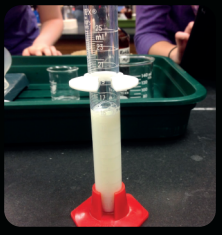
Principles of DAIRY CHEMISTRY

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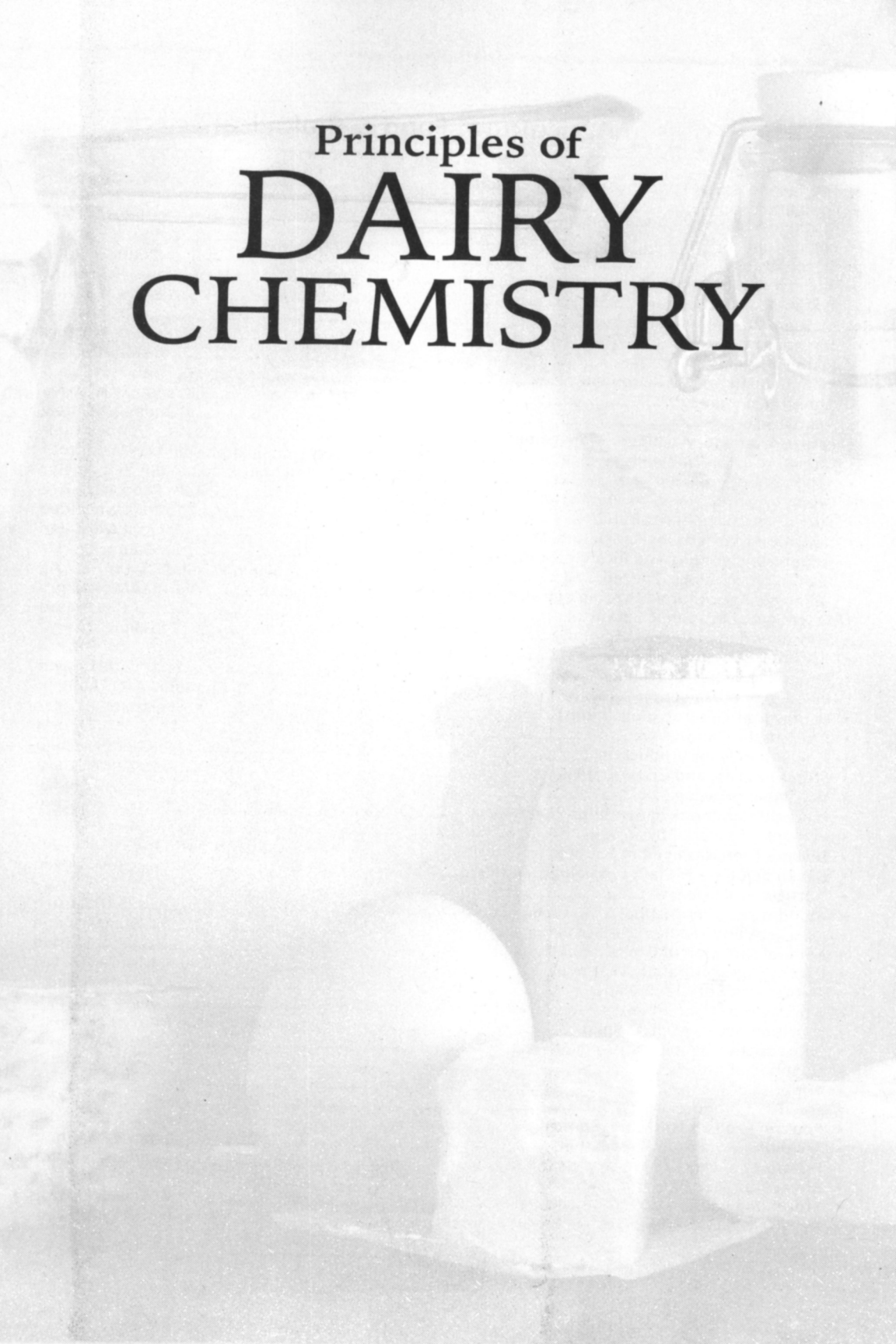

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Engaging Sciences—Developing Minds!

Principles of
DAIRY
CHEMISTRY

MEDTECH
A Division of
Scientific International Pvt. Ltd.

First Indian Reprint: 2018

ISBN: 978-93-86800-83-1

This book has been printed by an arrangement with Authors.

Published by: Vinod Kumar Jain, Scientific International (Pvt) Ltd.

Registered Office:
- 4850/24, Ansari Road,
  Daryaganj, **New Delhi**-110002

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- House No. 31, 1st Floor, KKB Road, Chenikuthi,
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Printed in India

PREFACE

Dairy technology is a broad domain that helps us to understand the significance of milk from agricultural and human health aspect. Marked improvement in the agriculture and dairy technology has made this scientific expansion possible. This edition has been updated to develop a body of knowledge that provides the biochemists, experts, researchers a book with solution allied with all the other aspects of the dairy technology.

The book commences with the composition of milk in a quantitative tabulation fashion. Unraveling the details of the composition of milk has presented chemists with an interesting and challenging problems. Milk lipids, proteins, salts and milk enzymes are represented in an exhaustive and concise manner. The special attention has been delivered in the consideration of physical properties of milk. This is followed by comprehensive description of physical chemistry of milk fat globules and caseinate-phosphates. The book also focuses on effect of heat and flavor and off-flavor in milk and also nutritive value of milk.

We acknowledge the fact that readers of this book belong to a broad spectrum from academic or professional scales to food technology experts, biochemists, and nutritionists. Therefore, we made enormous efforts to make the contents of this book as pleasant as possible for

broad range of readers. Researchers or academicians may use this book as a primary or reference material as a balanced literature of recent work in the dairy chemist for developing new models and practical applications. The organization of text in all the chapters is done with in-depth explanation of ideas and concepts. In this edition, we aim to serve a wide range of readers' groups with the intention of contributing towards technological advancement in the field of dairy chemistry.

The author has produced this book for the students who has had one year of inorganic and organic chemistry and a semester or quarter of biochemistry. At the same time, content has been adapted to keep serving the modern day requirements and its future possibility of dairy chemistry. Author is very grateful to those who have helped directly with this project and to many colleagues, associates, and friends in our own and other institutions who have shown interest and lent their encouragement.

Authors

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CHAPTER 1

THE COMPOSITION OF MILK

Interest in the composition of milk stems largely from its use as human food. Naturally, men have desired to assess the nutritive value of milk in terms of the individual nutrients that it contains. Furthermore, as the manufacture of various dairy products has developed as a means of extending the use of milk as food, problems have arisen which have demanded further knowledge of the composition and properties of milk.

Unraveling the details of the composition of milk has presented chemists with an interesting and challenging problem. Previous to 1850, milk had been found to contain fat, sugar, protein, and minerals. These classes had been subdivided into individual components to only a limited extent. Numerous studies in the past century have revealed the presence of a wide array of constituents in normal milk. Undoubtedly others await discovery. These constituents are present in the three physical states of solution, colloidal dispersion, and emulsion. Thus the chemistry of milk embraces a wide range of knowledge in physical, organic, and biochemistry.

This chapter presents a roughly quantitative tabulation of present knowledge of the composition of cow milk, a discussion of variations

in gross composition, and a summary of quantitative relationships among certain constituents.

THE CONSTITUENTS OF MILK

Table 1 gives a tabulation of individual constituents or groups of constituents that have been identified in normal cow milk as it is drawn from the udder. Some of these constituents actually have been isolated, others have been identified by specific tests. A few materials have

Table 1. APPROXIMATE CONCENTRATIONS OF THE CONSTITUENTS IN NORMAL COW MILK

Constituent or Group of Constituents	Approximate Concentration, weight per liter of milk
1. Water	860–880 g.
2. Lipides in emulsion phase	
a. Milk fat (a mixture of mixed triglycerides)	30–50 g.
b. Phospholipides (lecithins, cephalins, sphingomyelins, etc.)	0.30 g.
c. Cerebrosides	?
d. Sterols	0.10 g.
e. Carotenoids	0.10–0.60 mg.
f. Vitamin A	0.10–0.50 mg.
g. Vitamin D	0.4 μg.
h. Vitamin E	1.0 mg.
i. Vitamin K	trace
3. Proteins in colloidal dispersion	
a. Casein (α, β, γ fractions)	25 g.
b. β-lactoglobulin (s)	3 g.
c. α-lactalbumin	0.7 g.
d. Albumin probably identical to blood serum albumin	0.3 g.
e. Euglobulin	0.3 g.
f. Pseudoglobulin	0.3 g.
g. Other albumins and globulins	1.3 g.
h. Mucins (?)	?
i. Fat globule protein (?)	0.2 g.
j. Enzymes	?
1. Catalase	
2. Peroxidase	
3. Xanthine oxidase	
4. Phosphatases (acid and alkaline)	
5. Aldolase	
6. Amylases (α and β)	
7. Lipases and other esterases	

Table 1 (*Continued*)

Constituent or Group of Constituents	Approximate Concentration, weight per liter of milk
j. Enzymes (*Continued*)	
8. Proteases	
9. Carbonic anhydrase	
10. Salolase (?)	
4. Dissolved materials	
a. Carbohydrates	
1. Lactose (α and β)	45–50 g.
2. Glucose	50 mg.
3. Other sugars	traces
b. Inorganic and organic ions and salts	
1. Calcium*	1.25 g.
2. Magnesium*	0.10 g.
3. Sodium	0.50 g.
4. Potassium	1.50 g.
5. Phosphates* (as $PO_4^{=}$)	2.10 g.
6. Citrates* (as citric acid)	2.00 g.
7. Chloride	1.00 g.
8. Bicarbonate	0.20 g.
9. Sulfate	0.10 g.
10. Lactate (?)	0.02 g.
c. Water soluble vitamins	
1. Thiamine	0.4 mg.
2. Riboflavin	1.5 mg.
3. Niacin	0.2–1.2 mg.
4. Pyridoxine	0.7 mg.
5. Pantothenic acid	3.0 mg.
6. Biotin	50 μg.
7. Folic acid	1.0 μg.
8. Choline (total)	150 mg.
9. Vitamin B_{12}	7.0 μg.
10. Inositol	180 mg.
11. Ascorbic acid	20 mg.
d. Nitrogenous materials not proteins or vitamins (as *N*)	250 mg.
1. Ammonia (as *N*)	2–12 mg.
2. Amino acids (as *N*)	3.5 mg.
3. Urea (as *N*)	100 mg.
4. Creatine and creatinine (as *N*)	15 mg.
5. Methyl guanidine (?)	?
6. Uric acid	7 mg.
7. Adenine	
8. Guanine	
9. Hypoxanthine (?)	
10. Xanthine (?)	
11. Uracil-4-carboxylic acid (orotic acid)	50–100 mg.

