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Proteins of Milk\*

## INTRODUCTION

Proteins are of great importance in biochemistry, since they form a large proportion of the food and the body tissues of man and other animals. Research on proteins is extremely difficult because of the large size and complex structure of their molecules and aggregates, their insolubility in most common solvents, their usually noncrystalline form, and the readiness with which they undergo decomposition or other chemical change when attempts are made to isolate and purify them or to subject them to the usual methods of characterization. Proteins may be defined as substances composed principally of amino acids chemically combined. Carbon, hydrogen, nitrogen, oxygen, sulfur, and, in a few instances, phosphorus are the elements present in proteins. Cow's milk contains approximately 3.5% protein, or, as distributed, 2.9% casein and 0.6% whey proteins.

The chemistry of casein was reviewed by Jollès<sup>113</sup> in 1966, of the whey proteins by Garnier<sup>66</sup> in 1965, and of minor components of casein by Groves<sup>86</sup> in 1969. A comprehensive review of the chemistry of milk proteins by McKenzie<sup>137</sup> appeared in 1967, and a two-volume treatise on the chemistry and molecular biology of the milk proteins, edited by the same author,<sup>138</sup> was published in 1970 and 1971.

## NOMENCLATURE

In discussing the proteins of milk, it is necessary to distinguish between the proteins *in* milk and those obtained *from* milk by various chemical and physical fractionation procedures. Because of the ease with which casein can be isolated from milk, the earliest subdivision of milk proteins was to casein and whey proteins. However, this implies

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that casein exists in milk in the same form as it does in the isolated state, which is not true when casein is precipitated by acid, as is customary. The casein in milk is in the form of a complex or micelle, consisting of calcium caseinate plus phosphate, additional calcium, magnesium, and citrate. *Casein* may be defined most simply as the protein precipitated by acidifying skimmilk to a pH value near 4.6 at 20°C. The proteins remaining after casein has been removed from skimmilk are known as *whey proteins* or *milk-serum proteins*. They have been fractionated by salting-out methods to produce a lactalbumin fraction and a lactoglobulin fraction. Each of these—casein, lactalbumin and lactoglobulin—was generally considered to be a single chemical entity until Linderstrøm-Lang<sup>130,131</sup> in 1925 reported the fractionation of casein, and Palmer<sup>162</sup> in 1934 the further fractionation of the whey proteins. Since then research has resulted in the isolation of at least nine main components of milk proteins, several of which are still heterogeneous, as indicated by electrophoretic measurements.

Subdivisions of milk-serum proteins sometimes mentioned in milk protein literature are the *heat-labile milk-serum proteins* and the *proteose-peptone fraction*, the first being the portion of the milk-serum proteins rendered acid-precipitable at pH 4.6 to 4.7 by previous heat treatment of the milk or whey, the second being the portion not made precipitable by this means. *Commercial lactalbumin* is a mixture of the heat-labile, acid-precipitable milk-serum proteins. The so-called classic *lactalbumin fraction* is the portion of the milk-serum proteins that is soluble in neutral half-saturated ammonium sulfate solution or neutral saturated magnesium sulfate solution. In a classification advocated by Rowland,<sup>189</sup> *lactalbumin* is the portion of the heat-labile milk-serum protein that is soluble in saturated magnesium sulfate solution. The classic *lactoglobulin fraction* is the portion of the milk-serum proteins that is insoluble in neutral half-saturated ammonium sulfate solution or saturated neutral magnesium sulfate solution. In the Rowland classification, *lactoglobulin* is the portion of the heat-labile milk-serum protein that is insoluble in saturated magnesium sulfate solution. A complex of proteins and enzymes adsorbed on the surface of the fat globules of milk is usually designated as the *membrane proteins of the fat globules*.

The contemporary nomenclature of the principal proteins found in cow's milk, as reported<sup>188</sup> by a Committee of the American Dairy Science Association, appears in Table 3.1, in which there are listed distinguishing characteristics of the proteins. Some of the problems that have arisen in the naming of newly discovered milk proteins have been dealt with in previous reports of the Committee.<sup>110,34,214</sup> The present status is discussed cogently by Jenness.<sup>109</sup>

## DETERMINATION OF PROTEIN FRACTIONS

The separation of the proteins of skimmilk into the casein, lactalbumin, and lactoglobulin fractions that was employed for many years was preparatory to the determination, characterization, and study of each of these fractions. The methods employed were precipitation of casein by acidification to its isoelectric point, and salting-out methods for the lactalbumin and lactoglobulin fractions. Rowland<sup>189</sup> devised an analytical scheme to determine these fractions, one fraction consisting of proteoses and peptones, and the other consisting of nonprotein nitrogen compounds. The results are usually expressed in terms of the percentage distribution of nitrogen among these five fractions. The preparation of samples for study is incidental. The following is an outline of the Rowland scheme.

Total N(I) of the milk is determined by the Kjeldahl procedure. Non-casein N(II) is determined by precipitating casein by a mixture of acetic acid and sodium acetate, filtering, and analyzing the filtrate for N. Nonprotein N(III) is determined by precipitating the total protein by means of 15% trichloroacetic acid, filtering, and analyzing the filtrate for N. Nonprotein N plus proteose-peptone N(IV) is determined by boiling an aliquot of the filtrate II, adjusting the reaction to pH 4.75, filtering, and analyzing the filtrate for N. Lactoglobulin N(V) is determined by adjusting an aliquot of the filtrate II to pH 6.8-7.2, saturating it with magnesium sulfate, filtering off the lactoglobulin fraction after several hours, washing it with saturated magnesium sulfate solution, and analyzing it for N. Then, casein N = I - II, lactalbumin N = II - (IV + V), lactoglobulin N = V, proteose-peptone N = IV - III, and nonprotein N = III. Rowland, by use of his method, found the average nitrogen distribution in normal milk to be: 78.3% casein N; 9.1% lactalbumin N; 3.5% lactoglobulin N; 4.1% proteose-peptone N; and 5.0% nonprotein N.

The classic fractions obtained by the Rowland procedure were investigated electrophoretically by Larson and Rolleri.<sup>125,186</sup> The electrophoretic method not only reveals the complexity of the fractions produced by chemical partition, but gives somewhat different results, particularly in the distribution of the albumin and globulin fractions. The distribution percentages shown in Table 3.1 were compiled from both types of study which, incidentally, agree in assigning 2 to 6% of total skimmilk protein to the proteose-peptone fraction.

A newer chemical partition method employing sodium sulfate at controlled pH values for the preparation of fractions was developed by Aschaffenburg and Drewry.<sup>11</sup> This procedure gives results in substantial agreement with the electrophoretic data and also permits the

Table 3.1

Protein or Protein Fraction	Approx. % of Skimmilk Protein b (Peak Number) <sup>e</sup>	Occurrence in Electrophoretic Pattern	Reference to Preparation g	Electrophoretic Mobility j	pI <sup>i</sup>	Sedimentation Constant (S <sub>20</sub> ) <sup>n</sup>	Molecular Weight <sup>o</sup>	Components
I Casein (Precipitated from skimmilk by acid at pH 4.6)	76-86		101,224			1.3, 6.0-7.5 <sup>m,226</sup>	15,000 <sup>222</sup> 33,600 <sup>35</sup>	
α-Casein	53-70	1	101,224	-6.7 <sup>101</sup>	4.1 <sup>224</sup>	3.99 <sup>204</sup>	27,000 <sup>154</sup> 23,000 <sup>190</sup> 23,000 <sup>p</sup>	α <sub>s1</sub> -Variants A, B, C, D α <sub>s0</sub> , α <sub>s2</sub> , α <sub>s3</sub> , α <sub>s4</sub> , α <sub>s5</sub> -s Variants A <sup>1</sup> , A <sup>2</sup> , A <sup>3</sup> , B, C D, B <sub>z</sub>
α <sub>s</sub> -Casein	45-55	1f	213,226	-6.7 <sup>f101</sup>	4.1 <sup>110</sup>	3.99 <sup>204</sup>	24,000 <sup>89,173</sup> 176,184, <sup>204</sup>	
β-Casein	25-35	2	101,224	-3.1 <sup>101</sup>	4.5 <sup>224</sup>	1.57 <sup>204</sup>	19,000 <sup>q230</sup>	Variants A, B, sub-variants containing 0 to 5 carbohydrate chains
κ-Casein	8-15	1f	209,226,231	-6.7 <sup>f101</sup>	4.1 <sup>209</sup>	1.4 <sup>209</sup>		
γ-Casein	3-7	3	101	-2.0 <sup>101</sup>	5.8-6.0 <sup>101</sup>	1.55 <sup>151</sup>	21,000 <sup>r</sup>	Variants A <sup>1</sup> , A <sup>2</sup> , A <sup>3</sup> , B components R, S, TS (TS has several variants)
II Noncasein Proteins	14-24							
A—Lactalbumin (soluble in 1/2 saturated (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> solution)	7-12	6	101,162	-5.3 <sup>188</sup>	5.3 <sup>188</sup>	2.7 <sup>216,217</sup>	36,000 <sup>215,217</sup>	Variants A, ADR, B, BDR, C, D
β-Lactoglobulin	2-5	4	10,72,75	-4.2 <sup>72</sup>	4.2-4.5 <sup>123</sup>	1.75 <sup>72</sup>	14,440 <sup>30</sup>	Variants A, B in Zebu
α-Lactalbumin Blood Serum Albumin	0.7-1.3	7	177	-6.7 <sup>177</sup>	4.7 <sup>177</sup>	4.0 <sup>35</sup>	69,000 <sup>177</sup>	
B—Lactoglobulin (insoluble in 1/2 saturated (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> solution)	0.8-1.7	1	197	-1.8 <sup>151,197</sup>	6.0 <sup>151,197</sup>	8.77 <sup>151</sup>	180,000 <sup>197</sup> 252,000 <sup>151</sup>	
Euglobulin c	0.6-1.4	2	197	-2.0 to 2.2 <sup>k</sup>	5.6 <sup>151,197</sup>	8.06 <sup>151</sup>	180,000 <sup>197</sup> 289,000 <sup>151</sup>	

