant liquor is precipitated by adding solid ammonium sulfate to 3.3 *M*. The precipitated protein is removed by filtration and dissolved in water and again dialyzed at pH 5.2. A precipitate appears after exhaustive dialysis. This precipitate is composed of crude α-lactalbumin and some additional β-lactoglobulin. This insoluble material is extracted with small amounts of 0.1 *M* sodium chloride, which dissolves the β-lactoglobulin, leaving α-lactalbumin as an insoluble precipitate. The precipitate is removed by centrifugation and dissolved in 0.03 *N* ammonium hydroxide, and the solution is filtered. The filtrate is acidified by the addition of 0.1 *N* sulfuric acid to pH 4.0. The α-lactalbumin, which is precipitated, is removed by centrifugation and again dissolved in ammonium hydroxide. The pH is adjusted to 6.6, and to the clear solution an equal volume of saturated ammonium sulfate solution (adjusted to pH 6.6) is added dropwise with stirring. The precipitate, which is formed at half-saturation with ammonium sulfate, is amorphous and is removed by centrifugation. To the supernatant liquor, saturated ammonium sulfate solution is added as before. When the concentration of salt reaches 60 per cent saturation, crystallization of α-lactalbumin begins and is complete at about two-thirds saturation.

α-Lactalbumin crystallized at pH 6.6 is easily soluble in water, but when the solution is adjusted to pH 4.6, the protein is precipitated in an amorphous condition. It may be recrystallized, however, by dissolving it in dilute ammonia and again salting it out with saturated ammonium sulfate solution at pH 6.6. As yet, no method has been found to crystallize the α-lactalbumin from water at pH 4.6. α-Lactalbumin, as prepared by this method, contains 15.86 per cent nitrogen, 1.91 per cent sulfur, and only a trace of phosphorus. The tryptophan content of 7.2 per cent is unusually high for a milk protein. At pH 6.8 the optical rotation $[\alpha]_D^{25}$ is $-61°$.

c. Preparation and Properties of β-Lactoglobulin

In attempting to prepare crystalline lactalbumin by the described methods, Palmer,[15] by dialysis from aqueous salt solution, obtained the principal protein of whey in the form of large crystals. The method used for separating β-lactoglobulin from milk is indicated under the section on the preparation of α-lactalbumin. Its sedimentation constant of $S_{20} = 3.1$ is identical with the β-component of whey described by Pedersen,[13] who also determined its isoelectric point to be 5.2.[97]

Palmer[15] determined the solubility of β-lactoglobulin in water, dilute sodium chloride, and concentrated sodium sulfate solutions. He observed that a fourfold increase in the amount of solid phase did not change the solubility, indicating the purity of this protein. In further study of its solubility, Sørensen and Palmer[98] found that a threefold variation in the amount of solid phase did not change the solubility more than 1 per cent. These results on its solubility, the ease of obtaining large crystals, and the other unique properties of β-lactoglobulin have made it a favorite protein for investigation. Crowfoot[99] determined its crystal structure and unit cell by means of x-ray measurements. Assuming eight molecules per unit cell, she calculated a molecular weight of 40,000 for air-dried β-lactoglobulin,[100] which was close to Pedersen's[97] value of 39,000 for its molecular weight. A value of 35,800 for the anhydrous molecular weight was deduced from Crowfoot's data on the assumption that air-dried crystals contain 9.78 per cent moisture.[101] Senti and Warner[102] obtained a value of 35,400 for the molecular weight of β-lactoglobulin from x-ray measurements, which is also in agreement with the value obtained by Bull and Currie[103] from osmotic-pressure measurements

(97) K. O. Pedersen, *Biochem. J.* **30**, 961 (1936).
(98) M. Sørensen and A. H. Palmer, *Compt. rend. trav. lab. Carlsberg. Sér. chim.* **21**, No. 21 (1938).
(99) D. Crowfoot, *Chem. Revs.* **28**, 215 (1941).
(100) I. Fankuchen, *in* E. J. Cohn and J. T. Edsall, eds., Proteins, Amino Acids and Peptides, Reinhold Pub. Corp., New York, 1943, p. 328.
(101) T. L. McMeekin and R. C. Warner, *J. Am. Chem. Soc.* **64**, 2393 (1942).
(102) F. R. Senti and R. C. Warner, *J. Am. Chem. Soc.* **70**, 3318 (1948).
(103) H. B. Bull and B. T. Currie, *J. Am. Chem. Soc.* **68**, 742 (1946).

tary tract.[88] The finding that the immune globulin is not absorbed into the blood stream of the calf, if the calf is 24 hr. old at the time of feeding the colostrum,[82] indicates that the presence of trypsin inhibitor in colostrum is not of primary importance in assisting the immune globulin to reach the blood stream of the calf.

There is considerable evidence that the immune globulin of colostrum originates in the blood stream.[11] It has been demonstrated, however, that colostrum contains large numbers of plasma cells, and their number parallels the concentration of immune bodies.[90] It has also been shown

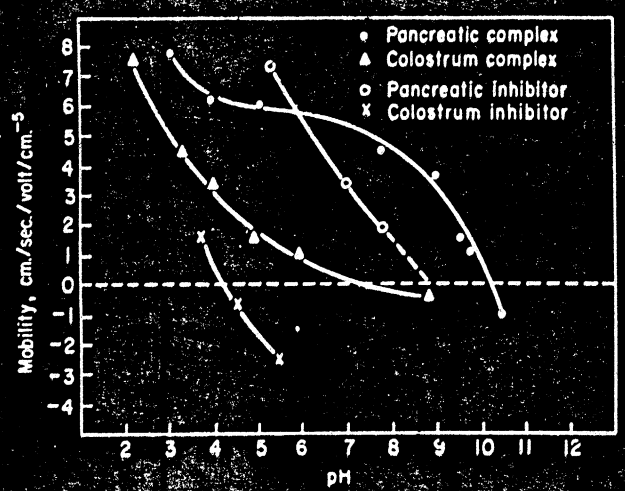


FIG. 10. Comparison of pH-mobility curves of trypsin inhibitor and its complex from colostrum and pancreas. (After Laskowski *et al.*)[88]

that plasmacytosis and an increase in immune bodies could be produced by cessation of milking. Comline *et al.*[91] have demonstrated that colostrum globulin reaches the general circulation through the lymph and not by the portal system.

b. "*Albumin*" *Fraction*

In studies on the distribution of milk proteins,[4] the portion of whey soluble in saturated magnesium sulfate or 0.5 saturated ammonium sulfate is usually designated the albumin fraction. Wichmann, in 1899,[92] crystallized lactalbumin from this fraction. Later Sjögren and Svedberg[93] obtained lactalbumin by the method described by Wichmann.

(90) B. Campbell, R. M. Porter, and W. E. Petersen, *Nature* **166**, 913 (1950).
(91) R. S. Comline, H. E. Roberts, and D. A. Titchen, *Nature* **167**, 561 (1951).
(92) A. Wichmann, *Z. physiol. Chem.* **27**, 575 (1899).
(93) B. Sjögren and T. Svedberg, *J. Am. Chem. Soc.* **52**, 3650 (1930).

The product was heterogeneous with respect to size, the molecular distribution being 12,000–25,000. Pedersen[13] found that milk whey contains three components, which he designated α, β, and γ. The α-component had a sedimentation constant $S_{20} = 1.9$, the β-component $S_{20} = 3.1$, and the γ-component $S_{20} = 7.1$. The α-component was identified with a crystalline fraction separated by Kekwick (unpublished). On the basis of these data, Svedberg[94] calculated the molecular weight of the α-component to be 17,600 and designated the protein as α-lactalbumin.

In 1939 Sørensen and Sørensen[95] isolated a crystalline protein from the albumin fraction of milk whey, which they designated a "crystalline-insoluble substance." The crystalline form of the insoluble substance obtained from ammonium sulfate solutions appeared to be identical with crystalline β-lactoglobulin, but it differed from the latter in its insolubility in dilute salt solutions and in its tryptophan content. This apparently new milk protein was not further characterized, and its presence in whey has not been generally accepted. It differs from that isolated by Wichmann[92] in that it is insoluble in water at pH 4.6.