Table 1 (*Continued*)

Constituent or Group of Constituents	Approximate Concentration, weight per liter of milk
d. Nitrogenous materials (*Continued*)	
12. Hippuric acid	30–60 mg.
13. Indican	0.3–2.0 mg.
14. Thiocyanate (?)	
e. Gases (milk exposed to air)	
1. Carbon dioxide	100 mg.
2. Oxygen	7.5 mg.
3. Nitrogen	15.0 mg.
f. Miscellaneous	
1. Esters of phosphoric acid not yet identified (as phosphorus)	0.10 g.
5. Trace elements (form of occurrence not elucidated)	
Usually present	
Rb, Li, Ba, Sr, Mn, Al, Zn, B, Cu, Fe, Co, I	
Occasionally present or questionable	
Pb, Mo, Cr, Ag, Sn, Ti, V, F, Si	

(?) Presence, identity, or concentration uncertain.
* Partly in colloidal dispersion.

been included in the table whose presence in milk is not entirely certain. Milk fat, lactose, the caseins, β-lactoglobulin, α-lactalbumin, and probably some of the other proteins are specifically characteristic of milk, being synthesized in the mammary gland. The other constituents, however, are not specific for milk, but are found in other biological sources. An extensive compilation of the composition of cow milk in comparison with that of human and goat milk has been made by Macy et al. (13).

Table 2. Distribution of the Elements Nitrogen, Phosphorus, and Sulfur among the Milk Constituents

Type of Compounds	Weight of Element per Liter of Milk		
	Nitrogen, g.	Phosphorus, g.	Sulfur, g.
1. Inorganic	0.01 (N_2)	0.70	0.04 ($SO_4^=$)
2. Water-soluble organic	0.25 (N.P.N.)	0.10 (ester)	0.02
3. Proteins			
a. Casein	4.00	0.20	0.20
b. Serum proteins	1.00	nil	0.10
4. Lipides	0.01	0.015	very low
Total	5.27	1.015	0.36

The important elements, nitrogen, phosphorus, and sulfur, occur in a number of different compounds in milk. In analytical work they are often partitioned among the four categories; inorganic, water-soluble organic, protein, and lipides. Table 2 gives the distribution of these elements among these four categories.

VARIATIONS IN THE GROSS COMPOSITION OF MILK

Milk is not a uniform article of commerce, but varies considerably in composition. The variations are caused for the most part by the interplay of a number of factors affecting the physiology of the cow, but some variation may result from treatments following milking. Since the extent of variation is appreciable, various analyses are necessary to insure that buying and selling are conducted on an equitable basis, and that minimum nutritive requirements as defined by legal standards are met. Furthermore, variations in the composition of milk must be compensated in processing operations such as the manufacture of cheddar cheese or of evaporated milk, where constant ratios among certain constituents are necessary in the finished product. The great practical and academic interest in the composition of milk has resulted in much research designed to elucidate the various factors responsible for the variations.

This section deals only with the variations in gross composition, that is, in the contents of water, fat, lactose, proteins, and ash (mineral constituents). Variations in fat content have been studied far more extensively than those in the other constituents because of the greater economic value that has been attached to the fat. The discussion will be confined for the most part to description of the variations that have been found to occur in the composition of commercial milk. The underlying physiological and biochemical mechanisms by which the various factors operate are not dealt with in detail. The reader is referred to various treatises and reviews for summaries of present knowledge on the biochemistry and physiology of milk secretion (8, 9, 18).

Variations Due to Analytical Methods

At the outset it must be pointed out that methods of analysis frequently influence the reported values for gross composition.

The water or total solids content of milk may be determined by

some gravimetric method involving evaporation of the water and weighing the residue. Such procedures are reasonably well standardized. On the other hand, total solids content frequently is calculated by one of various formulas relating it to fat content and specific gravity (see Chapter 8). The fact that several formulas have been devised attests to the difficulties that may be encountered in this procedure.

Fat is determined either volumetrically as in the Babcock or Gerber methods, or gravimetrically by extraction, evaporation of solvent, and weighing of the residue as in the Roese-Gottlieb or Mojonnier procedures. There are well-recognized systematic differences in results obtained by these two types of method. Most workers have found that the Babcock fat test is 0.05 to 0.10% higher than the Mojonnier result.

Proteins are almost universally determined by Kjeldahl analysis for nitrogen and multiplication of the nitrogen content by a factor representing $100/N$, where N is the percentage of nitrogen in the protein. The factor most commonly used for milk proteins is 6.38 (i.e., 100/15.65) but sometimes other factors have been employed. In reports of gross composition, protein is often calculated by multiplying total nitrogen by 6.38. Such values are about 5% higher than the true protein content since about 5% of the nitrogen of cow milk is in the form of non-protein constituents.

The content of mineral constituents in milk is usually measured by incinerating a sample and weighing the resulting ash. Since some of the salts are appreciably volatile, the amount of ash obtained will depend upon the temperature of ashing which must be closely controlled to obtain reproducible results. Furthermore, the ash does not truly represent the salt system present in the original milk because it includes some mineral elements originally present in the proteins, and it excludes organic ions such as citrate.

Lactose is usually measured by methods based on either its optical rotation or its reducing power. Lactose has sometimes been calculated by difference, that is, the difference between total solids and the sum of the contents of fat, protein, and ash. This procedure is not to be recommended for precise work. Lactose content calculated by difference is higher than the true value by the amount (about 0.2%) that "ash" is lower than "salts," and also by the amount of "minor" constituents not included in other categories. The student should be warned that some values reported for the lactose content of milk represent lactose monohydrate, others anhydrous lactose. It is not always possible to ascertain which is meant. Anhydrous lactose

= 0.95 × lactose monohydrate. Methods of determining lactose are discussed in Chapter 3.

It is obvious that some variation in gross composition of milk may be due to variation in the methods used for analysis. This factor must constantly be borne in mind when interpreting and comparing various sets of data. The Association of Official Agricultural Chemists has compiled and published standard methods suitable for these analyses (1).

The solid constituents of milk, other than fat, are often grouped as "solids-not-fat," the percentage of which is calculated as the difference between the total solids and fat contents. Variations in fat content would be reflected in variations in solids-not-fat content of the whole milk, even though the solids content of the fat-free plasma remained constant. Thus, in order to obtain a true picture of variations in solids-not-fat, they are best expressed as solids content of the fat-free plasma.

$$\text{Per cent solids in fat-free plasma} = \frac{\text{Per cent solids-not-fat in whole milk}}{100 - \text{per cent fat in whole milk}}$$

Overall Variation

Variation in the gross composition of milk is most appropriately expressed in terms of frequency distribution curves. Such curves are illustrated in Fig. 1, taken from data of Tocher (22), who analyzed 676 samples collected at random in Scotland during a period of about 14 months. Sampling in each district of the country was roughly proportioned to the dairy cow population. Each sample represented an entire single milking of one cow. These curves show the characteristic tendency for a rather broad variation in the fat content and a narrow variation in the content of ash. The curve for lactose shows the characteristic skewness toward the lower values while that for nitrogen exhibits a skewness toward the high values. These techniques of sampling and presentation of the data well describe the variability of milk within a geographical area over a definite period of time.

Inherited Variations

Variation among breeds. The several strains of dairy cattle known as breeds have, as a result of long-continued segregation and inbreeding, rather pronounced and characteristic differences in the

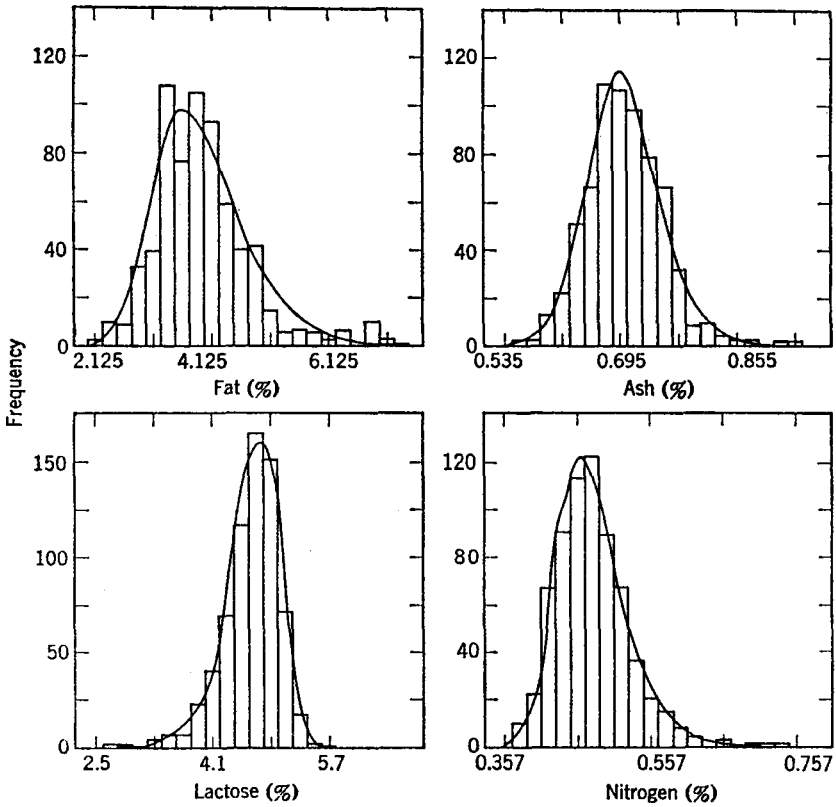

Fig. 1. Frequency distribution of fat, ash, lactose, and total nitrogen in 676 samples of Scottish milk. Data of Tocher (22).

composition of the milk that they produce. This is particularly true of fat content. Since advanced registry systems record fat tests, a wealth of information on variability of fat content is available. Figure 2 shows a compilation of such data in the form of frequency distribution curves made by Turner (24) from records of four of the major breeds in the United States. Each value used represented the average fat content of milk produced by a single cow during a lactation period. These distribution curves exhibit considerable overlapping, but also show the characteristic breed differences in the distribution. It will be noted that the extent of variability is greater among Guernseys and Jerseys than among Holsteins and Ayrshires. Data for the other constituents of milk are not so extensive as those for fat; however, available information indicates that the protein and lactose contents differ

among breeds in the same direction but to a smaller extent than fat content, whereas the ash content is relatively constant. Table 3 (p. 10), taken from the work of Overman et al. (14), illustrates this point.