IgG Immunoglobulins h	1-2	1 and 2						
IgG1	1-2	1 and 2						
IgG2	~ 0.2-0.5	1						
IgM Immunoglobulin	~ 0.1-0.2	2						
IgA Immunoglobulin	~ 0.05-0.1	2 (?)						
C—Protease-Peptide Fraction i (not precipitated at pH 4.6 from skim milk previously heated to 95-100°C, 30 min)	2-6	3,5,8	12,110,125	-3.8 to 9.3 <sup>188</sup>	3.3-3.7 <sup>188</sup>	0.8 to 4.0 <sup>188</sup>	4.100 to 20,000 <sup>188</sup>	Multiple, including glycoproteins

a Adapted from the reports of Thompson *et al.*<sup>214</sup> and Rose *et al.*<sup>188</sup>  
b Values compiled or calculated from Rowland nitrogen distribution data, relative areas of electrophoretic patterns, and protein yield studies.<sup>101,110,124,125,186,189,188</sup>  
c Euglobulin is not well characterized and is not included in the most recent revision of the nomenclature of the proteins of cow's milk.<sup>188</sup>  
d Pseudoglobulin is considered to be primarily IgG1.<sup>188</sup>  
e Free-boundary electrophoresis in veronal buffer at pH 8.6, r/2 = 0.1. Casein components designated in descending order of mobility in casein pattern; whey proteins, designated in ascending order of mobility in acid whey pattern.<sup>125</sup>  
f Value for whole  $\alpha$ -casein (i.e.,  $\alpha_1$ - and  $\kappa$ -casein complex).  
g See Reference 138 for other references concerning preparation of milk proteins.  
h Nomenclature and properties of immunoglobulins of the cow have recently been reviewed.<sup>37</sup>  
i Composition and physicochemical properties of protease-peptide fraction are summarized in Reference 188.  
j Electrophoretic mobility =  $10^{-4}$  cm<sup>2</sup> volts<sup>-1</sup> sec<sup>-1</sup> in Tiselius moving-boundary method, 2°C. in veronal buffer, pH 8.6, r/2 0.1, descending pattern.  
k Average of values from Smith<sup>147</sup> and Murthy and Whitney.<sup>151</sup>  
l Isoelectric point, or pH of zero electrophoretic mobility.  
m From bimodal sedimentation pattern.  
n Sedimentation coefficient,  $S_{20} = (dx/dt) (\omega^2 x)$  in Svedberg units ( $S = 1 \times 10^{-13}$ ) corrected to 20°C.  
o Refer to original literature for methods and conditions of determination.  
p From amino-acid sequence.<sup>147</sup>  
q Value for carbohydrate-free monomer; approximately 600 should be added for each carbohydrate chain.  
r From amino-acid analyses. Groves, M. L., and Gordon, W. G., unpublished data. For discussions of the minor proteins ( $\gamma$ , R, S, TS-casein) and their relationship to  $\beta$ -casein see References 71, 86, 188.  
s See References 3 and 105 for a discussion of these proteins.

further differentiation of the classic albumin fraction into  $\alpha$ -lactalbumin and  $\beta$ -lactoglobulin fractions.

The free-boundary electrophoretic method, when applied in 1939 by Mellander<sup>145</sup> to casein itself, showed that the protein was made up of at least three components, which were designated  $\alpha$ -,  $\beta$ -, and  $\gamma$ -casein in the order of decreasing mobility in a phosphate buffer. The more discriminating method of zonal electrophoresis, for example, starch gel electrophoresis as used by Wake and Baldwin,<sup>223</sup> reveals that casein is in fact a much more complex mixture of proteins.

Another method which has yielded important information about the proteins in milk is ultracentrifugation. It is mentioned here primarily in connection with the names of the individual whey proteins,  $\alpha$ -lactalbumin,  $\beta$ -lactoglobulin, and  $\gamma$ - or immuno-lactoglobulin. In the sedimentation diagrams of decalcified skim milk described by Pedersen,<sup>168</sup> the peaks were identified by Greek letters in the order of increasing sedimentation constants, the smallest whey protein being designated  $\alpha$ -lactalbumin, the next larger  $\beta$ -lactoglobulin (see Cannan *et al.*<sup>39</sup>), and the largest  $\gamma$ -lactoglobulin. Peaks with even larger constants were ascribed to components of casein.

The components of the milk protein fractions will be dealt with as individual proteins in later sections of this chapter.

## THE CASEINATE COMPLEX

The principal protein in milk, casein, is present in the form of micelles, or particles of macromolecular sizes. These are made up of the various components of casein bonded together as "calcium caseinate," and complexed further with calcium phosphate and magnesium and citrate ions. The casein complex is considered in detail elsewhere in this volume.

## CASEIN

### Elementary Composition

In the early years of protein chemistry the determination of the elementary constitution of isolated proteins, as of other organic chemicals, was an important method of characterization. With the recognition that proteins are very large molecules, composed essentially of the same amino acids, it became evident that differences in carbon and hydrogen content could not be great, and determinations of these elements are seldom made on proteins. Nevertheless, because casein was one of the first proteins to be prepared in purified form, the following

results of an analysis of acid-precipitated casein reported in 1883 by Hammarsten<sup>93</sup> are at least of historical interest: C, 53.0; H, 7.05; N, 15.65; S, 0.76; and P, 0.85%. The determinations of phosphorus, sulfur and nitrogen compare favorably with modern analyses. Estimations of these elements are still useful in the characterization of casein and other proteins, since phosphorus is an uncommon constituent of proteins, sulfur provides a measure of the total sulfur-containing amino acids present, and nitrogen percentage is a convenient rough index of the content of protein in foods, feeds, and other biological materials. It has been customary for many years to estimate the protein content of biological materials by determining their nitrogen content, usually by the Kjeldahl method, and multiplying by the factor 6.25, because purified proteins contain about 16% nitrogen. In the case of dairy products, the factor used is 6.38, this figure reflecting more accurately the nitrogen content of milk proteins.

### Amino Acid Composition

Many important discoveries regarding proteins and their amino acid composition were made in the nineteenth and early twentieth centuries by investigators who used the readily prepared casein as a typical protein. For example, a few amino acids were discovered in hydrolyzates of casein and were recognized later to be common constituents of all proteins.<sup>221</sup> Casein was also widely used in the development of methods for the quantitative amino acid analysis of proteins. Actually, however, it was another milk protein,  $\beta$ -lactoglobulin, that was the first large protein to have its complete amino acid composition established through the classic research of Brand *et al.*<sup>28</sup> in 1945. A few years later casein and its main components were also analyzed by similar methods.<sup>74</sup> These results, as well as later analyses of purified casein components and other milk proteins, are summarized in Table 3.2. Compilations from the older literature of the many quantitative determinations of amino acids in milk proteins have been made by Block and Weiss<sup>26</sup> and Orr and Watt.<sup>159</sup> More up-to-date tabulations may be found in the treatise by McKenzie.<sup>138</sup>

Milk and milk products provide food proteins of excellent quality for the nutrition of man and animals. Casein, the dominant protein, is a good source of amino acids indispensable to man. Commercial lactalbumin, or the heat-coagulable whey proteins, has an even higher biological value. Table 3.3 lists the content of essential amino acids (also cystine and tyrosine) in these proteins and in dried skim milk and dried whey. The data are taken from Table 2 of the Orr and Watt report.<sup>159</sup> The nutritive value of milk proteins is also reviewed by Henry.<sup>96</sup>

Table 3.2  
AMINO-ACID COMPOSITION OF COW'S MILK PROTEINS<sup>f</sup>  
(GRAMS PER 100 GM PROTEIN)

Constituent	$\alpha$ -Lactalbumin <sup>77</sup>	$\beta$ -Lactoglobulin <sup>88,175</sup>	Blood Serum Albumin <sup>205</sup>	Immune Globulin <sup>84,88,197</sup>	Casein <sup>73,74</sup>	$\alpha$ -Casein <sup>73,74</sup>	$\alpha$ s1-Casein Bdi <sup>147</sup>	$\kappa$ -Casein <sup>210</sup>	$\beta$ -Casein <sup>73,74</sup>	$\gamma$ -Casein <sup>73,74</sup>
Total N	15.9	15.6 <sup>2a</sup>	16.1	15.3-16.1	15.6	15.5	15.4	15.3	15.3	15.4
Total P	0.0	0.0	0.0	0.0	0.8	0.99	1.05	0.16 <sup>192</sup>	0.61	0.11
Total S	1.9	1.6 <sup>2a</sup>	1.9	1.0	0.9	0.72 <sup>101</sup>	0.68	0.70	0.86 <sup>101</sup>	1.03 <sup>101</sup>
Glycine	3.2	1.4	1.8	5.2	2.0	2.3	2.9	1.2	1.6	1.5
Alanine	2.1	7.0	6.3	4.8	3.2	3.8	3.4	5.4	2.0	2.3
Valine	4.7	6.1	5.9	9.6	7.2	6.3	5.5	6.3	10.2	10.5
Leucine	11.5	15.5	12.3	9.6	9.2	7.9	9.4	6.1	11.6	12.0
Isoleucine	6.8	6.9	2.6	3.0	6.1	6.4	6.1	7.1	5.5	4.4
Proline	1.5	5.1	4.8	10.0	10.6	7.5	8.3	11.0	15.1	17.0
Phenylalanine	4.5	3.5	6.6	3.9	5.0	4.6	5.6	3.9	5.8	5.8
Tyrosine	5.4	3.7	5.1	6.7	6.3	8.1	7.7	7.6	3.2	3.7
Tryptophan	(7.0) <sup>a</sup>	2.7	0.58	2.7	1.7	2.2	1.7	1.0 <sup>201</sup>	0.83	1.2
Serine	4.8	4.0	4.2	11.5	6.3	6.3	7.1	5.0	6.8	5.5
Threonine	5.5	5.0	5.8	10.5	4.9	4.9	2.5	6.7	5.1	4.4
Cystine + Cysteine	6.4 <sup>c</sup>	3.4	6.5	3.2	0.34	0.43	0.0	1.2 <sup>200</sup>	0.0	0.0
Methionine	0.95	3.2	0.81	0.9	2.8	2.5	3.2	1.7	3.4	4.1
Arginine	1.2	2.8	5.9	4.1	4.1	4.3	4.4	4.0	3.1	1.9
Histidine	2.9	1.6	4.0	2.1	3.1	2.9	3.2	2.4	3.1	3.7
Lysine	11.5	11.8	12.8	6.8	8.2	8.9	8.7	6.5	6.5	6.2
Aspartic acid	18.7	11.4	10.9	9.4	7.1	8.4	8.5	7.7	4.9	4.0
Glutamic acid	12.9	19.3	16.5	12.3	22.4	22.5	24.3	19.8	23.2	22.9
Amide N	1.4	1.1 <sup>2a</sup>	0.78	—	1.1	1.6	1.3	1.9	1.6	1.6

a By method of Spies and Chambers.<sup>203</sup>  
b By method of Spies;<sup>203</sup> more reliable value.  
c No cysteine present.  
d Based on amino acid sequence of protein.<sup>147</sup>  
e No cysteine present.  
f Table 3.2 does not include analyses of many other milk proteins whose amino acid compositions have been reported. The reader is referred to the treatise edited by McKenzie<sup>128</sup> for more inclusive compositional data.