By a method similar to that described by the Sørensens,[95] Gordon and Semmett[96] recently isolated a crystalline substance insoluble in water from milk whey which confirmed many of the Sørensens' observations. The crystalline protein isolated from whey by Gordon and Semmett has a mobility of -4.2 u in veronal buffer, pH 8.5 and 0.1 ionic strength.[14] G. L. Miller[14] has determined the molecular weight of the whey protein isolated by Gordon and Semmett and found it to be homogeneous in the ultracentrifuge. He also reported that the molecular constants for this protein are similar to those found by Pedersen[13] for the lactalbumin isolated by Kekwick. Sedimentation and diffusion determinations were made at a protein concentration of 1 per cent in a buffer of 0.18 ionic strength NaCl and 0.02 ionic strength phosphate at pH 7.5. A value of $S_{20} = 1.75$ was found for the sedimentation constant and $D_{20} = 10.6 \times 10^{-7}$ for the diffusion constant. From these results a value of 15,000 was calculated for the molecular weight of the whey protein isolated by Gordon and Semmett, the determined value of 0.735 being used for the partial specific volume. There can be no doubt that the protein isolated by Gordon and Semmett represents a major component of milk whey, as indicated in Table II, and is the same protein as the α-component of whey described by Pedersen,[13] with a sedimentation constant of $S_{20} = 1.9$. Since this protein is insoluble in water and appears to be

(94) T. Svedberg, *Nature* **139**, 1051 (1937).
(95) M. Sørensen and S. P. L. Sørensen, *Compt. rend. trav. lab. Carlsberg. Sér. chim.* **23**, No. 7 (1939).
(96) W. G. Gordon and W. F. Semmett, *Federation Proc.* **11**, No. 1, 220 (1952).

and the value of 35,700 by Halwer, Nutting, and Brice from light-scattering measurements.[104] Cecil and Ogston[105] and also Miller and Golder[106] have reported values of 35,400 for the molecular weight of β-lactoglobulin from sedimentation constants. These findings leave little doubt that the molecular weight of β-lactoglobulin is about 35,000.

The density and water content of single, large (5–15 mg.) β-lactoglobulin crystals were determined by McMeekin and Warner.[101] The density was determined in organic solvents of suitable density in which the crystal was insoluble. Values of 1.114–1.115 for the density of these crystals were found which differed greatly from the accepted value of 1.26–1.27.[54] The higher value of 1.26 for the density was obtained from equilibrium measurements when the crystal was suspended in salt and sucrose solutions. It was found that the higher densities were correct for β-lactoglobulin crystals suspended in these solutions, since the salt or sucrose penetrated the protein crystal and made it more dense. Calculated on the basis of the water content of the protein crystal, the concentration of salt in the crystal is about two-thirds of that of the suspending medium. The correct lower density values for β-lactoglobulin crystals obtained from measurements in organic solvents were applicable to the densities used in determining the molecular weight of β-lactoglobulin by x-ray.[100]

The water content of the protein crystal is also essential for determining its molecular weight by x-ray. The water content of the β-lactoglobulin crystals was determined by loss in weight. Individual crystals were removed from the supernatant liquid, and the surface water was removed by blotting. The loss of weight of the crystal was determined as a function of time, and the weight of the crystal at the time of removal from the suspending liquid was found by extrapolation as shown in Fig. 11. The water content of the crystal was obtained by subtracting the weight of the crystal dried at 70° in vacuum from the weight of the crystal at the time of its removal from the suspending liquid. By this procedure β-lactoglobulin crystals were found to contain 46 per cent water. The rate of loss of water by the crystal in air was found to follow a first-order equation, being proportional to the logarithm of the weight of water remaining in the crystal until 70 per cent of the water is lost (Fig. 11). This indicates that the vapor pressure of the water in the crystal does not change until most of the water is lost. This finding is in agreement with the results recorded in Fig. 7 showing that the specific volume of the crystal is proportional to the water content over the range of 14–46 per cent

(104) M. Halwer, G. C. Nutting, and B. A. Brice, *J. Am. Chem. Soc.* **73**, 2786 (1951).
(105) R. Cecil and A. G. Ogston, *Biochem. J.* **44**, 33 (1949).
(106) G. L. Miller and R. H. Golder, *Arch. Biochem. Biophys.* **36**, 249 (1952).

water. The value of 46 per cent for the water content of β-lactoglobulin crystals obtained by direct measurement compares with the value of about 20 per cent when calculated by the indirect method of Sørensen and Høyrup[107] which is based on a comparison of the concentration of salt and

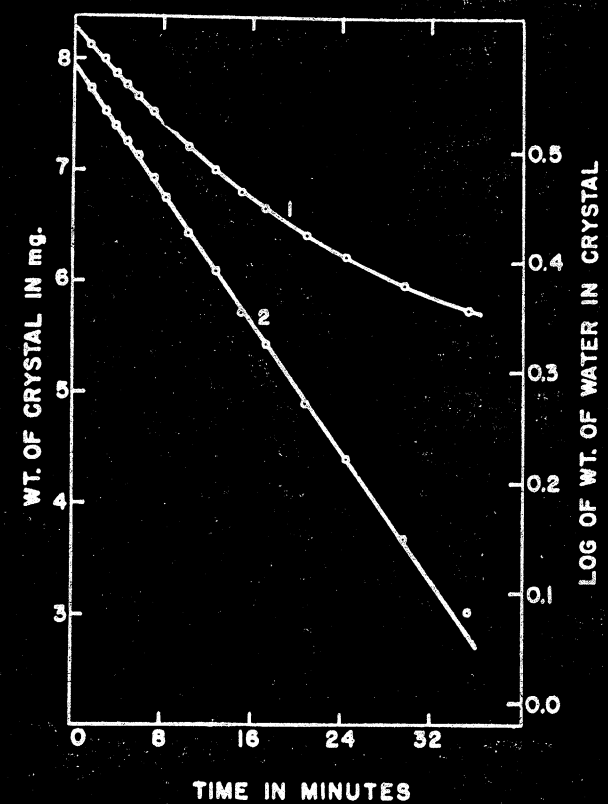


FIG. 11. Rate of loss of water by β-lactoglobulin crystal at room temperature. *1*, loss in weight of crystal as a function of time; *2*, logarithm of weight of water in the crystal shown in *1*. (After McMeekin and Warner.)[101]

water in the crystal with that of the suspending medium. The division of the water in the protein crystal into two kinds on the basis of its salt content when suspended in salt solutions has been suggested by Perutz.[108] There is evidence, however, which indicates that a portion of the salt combines with the protein in the crystal.[109]

(107) S. P. L. Sørensen and M. Høyrup, *Compt. rend. trav. lab. Carlsberg* **12**, 164 (1917).
(108) M. F. Perutz, *Trans. Faraday Soc.* **42B**, 187 (1946).
(109) T. L. McMeekin, Abstracts of Papers, 120th Meeting Am. Chem. Soc., New York, 1951, p. 10C.

The relationship between the composition and density of β-lactoglobulin crystals and that of sucrose solutions used as a suspending medium has been determined.[110] The solubility of β-lactoglobulin in sucrose solutions is small and does not affect these measurements. For comparative purposes the densities of β-lactoglobulin crystals equilibrated in serum albumin solutions were also determined. The density of the protein

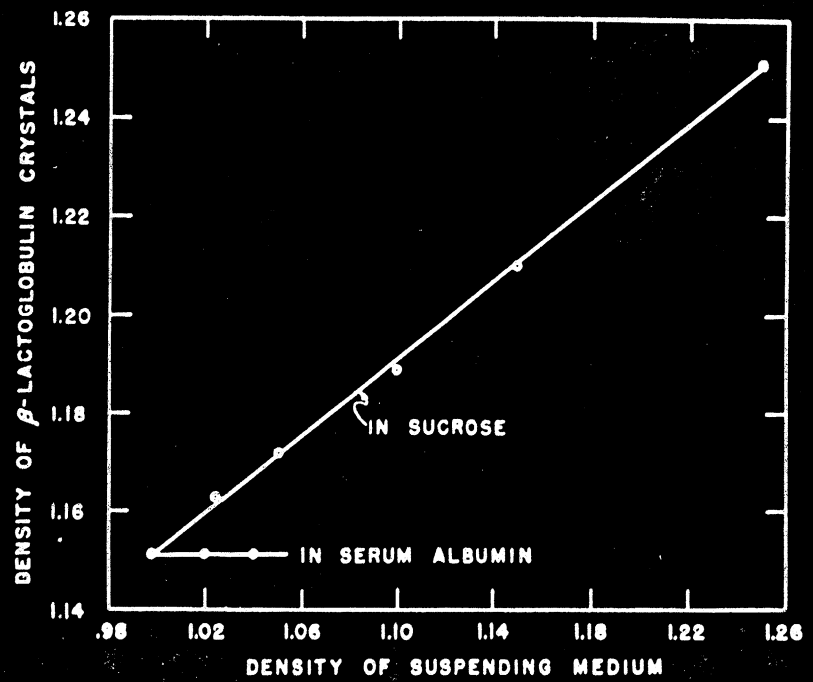


FIG. 12. Densities of β-lactoglobulin crystals equilibrated in sucrose and serum albumin solutions. (After McMeekin *et al.*)[110]

crystals was determined in bromobenzene–xylene mixtures after equilibrating in the suspending medium. It was found that the density of the protein crystal is proportional to the density of the sucrose suspending medium and that the serum albumin solutions have no effect, as shown in Fig. 12. The composition of the protein crystal suspended in sucrose solutions is graphically illustrated in Fig. 13 as a function of sucrose concentration. It can be seen that there is a reciprocal relationship between the amount of sucrose and water in the protein crystal. The total volume of the crystal, however, decreases with increasing concentrations of sucrose owing to loss of water not replaced by the volume of sucrose.