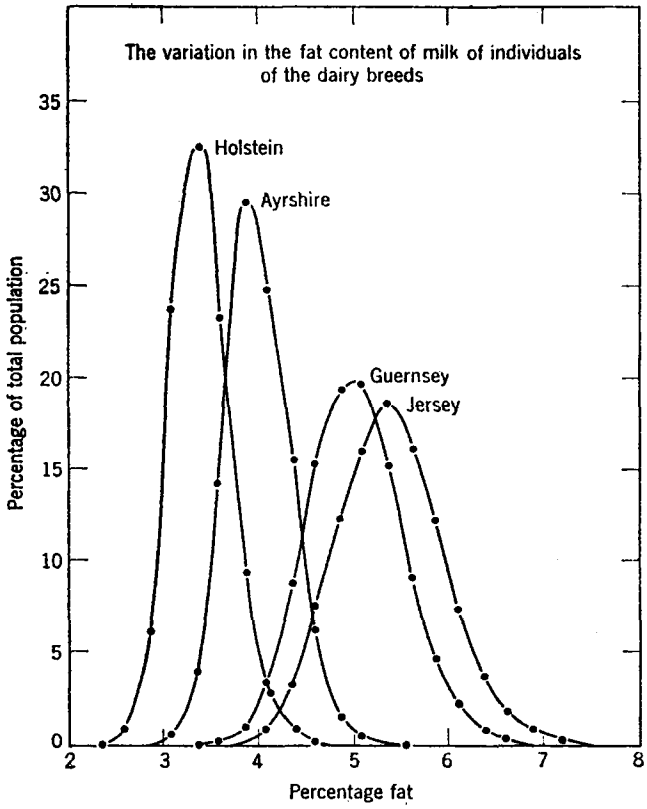

Fig. 2. Distribution of lactation average fat tests of milks of cows of four dairy breeds. Included are 2665 Ayrshire records averaging 4.03%, 36,861 Guernsey records averaging 5.05%, 26,773 Holstein records averaging 3.41%, and 31,297 Jersey records averaging 5.41%. Data of Turner (24).

Individual variations within a breed. The variability in the composition of milk among cows within a breed results partly from hereditary differences and partly from environmental factors. Discussion of the genetics of dairy cattle is outside the province of this book (21), but it is well known that the fat content of milk is an inherited characteristic. It seems definitely to be established that the fat content is inherited independently of milk producing ability. The mode

Table 3. COMPOSITION OF MILK OF VARIOUS BREEDS OF DAIRY CATTLE*

Breed	No. of Cows	No. of Samples	Fat		Protein†		Lactose‡		Ash		Total Solids	
			Mean	S.D.§	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
Holstein	19	268	3.55	0.57	3.42	0.51	4.86	0.27	0.68	0.04	12.50	1.04
Brown Swiss	17	428	4.01	0.60	3.61	0.53	5.04	0.39	0.73	0.05	13.41	0.95
Ayrshire	14	208	4.14	0.54	3.58	0.34	4.69	0.47	0.68	0.04	13.11	0.96
Jersey	15	199	5.18	0.80	3.86	0.43	4.94	0.36	0.70	0.04	14.69	1.11
Guernsey	16	321	5.19	0.71	4.02	0.46	4.91	0.30	0.74	0.05	14.87	1.10

* Data of Overman et al. (14).

† Total nitrogen multiplied by 6.38.

‡ Calculated by difference, i.e., total solids − (fat + protein + ash).

§ Standard deviation.

of inheritance evidently is not simple. It is controlled by a number of genetic factors. Until recently, very few studies of the heritability of the other constituents of milk had been made for the simple reason that suitable records had not been kept. Available data, particularly that with identical twins, indicates that the protein and lactose levels are genetically determined to some extent independently of the fat content. Since this is so, it is reasonable to expect that the solids-not-fat content of milk could be increased relative to the fat by selective breeding, if such were demanded by economic considerations. It appears that the quantity of each constituent synthesized by the mammary tissue (fat, proteins, and lactose) depends to some extent on separately inherited factors, and that the composition of milk merely represents the relation between the amount of each of these constituents and the total volume of milk that is produced. Heredity determines the capacity of an animal to produce a certain amount of milk of a certain composition. Environment and various physiological factors greatly influence the amount and composition of the milk that actually is produced.

In interpreting the effects of the physiological and environmental factors on milk composition, it is necessary to take cognizance of changes in milk yield that may occur. Thus, an increase in the percentage of fat may reflect a decreased milk yield, rather than an increased secretion of fat itself. The picture may be incomplete if only the percentage of a constituent is given without considering yield. The milk producer and the dairy physiologist are primarily interested in the weight of each constituent produced by a cow per unit of time. On the other hand, the commercial processor of milk is not especially

interested in such physiological considerations, but only in the percentage composition of milk as he receives it.

Nutrition of the Cow in Relation to the Composition of Milk

Plane of nutrition. Some study has been made of the effects on milk composition of altering the total energy intake of cows without changing the composition of the ration. In general, overfeeding above the level required for maintenance and maximum production causes no consistent change in the composition of milk. The major effect of overfeeding is to fatten the animal. Underfeeding, on the other hand, tends to reduce the yield of milk, and to deplete the animal's accumulated store of fat. In some cases the reduced yield may be accompanied by an increase in fat test, although the total secretion of fat is not generally greatly affected. Milk flow is much better maintained when underfeeding occurs early in the lactation. Cows often are not able to consume sufficient feed early in their lactation period to meet the demands of milk production and body maintenance. Such a condition may be described as "physiological underfeeding." It is accompanied by a marked increase in the fat percentage of the milk, especially when the cow is fat. The yield of milk fat actually is increased. Underfeeding has, in some cases, been observed to depress the level of solids-not-fat in milk to a small extent. Instances have been reported where both the protein and the lactose were decreased by underfeeding (20).

Specific composition of the ration. A great deal of work has been done in attempting to determine whether the specific composition of a ration adequate from the standpoint of energy influences the gross composition of milk. In particular, the possible effects of roughage, fat level, protein level, and minerals in the ration have been studied.

It has been well established that significant depressions in the fat content (of the order of 0.5%) of milk may occur with little change in milk yield when cows are fed rations low in roughage. The depression of fat content is not entirely consistent, being more pronounced in the early stages of lactation and depending on the type of carbohydrates in the concentrate (4). Such results suggest that rations low in roughage interfere with the usual synthetic processes occurring in the rumen, so that less fat is synthesized. Solids-not-fat content is not affected markedly or consistently by reducing roughage intake.

Milk fat is synthesized from carbohydrate on a diet devoid of fat, but small increases in both milk and fat yields may be obtained by

including fat in the ration. These increases tend to compensate each other, however, so that no consistent increase in fat tests actually occur when the fat content of the ration is raised. Feeding cod liver oil to cows in the amount of 6 to 8 ounces daily, for the purpose of enhancing the contents of vitamin A and D in the milk, depresses the fat content of the milk by about 25% without affecting milk yield. This effect is thought to be caused by highly unsaturated fatty acids in the cod liver oil, since it is not due to the unsaponifiable materials and since hydrogenation eliminates it. Menhaden oil produces a comparable effect, but shark and salmon oils do not contain the fat-depressing agent.

The protein content of the ration does not markedly affect the composition of milk, although some reports have indicated a small depression of the protein content of milk on low-protein rations.

The mineral content of the ration does not greatly affect the gross mineral composition of milk. Deficiencies of calcium or phosphate in the ration severe enough to limit the yield of milk do not alter the concentration of calcium and phosphate in milk. The cow withdraws these minerals from skeletal reserves to compensate for the deficiency in the ration. Conversely, supplementation with extra calcium or phosphate of rations already adequate in these minerals does not increase their concentration in milk. There have been many reports that pasture feeding increases the calcium, phosphate, and citrate in milk over the levels obtained on dry feed, but the whole subject is controversial. Likewise, it has been reported that feeding of materials high in calcium, phosphorus, and magnesium resulted in an increased fat percentage in milk; these claims have not been corroborated adequately.

It can be concluded that the cow has a strong tendency to produce milk of as nearly a constant composition as possible regardless of wide variations in the quantity and quality of food ingested. Nevertheless, variations in gross composition of the ration sometimes slightly influence the gross composition of commercial milk. On the other hand, the concentration of some of the minor constituents of milk is decidedly dependent on the feed. Vitamin A and carotene, iodine, and some of the trace metals may be cited as examples of such materials.