Table 3.3

AMINO ACIDS ESSENTIAL TO MAN IN COW'S MILK PRODUCTS  
(GRAMS PER 100 GM)

	Casein	Lactalbumin	Dried Nonfat Milk	Dried Whey
Tryptophan	1.3	2.2	0.50	0.15
Threonine	4.3	5.2	1.6	0.68
Isoleucine	6.6	6.2	2.3	0.73
Leucine	10.0	12.3	3.5	1.04
Lysine	8.0	9.1	2.8	0.77
Methionine	3.1	2.3	0.87	0.19
Cystine	0.38	3.4	0.32	0.25
Phenylalanine	5.4	4.4	1.7	0.32
Tyrosine	5.8	3.8	1.8	0.13
Valine	7.4	5.7	2.4	0.64

### Sulfur

Casein contains 0.78% sulfur; the amino acids cystine and methionine account for 0.09 and 0.69%, respectively, cysteine being absent.<sup>119</sup> When casein is fractionated, it is found that  $\alpha$ -casein contains all the cystine in casein.<sup>74</sup> On fractionation of  $\alpha$ -casein, most of the cystine is found in  $\kappa$ -casein,<sup>225,115</sup> some in the minor components  $\alpha_{\kappa 3}$ ,  $\alpha_{\kappa 4}$  and  $\alpha_{\kappa 5}$ -caseins.<sup>105</sup>

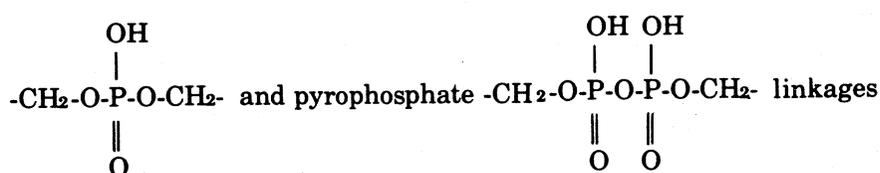
### Phosphorus

When casein is treated with proteolytic enzymes, rather large polypeptides, some of which contain phosphorus and resist further enzymatic degradation, are formed. It has been thought that these products, often called phosphopeptones, may play an important role in the nutrition of the young mammal. Thus, Mellander suggested that phosphopeptones can combine with other nutrients, such as calcium and iron, in this way favoring absorption of these elements, and, furthermore, that during subsequent digestion a unique mixture of amino acids is provided at the proper time for optimal utilization of protein.<sup>146</sup>

In 1927, Posternak<sup>179</sup> isolated a phosphopeptone from tryptic digests of casein and found that it contained 5.9% phosphorus, 11.9% nitrogen, and glutamic and aspartic acids, serine and isoleucine. He believed that all the phosphoric acid found had been linked to serine, presumably because serine was the only hydroxy-amino acid present in the hydrolyzate. That some phosphoric acid is indeed bound to serine was proved by the isolation of phosphoserine from a weak acid hydrolyzate of casein by Lipmann<sup>132</sup> in 1933. At about the same time Levene

and Hill hydrolyzed further a phosphopeptide from casein and isolated a dipeptide made up of phosphoserine and glutamic acid.<sup>128</sup> Twenty years later, in 1953, de Verdier<sup>220</sup> was able to prepare phosphothreonine from casein hydrolyzed by weak acid. From the preceding evidence and many other studies of a similar nature, it may be concluded that the phosphorus in casein is bound chiefly, if not entirely, in ester linkages with the hydroxyl groups of serine and threonine.

As to the nature of the ester linkages, Perlmann has postulated that not only orthomonophosphate ester linkages, but also diester



occur in casein, and that the principal electrophoretic components of casein,  $\alpha$ - and  $\beta$ -caseins, differ in the types of linkage present.<sup>169</sup> However, a considerable number of more recent investigations indicate that phosphorus is bonded in the same way in whole casein,  $\alpha$ -casein, or  $\beta$ -casein and that the bond is most likely the orthomonophosphate ester linkage.<sup>2,107,118,161,191</sup>

Work on the isolation of phosphopeptides and phosphopeptones has continued to provide important information concerning the structure of casein. This research was facilitated by the fractionation of casein into its several components and by the development of newer, more powerful methods of separating and characterizing products of partial hydrolysis of the components. For example, Peterson *et al.*<sup>174</sup> isolated a relatively large, electrophoretically homogeneous phosphopeptide from tryptic digests of  $\beta$ -casein. The phosphopeptide, with a molecular weight of about 3,000, consisted of 24 amino acid residues of 10 different amino acids, and 5 phosphoric acid groups presumably attached to the 4 serine and 1 threonine residues present. Essentially all the phosphorus of  $\beta$ -casein appeared to be concentrated in this portion of the molecule. In later work on the primary structure of  $\beta$ -casein A<sup>2</sup>, one of the genetic variants of this protein, the same phosphopeptide was isolated by Ribadeau-Dumas *et al.*<sup>184</sup> as tryptic peptide, T1. Subsequently, the same authors reported much of the primary structure of the entire  $\beta$ -casein molecule with peptide T1 positioned at the N-terminal end.<sup>184</sup> Similarly, in the case of  $\alpha$ -casein, a tryptic polypeptide of 35 amino acid residues with 7 phosphate groups was isolated and partly sequenced by Österberg in 1964.<sup>161</sup> A 21-residue phosphopeptide,

probably a portion of the Österberg peptide, was prepared from  $\alpha$ -casein by Schormüller *et al.*<sup>194</sup> in 1948. Presumably the same larger peptide was isolated later by Grosclaude *et al.*<sup>84</sup> from  $\alpha_{S1}$ -casein B. Now designated Tm1, the peptide was found to contain 8 of the 9 phosphate groups in this  $\alpha_S$ -casein polymorph. In 1970 Mercier *et al.*<sup>147</sup> worked out the partial sequence of the phosphopeptide and its position in the primary structure of  $\alpha_{S1}$ -casein B.

The localization of phosphorylated amino acids in limited regions of both the  $\alpha_{S1}$ - and  $\beta$ -casein molecules is an important outgrowth of this research. Such information will lead to a fuller understanding of the interactions among components of casein and the structure and behavior of casein micelles.

### Carbohydrate in Casein and Other Milk Proteins

The primary carbohydrate of whole unfractionated cow's milk is, of course, lactose, which occurs to the extent of 50 gm per l. Very little carbohydrate is associated with milk proteins through covalent linkage; however, the carbohydrate which is, seems to play an important role in stabilizing the casein micelle. The presence of small proportions of carbohydrate in casein has been detected by various investigators. For example, using the orcinol reaction, Sørensen and Haugaard<sup>199</sup> found highly purified, acid-precipitated casein to contain 0.31% hexose, believed to be galactose but later shown to be a mixture of hexose, hexosamine and sialic acid.<sup>48</sup> Nitschmann *et al.*<sup>158</sup> demonstrated that a large peptide, which was split off from casein by the action of rennet, contained considerable percentages of galactose, galactosamine and neuraminic acid. The peptide was called a glycomacropptide. The other major product of the rennet reaction is para-casein which is essentially devoid of carbohydrate. It was subsequently shown that the carbohydrate is concentrated largely in the  $\alpha$ -fraction of casein and more particularly in the  $\kappa$ -portion of  $\alpha$ -casein, from which the glycomacropptide may be prepared.<sup>1,115</sup> The carbohydrate is found in the carboxyl-terminal portion of the  $\kappa$ -casein molecule and is linked to the protein by an O-glycosidic linkage between the -OH groups of serine or threonine or both, and N-acetyl-galactosamine.<sup>60,218</sup> The other carbohydrate moieties are linked to the galactosamine, N-acetylneuraminic acid being terminal.<sup>115,116,218</sup> Variability in carbohydrate content of  $\kappa$ -casein is responsible for one type of heterogeneity of the protein, since from 0 to 5 carbohydrate chains may occur in the molecule.<sup>230</sup>

Other milk proteins are now known to have covalently bound carbohydrate. Aschaffenburg isolated a minor component of crystalline  $\alpha$ -lactalbumin B preparations which was analyzed by Gordon and found

to have the exact amino acid composition of  $\alpha$ -lactalbumin B. Brew and Hill showed that this component contains 1 mole of hexosamine per mole of protein.<sup>68</sup> Barman<sup>13</sup> observed that as much as 7% of an  $\alpha$ -lactalbumin preparation can be recovered as a glyco- $\alpha$ -lactalbumin after column chromatography. This heterogeneous glycoprotein has up to 15% carbohydrate, consisting of mannose, galactose, glucosamine, galactosamine, and N-acetylneuraminic acid. Hindle and Wheelock have reported similar findings.<sup>99</sup> A variant of  $\beta$ -lactoglobulin isolated<sup>17,18</sup> from Australian Droughtmaster beef cattle was shown to contain N-acetylneuraminic acid, hexosamine and hexose in a ratio of 1 : 4.3 : 2.7; otherwise, the protein is identical with  $\beta$ -lactoglobulin A.<sup>140</sup> The red protein, lactoferrin, which binds iron, has been shown to contain hexose, hexosamine and N-acetylneuraminic acid.<sup>70</sup> Other proteins containing carbohydrate are fat globule membrane proteins,<sup>108,211</sup> glycoprotein-a,<sup>88</sup> M-1 glycoproteins,<sup>20,21,22</sup> and the proteoseptone fractions.<sup>188</sup> In addition, several enzymes appearing in milk are glycoproteins; these include lactoperoxidase<sup>187</sup> and ribonuclease.<sup>23,24</sup> The carbohydrate, though found in relatively low percentages, seems to be distributed over a wide number and variety of proteins, and it may be inferred that, where it does occur in covalent linkage to protein, it plays an important biological role.