(110) T. L. McMeekin, M. L. Groves and N. J. Hipp, *J. Am. Chem. Soc.* **72**, 3662 (1950).

The similarity between the density of β-lactoglobulin crystals in sucrose suspensions and the sedimentation rates of tobacco mosaic virus in sucrose solutions, as reported by Schachmann and Lauffer,[111] has been taken to indicate that the environment affects proteins in solution in a similar manner to its effect on the protein crystal.[110]

The combination of β-lactoglobulin with acids and bases has been described in a comprehensive manner as that of a dissociating curve.[58,112] This curve expresses the relation between the pH of the protein solution

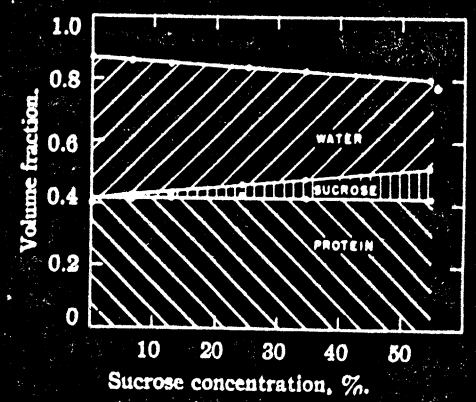


FIG. 13. Volume fractions of sucrose, protein, and water in crystals of β-lactoglobulin suspended in varying concentrations of sucrose solutions. (After McMeekin *et al.*)[110]

and the amount of acid or base bound to the protein, as shown in Fig. 14. The temperature and the salt and protein concentrations are important factors in determining the dissociation of a protein. An analysis of the curve for β-lactoglobulin[58] indicated the presence of 58 carboxyl, 34 amino, 6 imidazole, and 6 guanidino groups in 1 mole (40,000 g.) of β-lactoglobulin. By amino acid analyses,[113] 48 cationic and 64 anionic groups were found, which is in excellent agreement with the titration data.

Grönwall[114] found that the solubility of β-lactoglobulin in dilute salt solution varies with the method used for its preparation. In agreement with Palmer's results, the solubility did not increase more than 1 per cent when the solid phase was increased several times. As a test of purity, these solubility measurements were deficient in that a large excess of solid phase was used and there was no indication that the solubility values represent equilibrium values. The fact that different preparations of

(111) H. K. Schachmann and M. A. Lauffer, *J. Am. Chem. Soc.* **71**, 536 (1949).
(112) R. K. Cannan, *Chem. Revs.* **30**, 395 (1942).
(113) E. Brand, L. J. Saidel, W. H. Goldwater, B. Kassell, and F. J. Ryan, *J. Am. Chem. Soc.* **67**, 1524 (1945).
(114) A. Grönwall, *Compt. rend. trav. lab. Carlsberg. Sér. chim.* **24**, Nos. 8–11 (1942).

β-lactoglobulin gave different solubilities, however, indicates that the preparations were not identical. Li[115] found that β-lactoglobulin has three components, as determined by electrophoresis at pH's 4.8 and 6.5 in acetate buffer of 0.1 ionic strength. β-Lactoglobulin was fractionated by

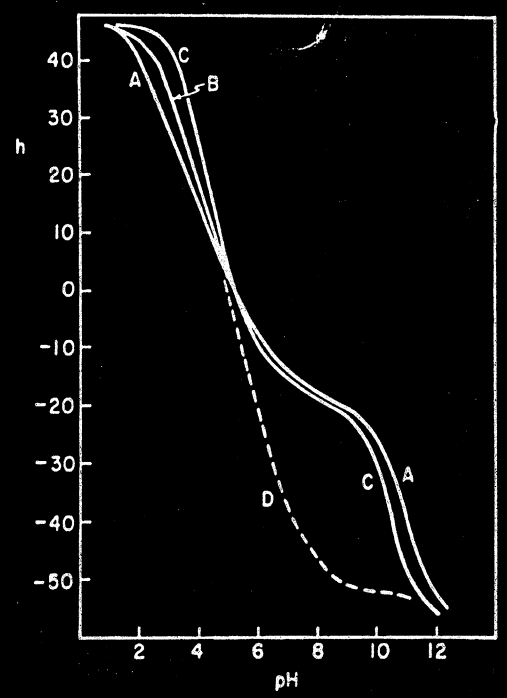


FIG. 14. Dissociation curves of β-lactoglobulin. Curves *A*, 0.019 *M* potassium chloride, 0.5 per cent protein; curve *B*, 0.135 *M* potassium chloride, 0.5 per cent protein; curve *C*, 0.67 *M* potassium chloride, 0.5 per cent protein; curve *D*, 1 *M* formaldehyde, 2 per cent protein. (After Cannan.)[112]

dialysis from solution in acetate buffers of varying pH to give crystalline preparations that varied in electrophoretic composition when determined at pH 4.7.[116] The solubility of these fractions in water and dilute salt varied in a consistent manner with the electrophoretic composition. Further fractionation of β-lactoglobulin by means of alcohol at low temperatures resulted in a crystalline preparation that contained only one electrophoretic component under all conditions studied.[117] The electro-

(115) C. H. Li, *J. Am. Chem. Soc.* **68**, 2746 (1946).
(116) T. L. McMeekin, B. D. Polis, E. S. DellaMonica, and J. H. Custer, *J. Am. Chem. Soc.* **70**, 881 (1948).
(117) B. D. Polis, H. W. Shmukler, J. H. Custer, and T. L. McMeekin, *J. Am. Chem. Soc.* **72**, 4965 (1950).

phoretically homogeneous β-lactoglobulin differed from ordinary β-lactoglobulin only in its solubility and mobility. No difference was found in amino acid composition, molecular weight, and optical rotation. A comprehensive comparison of the amide nitrogen of samples of β-lactoglobulin has been reported.[118] A value of 32.5 amide groups per mole (42,000) was found on two preparations of β-lactoglobulin made by different methods. This value is in agreement with the usually reported value, though it is considerably lower than the value of 34.6 reported earlier from the same laboratory.[119]

β-Lactoglobulin resembles serum albumin in that it binds fatty acids and other small molecules in solution.[120] A crystalline compound of β-lactoglobulin with two equivalents of dodecyl sulfate has been isolated.[121] This derivative is apparently undissociated, since the dodecyl sulfate was not removed by barium ion. The solubility of the derivative in water and in dilute salt is about one-half as large as that of β-lactoglobulin.

The rate of denaturation of β-lactoglobulin has been determined under several conditions. Heat denaturation of β-lactoglobulin solution at pH 7.0 in buffer solution of 0.1 ionic strength has been shown to involve at least two reactions that could be followed by electrophoretic mobilities.[122] The first process, initiated only above 65°, was accompanied by a fourfold increase in particle weight as determined by diffusion and sedimentation-constant measurements. The second process, which takes place only after the first process has occurred, was accompanied by a marked increase in mobility and had the characteristics of a second-order reaction. The second reaction was repressed at 75°C. and did not take place at 99°.

It has been found that the denaturation of β-lactoglobulin in 38 per cent urea as measured by insolubility in salt at the isoelectric point has a high negative temperature coefficient being faster at 0° than at 37°.[123] The reversal of denaturation produced at 0° in urea solution was rapid when the temperature was raised to 37°. Linderstrøm-Lang[124] has shown that denaturation precedes hydrolysis when β-lactoglobulin is hydrolyzed by trypsin. A very large contraction in solution volume was also found when the first peptide bonds of β-lactoglobulin are hydrolyzed by trypsin. This change in volume was considered to be associated with the collapse

(118) C. F. Jacobsen, *Compt. rend. trav. lab. Carlsberg. Sér. chim.* **26**, 455 (1949).
(119) R. D. Hotchkiss, *J. Biol. Chem.* **131**, 387 (1939).
(120) B. D. Davis and R. J. Dubos, *J. Exptl. Med.* **86**, 215 (1947).
(121) T. L. McMeekin, B. D. Polis, E. S. DellaMonica, and J. H. Custer, *J. Am. Chem. Soc.* **71**, 3606 (1949).
(122) D. R. Briggs and R. Hull, *J. Am. Chem. Soc.* **67**, 2007 (1945).
(123) C. F. Jacobsen and L. K. Christensen, *Nature* **161**, 30 (1948).
(124) K. Linderstrøm-Lang, *Cold Spring Harbor Symposia Quant. Biol.* **14**, 117 (1950).