Seasonal Variations and the Effect of Temperature

Rather pronounced seasonal variations occur in the composition of milk, at least in the temperate regions. Obviously, samples of herd

milk or, better still, samples representing several herds must be used to determine seasonal variations in order to reduce to a minimum individual peculiarities and the effect of the stage of lactation. The fat content exhibits a pronounced seasonal trend, being higher in winter than in summer. The content of solids-not-fat displays the same tend-

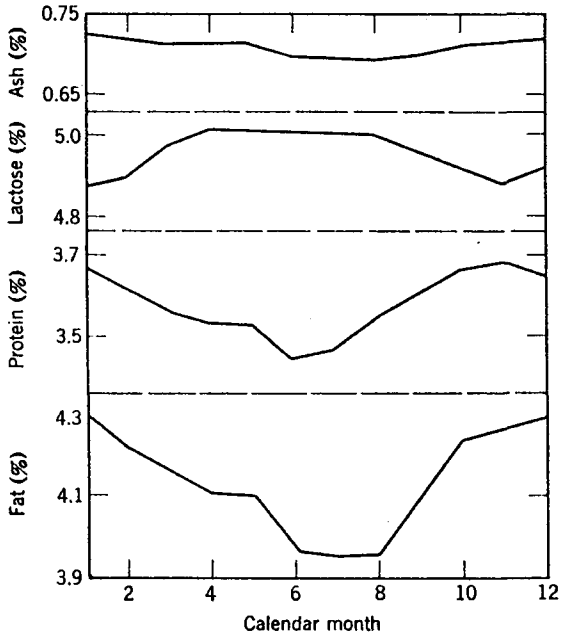

Fig. 3. Seasonal variation in fat, protein, lactose, and ash contents of milk. Data represent average values for 2426 three-day composite samples (208 Ayrshire, 428 Brown Swiss, 321 Guernsey, 268 Holstein, 199 Jersey, and 1002 Guernsey-Holstein crossbreds) collected at four- to five-week intervals during the lactation period. Averages weighted by milk production. Data of Overman (14).

ency, although to a lesser degree and with considerably more irregularity. Figures 3 and 4 give a typical picture of the nature and extent of these variations. Of the individual solids-not-fat, the protein and mineral contents are lower in summer than in winter, but the lactose content does not seem to follow a consistent seasonal trend. A slight tendency for calcium and phosphate to be lower in summer than in winter has been reported, whereas the chloride content may exhibit the opposite tendency.

Variations in environmental temperature have been suggested as being at least partially responsible for seasonal fluctuations. In sup-

port of this reasoning significant negative correlations have been demonstrated between temperature and the fat and solids-not-fat contents of milk produced in a given herd or delivered at a given plant. Much more conclusive evidence of the effect of temperature is the results of experiments in which cows were kept in rooms of controlled temperature, on a ration of constant composition. Such studies, notably those at the Missouri Agricultural Experiment Station (7), have

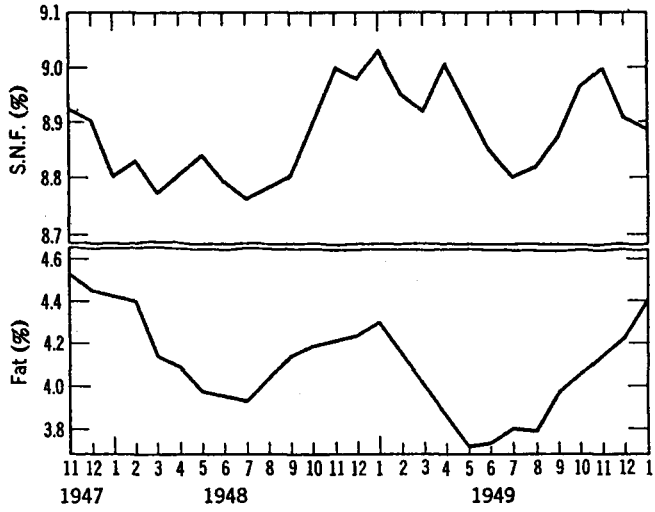

Fig. 4. Monthly variations in fat and solids-not-fat contents of California milk from November 1947 to January 1950. Data represent 20,694 samples collected from all areas of the state as it was delivered to creameries. Data of Jack et al. (10).

shown that variations in environmental temperatures from 30° to 75° F. do not materially influence the yield or the composition of milk produced by cattle of European ancestry. The principal change in this range is a decrease in fat content of a few tenths of 1% as the temperature is raised. Between 85° F. and 105° F., milk yield decreased, the fat content increased, solids-not-fat decreased, total nitrogen decreased, lactose decreased, and chloride increased. The fall in solids-not-fat content was more pronounced with Holsteins than with Guernseys. Low environmental temperatures from 40° down to 5° F. caused fat content to increase, solids-not-fat to increase, and total nitrogen to increase, but had no effect on chloride and lactose. Both high and low temperatures lowered the ratio of solids-not-fat to

fat. The degree of change varied considerably among individual cows. The maximum fluctuations in fat, solids-not-fat, and lactose that have been produced by varying temperatures are of the order of 1%. Chloride content has been doubled by high temperature. There seems to be no question as to the important influence of environmental temperature on milk composition.

Age of the Cow

The fat content of milk tends to decline with successive lactation periods. A negative correlation has been demonstrated between the age of cows and the average fat content for the lactation period. This effect is small, averaging not more than 0.2% fat over the entire productive lifetime of a cow. Furthermore, age is of little or no importance insofar as the fat content of commercial mixed milk is concerned, since its effect is so small and since herds include cows of varying ages. The percentage of solids-not-fat likewise decreases with successive lactation periods, but here again the effect is not of commercial significance.

Stage of Lactation

The composition of milk produced by a cow changes considerably with the progress of lactation, the greatest changes occurring at the beginning and at the end of the period. Colostrum, the initial secretion after parturition, differs from normal milk in that it contains more mineral salts (ash), total protein, casein, serum protein, and less lactose. Its fat content may be higher or lower than that of milk. Colostrum from different cows varies much more in composition than does milk. With successive milkings, the composition rapidly approaches that of milk, and the variability decreases. This fact is exemplified by the data presented in Figs. 5 and 6 taken from the work of Parrish et al. (17). The rate of change of the concentrations of some of the constituents (solids-not-fat, serum proteins, total protein) seems to proceed logarithmically with time, at least in the early stages. Of the individual mineral constituents, calcium, magnesium, phosphorus, and chloride are present in higher concentrations in colostrum than in milk, but potassium is present at a lower level. When cows are milked continuously right up to parturition, the composition of the milk is not greatly altered by parturition except that an increase occurs in the content of serum protein. The transition from colostrum

to a composition within the range of variation of normal milk is complete in about four days, the protein content being the slowest to complete the transition.

For legal and regulatory purposes, milk is often defined as the whole, fresh secretion obtained by the complete milking of one or more

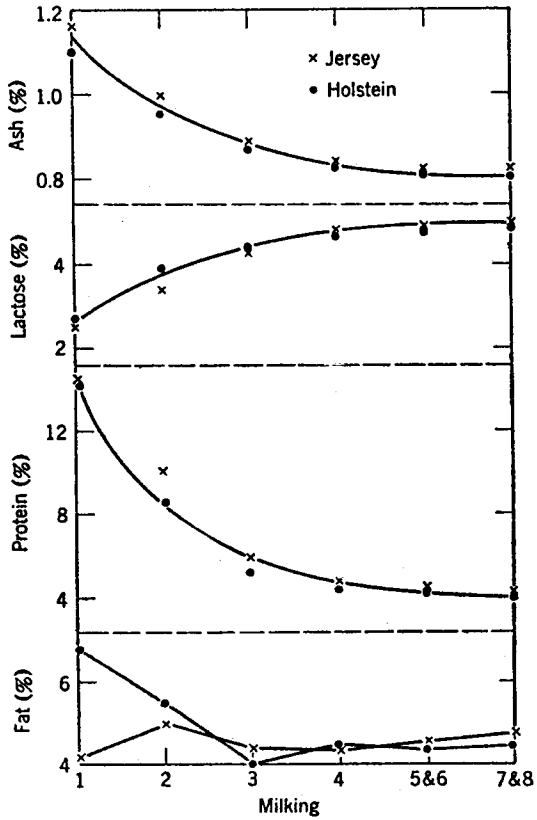

Fig. 5. Change in the composition of milk at successive milkings after parturition. Data represent averages for a number of cows. Data of Parrish et al. (17).

healthy cows, excluding that obtained within 15 days before and five days after calving or such longer period as may be necessary to render the milk practically colostrum free. Although the secretion is more or less "normal milk" within four days after parturition, the trends of the colostrum period continue, though at a reduced rate, for several weeks or months. The solids-not-fat, total protein, casein, serum protein,

ash, calcium, phosphorus, and chloride all exhibit similar tendencies to decline in concentration during the first few weeks of lactation. Following this, they remain constant or rise gradually until close to the end of the period, when a considerable increase occurs.

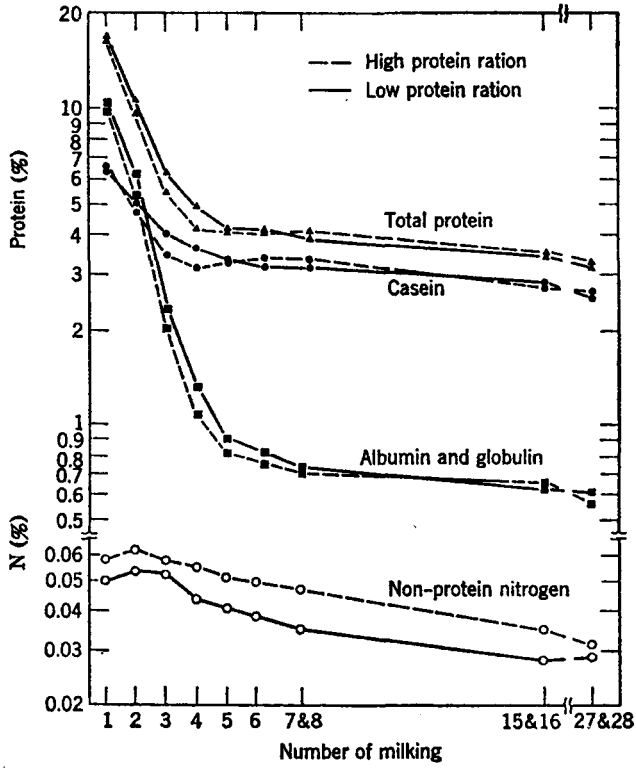

Fig. 6. Changes in the protein and non-protein nitrogenous constituents of milk at successive milkings after parturition. Data obtained from two groups of five cows each, on high and low protein rations respectively. Data of Parrish et al. (17).