### Solubility

Casein is dissolved by aqueous solutions of acids, alkalies, and alkaline salts. The amount dissolved in a definite weight of solvent depends on the pH value of the solvent. In acid solutions of a pH on the acid side of the isoelectric point of casein, compounds are formed with the nonmetallic element or radical of the acid, and, in alkaline solutions, compounds are formed with the metal of the alkali or alkaline salt. Hence, such solutions are not solutions of casein in a strict sense. Solubility of casein, strictly speaking, is the solubility of chemically unaltered hydrogen caseinate, and hence is the solubility of casein in solutions at its isoelectric point. That point is usually considered to be pH 4.6, but it is shifted by the presence of neutral salts in solution and may be a point in a zone extending from pH 4.0 to pH 4.8, approximately.<sup>81</sup> Much of the literature on solubility of casein does not state whether the determinations were carried out under isoelectric conditions and consequently must be accepted with reservations. Because the amount of casein dissolved by many solvents varies with the quantity of casein added to a definite quantity of solvent, many available data have limited usefulness. This is a necessary consequence of the fact that the casein preparations studied do not consist of a single molecular species, and therefore no true solubility constant for casein

can be expected. The solubility of casein in water at the isoelectric point has been reported as 0.05 gm/l at 5°C,<sup>170</sup> and 0.11 gm/l at 25°C.<sup>43</sup> Its solubility in various organic acids, mixtures of water and certain organic solvents, and aqueous solutions of a variety of salts has been investigated. Appropriate references to these studies in the older literature may be found in the preceding edition (1965) of this book.

### Optical Rotation

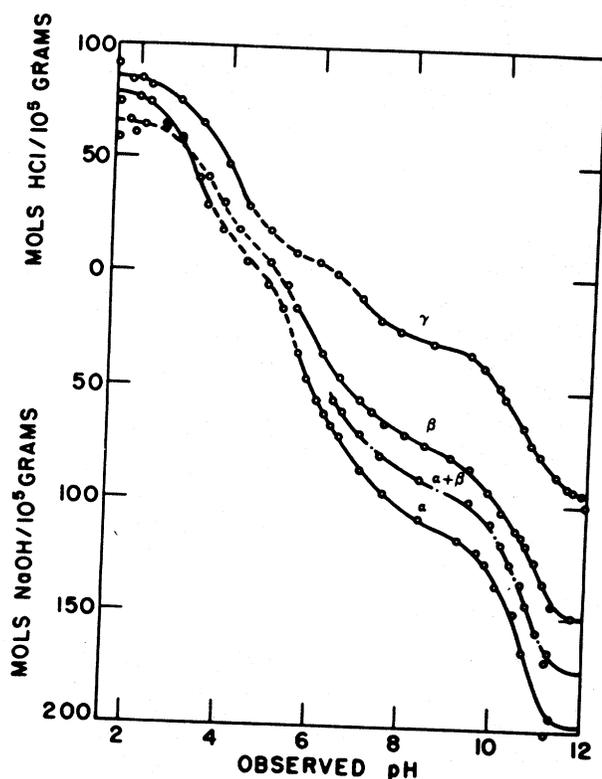
Solutions of casein rotate polarized light in the levo direction. The specific rotation of casein varies with samples prepared in different ways and with the solvent. Gould<sup>78</sup> found  $[\alpha]_{D}^{30^{\circ}} = -81.7^{\circ}$  for 2% Hammarsten casein in 10% sodium acetate solution; values for commercial caseins under the same conditions differed over the range of  $-70^{\circ}$  to  $-90^{\circ}$ . Hipp *et al.*<sup>101</sup> determined  $[\alpha]_{D}^{25^{\circ}}$  for casein and purified fractions in 1% solutions in veronal buffer, pH 8.4, ionic strength 0.1; the values were  $-105^{\circ}$  for whole casein,  $-87.4$  to  $-90.5^{\circ}$  for  $\alpha$ -casein prepared by different methods,  $-125^{\circ}$  for  $\beta$ -casein, and  $-132^{\circ}$  for  $\gamma$ -casein.

### Combining Capacity with Acids and Bases

Much effort has been spent in determining the combining capacity of casein as an acid and as a base. Electrometric titration,<sup>44</sup> determination of the minimal amount of acid or base required to dissolve a given weight of casein,<sup>170</sup> and conductivity represent some of the methods employed. The results obtained for the combining capacity of casein have varied greatly depending on the method of preparing the casein and the method used to determine its combining capacity. Variations in the combining capacity of casein can in part be explained by the now known heterogeneity of the casein and its lability.

From the acid- and base-combining capacity of casein Cohn and Berggren<sup>44</sup> calculated the number of dissociating groups. They found that this number could be correlated with the amino acid constitution of the casein. More recently, Hipp *et al.*<sup>102</sup> determined the acid- and base-binding capacities of  $\alpha$ -,  $\beta$ - and  $\gamma$ -caseins from their titration curves (Fig. 3.1).

By selecting the pH values where the ionic groups of the amino acid residues would be expected to dissociate, the number of ionizing groups was estimated from the titration curve. Thus, at pH 6.35, carboxyl groups plus one equivalent of phosphoric acid are considered to be dissociated and were estimated by the equivalents of alkali combined at this pH. Similar estimates for the remaining dissociating groups in casein were made at other pH values. The estimates of the



After Hipp *et al.*<sup>102</sup>

FIG. 3.1. ACID-BASE TITRATION CURVES FOR  $\alpha$ -,  $\beta$ -, and  $\gamma$ -CASEIN AND AN EQUAL MIXTURE OF  $\alpha$ - and  $\beta$ -CASEIN AT PROTEIN CONCENTRATION OF 1% 0.05 IONIC STRENGTH AND 25°C

Ordinates are moles bound/10<sup>5</sup> gm. 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  $\alpha$ - and  $\beta$ -casein is given.

number of ionic groups in  $\alpha$ -,  $\beta$ -, or  $\gamma$ -casein arising from its amino acid residues were consistent with the number of ionic groups calculated to be present from the amino acid compositions of these proteins, as reported by Gordon *et al.*<sup>73,74</sup> and given in Table 3.2. The maximum acid- and base-combining capacities, as determined from the titration curves (Fig. 3.1) of  $\alpha$ -,  $\beta$ -, and  $\gamma$ -caseins, were calculated to be 78, 66, and 85 moles of acid and 198, 150, and 96 moles of base per 10<sup>5</sup> gm, respectively. These values for the base-binding capacity are somewhat higher than the values of 176 and 128 moles per 10<sup>5</sup> gm for  $\alpha$ - and  $\beta$ -casein calculated from their amino acid constitutions. However, the values determined for the base-combining capacity of

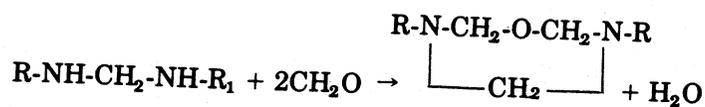
$\alpha$ -,  $\beta$ -, and  $\gamma$ -casein by titration (Fig. 3.1) lead to a calculated value of 183 moles per  $10^5$  gm of casein based on the constitution of unfractionated casein being 16 parts  $\alpha$ -, 4 parts  $\beta$ -, and 1 part  $\gamma$ -casein, which is in agreement with the value reported by Cohn and Berggren<sup>44</sup> for the base-combining capacity of several casein preparations.

### Derivatives of Casein

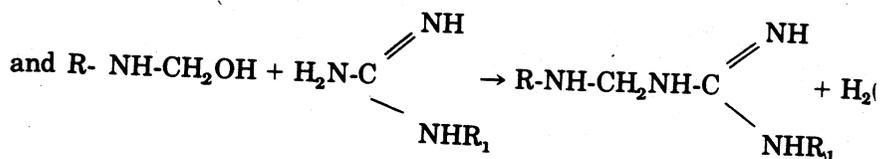
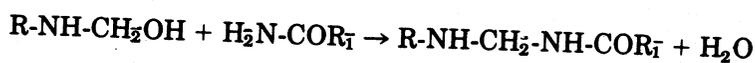
Earlier editions of this volume dealt at some length with many different compounds made from casein. In numerous studies, casein, because of its ready availability in purified form, was used as a model protein for the preparation of various derivatives. Furthermore, since large quantities of the protein were manufactured, some of the unique properties of the native protein or its derivatives were exploited for diverse industrial applications. Derivatives that were prepared include halogenated caseins, desaminocasein, nitrocasein, methylated casein, a number of acylated caseins, formaldehyde casein, carbamidocasein, casein treated with sulfur-containing reagents and metal compounds of casein. References to original papers are listed in the preceding edition (1965) and in the book by Sutermeister and Browne<sup>205</sup> published in 1939. Most of this work has been outdated but the reaction of casein with formaldehyde is still of some importance, as are also certain industrial uses of casein. These topics will be discussed presently.