of the protein structure. Further hydrolysis of the protein gave a contraction of 20 cc. per peptide bond split, which is the value given by synthetic peptides. The influence of temperature on the denaturation of β-lactoglobulin in urea was also measured by changes in optical rotation.[125] In an analysis of denaturation in urea, a distinction was made between a reversible denaturation with a negative temperature coefficient

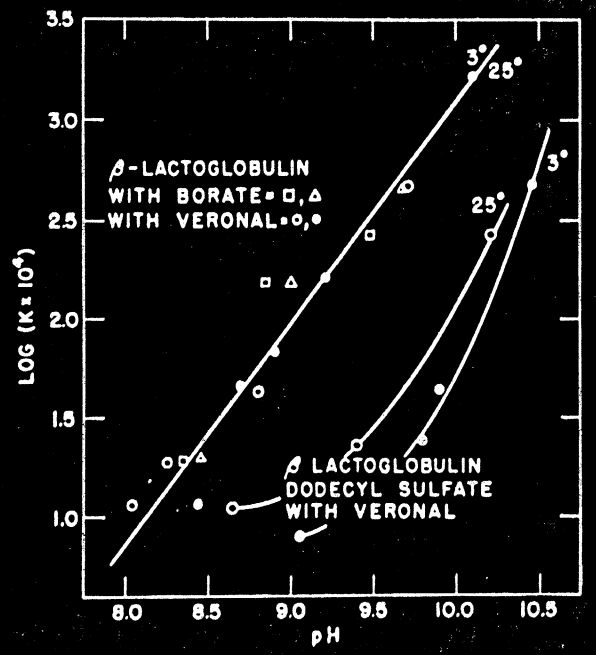


FIG. 15. Comparison of the effects of pH and temperature on the velocity constant of denaturation of β-lactoglobulin and β-lactoglobulin dodecyl sulfate. □, borate at 25°; △, borate at 3°; ○, veronal at 25°; ●, veronal at 3°. (After Groves *et al.*)[126]

and an irreversible denaturation with a positive temperature coefficient. The irreversible portion of the reaction is associated with the oxidation of sulfhydryl groups unmasked in reversible denaturation.[125] It was also shown that trypsin caused a rapid denaturation of β-lactoglobulin at pH 9.3 in the absence of urea. The rate of denaturation of β-lactoglobulin has been found to be the same at 3° and at 25° from pH 8.0 to 10.0, as shown in Fig. 15.[126] Under these conditions the rate of denaturation is inversely proportional to the 1.1 power of the hydrogen-ion concentration. β-lactoglobulin containing two molecules of dodecyl sulfate, however, was much more slowly denatured at these pH values than was β-lactoglobulin, and the rate of denaturation was greater at 25° than at 3°.

(125) L. K. Christensen, *Compt. rend. trav. lab. Carlsberg, Sér. chim.* **28**, 37 (1952).
(126) M. L. Groves, N. J. Hipp, and T. L. McMeekin, *J. Am. Chem. Soc.* **73**, 2790 (1951).

TABLE VI
AMINO ACID COMPOSITION OF MILK PROTEINS[a]

Constituent	Human casein[d]	Cow[b] casein[10]	Cow β-lactoglobulin	Cow colostrum globulin	Immune globulin of cow milk
Total N	14.89	15.63	15.60[118]	15.63[17]	15.67[c,11]
Total S	0.88	0.76[1]	1.60[118]	1.1[128]	1.00[c,87]
Total P	0.57	0.86			
Amino N		0.93	1.24[118]		
Amide N		1.6	1.07[118]		
Glycine	0.0	2.0	1.4[118]		
Alanine	2.0	3.2	7.4[127]		
Valine	5.0	7.2	5.8[118]	10.2[17]	9.6[87]
Leucine	12.2	9.2	15.6[118]	8.9[17]	9.6[87]
Isoleucine	6.3	6.1	6.1[87]	2.77[87]	3.03[c,87]
Proline	8.9	10.6	4.1[118]	10.0[128]	
Phenylalanine	5.8	5.0	3.5[118]	3.6[17]	3.88[87]
Cysteine			1.11[118]		
Cystine	0.64	0.34	2.29[118]	3.26[87]	3.15[c,87]
Methionine	2.4	2.8	3.22[118]	0.89[87]	0.90[c,87]
Tryptophan	1.4	1.7[d]	1.94[118]	2.74[17]	2.70[87]
Arginine	3.4	4.1	2.88[118]	4.36[87]	4.05[c,87]
Histidine	1.8	3.1	1.58[118]	1.99[87]	2.05[c,87]
Lysine	5.4	8.2	11.4[118]	6.3[87]	6.8[c,87]
Aspartic acid	4.6	7.1	11.4[118]	9.4[128]	
Glutamic acid	20.9	22.4	19.5[118]	12.3[128]	
Serine	5.4	6.3	5.0[118]		
Threonine	4.5	4.9	5.85[118]	9.4[87]	10.5[c,87]
Tyrosine	5.7	6.3	3.78[118]	6.7[128]	
Totals	96.3	110.5	113.85	92.81	56.26

[a] Grams per 100 g. protein.
[b] Except for sulfur.
[c] Average of milk euglobulin and milk pseudoglobulin.
[d] Spies method.

(127) A. S. Keston, S. Udenfriend, and R. K. Cannan, *J. Am. Chem. Soc.* **68**, 1390 (1946).
(128) R. G. Hansen, R. L. Potter, and P. H. Phillips, *J. Biol. Chem.* **171**, 229 (1947).

IV. Amino Acid Composition of Milk Proteins

The results of recent amino acid analyses on milk proteins are included n Table VI. Values for the amino acid analysis for the total casein fraction are important as well as those of the electrophoretic components (see Chap. 3 by Tristram, Vol. I of this series), since the composition of casein rom cow's milk does not appear to vary. Human and cow milk caseins ppear to have closely related, but not identical, amino acid compositions. The phosphorus content of human casein is much lower than that of cow ilk casein. The sulfur content of human casein appears to vary widely, epending on the method used for separating the casein.[1] Milk proteins ave a well-balanced distribution of amino acids and are an excellent ource of the eight essential amino acids needed for human nutrition.

The nitrogen terminal groups have been estimated in β-lactoglobulin nd in α- and β-casein by the dinitrofluorobenzene method of Sanger.[129] -Lactoglobulin was found to have three terminal leucyl residues.[130] It as also reported that only 19 of the 31 total ε-amino groups of lysine are vailable for reacting with fluorodinitrobenzene, but that on denaturing, l of the amino groups react with this reagent. By this method it was so found that only two of the four histidyl residues in native β-lactoobulin are reactive and that all of the histidyl groups are reactive on the enatured protein.[131] It has been estimated by the Sanger method that casein contains 10.7 terminal arginyl groups and 1.5 terminal lysyl roups and that β-casein contains 5.3 terminal arginyl groups and 2.4 rminal lysyl groups.[132]

V. Enzymes in Milk

The historical aspects of the discovery of enzymes in milk have been ummarized recently.[133] The origin and function of milk enzymes are oorly defined, since an enzyme present in milk may be derived from bacria or leucocytes. The amounts of enzymes present in milk are comaratively small, but at least one milk enzyme has been crystallized.[134] estruction of the enzyme phosphatase is used as a test for the adequacy pasteurization, since under the conditions for its destruction pathonic organisms are also destroyed. The following enzymes have been und in cow milk: protease, amylase, lactase, lipase, xanthine oxidase, hosphatase, aldolase, catalase, and lactoperoxidase.

(129) F. Sanger, *Biochem. J.* **39**, 507 (1945).
(130) R. R. Porter, *Biochim. et Biophys. Acta* **2**, 105 (1948).
(131) R. R. Porter, *Biochem. J.* **46**, 304 (1950).
(132) E. F. Mellon, A. H. Korn, and S. R. Hoover, *J. Am. Chem. Soc.* **75**, 1675 (1953).
(133) T. L. McMeekin and B. D. Polis, *Advances in Protein Chem.* **5**, 201 (1949).
(134) H. Theorell and Å. Åkeson, *Arkiv Kemi, Mineral. Geol.* **17B**, No. 7, 1 (1943).