The fat content tends to follow the pattern exhibited by the solids-not-fat, but the fat content of early lactation milk depends greatly on the condition of the cow at parturition. Cows in good condition at this time produce milk of relatively high fat content initially, particularly if they are somewhat underfed. The fat percentage then decreases for a month or more and this phase is followed by a period of gradual rise. On the other hand, cows that are thin at parturition produce low fat

milk at first and the fat percentage rises gradually with the progress of lactation. Average figures for large numbers of cows generally exhibit a decline in fat tests for a few months, followed by an increase. Fat content does not, as a rule, exhibit a sharp rise at the end of lactation. The lactose content is relatively constant throughout lactation, but declines slightly near the end of the period. The pattern of change generally exhibited by the fat, and of the solids, except lactose, is essentially the inverse of that followed by milk yield, which rises to a maximum in about a month and then declines gradually. Lactation patterns for fat, protein, and lactose are shown in Fig. 7.

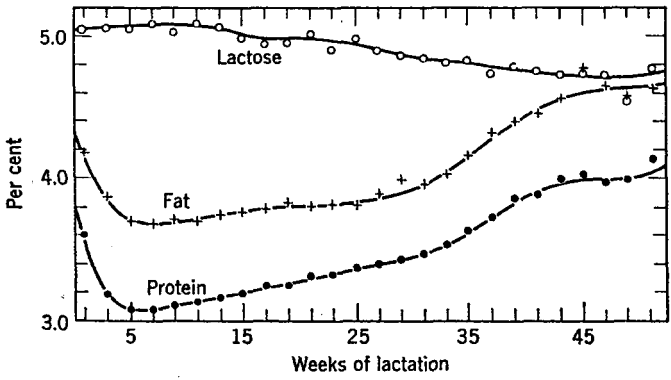

Fig. 7. Changes during lactation of the fat, protein, and lactose contents of milk. Data represent 2152 samples of milk taken biweekly and representing 58 cows. Lactose was calculated by difference. Data of Bonnier et al. (5).

It has often been stated that pregnancy is without effect on the changes in milk composition during the lactation period, but that it may exert an indirect influence by bringing lactation to an earlier termination. This idea is based on early experiments by Palmer and Eckles (16), in which the lactation patterns of four farrow cows for protein, fat, and lactose did not appear to differ decidedly from those of cows which were pregnant during the lactation period. Furthermore, breeding at various dates during lactation did not alter the pattern of ten cows appreciably. It seems possible, however, that this idea may need to be revised in the light of other studies. More recent work (5) has indicated that the increase in fat and solids-not-fat during lactation occurs only when the cow is pregnant.

Cows milked continuously for several years without calving con-

tinue to yield milk within the normal range of composition and properties.

Infection of the Udder

Udder infections greatly influence the composition of milk. The principal effects are lowering of the concentrations of fat, solids-not-fat, lactose, and casein and increasing the serum protein and chloride contents. The ability to synthesize casein, lactose, and fat is impaired, salts of blood pass into the milk to offset the osmotic pressure deficiency caused by lowered lactose level, and the tissue seems to become permeable to blood proteins. The net result is as though the infected tissue acted more like a simple filter than does healthy tissue. Infections severe enough to be detectable clinically result in milk which is outside the range of composition of normal milk. However, more interest centers in the effects on milk composition of subclinical infections. Considerable study has been made of the relation between incidence of infection as determined by bacteriological examination and the extent of alteration of the composition of milk. Unfortunately, the bacteriological diagnosis is complicated by the facts that many different kinds of organisms are capable of attacking the udder tissue, and that the mere presence of organisms in the milk does not necessarily signify extensive attack of the tissue. Formerly it was held that the principal causative organism of mastitic infections is the hemolytic streptococcus *S. agalactiae.* Consequently, many attempts were made to establish the existence of a relation between incidence of this organism and specific changes in milk composition. The objective of much of this work was to establish a means of diagnosing the subclinical mastitis by chemical analysis of the milk. At this point, another difficulty arises, the problem of deciding just where to draw the dividing line between normal and abnormal milk. The limits of variability in composition of normal milk have not, in most cases, been established adequately. Furthermore, when it is considered that the composition of so-called "normal" samples may have been influenced by undetected infections, the real magnitude of the problem becomes apparent. These difficulties must be taken into account in interpreting studies on milk from infected udders. The chemical changes that have been suggested and used in attempting to diagnose mastitis are catalase content, casein number, solids-not-fat content, chloride content, lactose content, and chloride-lactose ratio. Catalase is discussed in Chapter 6.

In normal milk, casein ordinarily accounts for 78% or more of the total protein. Some attempts have been made to use this as a criterion of normality. Low casein numbers in the milk of cows late in lactation do not necessarily signify infection.

Depression of solids-not-fat is likewise produced by infection as a result of the impairment in the synthesis of casein and lactose. However, solids-not-fat varies so greatly among normal samples that it is not an especially satisfactory criterion for infection.

The chloride content has been widely used in the studies of mastitis. The limits to be specified for separating normal from abnormal milk depend somewhat on the method used to determine chloride. Direct titration with silver nitrate yields higher results than the more accurate Volhard titration. A value of 0.14 or 0.15% is usually considered the limit of chloride as determined by direct titration, whereas 0.12% seems to be more nearly approximated when the Volhard method is used.

Udder infection lowers the lactose content which otherwise is rather constant. Lactose content alone has not been used to any extent as a criterion for distinguishing normal and abnormal milks, but the chloride-lactose number [(100 × per cent chloride)/per cent lactose] proposed by Koestler has received some attention. Since infection causes increase in chloride and decrease in lactose, their ratio changes proportionately more than either alone. The chloride-lactose number is generally 1.5 to 3.0 for normal milks, but increases markedly in mastitis cases.

Variation among the Quarters of the Udder

It is well known that the four quarters of the cow's udder are anatomically separate and distinct. They are also functionally separate, each producing milk independently of the others. A number of rather limited studies have been made of the composition of milk obtained by complete and separate milking of the four glands. These have dealt either with a single cow through a lactation period or a few cows for short periods of time. Interest has been primarily in yield and fat content, although in some cases other constituents were determined as well. All of these studies indicated that the milks obtained from individual glands of the udder may differ considerably in composition. Unfortunately, the results probably do not truly represent the composition of milk as secreted by the individual glands, because the quarters undoubtedly were milked in some sort of rotation. This procedure in

itself alters the composition of the milk of the quarters milked last as regards fat content. Only by simultaneous milking of the four quarters can a true representation be obtained of the fat content of the milks secreted by each. When all quarters are milked simultaneously, no tendency has been observed from any quarter to secrete milk richer in fat than the others. The differences are random rather than following any patterns. It is logical to conclude that variability in fat tests among quarters is due to accidental effects occurring during the growth, development, and lactation of the animal, which permanently or temporarily affect the secretory tissue. Bruises or other injuries may temporarily reduce the milk production in the quarter with a resulting increase in fat percentage. Infection by microorganisms greatly influences the composition of milk, and since infection in one quarter does not necessarily extend to the others, it may be an important cause of differences among quarters. Turner (23) has summarized information on the variability among quarters.

Variations Due to the Milking Procedure

The foregoing sections have dealt with the factors affecting the composition of milk as secreted. Attention now will be focused on variations in composition arising from peculiarities in the milking procedure. It is well known that the fat content of milk increases continuously during the milking process, the fore milk being very low and the strippings rich in fat. The solids-not-fat calculated as percentage of the fat-free plasma does not change during the milking process.

The fat globules appear to be partially trapped in the alveoli so that their passage into the milk is hindered. The magnitude of the effect differs greatly from cow to cow; it is much more pronounced in high-producing cows. The effect has been demonstrated admirably by Johansson (12) with a special apparatus which made it possible to measure milk flow and, at the same time, to take samples continuously during the process of milking. This work showed a continuous increase in fat content during the process of milking.

It follows from these considerations that at an incomplete milking the fat content of the milk will be lower than normal, but if the udder is subsequently milked normally the fat content will be higher than normal. Johansson verified this and also found that if incomplete milking is practiced for several successive days, the fat percentage is markedly decreased only on the first day of incomplete milking.

The interval between milkings influences the fat content of milk,

but has no effect on the solids-not-fat. When the intervals are unequal, the milk yield is greater and the fat content is lower following the longer interval. Since, under usual management practices, the night interval is longer than the day, milk obtained at the morning milking contains less fat than evening milk. Some tendency has been observed for morning milk to be lower in fat content than evening milk, even when the intervals between milkings are equal, but this effect does not seem to be entirely substantiated.

The variations due to milking procedure undoubtedly account for some of the variations in fat content of an individual patron's milk as delivered at the dairy plant.

Unusual excitement of the cow at milking time may impede the "let-down" of milk and thus alter the fat content of the milk obtained.

QUANTITATIVE RELATIONS AMONG THE MILK CONSTITUENTS

The existence of general quantitative relations among the constituents of milk is of both physiological and commercial interest. If, for example, two constituents occur in a constant ratio, the implication is that a connection may exist between the mechanisms by which they are secreted. On the practical side, general relations have been sought as a means of calculating difficultly determined constituents or properties from more readily determined ones. In this section, a few important relations are discussed.

Fat, Energy, and Milk Yield

The calorific value of milk is important as a measure of: (*a*) the human food value of the milk; (*b*) the amount of feed required by the cow for lactation; and (*c*) the productivity of the cow in connection with studies of the inheritance of milk production.