### Formaldehyde Casein

The reaction of formaldehyde with casein is of practical as well as of theoretical interest, since formaldehyde is used as a hardening agent for plastic casein in industry. It is agreed that the free amino groups of casein are the points initially attacked, but several ideas have been advanced as to the reactions involved. Blum<sup>27</sup> believed that methylene caseins,  $R-N=CH_2$  and  $R-NH-CH_2-NH-R_1$ , were formed with loss of water. Benedicenti<sup>19</sup> suggested that formaldehyde might be added, without elimination of water, to form a compound of the formula  $R-NH-CH_2OH$ . The probability that this reaction takes place is supported by the fact that much of the formaldehyde can be removed from formaldehyde casein by steam distillation, but the fact that heat treatment fixes part of the formaldehyde on the casein indicates that other reactions take place.<sup>155, 157, 208</sup> According to Wormell and Kaye,<sup>229</sup> formaldehyde, under neutral conditions, first becomes attached to each lysine side chain, and, in a second stage, cyclic compounds may be formed when two  $NH_2$  groups have been linked by a methylene group in the first step:



Under acid conditions, amino and amide groups are linked through methylene groups. Fraenkel-Conrat and Olcott<sup>64</sup> have offered evidence that, in neutral or slightly acid solutions, CH<sub>2</sub>O introduces -CH<sub>2</sub> bridges between amino groups and reactive =CH groups of phenolic and imidazole rings of proteins, and that the hardening action of formaldehyde is not due to a primary reaction, such as that suggested by Benedicenti, but is due to a secondary reaction which transforms methylol, -CH<sub>2</sub>OH, groups into cross-linking methylene bridges that link amino and primary amide or guanidyl groups:



### Browning Reaction

Concentrated and dried forms of milk, when held for considerable time in storage, gradually develop a brown color. Very often, even under conditions when browning does not occur, the forerunner of discoloration, especially in high moisture dry milks, is off-flavor. In either case, whether browning takes place or not, the products are usually unfit for human consumption. The reaction involved has been studied by many investigators.<sup>45,126,127,149,163</sup> Considerable evidence<sup>47,165</sup> has been presented in support of the view that browning in milk is caused by a Maillard-type interaction<sup>135</sup> between the free amino groups of milk proteins and the aldehyde group of lactose. Early formation of a lactose-casein complex has been postulated<sup>79,166</sup> and the involvement of the ε-amino group of lysine was demonstrated by the experiments of Henry *et al.*<sup>97</sup> and Lea and Hannan<sup>127</sup>; more recently, a lysine derivative was isolated from heated milk powders.<sup>61,32</sup> Participation of other amino acids (arginine, histidine, methionine, and tyrosine) has also been documented.<sup>127,163,56,164</sup> The browning reaction is accelerated by increases in temperature, pH value, and water content of the protein-containing material, or in the relative humidity to which a solid protein

is exposed. The reactions appear to be similar to those between casein and formaldehyde, not only in that the primary reaction is between aldehyde and amino groups, but also in that there is a secondary reaction, which, in this instance, produces color and decreases the dispersibility of the protein.

The isolation and identification of products of browning, such as maltol, 5-hydroxy-methyl-2-furfuraldehyde and furfuryl alcohol, have been reported.<sup>165,167</sup> These have been shown to arise from the casein-catalyzed degradation of lactose. The isolation and identification of 40 compounds among the products arising from the browning reaction of casein-lactose in the dry state were reported.<sup>58,59</sup> These compounds have not as yet been evaluated for flavor, but such studies will be important in correlating the development of off-flavor with manifestations of the browning reaction. The chemistry of the browning reaction was reviewed by Danehy and Pigman,<sup>49</sup> by Hodge<sup>108</sup> and, with particular reference to milk and dairy products, by Patton.<sup>165</sup>

One interest of the dairy industry in the browning reaction is its relation to nutrition. The economic interest in browning and production of color and concomitant off-flavor is obvious. Browning results in lowered consumption of the foods involved because of poor palatability, appearance and physical properties, loss of nutritional value from destruction of essential amino acids and vitamins, and loss of biological value and digestibility of protein; finally, browning may produce toxic substances and metabolic inhibitors. The nutritive value of milk and milk products has been reviewed.<sup>122,96</sup>

### **Precipitation of Casein**

It might be expected that all the casein in a sample of milk would be precipitated simply by adding sufficient acid to bring the pH value to approximately 4.6. However, the reaction of acid with caseinate complex is not instantaneous and the pH will tend to rise slowly with time. If a considerable excess of strong acid, such as hydrochloric, is added, the complex will be broken down more rapidly, but some of the separated casein will dissolve as chloride and may not reprecipitate completely on readjustment of the pH to the isoelectric point. To stabilize the pH, a buffer mixture of acetic acid and sodium acetate may be used. In order to increase the rate of precipitation, the acetic acid is added alone to give a pH value slightly on the acid side of the isoelectric point. After a few minutes, the sodium acetate is added and thus the pH is brought to the isoelectric point and stabilized there. Because of the buffer action of the acetate mixture, the same proportions of acid and salt are equally effective with milks of different percentages

of caseinate complex. According to Rowland<sup>189</sup> maximal precipitation of casein from milk is effected by adding for each 10 ml milk, 80 ml water at 40°C, then 1.0 ml 10% acetic acid, and, after 10 min, 1.0 ml normal sodium acetate.

### Preparation of Casein for Research Purposes

The earliest accepted method for the preparation of so-called "pure casein" was that of Hammarsten.<sup>93</sup> Many modifications of this method and a few somewhat different methods have been proposed. Brief reference is made below to several of these, but the original publications should be consulted for details.

Casein "nach Hammarsten" is prepared by the following method. Skimmilk is diluted with 4 times its volume of water and the casein precipitated by the addition of dilute acetic acid. The casein is then repeatedly dissolved in water containing the least amount of alkali that will dissolve it, the solution filtered to remove insoluble substances, the casein reprecipitated with dilute acetic acid and washed with water. The casein must not be exposed to high concentrations of hydroxyl ion nor of hydrogen ion because of the danger of racemization or hydrolysis under these conditions. Without previous drying, the casein is triturated with absolute ethanol, the ethanol removed by vacuum, the casein treated with anhydrous ether, the ether removed, and the product dried either over sulfuric acid at reduced pressure or over calcium chloride at atmospheric pressure. The first method of drying yields a product that is difficult to wet with water; the second gives a product that contains traces of water and is therefore readily wetted.

Dunn<sup>51</sup> precipitated casein at pH 4.8 by adding 0.5*N* hydrochloric acid to dilute skimmilk, and then washed the precipitate with water, ethanol, and ether. Warner<sup>224</sup> obtained raw milk at time of milking, added toluene as preservative, and chilled the milk immediately to 2°C. All subsequent operations were carried out at that temperature. The milk was skimmed and the casein precipitated at pH 4.6 by adding 0.1*N* hydrochloric acid. It was then washed with ice-water, dissolved with sodium hydroxide to pH 6.5, and the solution extracted with ether. The solution was diluted and the casein reprecipitated with 0.01*N* hydrochloric acid. Finally, it was washed thoroughly with water. A dry product was also made using ethanol and ether. This casein contained 0.86% phosphorus.

Precipitation of casein at pH 4.1, as in the commercial grain-curd process described later, followed by thorough washing with slightly acidulated water, removes all but about 0.20% calcium oxide and all the phosphorus pentoxide in excess of 1.80% (0.79% phosphorus). For

most purposes, the small quantity of extraneous ash remaining—less than 0.50%—is not objectionable, and, what is of considerable importance, the casein is not exposed to alkalinities greater than that of milk itself.

Casein that is practically free of both ash and vitamins may be prepared by a method of Block and Howard,<sup>25</sup> which avoids exposure of casein to alkaline pH values. Purified sulfur dioxide is bubbled through skimmilk held at approximately 36°C until the pH value is 4.5 to 4.6. The precipitated casein is removed by filtration and washed with water at pH 4.5 to 4.6. The casein is redissolved by stirring it in suspension in water and passing in sulfur dioxide until the pH value is 1.8 to 1.9. It is reprecipitated by adding dilute alkali until the pH value is again 4.5 to 4.6; it is then separated by filtration, washed with water, and dried by use of anhydrous solvents as described above.

### Preparation of Casein for Industrial Use

Commercial casein is made by either of two general methods—precipitation by acid or coagulation by rennin. The latter method is used almost exclusively for producing casein for the manufacture of plastics, since rennin casein has the peculiar properties considered essential for this product. Acid precipitation is employed for producing casein for its other commercial uses, for most of which adhesive properties are the most important requirement.

In the manufacture of rennin casein,<sup>152</sup> a fresh, low-fat, skimmilk is warmed to 35.5°C and curdled with about 4 oz rennin extract to each 100 gal milk. The coagulation should be complete in 15 to 20 min., after which the curd is broken up and gradually heated to 55 to 65°C. After it has settled for 10 min, the curd is drained of whey and washed several times with water at 26 to 32°C. It is then pressed for an hour, shredded, and dried in thin layers at 43 to 46°C. Rapid drying at a low temperature is essential if the product is to be of a light color.

Acidification methods<sup>227</sup> may be of the self-sour type, in which the acid is formed in the skimmilk by bacterial fermentation of lactose to lactic acid, or of the type in which acids are added to the milk in sufficient quantity to precipitate the casein and attain the desired degree of acidity. There are numerous modifications, depending on the acid and the temperatures used and on the mechanical equipment. Several procedures employ continuous precipitation, washing and drying, which, as is to be expected, give a product of remarkable uniformity in composition, color, and adhesiveness.

In the self-sour processes, the milk is inoculated with lactic acid

bacteria and allowed to stand at a favorable temperature until curdling takes place. The curd at this acidity—about pH 4.7—is soft and fine; in order to agglomerate it, heat is employed. Excessively high temperatures produce a rubbery curd that is impossible to wash successfully unless it is chopped. After draining, the curd is washed several times, drained, pressed overnight, shredded, and dried at 54°C. Casein of this type, unless exposed to acidity greater than pH 4.7, contains a somewhat greater percentage of ash than the grain-curd casein described below.

The above-described process may be carried out more rapidly if the curd is precipitated by adding dilute acid with moderate stirring; but if a mineral acid, such as hydrochloric or sulfuric, is added to the skim milk at above 35°C in sufficient quantity to bring the reaction to pH 4.1 (apparently pH 4.6, if methyl red is used as indicator), a curd of coarse, granular texture is formed. This is known as grain-curd; because of its open texture and the fact that calcium phosphate is completely in solution at this acidity, it is easily washed to produce a low-ash, low-acid casein. In the continuous adaptation of this process, temperatures of coagulation as high as 43°C are employed and the chewing gum-textured curd produced is chopped before washing. The washing, pressing, shredding, and drying operations are the same for all types of curd. Casein for paper coating should be ground to 20 to 30 mesh, the proportion of finer particles being kept as small as possible.