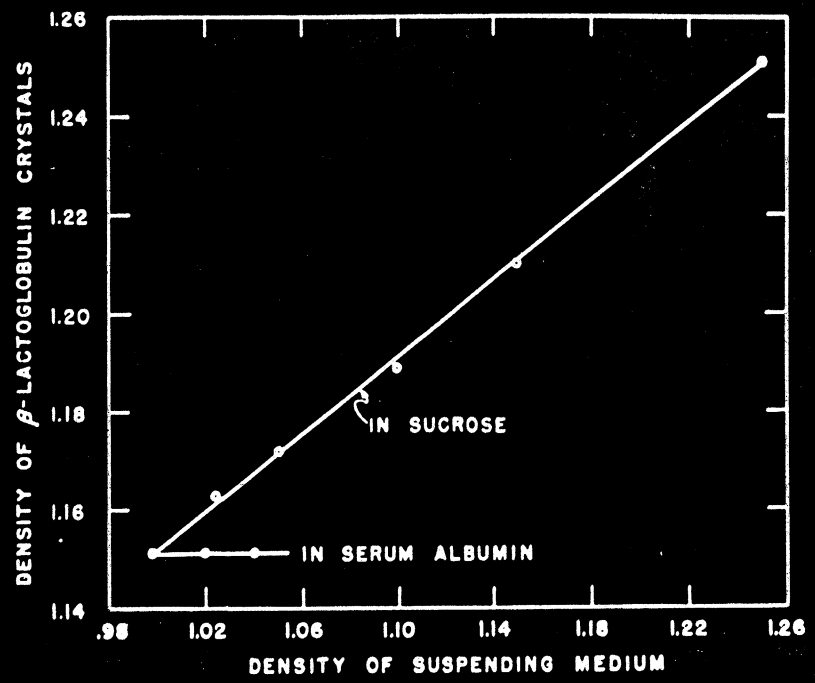
Protease. The presence of a proteolytic enzyme in milk was reported by Babcock and Russell.[135] Warner and Polis[87] showed that it is precipitated in the casein fraction of milk and is present in commercial casein. The enzyme had optimal activity at pH 9.2 in borax buffer and is tryptic in nature, hydrolyzing casein down below the peptone stage. By precipitating the dialyzed casein enzyme digest at pH 4.5, and fractionation of the filtrate with ammonium sulfate, a 150-fold increase in activity of the enzyme was obtained. This protease is resistant to the action of heat, though it can be destroyed by heating in borax solution at 80° for 10 min. In the fractionation of casein by means of alcohol and urea,[12] the protease is concentrated in the α-casein fraction. However, the protease can be removed from α-casein by fractional precipitation with alcohol at pH 6.0.

Lactoperoxidase. This enzyme was obtained in the crystalline state by Theorell and Åkeson[134] and is the only milk enzyme that has been crystallized. The yield was about 0.2 g. from 100 l. of milk, or 2 per cent of the total amount present. This result indicates that lactoperoxidase constitutes 0.2 per cent of the total protein of milk. The enzyme is a hemin protein with an iron content of 0.07 per cent which is approximately one mole of iron per mole of enzyme.[136] It was found to be homogeneous with a molecular weight of 93,000 as determined by sedimentation and diffusion measurements.[136] The method of preparing the pure enzyme involved the removal of casein with ammonium sulfate, heating for 15 min. at 70°C. to remove inert protein, precipitation of other impurities with basic lead acetate, removal of red impurities with acetone, and final purification by electrophoresis.

An alternative method for the purification of lactoperoxidase has recently been described by Polis and Shmukler.[137] Casein was removed by coagulation with rennet. The enzyme was precipitated at a concentration of 2.8 *M* ammonium sulfate at pH 6.0. This fraction was purified by dissolving in sodium tetraborate solution and fractional precipitation with ammonium sulfate. Further purification was obtained by means of displacement chromatography on columns of calcium phosphate and silica (Celite). Crystalline lactoperoxidase was obtained by crystallizing from 2.2 *M* dipotassium acid phosphate. Lactoperoxidase prepared by this method was qualitatively similar in many of its properties to the lactoperoxidase described by Theorell and Åkeson.[134] The molecular weight as determined by light scattering was found to be 82,000 and the

(135) S. M. Babcock and H. L. Russell, 14th Ann. Rept Wis. Agr. Expt. Sta., 1897, p. 161.
(136) H. Theorell and K. O. Pedersen, The Svedberg Memorial Volume, Almquist Wiksells, Uppsala, 1944, p. 523.
(137) B. D. Polis and H. Shmukler, *J. Biol. Chem.* **201**, 475 (1953).

The relationship between the composition and density of β-lactoglobulin crystals and that of sucrose solutions used as a suspending medium has been determined.[110] The solubility of β-lactoglobulin in sucrose solutions is small and does not affect these measurements. For comparative purposes the densities of β-lactoglobulin crystals equilibrated in serum albumin solutions were also determined. The density of the protein



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(110) T. L. McMeekin, M. L. Groves and N. J. Hipp, *J. Am. Chem. Soc.* **72**, 3662 (1950).

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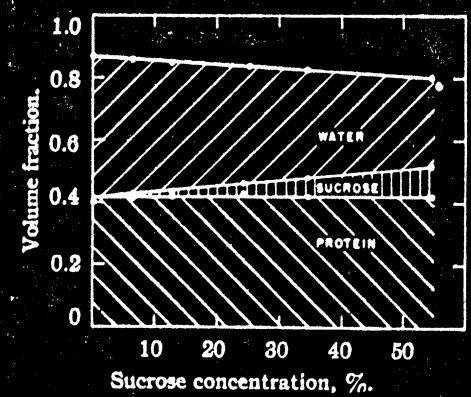

FIG. 13. Volume fractions of sucrose, protein, and water in crystals of β-lactoglobulin suspended in varying concentrations of sucrose solutions. (After McMeekin *et al.*)[110]

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(111) H. K. Schachmann and M. A. Lauffer, *J. Am. Chem. Soc.* **71**, 536 (1949).
(112) R. K. Cannan, *Chem. Revs.* **30**, 395 (1942).
(113) E. Brand, L. J. Saidel, W. H. Goldwater, B. Kassell, and F. J. Ryan, *J. Am. Chem. Soc.* **67**, 1524 (1945).
(114) A. Grönwall, *Compt. rend. trav. lab. Carlsberg. Sér. chim.* **24**, Nos. 8–11 (1942).

Catalase. The presence of catalase in milk was demonstrated by Grimmer[152] and by Harden and Lane-Claypon.[153] Increased bacterial or leucocyte counts, however, are invariably followed by higher catalase contents. The catalase activity is precipitated with the casein.

Milk contains other proteins in small quantities, which have been shown by some unique chemical, physical, or biological property. Sørensen and Sørensen described a red protein in whey and devised a method for its partial purification.[95] The copper of milk is almost entirely combined with protein. Dills and Nelson[154] separated a protein from whey that contains 0.19 per cent copper. The copper is nonionic and could not be removed by dialysis at pH 6.5; however, it could be removed by dialysis at pH 3.5. No reaction was found that was catalyzed by the copper protein. It had no ascorbic acid oxidase activity, which is of interest, since milk loses ascorbic acid activity on standing.

The isolation and characterization of a milk protein fraction with marked surface activity has been described by Aschaffenburg and Ogston.[155] This protein was obtained from the filtrate of whey after removing the heat-coagulable proteins. In the ultracentrifuge it was found to contain two components, with a molecular weight of 4900 and 23,900. The separation of a fraction from whey capable of being photosensitized to produce the solar-activated "burnt" flavor of homogenized milk has also been described.[156]

VI. Antibacterial Factor of Milk

The ability of milk to inhibit the growth of several species of bacteria has long been known. The factor responsible for this effect is a heat-labile, nondialyzable component of whey, which was named "lactenin" by Jones and Simms.[157] Recent studies by Wilson and Rosenblum[158] have shown that oxygen is necessary for the antibacterial activity of lactenin and that reducing agents such as glutathione or thioglycolic acid inhibit its activity. It was found that the antibacterial activity of milk is not due to peroxidases, xanthine, lysozyme, or an antibiotic in milk.

(152) W. Grimmer, *Biochem. Z.* **53**, 429 (1913).
(153) A. Harden and J. E. Lane-Claypon, *J. Hyg.* **12**, 144 (1912).
(154) W. L. Dills and J. M. Nelson, *J. Am. Chem. Soc.* **64**, 1616 (1942).
(155) R. Aschaffenburg and A. G. Ogston, *J. Dairy Research* **14**, 316 (1949).
(156) B. R. Weinstein, C. W. Duncan, and G. M. Trout, *J. Dairy Sci.* **34**, 570 (1951).
(157) F. S. Jones and H. S. Simms, *J. Exptl. Med.* **50**, 279 (1929); *ibid.* **51**, 327 (1930).
(158) A. T. Wilson and H. Rosenblum, *J. Exptl. Med.* **95**, 25, 39, 51 (1952).