Direct calorimetric measurements of milk energy have seldom been made. The tendency has been to establish formulas for calculating milk energy from analytical data. The calorific value can be calculated from the fat, protein and lactose contents employing standard values for the heats of combustion of each (i.e., 9.11, 5.86, and 3.95 kilocalories per gram respectively). Of course, calorific values of milk or its constituents determined in a calorimeter are not exactly equivalent to their energy value in the animal body because the con-

stituents are not oxidized completely in animal metabolism. A relation between energy and fat content would be useful since fat is readily and routinely determined. In the few studies in which direct calorimetric measurements have been made, very high correlations have been obtained between fat content and energy. These are to be expected since fat itself is a heavy contributor to energy content and is highly correlated with protein, one of the other energy yielding solids. The high correlation coefficients suggest the possibility of setting up an equation to calculate energy from fat content with a high degree of accuracy. However, it must first be established whether a single equation describes the relation between fat and energy for all groups of samples, or whether different equations must be employed for various groups. There has been some question as to whether the relation between fat and energy is entirely linear, and some disagreement as to the amount of change in energy per unit change in fat. But, in general, the equation of Overman and Sanmann (15), $E = 115.33(2.51 + F)$, expresses the relation with reasonable accuracy.

Fat, Protein, and Energy

A rather high positive correlation exists between the fat and protein contents of milk. Most workers deal with this relation as linear, although not all would agree that it is linear. Treating the relation as being linear, Overman and co-workers (14) calculated a general regression equation of $P = 1.93 + 0.42F$ for 2426 samples representing several breeds. When each breed was considered separately, however, different equations were obtained for each. This would indicate that the relation between fat and protein is determined, to some extent at least, by separately inherited factors. As might be expected, protein content and milk energy are positively correlated, but the relation is not so close as between fat and energy, because protein is a smaller contributor to energy than is fat.

Fat and Lactose

Fat and lactose contents of milk are virtually independent. The lactose content is practically constant regardless of the fat content. Such trend as there is seems to be negative; low lactose contents tend to be associated with higher fat percentages. However, the correlation coefficients are small and the trend is of no practical importance.

Fat and Solids-Not-Fat

The relation between the contents of fat and solids-not-fat in milk is of paramount importance in the establishment of plans for pricing milk which attempt to take the solids-not-fat into consideration. If a constant relation existed between these two variables for all samples of milk, it would be a simple matter to establish a pricing formula, giving due recognition for the value of the solids-not-fat and to apply this formula by determining the fat content only. With the increasing emphasis on the nutritional importance of the solids-not-fat in recent years, it is natural that attempts should be made to reflect their value in the price of milk, and that the question of the relation between fat content and solids-not-fat content should come to the fore. Another reason for interest in the relation between fat and solids-not-fat is that legal standards for composition of milk usually specify a minimum level of fat and solids-not-fat. In some cases the legal minimum for solids-not-fat content is higher than the solids-not-fat content of actual milk of the minimum fat content. Present legal requirements of the various states are summarized in Table 4 (p. 26). Many of these requirements specify a minimum solids-not-fat content of 8.5% and a minimum fat content of 3 to 3.25%. Great Britain also has a legal standard of 3% fat and 8.5% solids-not-fat. Extensive surveys of the fat and solids-not-fat contents of commercial milk involving samples of known origin have been made by Jacobson (11) on over 100,000 samples of New England milk, and by Jack et al. (10) on over 20,000 samples of California milk. The overall relations computed by these workers were found to be linear. They are given in Fig. 8, along with a curvilinear relation from data compiled by a committee of the American Dairy Science Association (2). A glance at this figure indicates plainly that milk of 3 to 3.25% fat contains on the average less than 8.5% solids-not-fat. Such milk can be brought up to the legal minimum only by blending with richer milk or by adding condensed or dry skimmilk. The latter process is not legal. The minimum standard of 3.25% fat and 8% solids-not-fat, recommended by the U. S. Public Health Service, more nearly conforms to actual milk.

The positive correlation between fat and solids-not-fat is expected since protein content is positively correlated with fat content and lactose is relatively constant. The relations shown in Fig. 8 are averages representing many samples. However, Jack et al. (10), as well as Richardson and Folger (19), in a review of the whole question emphasize that different groups of samples exhibit different relations between

fat and solids-not-fat. The discrepancies are due, at least in part, to inheritance, udder infection, and feed.

It has been pointed out previously that fat content and solids-not-fat content are not perfectly correlated with each other; to some extent they are determined by separately inherited factors. Consequently, it

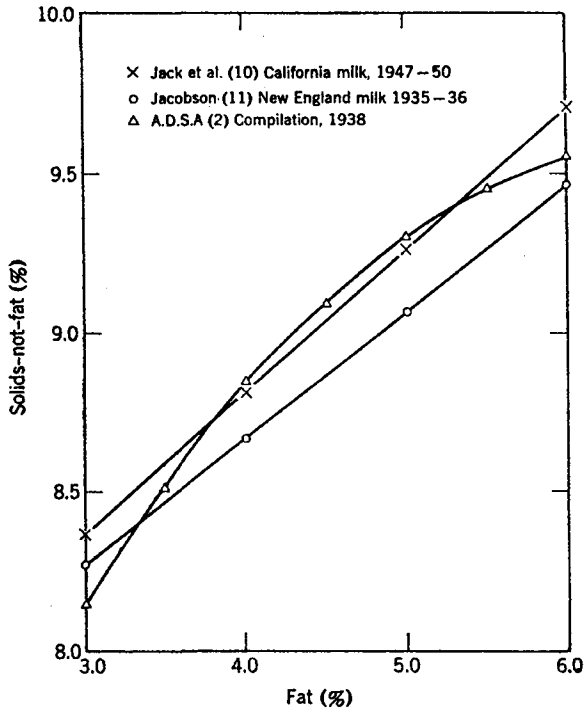

Fig. 8. Average relation between fat and solids-not-fat contents from three sets of data.

is reasonable to expect that the different relations between the two would be obtained when different cow populations are examined. Certainly the relation differs among breeds of cow. For example, the overall relation found by Jack et al. (10) and shown in Fig. 8 is: Solids-not-fat (S.N.F.) = 7.07 + 0.444F, but for Holstein and Jersey samples in the same study, the equations were respectively:

Holstein	S.N.F. = 6.60 + 0.554F
Jersey	S.N.F. = 8.09 + 0.253F

Similar breed differences were found by Overman et al. (14) in Illinois.

Table 4. MINIMUM LEGAL STANDARDS OF THE SEVERAL STATES FOR FAT, NON-FAT MILK SOLIDS, AND TOTAL MILK SOLIDS OF MILK

State	Milk Fat, %	Non-fat Milk Solids, %	Total Milk Solids, %
Federal	...	...	...
Alabama	3.25	8.5	11.75
Alaska	3.25	8	11.25
Arizona	3.25	8	11.25
Arkansas	3.25	8	11.25
California	3.5	8.15	...
Colorado	3.2	...	...
Connecticut	3.25	8.5	11.75
Delaware	3.5	8.5	...
District of Columbia	3.5	...	11.5
Florida	3.25	8.5	...
Georgia	3.25	8.5	11.75
Hawaii	3	8.5	...
Idaho	3.2	8	11
Illinois	3	8.5	11.5
Indiana	3.25	8	...
Iowa	3	8.5	11.5
Kansas	3.25	...	...
Kentucky	3.25	8	...
Louisiana	3.5	8.5	12
Maine	3.25	8.5	11.75
Maryland	3.5	...	12
Massachusetts	3.35	...	12
Michigan	3	8.5	...
Minnesota	3.25	...	...
Mississippi	3	8.5	11.75
Missouri	3.25	8	...
Montana	3.25	8	11.25
Nebraska	3	...	...
Nevada	3.25	8.5	11.75
New Hampshire	3.35	...	11.85
New Jersey	3	...	11.5
New Mexico	3.25	8.5	11.75
New York	3	...	11.5
North Carolina	3.25	8.5	11.75
North Dakota	3.25	...	11.5
Ohio	3	...	12
Oklahoma	3.25	8	11.25
Oregon	3.2	8.5	11.7
Pennsylvania	3.25	...	12

Table 4 (*Continued*)

State	Milk Fat, %	Non-fat Milk Solids, %	Total Milk Solids, %
Puerto Rico	3	9	12
Rhode Island	3.25	...	12
South Carolina	3.8	8	11.25
South Dakota	3.25	8.5	...
Tennessee	3.5	8.5	12
Texas	3.25	8	...
Utah	3.2	8.3	11.5
Vermont	3.25	8.5	11.75
Virginia	3.25	8.5	11.75
Washington	3.25	8	...
West Virginia	3.5	8	11.5
Wisconsin	3	8.25	...
Wyoming	3.25	8.25	11.5

From *U.S.D.A. Agriculture Handbook* 51, 1953 (3).

Udder infection profoundly influences the composition of milk; it is not known to what extent infection has contributed to the differences in relations between fat and solids-not-fat found by various workers in the past. Recent studies indicate the possibility that feed may influence the relations.

Although general relations such as shown in Fig. 8 can be calculated from large numbers of samples, they will not necessarily give a correct calculation of the solids-not-fat content of a given patron's milk from its fat content. The derivation and applicability of equations designed to calculate solids-not-fat and total solids of milk from the fat content and specific gravity are discussed in Chapter 8.

Lactose and Chloride

The complementary relation between lactose and chloride has long been recognized. It arises from the tendency of the mammary gland to maintain milk isotonic with blood regardless of the extent of synthesis of lactose. Thus, if the lactose synthesizing ability is impaired, the osmotic deficiency is compensated by an increase in chloride (and sodium). Such increases in sodium and chloride occur, as has been noted, in cases of udder infection and in late lactation. Various workers have calculated linear regressions between lactose and chloride contents, but there is considerable discrepancy among the various

groups of samples in the exact relation found. Several indices based on the lactose-chloride relation have been suggested as a means of detecting abnormal or adulterated milk. The chloride-lactose number as suggested by Koestler was discussed in connection with detection of milk from infected udders. This ratio generally has a value of 1.5 to 3.0 for normal milks but increases markedly when the chloride increases and the lactose decreases in mastitis or in late lactation.

As progress is made in our understanding of the genetics and mechanics of milk secretion, many of the variations in milk secretion that seem anomalous at present will be clarified.