Short descriptions of more up-to-date methods for preparing caseins of these types, as well as coprecipitated casein (casein + whey protein), low-viscosity casein and sodium caseinate, are given by Fox.<sup>62</sup> Coprecipitate manufacture is also discussed by Beeby *et al.*<sup>14</sup> A comprehensive review of the manufacture and uses of casein and coprecipitate, with special emphasis on developments since 1953, has been published by Muller.<sup>150</sup>

### Uses of Casein

The most extensive nonfood use of casein in the United States, some 34 million pounds<sup>62</sup> in 1967, is in coating paper for books and magazines. For this use it is dissolved in alkali, mineral pigments are added, and the suspension is applied to the paper by means of rollers or brushes. The casein binds the pigment to the surface of the paper and renders the surface smooth and nonabsorptive, thus making the paper suitable for fine printing. Formaldehyde may be used to make the casein-bound coatings waterproof, as on playing cards and wall papers. Casein is used in the paper industry also as a dispersing agent for the rosin used for sizing paper.

Casein glues are used chiefly in the woodworking industries. They consist of casein, a solution of alkali as solvent and a calcium compound. The calcium caseinate formed becomes insoluble on drying and causes the glue to become waterproof. About 10 million lb of casein were used in 1967 in this manner<sup>62</sup> and smaller quantities in the following ways.

Plastic casein is made by stirring dry rennin casein, pigments, and a small proportion of water into a heavy dough and extruding this dough through dies under pressure. Formaldehyde is used as an insolubilizer and hardening agent, being applied usually to the finished article.

Casein-containing, water-thinned paints may consist of pigments, an insolubilizer and a solution of casein in alkali, or may contain oil in the form of an oil-in-water emulsion. The first type, of which whitewash made from skim milk and lime is the simplest example, is used mostly on exterior surfaces; the second type is popular for use on interior walls and ceilings.

Casein is used in the textile industry for many purposes, such as fixing colors, loading, sizing, softening, and waterproofing. In the leather industry it is used in solution in a minimum amount of alkali to give a gloss to light leathers. A mixture of lime and casein is used as an adhesive and spreader in applying insecticides. Many more uses of minor importance are listed in Sutermeister and Browne.<sup>205</sup>

Ground casein can be converted into fibrous forms by extruding an alkaline solution of the protein into an acid coagulating bath, or by extruding a heated mixture of casein and water into air. The term casein fiber is reserved for the fine wool-like filaments obtained by the first method. The coarser product of the second method is called casein bristle. Casein fiber was produced commercially in the United States for a number of years as a wool substitute during war-time scarcity. As an example of the research which was carried out with the goal of improving the properties of the fiber, the description of continuous-filament casein yarn by Peterson *et al.* in 1948 may be cited.<sup>172</sup> The preparation of casein fibers and bristles was reported by Whittier *et al.*<sup>227A</sup> and by McMeekin *et al.*<sup>103,144,171</sup> and these attained limited commercial production.

Nonfood uses of casein have been described at somewhat greater length by Salzberg.<sup>190</sup>

Of the approximately 100 million pounds of casein and sodium caseinate consumed annually in the United States, about one-third is used by the food industry in the manufacture of a variety of products. This topic is dealt with in the chapters by Fox<sup>62</sup> and Beeby *et al.*,<sup>14</sup> and in the review by Muller.<sup>150</sup>

## Fractions and Components of Casein

Casein occurs in milk as a colloidal calcium phosphate complex which contains about 7% inorganic material.<sup>182</sup> It has been realized for some time that although casein can be prepared and purified in a readily reproducible fashion, the product cannot be regarded, chemically, as a single protein; rather it is made up of a number of different proteins which can be separated and shown to have distinctive properties. The starting point for the fractionation of casein is, of course, the so-called whole casein preparation. This material is generally obtained in any one of three ways. The earliest and most widely-used method is that of acid precipitation under controlled conditions of temperature and pH. These methods eventually find their antecedent in the method of Hammarsten.<sup>93</sup> A second method employs centrifugation of skim milk in the presence of added  $\text{CaCl}_2$ , with appropriate consideration of conditions of temperature, pH and calcium-ion concentration.<sup>222,226</sup> Finally, a method of salt precipitation using 264 gm  $(\text{NH}_4)_2\text{SO}_4$  per l of skim milk held at 2°C has been described.<sup>137,139</sup> Each of the three methods of preparation is carefully considered by McKenzie.<sup>137,139</sup>

An early observation of the heterogeneity of casein was made by Osborne and Wakeman<sup>160</sup>, who isolated a small amount of casein with unique properties from alcohol extracts obtained in drying casein. Casein has since been fractionated on the basis of differential solubility in solutions of hydrochloric acid,<sup>131</sup> acidified ethanol,<sup>130</sup> and 50% ethanol,<sup>160</sup> by precipitation from solutions containing ammonium chloride by means of acetone,<sup>41,42</sup> and by successive precipitations by other methods.<sup>82,112</sup> Fractions of varying phosphorus, tyrosine, and tryptophan content were obtained, but no evidence of homogeneity of the fractions was presented, nor was complete separation into distinct components claimed. In 1939, Mellander, using the method of electrophoresis developed by Tiselius, showed the presence of three components in casein which he designated  $\alpha$ -,  $\beta$ -, and  $\gamma$ -casein in decreasing order of mobility.<sup>145</sup> The method enabled him to isolate small amounts of  $\alpha$ - and  $\gamma$ -caseins, which were found to be quite different in their phosphorus-to-nitrogen ratios.

The separation of casein into  $\alpha$ - and  $\beta$ -caseins, its principal components, was achieved by Warner<sup>224</sup> by preparing a very dilute solution of acid-precipitated casein, 0.12 to 0.3%, at pH 3.5 and 2°C and then adding 0.01N sodium hydroxide to pH 4.4. Under these conditions,  $\beta$ -casein is more soluble and remains in solution, whereas  $\alpha$ -casein is precipitated. By reprecipitating  $\alpha$ -casein in like manner at least 6 times, it is obtained free of  $\beta$ -casein as shown by electrophoresis.

From the supernatant solution  $\beta$ -casein is precipitated by adjusting the pH to 4.9 at room temperatures. It too can be readily purified so as to be free of other electrophoretic components. Warner's preparations were not electrophoretically homogeneous under all conditions, but neither component contained any of the other.

Two important methods for the separation of  $\alpha$ - and  $\beta$ -casein, and for the preparation of  $\gamma$ -casein, have been described by Hipp *et al.* In the first,<sup>101</sup> the fractionation is carried out with solutions of casein in 50% ethanol by varying temperature, pH, and ionic strength. In the second,<sup>101</sup> casein is dissolved in 6.6*M* urea, and the fractionation of components is achieved by the addition of water. The order of precipitation of the casein fractions by both methods is  $\alpha$ -,  $\beta$ -, and  $\gamma$ -casein, indicating that the charge on the casein component is the solubility-determining factor under these conditions.

The development of chemical methods for the preparation of the principal electrophoretic components of casein has enabled numerous investigators to study their properties and composition. A few of the distinctive properties are listed in Table 3.1, and amino acid compositions in Table 3.2.

That  $\alpha$ -casein is not always homogeneous in its electrophoretic behavior has already been mentioned. Evidence had accumulated that the protective colloid for the stabilization of the micelles in milk is  $\alpha$ -casein, and that it is the component on which the enzyme rennin acted.<sup>40</sup> Nitschmann and Keller<sup>156</sup> found that nonprotein nitrogen is produced from only the  $\alpha$ -casein component by the action of rennin. In 1955 and in subsequent publications, von Hippel and Waugh<sup>222, 225, 226</sup> described the separation of casein from milk by high-speed centrifugation and its fractionation by means of calcium ion. They showed that  $\alpha$ -casein is really made up of subfractions:  $\alpha$ -casein, precipitable by calcium ion under certain conditions and also called "calcium-sensitive casein" and  $\kappa$ -casein (calcium-insensitive casein) not precipitable by calcium ion. They concluded<sup>226</sup> that the  $\kappa$ -casein fraction is the one acted upon by rennin and is responsible for micelle stability. The two points of view concerning the action of rennin were reconciled by McKenzie and Wake<sup>141</sup> on the premise that the  $\alpha$ -casein samples examined prior to 1956 contained both  $\alpha_s$ -casein and  $\kappa$ -casein. They showed that  $\kappa$ -casein is concentrated with  $\alpha_s$ -casein in fraction A during the alcohol fractionation procedure of Hipp *et al.*<sup>101</sup> On the other hand, fraction B contains  $\alpha_s$ -casein essentially free of  $\kappa$ -casein. The  $\alpha$ -casein obtained in the urea fractionation procedure<sup>101</sup> was found to consist of a mixture of  $\alpha_s$ - and  $\kappa$ -caseins. Thus, only alcohol fraction B was a suitable source for preparing  $\alpha_s$ -casein. Those  $\alpha$ -casein fractions con-

aining  $\alpha_s$ - and  $\kappa$ -casein contain the protective colloid, whereas those containing only  $\alpha_s$ -casein have no protective properties.

The heterogeneity of the  $\alpha$ -casein fraction was also investigated by McMeekin *et al.*<sup>100,104,142</sup> using calcium chloride and ammonium sulfate to separate the components. These investigators isolated three fractions designated  $\alpha_1$ -,  $\alpha_2$ -, and  $\alpha_3$ -casein which, in their properties, appear to be similar to Waugh's  $\alpha_s$ -casein,  $\lambda$ -casein,<sup>133</sup> and  $\kappa$ -casein, respectively.