VII. Relationship of Milk Proteins to Serum Proteins

The proteins of milk have often been compared with the blood serum proteins.[73,76,77,82] No protein has been found in serum that resembles casein chemically or biologically.[73,95] That casein is synthesized in the mammary gland from amino acids rather than from a glycoprotein[159] has been demonstrated recently by Barry.[160] He found that labeled tyrosine, lysine, and inorganic phosphorus were removed from the blood stream by the mammary gland and utilized in the synthesis of casein. In

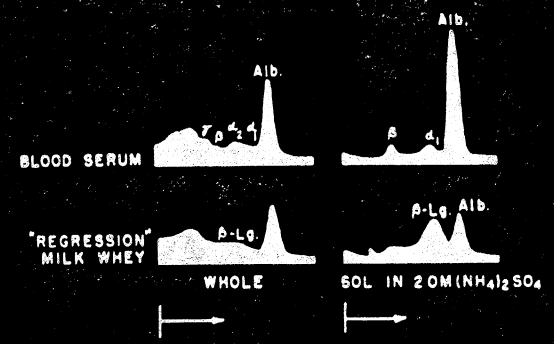

FIG. 16. Electrophoretic patterns of "regression" milk whey (27 days before parturition) and blood serum; veronal buffer, pH 8.35; ionic strength 0.1. Alb., albumin; β-Lg., β-lactoglobulin. (After McMeekin.)[9]

the goat it required about 2 hr. for the amino acid to pass from the blood stream into the casein in the milk. Crowther and Raistrick[76] obtained evidence indicating that the globulin of colostrum is present in normal milk whey and resembles the globulin of ox serum. This view of the relations of the immune globulin of whey has found support in the extensive comparisons by Smith *et al.*[17,87] of the properties of purified preparations of immune lactoglobulin from whey, colostrum, and serum by means of electrophoresis and sedimentation as well as by chemical analyses. He found that the immune protein of whey is the predominant protein of colostrum and that this globulin resembles the γ-globulin of serum. The finding of Campbell *et al.*[90] that plasmacytosis synchronizes with the heightened periods of immunity indicates that the immune globulin of colostrum is of cellular origin.

McCance and Widdowson[161] have drawn attention to the similarities between the composition of colostrum and that of "regression" milk, the

(159) E. P. Reincke, M. B. Williamson, and C. W. Turner, *J. Biol. Chem.* **138**, 83 (1941).
(160) J. M. Barry, *J. Biol. Chem.* **195**, 795 (1952).
(161) R. A. McCance and E. M. Widdowson, *Nature* **167**, 722 (1951).

of the protein structure. Further hydrolysis of the protein gave a contraction of 20 cc. per peptide bond split, which is the value given by synthetic peptides. The influence of temperature on the denaturation of β-lactoglobulin in urea was also measured by changes in optical rotation.[125] In an analysis of denaturation in urea, a distinction was made between a reversible denaturation with a negative temperature coefficient

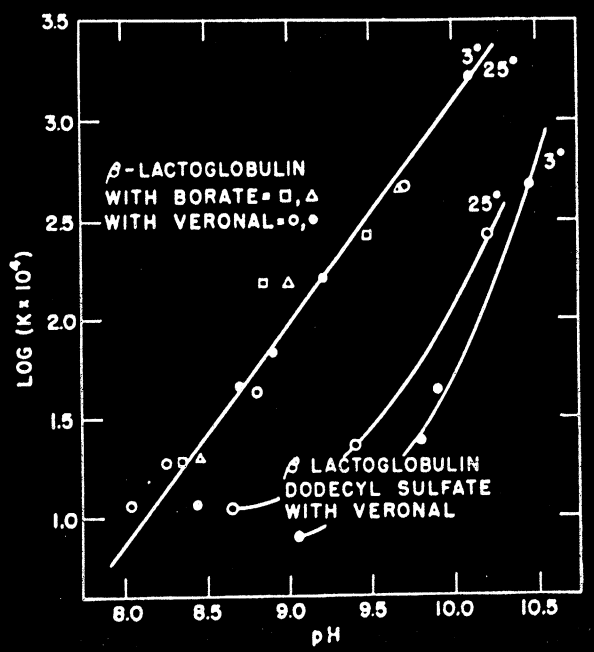

FIG. 15. Comparison of the effects of pH and temperature on the velocity constant of denaturation of β-lactoglobulin and β-lactoglobulin dodecyl sulfate. □, borate at 25°; △, borate at 3°; ○, veronal at 25°; ●, veronal at 3°. (After Groves *et al.*)[126]

and an irreversible denaturation with a positive temperature coefficient. The irreversible portion of the reaction is associated with the oxidation of sulfhydryl groups unmasked in reversible denaturation.[125] It was also shown that trypsin caused a rapid denaturation of β-lactoglobulin at pH 9.3 in the absence of urea. The rate of denaturation of β-lactoglobulin has been found to be the same at 3° and at 25° from pH 8.0 to 10.0, as shown in Fig. 15.[126] Under these conditions the rate of denaturation is inversely proportional to the 1.1 power of the hydrogen-ion concentration. β-lactoglobulin containing two molecules of dodecyl sulfate, however, was much more slowly denatured at these pH values than was β-lactoglobulin, and the rate of denaturation was greater at 25° than at 3°.

(125) L. K. Christensen, *Compt. rend. trav. lab. Carlsberg. Sér. chim.* **28**, 37 (1952).
(126) M. L. Groves, N. J. Hipp, and T. L. McMeekin, *J. Am. Chem. Soc.* **73**, 2790 (1951).

TABLE VI
AMINO ACID COMPOSITION OF MILK PROTEINS[a]

Constituent	Human casein[d]	Cow[b] casein[49]	Cow β-lactoglobulin	Cow colostrum globulin	Immune globulin of cow milk
Total N	14.89	15.63	15.60[118]	15.63[17]	15.67[c11]
Total S	0.88	0.76[1]	1.60[118]	1.1[128]	1.00[c87]
Total P	0.57	0.86			
Amino N		0.93	1.24[118]		
Amide N		1.6	1.07[118]		
Glycine	0.0	2.0	1.4[118]		
Alanine	2.0	3.2	7.4[127]		
Valine	5.0	7.2	5.8[118]	10.2[17]	9.6[87]
Leucine	12.2	9.2	15.6[118]	8.9[17]	9.6[87]
Isoleucine	6.3	6.1	6.1[87]	2.77[87]	3.03[c87]
Proline	8.9	10.6	4.1[118]	10.0[128]	
Phenylalanine	5.8	5.0	3.5[118]	3.6[17]	3.88[87]
Cysteine			1.11[118]		
Cystine	0.64	0.34	2.29[118]	3.26[87]	3.15[c87]
Methionine	2.4	2.8	3.22[118]	0.89[87]	0.90[c87]
Tryptophan	1.4	1.7[d]	1.94[118]	2.74[17]	2.70[87]
Arginine	3.4	4.1	2.88[118]	4.36[87]	4.05[c87]
Histidine	1.8	3.1	1.58[118]	1.99[87]	2.05[c87]
Lysine	5.4	8.2	11.4[118]	6.3[87]	6.8[c87]
Aspartic acid	4.6	7.1	11.4[118]	9.4[128]	
Glutamic acid	20.9	22.4	19.5[118]	12.3[128]	
Serine	5.4	6.3	5.0[118]		
Threonine	4.5	4.9	5.85[118]	9.4[87]	10.5[c87]
Tyrosine	5.7	6.3	3.78[118]	6.7[128]	
Totals	96.3	110.5	113.85	92.81	56.26

[a] Grams per 100 g. protein.
[b] Except for sulfur.
[c] Average of milk euglobulin and milk pseudoglobulin.
[d] Spies method.

(127) A. S. Keston, S. Udenfriend, and R. K. Cannan, *J. Am. Chem. Soc.* **68**, 1390 (1946).
(128) R. G. Hansen, R. L. Potter, and P. H. Phillips, *J. Biol. Chem.* **171**, 229 (1947).

soluble material was discarded, and the rennet in the filtrate was precipitated at 2° y dropwise addition of saturated ammonium sulfate over a period of three days. The olution obtained was clarified by the addition of sufficient sodium chloride to cause a light precipitate. Rennet was crystallized from the filtrate at 2° by slowly adding a aturated magnesium sulfate solution with stirring over a period of about 18 hr. A urther crop of crystals was obtained by the slow addition of saturated ammonium ulfate solution. Crystalline rennet prepared in this manner was about ten times more ctive than the starting material. It is very insoluble in water and is soluble only to he extent of 0.06 per cent in dilute salt solutions. From solubility experiments in which the ratio of the protein to solvent was varied, it was concluded that crystalline ennet is homogeneous. An unusual property of crystalline rennet is its apparent tability to drying with only a small decrease in activity.