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CHAPTER 2

MILK LIPIDES

INTRODUCTION

One of the most important constituents of milk is the lipide material. In order to orient the student on the practical implications of the chemistry of milk lipides, it is desirable to give a brief picture of the significant roles which they play in milk and milk products. These roles fall primarily into four main categories, namely, economics, nutrition, flavor, and physical properties.

The milk lipides or milk fat* have always had an important bearing on the economics of milk and milk products. In fact, it has not been until the last decade that the true worth of the non-fat milk solids has been recognized. Prior to that time, most price plans for milk were based largely, if not solely, on the milk fat content. Disposal of skim-milk generally was a problem and often the best fate it could expect was to be used as animal feed. Although this picture has changed considerably in recent years, milk fat still plays a very significant role in determining the price of milk both with regard to base price and differential for milk fat content exceeding the base price arrangement.

* The terms *lipide*(*s*) and *fat*(*s*) are used more or less interchangeably throughout this text.

Since milk fat is still relatively expensive compared to the other milk constituents, it is obvious that the cost of dairy products and food products that contain milk solids will depend to a considerable extent on the amount of milk fat which they contain.

Although the nutritive value of milk fat is a substantial subject in itself, we merely wish to touch upon the generally acknowledged part that this material may have in nutrition. First, as with other fats, it serves as a rich source of energy, yielding approximately 9 kilocalories per gram. Second, it serves as a carrier of the fat soluble vitamins A, D, E, and K. Third, milk fat contains significant amounts of so-called essential fatty acids (linoleic and arachidonic).

The most distinctive role which the milk lipides play in dairy products concerns flavor. The rich pleasing flavor of milk lipides is not adequately duplicated by any other type of fat. For this reason, milk fat in the form of butter, ice cream, coffee, and whipping cream has stood up remarkably well under the competitive onslaught of cheaper fats. It is also notable from a practical standpoint that the milk lipides are significant in many of the flavor problems which arise with dairy products (see Chapter 12). The subject of body and texture in dairy products is closely related to flavor so far as consumer acceptability is concerned. Undoubtedly the fine body and texture in most dairy products is one factor which makes them so appealing to most consumers. This characteristic in many instances is determined primarily by the milk lipides. They impart soft, smooth, and rich tasting qualities and overcome flat, hard, grainy, or watery characteristics which are normally encountered in their absence.

Thus it is clear that the milk lipides are of diverse practical importance. Moreover, it is evident that the contributions of this material in dairy products are interrelated. The fine flavor and physical properties which milk lipides impart to dairy products, and to food products in general, are important factors in the economics of milk. In addition, since milk lipides undoubtedly enhance the consumer acceptability of foods, they also serve the best interests of human nutrition through the incentive of eating what tastes good.

Terminology and definitions. There is some disagreement regarding terminology in the field of lipides and, to some extent, the confusion has been compounded by special terms employed in the dairy field. For the sake of clarity, it is necessary to define some of these terms. In the broad sense, lipides are defined as esters of fatty acids and related compounds. Other terms (lipoids, lipins, and fat) are similarly employed and have essentially the same meaning. The principal dis-

tinguishing characteristic of the lipides as food constituents is their solubility in organic solvents, particularly ethyl and petroleum ethers, and their insolubility in water. Generally the lipides are classified as simple, compound, or derived. The simple lipides are esters of fatty acids with various alcohols and as a category are composed of the fats, which are esters with glycerol, and the waxes, which are esters with long chain alcohols. Compound lipides are similar to the simple lipides with the exception that they contain other components in addition to alcohols and acids. In this class are found the various phospho- and glycolipides. The derived lipides are substances that are obtained from the simple and compound lipides and that have the general characteristics of lipides. Included in this category are the fatty acids, alcohols, hydrocarbons, and nitrogenous bases.

One factor of confusion in the field of lipides stems from use of the terms *fats* and *oils*. The main inference of these two terms is that some naturally occurring lipides are solids (fats) at room temperature and others are liquids (oils). This is a relative matter at best and most fats are plastic masses, whose degree of hardness or softness depends upon variable amounts of low melting constituents. Moreover, depending upon the temperature, any fat can be an oil and vice versa. The term *oil* introduces some additional implications in that there are oils originating from petroleum, such as mineral, fuel, and lubricating oils, as well as essential oils which are important in the field of flavor and perfumery. Although there is some relation between the lipides and the petroleum oils, based on solubility characteristics and the presence of trace amounts of hydrocarbons in some lipides, the subjects are distinctly different fields of study. The same is true of the lipides and essential oils. The classic test for differentiating these latter two groups of materials concerns their volatility, the lipides being capable of leaving a permanent grease spot on paper, whereas the essential oils, such as oil of orange or oil of rose, will evaporate in time, leaving no such spot.

In the field of Dairy Science, a number of terms relating to the milk lipides are used. These include *butterfat, butteroil, milk fat, dry milk fat,* and *anhydrous milk fat,* to mention a few. For the most part, such names are used interchangeably, a practice which is frequently confusing. For example, analyses of milk and milk products for fat content are ordinarily called "butterfat" tests even though the relation in this instance with butter may be distant. Use of the term butterfat is so deeply ingrained in the dairy field that there can be little hope of effecting a change in usage for some time to come. The designation

"milk fat" or "milk lipide" appears basic and well recommended for use in connection with milk and its products. The primary origin of all the lipides in pure dairy products is milk. However, there appears to be some merit in defining milk lipides in terms of the product from which it is derived since such definition implies a certain history for such lipides. For example, the terms *cheese fat* or *butter fat* might well be used for the lipide material recovered from these products. Use of the two terms dry milk fat and anhydrous milk fat interchangeably should be avoided. Dry milk fat is better reserved for the lipide material recovered from dry milk, whereas anhydrous milk fat should be used in describing the lipide material recovered from fresh fluid milk (or cream). Butteroil is a commercial designation which is applied to milk lipides containing 0.5% or less of moisture. The material does not always come from butter nor is it necessarily an oil. Use of the word butteroil seems to be giving way gradually to the more appropriate anhydrous milk fat.

As is true of most substances of biological origin, milk lipides are complex in composition and unique in character. In the present chapter an effort will be made to treat the following aspects of the subject: the composition of milk fat, physical and chemical properties of milk lipide components, and synthesis of milk lipides in the mammary gland. No effort will be made to treat the various quantitative methods for measuring the lipide content of milk and milk products since these are adequately treated elsewhere (20).

COMPOSITION OF MILK FAT

An adequate understanding of milk lipides requires some knowledge of their physical state in milk. The bulk of the fat in milk exists in the form of small globules, which average approximately 2 to 5μ in size. This is an oil in water-type emulsion. The surface of these fat globules is coated with an adsorbed layer of material commonly known as the fat globule membrane. This membrane contains phospholipides and proteins in the form of a complex. In addition to stabilizing the emulsion of the fat in milk and preserving the individual identities of the globules, the phospholipide-protein complex is important in a number of processes and problems of milk and milk products. These considerations, as well as details of the physical chemistry of milk fat globules, are considered in Chapter 9. Small quantities of lipides also occur in the milk serum, presumably in combination with protein.

Table 1. THE LIPIDES OF MILK

Constituent	Range of Occurrence	Location in Milk
Triglycerides	98–99%*	Fat globules
Phospholipides (lecithin, cephalin, sphingomyelin)	0.2–1.0%*†	Globule membrane and serum
Sterols (cholesterol, lanosterol)	0.25–0.40%*	Fat globules, globule membrane and milk serum
Free fatty acids (various)	Traces	Fat globules and milk serum
Waxes	Traces	Fat globules
Squalene	Traces	Fat globules
Fat-soluble vitamins	Traces	Fat globules
Vitamin A	7.0–8.5 μg./g. fat	
Carotenoids	8.0–10.0 μg./g. fat	
Vitamin E (tochopherols)	2–50 μg./g. fat	
Vitamin D	Trace	
Vitamin K	Trace	

* Based on total weight of the lipides.

† Calculated as lecithin on the basis of lipide phosphorus.

Thus, milk lipides are found in three distinctly different phases of milk; namely, the fat globules, the membrane surrounding these globules, and the milk serum. Table 1 presents the various lipide materials in milk, their range of occurrence, and their disposition in milk with respect to the three categories mentioned.

In order that the data in Table 1 are not misconstrued, it should be emphasized that some of the materials listed as constituents actually represent groups of compounds. For example, there are several classes of phospholipides in milk. There are several sterols in milk, the principal one being cholesterol. Many of the trace lipides have never been measured quantitatively, or analyses for them have been so limited that stating reliable ranges of concentration is not possible at present.

SAPONIFIABLE MATTER

One convenient method of classifying lipide material is on the basis of saponification, that is, whether or not it can be hydrolyzed with

base. Since in most fats the greater portion is made up of glyceride esters, the saponifiable material is large relative to the unsaponifiable matter. In addition to the triglycerides the saponifiable fraction includes the phospholipides and other esters, such as those of cholesterol. Although not saponifiable in the classic sense, free fatty acids bind base and thus are disposed with the saponifiable matter.

Triglycerides and their component acids. As mentioned in the section dealing with the physical and chemical properties of lipides, no entirely suitable procedures for isolating in pure form and characterizing the triglycerides of a fat are available. As a consequence, the composition of triglycerides in milk fat must be discussed largely in terms of the component fatty acids. The triglycerides of milk fat probably contain a greater variety of fatty acids than any other natural fat. Table 2 presents quantitative data for the major component fatty acids of milk fat as determined by several investigators and compiled by Jack and Smith (12). The data in this table were secured by ester fractionation techniques which generally have improved in the course of time. In addition to the major acids shown in Table 2, milk fat triglycerides also contain significant quantities of unsaturated fatty acids with more than one double bond. A compilation of values for these acids made by Smith and Jack is presented in Table 3.

Minor (trace) acid components. As analytical tools have become more refined, and as the knowledge of the chemistry of fatty acids has increased, evidence for the presence of many additional milk fat acids in relatively trace amounts is being secured. These acids embrace not only ones with odd numbers of carbons and branched chains but acids which are isomeric with regard to position and configuration (*cis-trans*) of double bonds. In many instances the findings regarding such acids are at present isolated observations; moreover, the methods of isolation and measurement have been developed only recently. The information which follows should be considered as somewhat tentative until more extensive studies have been made.