Mellander,<sup>145</sup> as has already been mentioned, demonstrated the existence of three electrophoretic fractions of casein, designated  $\alpha$ -,  $\beta$ -, and  $\gamma$ -casein. Hipp *et al.*<sup>101</sup> obtained a  $\gamma$ -casein preparation which was electrophoretically homogeneous on the alkaline side of the isoelectric point, pH 5.8 to 6.0. The preparation, low in phosphorus and high in sulfur relative to  $\alpha$ - and  $\beta$ -caseins, was thought to be similar to the casein isolated by Osborne and Wakeman.<sup>160</sup> Groves *et al.*<sup>91</sup> obtained essentially homogeneous  $\gamma$ -casein by the method of column chromatography, using DEAE-cellulose columns and phosphate buffers.  $\gamma$ -Casein was eluted with 0.02M phosphate, pH 8.3. A further examination of this  $\gamma$ -casein fraction<sup>90</sup> has revealed the existence of at least three other minor proteins designated S-, R-, and TS-caseins. The isolation, characterization and some properties of the components of the  $\gamma$ -casein fraction have been reviewed.<sup>86,139,188</sup> Further, the interesting relationship between  $\beta$ -casein and the members of the  $\gamma$ -casein fraction has been reported.<sup>71</sup>

There are other  $\alpha_s$ -caseins (i.e., caseins which are calcium-sensitive and stabilized by  $\kappa$ -casein) which have been designated  $\alpha_{S0}$ -,  $\alpha_{S2}$ -,  $\alpha_{S8}$ -,  $\alpha_{S4}$ -,  $\alpha_{S5}$ -caseins,<sup>3,105</sup> to distinguish them from the genetically variable  $\alpha_{S1}$ -casein.<sup>213</sup> Annan and Manson<sup>3</sup> isolated some of these  $\alpha_s$ -caseins by use of SE-Sephadex chromatography in 8M urea at pH 4.0. They described some of the physical chemical properties of these proteins. The relationship of  $\alpha_{S3}$ -,  $\alpha_{S4}$ -, and  $\alpha_{S5}$ -caseins has been partially clarified by the report of Hoagland *et al.*<sup>105</sup> It appears that  $\alpha_{S5}$ -casein gives rise to  $\alpha_{S8}$ - and  $\alpha_{S4}$ -caseins upon reduction, and that  $\alpha_{S8}$ - and  $\alpha_{S4}$ -caseins are very similar, based on amino acid molar ratios. More information is to be anticipated regarding these calcium-sensitive proteins.

A thorough discussion of casein components can be found in two recent publications concerned with milk proteins.<sup>137,139</sup> It is evident that whole casein is an extraordinarily complicated chemical mixture. This is readily apparent, as mentioned previously, from the analyses of zone electrophoresis in concentrated urea solutions described by Wake and Baldwin.<sup>223</sup> These authors report that about 20 components

can be resolved in a single starch-gel analysis of casein. It is equally apparent from what has been written here that many investigators are making important contributions to clarify the complexity of whole casein. Still another aspect of the complexity of casein was brought to light by Aschaffenburg.<sup>6</sup> Caseins prepared from the milks of individual cows and inspected by electrophoretic techniques were found to show genetically controlled polymorphism in the  $\beta$ - and  $\gamma$ -caseins. Polymorphism in  $\alpha_{s1}$ -caseins was demonstrated by Thompson and Kiddy<sup>213</sup> and genetic variation has now been confirmed in all the major and some of the minor casein components.<sup>86,139,134,212</sup> The biological significance of milk protein polymorphism has also been considered.<sup>57</sup>

Individual casein components have been isolated and purified to such a degree that the complete linear sequence of the polypeptide chain can be determined. Such studies have been undertaken on  $\alpha$ -,  $\beta$ -,  $\gamma$ -, and  $\kappa$ -caseins. In fact, the complete sequence for  $\alpha_{s1}$ -casein has been reported,<sup>147</sup> and the exact location of amino-acid substitutions and deletions is now known, as well as the monoester linkage of each phosphate group.<sup>84,147</sup> Similarly, the French workers have also determined the position of each amino acid in the primary structure of  $\beta$ -casein, and the amino-acid substitutions of several of the polymorphic forms of the protein.<sup>31,183,184</sup> The partial sequence of some of the  $\gamma$ -casein variants, as well as of the minor components of the  $\gamma$ -casein fraction, has also been reported,<sup>71</sup> and the possible relationship of these proteins to  $\beta$ -casein is discussed in the same report. Finally, the partial sequence of  $\kappa$ -casein has also been published.<sup>114</sup> The rennin-sensitive bond was shown to be Phe-Met.<sup>50,114</sup> It would seem that the sequences of all the major caseins will be known, and such information will be of great value in a rational approach to the study of the biological and physicochemical properties and industrial uses of milk and its protein components.

## WHEY PROTEINS

Prior to 1934, it was generally considered that whey protein consisted of two main components, named lactalbumin and lactoglobulin according to contemporary terminology, and comparatively small proportions of other protein-like substances. But in 1934, Palmer<sup>162</sup> reported the isolation from the lactalbumin fraction of a protein having the characteristics of a globulin. This protein, which comprises more than half the lactalbumin fraction, was at first called Palmer's globulin, but is now named  $\beta$ -lactoglobulin. This and several other proteins have been isolated from the classic fractions by methods to be described.

### Preparation of Whey Protein

The proteins of cheese whey, which include some casein in addition to globulins and albumins, may be precipitated together and recovered efficiently by procedures that require careful control of temperature and acidity.<sup>36,92,136</sup> If a product free from casein is desired, the whey should be from the acid precipitation of casein from skim milk, as previously described. The whey is put through a cream separator to remove as much fat as possible, its reaction adjusted to pH 6.3 to 6.5 by addition of 10*N* sodium hydroxide, and the whey heated to above 90°C with constant stirring to render the protein precipitable by acid. Then, 100 ml 33% acetic acid is added rapidly for each 100 lb whey and the stirring stopped as soon as the acid is thoroughly distributed. When the coagulum has collected, it is removed by filtration and, if desired, washed, drained, and dried. Solutions of other acids may be employed as precipitants in place of the acetic acid, the reaction being brought to pH 4.8 to 5.3. The whey protein prepared in this way is suitable for use in food products.

A soluble whey protein concentrate with excellent nutritional properties can be prepared following precipitation of the proteins by polymeric phosphate.<sup>228</sup> The product is available commercially.

Other methods for the preparation of whey proteins in both denatured and undenatured form, and in the form of coprecipitate with casein, have been proposed. Some of these have been described by Fox,<sup>62</sup> by Beeby *et al.*,<sup>14</sup> and by Muller.<sup>150</sup>

### Preparation of the Lactoglobulin and Lactalbumin Fractions

A method typical of older procedures used for the preparation of the lactoglobulin fraction is that of Rowland. In his scheme for the partition of milk proteins, Rowland<sup>189</sup> obtained precipitation of the lactoglobulin fraction as follows. Casein was removed from milk by means of acetic acid and sodium acetate, as described earlier in this chapter, the reaction of the filtrate was adjusted to pH 6.8 to 7.2, and the liquid was then saturated with magnesium sulfate to precipitate the lactoglobulin.

The method of Sjögren and Svedberg<sup>196</sup> for preparing the classic lactalbumin fraction is, similarly, illustrative of older procedures. It consisted of half-saturating skim milk with ammonium sulfate, adding acetic acid to give a pH value of 5.2, removing the precipitated casein and lactoglobulin fractions by filtration, and increasing the ammonium sulfate concentration to 80% of saturation. The resulting precipitate was removed by centrifuging. It was dissolved in water and re-

precipitated by adding ammonium sulfate and sulfuric acid. The precipitate was again dissolved in water, salts were removed by dialysis, and the protein was dried.

### **$\beta$ -Lactoglobulin**

$\beta$ -Lactoglobulin, a protein of molecular weight 36,000, occurs in milk to the extent of 3.0 g/l. In 1934, Palmer<sup>162</sup> discovered that a crystalline protein, insoluble in water, could be prepared from the classic lactalbumin fraction. The protein was named  $\beta$ -lactoglobulin and was shown to be the most abundant of the whey proteins. It was widely used in protein chemistry in research requiring a pure, crystalline protein. It was used for the first complete amino acid analysis of a fairly large protein; for the calculation of correlation between composition and titration curves of proteins with acids and bases; for the determination of the hydration of a protein crystal; for the investigation of the diffusion of electrolytes and nonelectrolytes into protein crystals; and for other important physicochemical studies. The extensive literature concerning the chemical and physical properties of  $\beta$ -lactoglobulin has been summarized.<sup>137,140,215</sup> A few pertinent data are given in Tables 3.1 and 3.2.

Palmer's original method for preparing  $\beta$ -lactoglobulin had as its starting point whey, which was obtained by adjusting skimmilk to pH 4.6 by adding hydrochloric acid and subsequently removing the precipitated casein. Crystalline  $\beta$ -lactoglobulin was obtained after fractionation of the whey with ammonium sulfate at pH 6.0 and dialysis at pH 5.2 of the precipitate obtained when the whey was saturated with ammonium sulfate. In 1957, Aschaffenburg and Drewry<sup>10</sup> prepared  $\beta$ -lactoglobulin in a somewhat different manner. Whey is obtained after precipitation of casein, globulins, other protein and fat by means of sodium sulfate (200 g/l).  $\alpha$ -Lactalbumin, serum albumin and other proteins are removed by acidification of the filtrate to pH 2.0. The  $\beta$ -lactoglobulin is salted out from the filtrate by adjusting the pH to 6.0 and adding ammonium sulfate (200 g/l). The protein is then crystallized by dialysis. Other methods reported<sup>5,7,63,185</sup> are variations of the general scheme of Aschaffenburg and Drewry. These methods have been compared and evaluated.<sup>137,140</sup> Modifications generally are related to whether  $\beta$ -lactoglobulin or  $\alpha$ -lactalbumin is the protein desired. Chromatographic methods<sup>4,175,180</sup> for the separation of whey proteins have also been utilized.

That even well-purified  $\beta$ -lactoglobulin, recrystallized many times, a single substance in both electrophoresis and ultracentrifugation,<sup>168</sup> was not truly homogeneous became apparent from the solubility studies

of Grönwall<sup>83</sup> and electrophoretic experiments of Li<sup>129</sup> and McMeekin *et al.*<sup>143</sup> Polis *et al.*<sup>178</sup> isolated a homogeneous, crystalline component,  $\beta_1$ -lactoglobulin, upon fractionation of crystalline  $\beta$ -lactoglobulin. An understanding of the heterogeneity came with the discovery by Aschaffenburg and Drewry<sup>8,9</sup> that individual cows produce either a mixture of two electrophoretically distinct  $\beta$ -lactoglobulins or only one or the other of these. The capacity to produce the different types was genetically controlled, and the two forms of the protein became known as  $\beta$ -lactoglobulins A and B.