Nitschmann and Lehmann[70] have reported a change in the electrophoretic pattern of casein due to the action of rennet on the casein. They heated sodium caseinate for 30 min. at 35°C. with rennet at pH 6.6. The electrophoretic pattern of the rennet-treated casein differed from that given by the same casein solution treated with boiled rennet in that the ascending boundary of the α-casein was split into two peaks. When a relatively large amount of crystalline rennet was used,[71] the casein was extensively hydrolyzed. The time involved in obtaining these hydrolytic changes in casein is greatly in excess of the time required for coagulation; consequently the question concerning the primary action of rennet remains open. Since α- and β-casein are clotted with rennet[38] and γ-casein does not form stable calcium caseinate suspensions, it is apparent that any theory of clotting based on the action of rennet on one of the components of casein[34,70] is inadequate.

Calcium or a closely related ion has generally been found necessary for milk coagulation. According to Hammarsten, calcium coagulates the casein after the action of rennet. Lundsteen,[72] however, produced clotting of calcium-free casein dissolved in solutions of citrate buffer at pH 5.4. At this pH, paracasein is stated to be less soluble than casein, and coagulates when formed. Linderstrøm-Lang's[34] casein preparation K_0 was used for these clotting experiments without calcium.

f. Casein from the Milk of Different Mammals

The earlier literature indicated that the composition of casein prepared from the milk of different mammals was identical or nearly so. Immunological evidence shows that the caseins from different species of animals are closely related.[73] With the improved methods for amino

(70) H. Nitschmann and W. Lehmann, *Experientia* 3, 153 (1947).
(71) H. Nitschmann and R. Varin, *Helv. Chim. Acta* 34, 1421 (1951).
(72) E. Lundsteen, *Biochem. Z.* 271, 259 (1934); *Compt. rend. trav. lab. Carlsberg. Sér. chim.* 22, 338 (1938).
(73) H. G. Wells and T. B. Osborne, *J. Infectious Diseases* 29, 200 (1921).

acid analysis, however, differences are found[4] in amino acid composition of caseins from milk of different species. The electrophoretic method of analysis also has shown differences in caseins from different sources. Human casein contains α- and β-casein, as does cow milk casein. The effect of pH on the mobilities and distribution of the components of these caseins from different sources, however, indicates differences in chemical composition. Dovey and Campbell[74] found that goat milk casein is composed of equal parts of α- and β-casein, that rabbit milk casein contains 11 per cent β-casein, and that cow milk casein contains 84 per cent α-casein and 16 per cent β-casein. Each of these caseins contains a small amount of γ-casein. The mobilites of the casein components also differ, depending on the species.

2. Proteins of Whey

Milk whey is obtained by precipitating casein with acid or by clotting with rennet. The concentration of protein in whey varies considerably with the species, but for cow milk it is about 0.6 per cent (Table I). The number of major components and the relative amounts of each as shown by electrophoresis are given in Table II. There are, however, numerous proteins in whey in small quantities, such as enzymes, which are not shown by electrophoretic patterns of whey. The proteins of whey are divided into a globulin fraction and an albumin fraction by means of their solubility in saturated magnesium sulfate or one-half-saturated ammonium sulfate solution.

a. Globulin Fraction

Since the work of Sebelien,[75] the classical globulin of milk has been prepared by saturating milk with magnesium sulfate. It was found that milk globulin, like blood globulin, could be separated into euglobulin and pseudoglobulin by dialysis.[76] The globulin of whey precipitated by magnesium sulfate was shown by Smith[11,77] to be a complex mixture. He devised a method for preparing an electrophoretically homogeneous globulin by fractionation with ammonium sulfate. In his method the crude globulin fraction is precipitated by 0.5 saturation with ammonium sulfate. The precipitate is dissolved and reprecipitated at pH 4.6 by 0.25 saturation with ammonium sulfate. This precipitate, which contains some casein, is discarded, and the globulin fraction is precipitated from the filtrate at 0.4 saturation with ammonium sulfate at pH 6.0. An

(74) A. Dovey and P. N. Campbell, *Nature* 169, 1014 (1952).
(75) J. Sebelien, *Z. physiol. Chem.* 9, 445 (1885).
(76) C. Crowther and H. Raistrick, *Biochem. J.* 10, 434 (1916).
(77) E. L. Smith, *J. Dairy Sci.* 31, 127 (1948).

on content to be 0.069 per cent. This lactoperoxidase contained two ectrophoretic components which were separated. Some evidence indiates that there is an equilibrium between these two forms of ctoperoxidase.

Amylase. The concentration of this starch-splitting enzyme in milk is onstant. It appears to be associated principally with cream, although kim milk has some amylase activity.[138]

Lactase. This enzyme acts on lactose, splitting the molecule into lucose and galactose, and has been reported to be a constituent of normal milk.[139]

Lipase. The presence of a true lipase resembling pancreatic lipase was demonstrated to be present in cow's milk.[140] The amount of this nzyme in milk usually increases with advanced lactation.

Xanthine Oxidase. Morgan, Stewart, and Hopkins[141] showed that milk is a good source of an enzyme capable of converting xanthine to uric acid. It was shown by Ball[142] that the xanthine oxidase of milk is largely associated with the cream fraction. A method was devised for its purification which resulted in a preparation 500 times more active per unit of dry weight than whole milk. Cream was extracted with an equal volume of 0.2 *M* disodium acid phosphate at 38°, and the aqueous layer was removed after centrifugation at a low temperature. After incubating the solution with pancreatic lipase for 3½ hr. at 38°, a solution of calcium chloride was added which produced a precipitate of calcium phosphate. The yellow solution obtained after the removal of calcium phosphate was made to 60 per cent saturation with ammonium sulfate and allowed to stand at 0°. The precipitate was removed at 0° and dissolved in water. The solution was made to 33 per cent saturation with ammonium sulfate, and the precipitate was removed at 0° and discarded. The xanthine oxidase in the supernatant was precipitated by making to 42 per cent saturation with ammonium sulfate and then removed by centrifugation at 0°. Xanthine oxidase was found to have an isoelectric point at pH 6.2 as determined by cataphoresis.

On the basis that each molecule of protein combines with one molecule of flavin, and purity considerations lead to an estimate of 74,000 for the maximum value for the molecular weight of xanthine oxidase. The flavin group was split off from the protein reversibly by prolonged dialysis

(138) C. J. Koning, *Milchw. Zentr.* **4**, 156 (1908); (*C. A.* **2**, 1986).
(139) A. J. J. Vandevelde, *Biochem. Z.* **11**, 61 (1908).
(140) F. E. Rice and A. L. Markley, *J. Dairy Sci.* **5**, 64 (1922).
(141) E. J. Morgan, C. P. Stewart, and F. G. Hopkins, *Proc. Roy. Soc.* (London) *Ser.* **B94**, 109 (1922).
(142) E. G. Ball, *J. Biol. Chem.* **128**, 51 (1939).

against water at 0°. Corran *et al.*[143,144] obtained a preparation of xanthine oxidase 1000 times more active than milk. Their method involves fractionation of milk with ammonium sulfate, precipitation of the active fraction with alcohol, absorption and elution of the enzyme from alumina gel, and further fractionation with ammonium sulfate. The flavin group of xanthine oxidase prepared in this manner was not split off by dialysis. This preparation of xanthine oxidase contained three components as determined in the ultracentrifuge. Most of the material with an absorption of 475 mμ had a sedimentation constant of $S_{20} = 12.6$.[145]

Phosphatase. Raw milk contains a phosphatase that has the characteristics of mammalian phosphatase in its activation by magnesium ions and pH activity relations.[146] Zittle and DellaMonica,[147] by comparative tests with inhibitors, have concluded that milk phosphatase bears a closer relationship to bone and kidney phosphatase than to intestinal phosphatase. They also determined the effects of anions, cations, and amino acids on milk phosphatase and devised methods for its purification. The greater heat resistance of bacterial phosphatase has been used as a means of its differentiation from milk phosphatase.[148]

Milk phosphatase is destroyed more slowly by heat than many bacteria, so that the phosphatase test is widely used as a test for pasteurization. Milk has been shown to contain small amounts of acid phosphatase which is more heat-resistant than the alkaline phosphatase.[149] From a study of the phosphatase activity of raw milk and its distribution in various milk fractions, it has been concluded that phosphatase is present to a large extent but not entirely at the fat–serum interphase.[150] It was found that cream contains about eight times as much phosphatase as skim milk and that buttermilk derived from making butter is particularly high in phosphatase.