At the present time 6 genetic variants of  $\beta$ -lactoglobulin are known. The C variant, found in Jersey cows, was discovered by Bell;<sup>15,16</sup> the D polymorph was shown to be present in Montbeliarde cattle in France.<sup>85</sup> The final two variants are identical with  $\beta$ -lactoglobulins A and B, but contain covalently bound carbohydrate.<sup>17,18,137</sup> These have so far been found only in Australian Droughtmaster beef cattle; hence the designation  $\beta$ -lactoglobulin A<sub>DR</sub> and B<sub>DR</sub>.<sup>188</sup> The distribution of the various  $\beta$ -lactoglobulins in different breeds is considered by McKenzie,<sup>140</sup> who has also listed the amino-acid compositions of the proteins and the differences in number of amino-acid residues per monomer. As an example, Gordon *et al.*<sup>69</sup> and Piez *et al.*<sup>175</sup> found that  $\beta$ -lactoglobulin A had one more residue of aspartic acid and valine and one less residue of glycine and alanine than  $\beta$ -lactoglobulin B in a total of 160 amino acids per monomer (18,500). The amino acids are substituted as aspartic acid ( $\beta$ -A)  $\rightarrow$  glycine ( $\beta$ -B) and valine ( $\beta$ -A)  $\rightarrow$  alanine ( $\beta$ -B).<sup>117</sup> A partial sequence for  $\beta$ -lactoglobulins A and B has been published.<sup>65</sup> Many investigations have been carried out on  $\beta$ -lactoglobulin concerning the location and reactivity of cystine, cysteine, tyrosine, and tryptophan residues.<sup>140</sup> In addition, much research has been reviewed<sup>137,140</sup> on its electrochemical properties, such as pH titration curves, isoionic points and ion binding, and electrophoresis; its solution properties, such as molecular size and conformation; the effect of heat, detergents and other denaturants; the interaction of the protein with  $\kappa$ -casein; and its X-ray crystallographic structure.

$\beta$ -Lactoglobulins have proved of great interest in protein chemistry because they are a family of simply prepared, crystalline proteins. Their interactions with  $\kappa$ -casein in milk are of technological importance, and these reactions may also be relevant to problems of allergenicity. In milk,  $\beta$ -lactoglobulin is the most abundant protein having a free sulfhydryl group in the form of a cysteine residue.

#### $\alpha$ -Lactalbumin

$\alpha$ -Lactalbumin, second in concentration to  $\beta$ -lactoglobulin among the

whey proteins, can also be crystallized from the lactalbumin fraction. The name was first used in connection with a "lactalbumin" isolated by Kekwick and with its behavior in the ultracentrifugal investigations of Svedberg and Pederson.<sup>206,207</sup> It is most likely that the same protein was crystallized by Sørensen and Sørensen<sup>200</sup> in 1939 and designated "crystalline insoluble substance" because of its insolubility in water at pH 4.6. A method for the isolation of the protein, based on the observations of Sørensen and Sørensen, was reported by Gordon and Semmett,<sup>72</sup> who also characterized the protein and proposed that it be known as  $\alpha$ -lactalbumin. The method was later modified<sup>75</sup> and described in detail.<sup>76</sup> In principle, the original Palmer method is used to remove casein and the lactoglobulin fraction from skim milk and to crystallize  $\beta$ -lactoglobulin from the lactalbumin fraction. The mother liquor from the crystallization is adjusted to pH 4 and ammonium sulfate is added to precipitate crude  $\alpha$ -lactalbumin. It is dissolved at pH 8, reprecipitated at pH 4, dissolved again, and crystallized by the addition of ammonium sulfate to  $\frac{2}{3}$  saturation at pH 6.6; it is recrystallized by repeating these steps. Other more convenient procedures which have been developed for the preparation of  $\alpha$ -lactalbumin were listed and described by Gordon.<sup>87</sup>

Table 3.1 shows some of the chemical properties of  $\alpha$ -lactalbumin and Table 3.2 its amino-acid composition. It is of interest that  $\alpha$ -lactalbumin contains no free sulfhydryl groups, though its content of cystine is high. It is also rich in tryptophan.  $\alpha$ -Lactalbumin occurs in two forms, genetic polymorphs A and B. In milks from Western breeds of dairy cattle the variant found is B, while in milks from African and Indian Zebu and Australian Droughtmaster cattle, both forms occur. Chemically, A differs from B by the simple substitution of glutamine for arginine.<sup>87</sup>

Until 1966  $\alpha$ -lactalbumin was considered to be a protein of good nutritive value but of little importance because of its low concentration in milk. However, in that year Ebner *et al.*<sup>54</sup> reported that an enzymatic role could be assigned to  $\alpha$ -lactalbumin, since it was identified as one of the two subunits of lactose synthetase. Beginning with this discovery, much research was devoted not only toward elucidation of the reactions involved in the biosynthesis of lactose, but also to the chemical structure of  $\alpha$ -lactalbumin and to the correlation of its structure with its biological function. Thus the complete amino-acid sequence of the protein was worked out by Brew *et al.*,<sup>29</sup> and the positions of the disulfide bonds were established by Vanaman *et al.*<sup>219</sup> Surprising similarities in primary structure and possibly in conformation of  $\alpha$ -lactalbumin and chicken egg white lysozyme were brought to light. Furthermore, it was found that the "A protein" subunit of lactose synthetase acts primar-

ily as a galactosyl transferase to produce N-acetyllactosamine; the function of  $\alpha$ -lactalbumin (the "B protein" subunit) is to modify the enzyme so as to increase greatly its activity as a lactose synthetase. Many of the developments in this very active field of research have been reviewed by Ebner,<sup>52,53</sup> Hill *et al.*<sup>98</sup> and Gordon.<sup>67</sup>

### Bovine Immunoglobulins

These proteins are present in ordinary milk in low concentration but they occur in colostrum—the milk secreted for a few days after parturition—in much larger percentages. They are of unique importance to the new-born calf because they are absorbed into its circulation, where they fulfill, temporarily, the immunological functions of blood gamma-globulin. The previous designation of immune globulins of milk as eu- and pseudo-globulins has now been superseded by a more general nomenclature.<sup>37, 188</sup> The early work of Smith *et al.*<sup>197, 198</sup> has also been reassessed in light of more recent developments in the field of immunology. These developments have been reported in a symposium on the bovine immune system.<sup>38</sup>

Three distinct classes of bovine immunoglobulins occur in milk; they are designated IgM ( $\gamma$ M), IgA ( $\gamma$ A), and IgG ( $\gamma$ G). IgG is subdivided into IgG1 and IgG2. IgG1 is selectively accumulated in the colostrum and milk of cows. IgM is a 19S protein with 12.3% carbohydrate which has been partially characterized after isolation from a fraction of whey insoluble in 33% ammonium sulfate.<sup>80</sup> The isolation of bovine IgA from milk has been reported by many investigators. It contains 8 to 9% carbohydrate, has a sedimentation coefficient of 10 to 12S, and can be eluted from Sephadex G-200 in the fractionation of whey. Glycoprotein-a<sup>88</sup> occurs free and bound to lacteal IgA. Hence, glycoprotein-a and lacteal IgA are probably homologous to the "secretory piece" and "secretory IgA," respectively, described for other species. IgG1 and IgG2 have about 2 to 4% carbohydrate and a sedimentation constant of about 7S. IgG2 molecules are somewhat more basic than IgG1 and are not retained on DEAE-cellulose columns at low ionic strength (pH 8.3), in contrast to IgG1 which is the more abundant protein in lacteal secretions. The two subclasses also differ in amino acid composition.<sup>88, 120, 148</sup>

The two subclasses have been correlated<sup>37</sup> with the early preparations of Smith. Smith's pseudoglobulin contains mostly IgG1 and also "secretory IgA." The euglobulin consists of IgG2-like globulins, slower IgG1 globulins, IgA and IgM. Amino-acid analyses of older  $\gamma$ -globulin

preparations are shown in Table 3.2. Butler has extensively reviewed the immunoglobulins of the cow.<sup>37,188</sup>

### Blood Serum Albumin

By repeated fractionations of the mother liquor remaining after crystallization of  $\beta$ -lactoglobulin from the crude lactalbumin fraction, Polis *et al.*<sup>177</sup> succeeded in isolating and crystallizing a true, water-soluble milk albumin. When this was compared with crystalline bovine serum albumin, it was found that the two are identical in physical properties and composition; some of these data are shown in Tables 3.1 and 3.2. It was demonstrated by Coulson and Stevens that the proteins are immunologically indistinguishable.<sup>46</sup>

### Other Milk Proteins

Table 3.1 makes reference to a proteose-peptone fraction, amounting to about 2 to 6% of the total protein present in milk. The fraction is operationally defined as that portion of the protein system that is not precipitated by heating at 95 to 100°C for 20 min and subsequent acidification to pH 4.7, but is precipitated by 12% (w/v) trichloroacetic acid.<sup>189</sup> Various workers have prepared substances of this nature from milk under such names as minor-protein fraction, sigma-proteose, and milk component 5, as well as proteose-peptone. The principal components of the proteose-peptone fraction have been designated milk-serum components "3," "5," and "8," in ascending order of mobility in moving-boundary electrophoresis.<sup>125</sup> More recently, the proteose-peptone fraction has been separated into four principal components designated "3," "5," and "8-slow," and "8-fast."<sup>121,153</sup> Each component is a glycoprotein and has been isolated from both heated and unheated skim milk. The properties of the proteose-peptone components have been summarized.<sup>188</sup>

The interphase between fat globules and milk plasma, the fat globule membrane, is of great interest in milk chemistry and has been the subject of several review articles.<sup>33,111,181</sup> The reader is also referred to Chapter 10 and to the review and discussion by Groves<sup>87</sup> of the proteins of the fat globule membrane. In addition, the same author<sup>87</sup> has considered the great variety of minor proteins and enzymes which occur in milk. Among these are lactoferrin (red protein), blood serum transferrin, lactollin, kininogen, M-1 glycoproteins; also nucleases, lactoperoxidase, xanthine oxidase, lipases, esterases, amylases, phosphatases, lysozyme and other enzymes. More refined methods of

isolation, fractionation, analysis, and characterization have stimulated interest in proteins occurring in almost trace amounts. This is true because many of these proteins are of great importance in catalyzing reactions of biological interest, in binding minerals and vitamins, and in affecting the stability and flavor of milk and milk products.

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