Aldolase. The muscle enzyme aldolase, which reversibly splits fructose 1,6-diphosphate into dihydroxyacetone phosphate and phosphoglyceric aldehyde, was reported by Polis and Shmukler to be present in milk.[151]

(143) H. S. Corran and D. E. Green, *Biochem. J.* **32**, 2231 (1938).
(144) H. S. Corran, J. W. Dewan, A. H. Gordon, and D. E. Green, *Biochem. J.* **33**, 1694 (1939).
(145) J. St. L. Philpot, *Biochem. J.* **33**, 1707 (1939).
(146) W. R. Graham, Jr., and H. D. Kay, *J. Dairy Research* **5**, 54, 63 (1933).
(147) C. A. Zittle and E. S. DellaMonica, *Arch. Biochem.* **26**, 112 (1950); *ibid.* **26**, 135 (1950); *ibid.* **35**, 321 (1952); *Proc. Soc. Exptl. Biol. Med.* **76**, 193 (1951).
(148) R. P. Tittsler, D. S. Sager, and G. P. Sanders, *J. Dairy Sci.* **31**, 705 (1948).
(149) J. E. C. Mullen, *J. Dairy Research* **17**, 288 (1950).
(150) J. H. Hetrick and P. H. Tracy, *J. Dairy Sci.* **31**, 867 (1948).
(151) B. D. Polis and H. W. Shmukler, *J. Dairy Sci.* **33**, 619 (1950).

An outstanding study of the proteins of colostrum has been made by nith.[11,17,77,87] Whole colostrum and fractions isolated by precipitation rocedures were studied electrophoretically as shown in Fig. 9. He olated an electrophoretically pure globulin from colostrum in high yield precipitation with ammonium sulfate in a manner similar to that scribed for the separation of globulin from normal whey and indicated

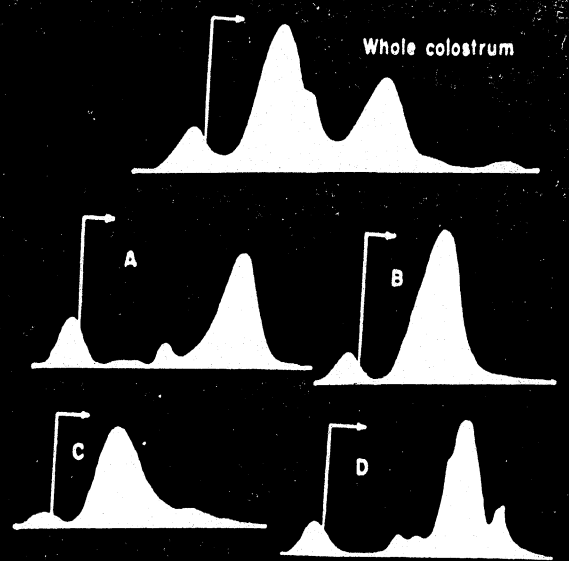

FIG. 9. Electrophoretic patterns of the descending boundaries of whole colostrum d of fractions derived from it. *A* is the casein; *B*, *C*, and *D* are ammonium sulfate ctions obtained between 0 and 0.3 saturation (*B*), between 0.3 and 0.5 (*C*), and tween 0.5 and 0.9 (*D*), respectively. Fraction *B* consists entirely of immune globu- and *C* mainly of this protein. The principal component of *D* is β-lactoglobulin, ich could be obtained in crystalline form. Electrophoresis was for 200 min. in onal buffer at pH 8.3–8.4 at an ionic strength of 0.1. (After Smith.)[17]

the legend for Fig. 9. By exhaustive dialysis, colostrum globulin uld be separated into water-insoluble euglobulin and water-soluble eudoglobulin fractions. The euglobulin migrated with a velocity of 1.9 *u* at pH 8.5 in veronal buffer of 0.1 ionic strength, as compared with 2.2 *u* for the pseudoglobulin. Both fractions had immune activity. e molecular weight of the colostrum globulin was reported to be about 0,000–190,000, as determined by the ultracentrifuge.

Relatively large amounts of trypsin inhibitor have been found in

(87) E. L. Smith and N. H. Coy, *J. Biol. Chem.* 164, 367 (1946); E. L. Smith and R. D. Greene, *ibid.* 171, 355 (1947); E. L. Smith and R. D. Greene, *ibid.* 172, 111 (1948); E. L. Smith, R. D. Greene, and E. Barther, *ibid.* 164, 359 (1946).

colostrum by Laskowski and Laskowski.[88] Trypsin inhibitor also occurs in traces in cream from normal milk, but the concentration in colostrum is many times greater. Trypsin inhibitor was purified and crystallized by procedures that are similar to those developed for the purification of trypsin inhibitor from pancreas.

After diluting the colostrum with one volume of water, the principal proteins were removed by making the solution to 2.5 per cent trichloroacetic acid and heating to 80° for 5 min. The heavy precipitate formed under these conditions is removed by filtration and the trypsin inhibitor precipitated from the filtrate by making to 80 per cent saturated by adding solid ammonium sulfate. The precipitate is removed by filtration and the filtrate is discarded. The precipitate is dissolved in water and inert material removed by again precipitating in 2.5 per cent trichloroacetic acid at 80°, and the trypsin inhibitor again precipitated by making to 80 per cent saturated with ammonium sulfate. The precipitate is dissolved in water and made to 30 per cent saturation with ammonium sulfate. The material insoluble in 30 per cent ammonium sulfate is discarded, and the trypsin inhibitor is again precipitated from the filtrate by making to 80 per cent saturated ammonium sulfate. The precipitate is dissolved in water and made to 2.5 per cent trichloroacetic acid and then extracted with ether in a separatory funnel. The aqueous layer is made to 30 per cent saturation with ammonium sulfate. Trypsin inhibitor is in the precipitate. The material is further purified by precipitation from salt-free solution at pH 5.5 and −18° by adding an equal volume of methanol. The insoluble material is removed by centrifugation and discarded, and the supernatant is treated with four times the previous volume of methanol. The precipitate obtained under these conditions is used to make the trypsin–trypsin inhibitor compound. After dissolving it in water, the calculated amount of crystalline trypsin, dissolved in a small volume of 0.0025 *N* hydrochloric acid, is added. The solution is brought to pH 8.0 with borate buffer. After cooling to 2° it is adjusted to pH 5.5 and any precipitate formed is discarded. The solution is made to 50 per cent saturation with ammonium sulfate and seeded with crystals of trypsin–trypsin inhibitor and allowed to crystallize at room temperature. The free trypsin inhibitor is obtained from its trypsin complex by precipitating the trypsin portion from solution with 5 per cent trichloroacetic acid. The inhibitor is removed from the filtrate by precipitating with 80 per cent saturated ammonium sulfate. The inhibitor is obtained in the crystalline form at pH 5.5 in the presence of 50 per cent saturated ammonium sulfate.

Trypsin inhibitor from colostrum differed from that obtained from pancreas in electrophoretic mobility and isoelectric point[89] as shown in Fig. 10. It also differed in activity per unit weight and amino acid composition. The molecular weight of colostrum–trypsin-inhibitor complex was found to be 89,000 by light scattering, which compares with a value of 36,000 obtained for the trypsin-inhibitor complex from pancreas.

The suggestion is made that the function of trypsin inhibitor in colostrum is to prevent the hydrolysis of the immune proteins in the alimen-

(88) M. Laskowski, Jr., and M. Laskowski, *J. Biol. Chem.* 190, 563 (1951).
(89) M. Laskowski, Jr., P. H. Mars, and M. Laskowski, *J. Biol. Chem.* 198, 745 (1952).

fluid that can be pressed from the udder during the dry period. The concentrations of the enzymes cholinesterase and phosphatase in colostrum and in "regression" milk of humans and dogs is many times greater than in normal milk. The marked similarity of the electrophoretic patterns of the proteins of cow's "regression" milk 27 days before parturition[9] and that of bovine blood serum drawn at the same time is shown in Fig. 16. Another point of similarity between regression milk and blood serum is that the pH of "regression" milk is the same as that of blood, pH 7.4. The pH of "regression" milk changes rapidly, however, and on the day of parturition has the pH value of normal milk, pH 6.5.

The component of whey in Table II with a mobility of $-6.4\,u$ amounting to 5 per cent of the whey protein, has a mobility close to that of serum albumin. Based on immunological studies, Peskett[162] reported that normal milk contains a small amount of serum albumin and that it increases during the secretion of abnormal milk. Electrophoretic studies showed that the amount of the component with a mobility of $-6.2\,u$ in milk began increasing about 70 days before parturition. This component has been isolated from normal milk whey[163] by fractionation with ammonium sulfate and with alcohol. In properties and composition it was shown to be identical with bovine serum albumin. This crystalline albumin from milk was shown by Coulson and Stevens to be also immunologically equivalent to serum albumin.[164]

(162) G. R. Peskett, Ann. Rept Natl. Inst. Res. Dairying, Reading, England, 1932, p. 52.

(163) B. D. Polis, H. W. Shmukler, and J. H. Custer, *J. Biol. Chem.* **187**, 349 (1950).

(164) E. J. Coulson and H. Stevens, *J. Biol. Chem.* **187**, 355 (1950).