Trace amounts of acetic acid recently have been detected in milk fat. New Zealand workers have isolated branched chain fatty acids from milk fat containing 14, 15, 17, and 20 carbon atoms. They also have demonstrated the presence of the 11 and 15 carbon straight chain fatty acids (12). Bosworth and Helz (3) have presented evidence of a monohydroxypalmitic acid in milk fat. Their observations appear to be the only direct evidence of hydroxy fatty acid in milk fat although there is some indirect evidence from other investigations (12). The C_{10} to C_{18} monounsaturated fatty acids shown in Table 2 have the

Table 2. MAJOR FATTY ACID COMPONENTS OF MILK FAT*

	Component Acids—Mole %					
	Iodine Value 27.99	Iodine Value 37.5	Iodine Value 46.9	Iodine Value 36.6	Iodine Value 39.6	Iodine Value 32.42
	(Calculated from weight, %)					
Saturated						
C_4	8.7	8.1	10.2	9.7	10.5	9.2
C_6	2.8	2.8	2.5	1.2	3.9	2.8
C_8	1.7	2.5	1.3	1.6	1.8	2.7
C_{10}	2.6	3.7	1.5	2.5	3.3	3.5
C_{12}	8.4	4.4	3.4	3.0	3.8	5.2
C_{14}	24.2	12.5	8.6	12.5	9.8	14.8
C_{16}	15.8	23.2	21.1	22.1	23.3	27.2
C_{18}	10.0	7.6	9.9	9.8	} 11.6	8.5
C_{20}	...	1.0	0.7	0.8	}	1.2
Monounsaturated						
C_{10}	...	0.4	0.2	0.3	0.3	0.3
C_{12}	...	0.9	0.2	0.3	0.3	0.2
C_{14}	...	1.7	0.9	1.0	1.2	1.5
C_{16}	...	3.7	2.8	3.0	1.9	5.2
C_{18}	27.2	24.8	31.4	30.5	26.8	15.3
C_{20}	...	0.2	0.5	0.6	1.6	0.7
Octadecadienoic	...	2.9	4.9	1.0	...	1.7

* Data from six investigations compiled by Jack and Smith (12).

Table 3. THE PERCENTAGE RANGES OF POLYUNSATURATED FATTY ACIDS IN MILK FAT AS SHOWN BY A NUMBER OF STUDIES*

	Conjugated			Nonconjugated		
	Diene	Triene	Tetraene	Diene	Triene	Tetraene
1	0.6–3.7	...	...	0.8–2.0	0.7–2.0	...
2	...	...	...	2.62–2.71	0.77–1.17	...
3	0.64–1.45	0.010–0.030	0.0012–0.0044	...	...	...
	av. 1.00	av. 0.019	av. 0.0030	...	...	...
4	0.65–2.1	0.035–0.07	0.0046–0.0072	...	...	...
5	0.7–1.4	0.02–0.04	Nil	0.2–0.7	0.5–1.0	0.3–0.4
6	0.74–1.08	0.02–0.03	0.002–0.004	1.16–1.59	0.73–0.97	0.28–0.44
	av. 0.89	av. 0.02	av. 0.003	av. 1.45	av. 0.83	av. 0.35

* Data from six investigations compiled by Jack and Smith (12).

double bond in the 9 position. However, the presence of at least one isomer of these is well established. This is 11-octadecenoic (vaccenic) acid. In addition to the acids containing 1, 2, 3, and 4 double bonds reported in Table 3, indications of long chain fatty acids containing 5 and 6 double bonds have been secured (28).

The chances for revealing trace amounts of hitherto undetected fatty acids in milk fat have been heightened considerably through the development of vapor phase chromatography. The great promise which this analytical tool holds is revealed by the observations of James and Martin (13) on the lower saturated fatty acids of goat milk fat. Vapor phase chromatographic patterns obtained for the methyl esters from this fat reveal an astounding number of branched and odd-numbered fatty acids. Although conclusive identification of all components was not accomplished, the presence of many heretofore unreported fatty acids in goat milk fat seems revealed beyond question. Considering the relatively great resolving power of vapor phase chromatography as compared with other methods of separation, it is probable that this approach will be very rewarding in both research and routine analysis for fatty acids in milk lipide-containing products.

Variations in fatty acids. In general, the fatty acids of milk fat present a characteristic pattern of compositon that is recognizable in any sample of milk. However, from a specific quantitative standpoint, the fatty acid composition shows variations with a number of factors just as do the other constituents of milk. This point is emphasized by the range of values revealed in Tables 2 and 3. Unfortunately, work in this area has been limited to date and in most cases has been confined to groups of acids revealed by such fat constants as iodine number, Reichert-Meissl value, etc., rather than to exploration of individual fatty acids. Of course, variations in these groups of acids are significant; however, the possibility of compensating changes between individual acids in one group is not precluded by such measurements. A further difficulty involved in this work concerns controlling all of the variables which bear on the cow and constitute factors in the synthesis of the fatty acids. The major factor which appears to influence variations in the fatty acid composition of milk fat is the feed. Both the nature of the feed and the plane of nutrition can affect composition of the fat. When feeds high in certain fatty acids are fed at elevated levels, these same acids occur at increased levels in the milk fat. When cows are underfed, the body fats are drawn on for milk production, which results in a lower level of the volatile fatty acids, an increase in the non-volatile fatty acids, and

an increase in the degree of unsaturation of the fat. Specific findings on this subject have been reviewed elsewhere (12).

Numbers of triglycerides. Bearing in mind the substantial numbers of fatty acids present in milk fat and that the levels of these are subject to variation, it is worth considering the number of different glycerides which may be present in milk fat. Assuming that there are 18 different fatty acids constituting the triglycerides of milk fat, it has been shown by Sommer (30) that 5832 different triglycerides are possible. It must also be remembered that 18 for the number of fatty acids is so conservative an estimate as to be grossly incorrect for reasons mentioned previously. The available evidence indicates that the fatty acids are randomly distributed in the triglycerides, which also supports the contention that the glyceride makeup of milk fat is extremely complex. There is a tendency on the part of some to overlook the complexity of natural fats, particularly in comparison with proteins. Perhaps from the standpoint of structure there is limited justification for this outlook, but lipides, and milk fat in particular, are among the most complex mixtures of compounds that are encountered in nature.

Phospholipides. The phospholipides of milk exhibit three properties which have made them particularly difficult from an investigational standpoint. These are: (*a*) there are several types rather than one type of phospholipide present; (*b*) they exist in complex with protein; and (*c*) they are highly unstable toward oxygen. When milk is centrifugally separated, approximately one-third of the lipide phosphorus is found in the skimmilk and the other two-thirds is disposed with the cream. Most of the methods for measuring the phospholipides of milk and dairy products involve recovery of lipide phosphorus by extraction with organic solvents. The phosphorus in the wet- or dry-ashed fat sample is determined colorimetrically as a blue phosphomolybdate complex. By using a factor, the result for lipide phosphorus is usually expressed as lecithin. The procedure of Deniges as employed for dairy products by Horrall (11) is representative of this method of analysis.

The values for phospholipides in milk and milk products show considerable variation. This is naturally to be expected since the phospholipides exist in a surface-active complex with protein which must be broken in order that organic solvents can recover the lipide phosphorus. It has been shown (31) that milk fat prepared by melting and washing butter granules contains very little lipide phosphorus (0.02% as lecithin); the same was found true for milk fat prepared by de-

emulsifying heavy cream with surface active agents. On the other hand, Mojonnier extraction of whole milk shows values of the order of 0.6 to 1% of the total lipides for phospholipide content as lecithin. So far as the Mojonnier reagent is concerned it is the ethyl alcohol which accomplishes dissociation of the protein from the phospholipides. Parallel behavior by butyl alcohol seems indicated in view of its capacity to liberate the enzymes associated in fat globule membrane material and its utility in fat-testing procedures.

The expression of phospholipides as lecithin in milk and its products is misleading. Completely adequate compositional data on the phospholipides of milk are not available at the present time. Moreover, pure lecithin of precise chemical structure has not been isolated from the milk phospholipides. Although a substantial fraction of lecithin-like components containing phosphoric acid and choline has been shown, there is increasing evidence that significant amounts of cephalin are present, as well as smaller quantities of sphingomyelin.

The most recent findings on this subject are those of Rhodes and Lea (25). They analyzed the phospholipides of skimmilk, buttermilk, and butter from one lot of Friesian milk with the aid of chromatographic methods and found no major differences between the three preparations. The mole % composition of buttermilk phospholipides was phosphatidylethanolamine (cephalin)-29, phosphatidylserine-10, phosphatidylcholine (lecithin)-33, sphingomyelin-19. Lipide-bound inositol and plasmologens were found in amounts of 5 and 3 moles % respectively. Nitrogen associated with the cephalin fraction, equivalent to 14 moles % of the lipide P, was not identified. Lysoglycerophospholipides and cerebrosides could not be demonstrated. Unsaturated fatty acids were shown present in both the α- and β-positions of the milk glycerophospholipides. This is in contrast to those from other animal sources, which usually contain unsaturated acids in the α-position and predominantly saturated acids in the β-position.

Hilditch (10) presents data on the fatty acid composition of milk phospholipides. The preparations on which these analyses were made are admittedly crude. However, the data made clear that the acids in the phospholipide fraction are distinct from those characteristic of milk fat. The shorter chain acids are absent and the pattern of acids present seems to resemble closely those characteristics of phospholipides from various portions of the animal body. The presence of significant amounts of polyunsaturated fatty acids in milk phospholipides seem well established on the basis of polybromo addition com-

pounds, high iodine values, and extreme susceptibility to oxidative deterioration.

Minor saponifiable constituents. In addition to triglyceride and phospholipide material, there are several minor components of the milk lipide system which can be classed under the heading of saponifiable. These include cholesterol esters and vitamin A esters, whose presence in milk fat must be considered tentatively established; free fatty acids; and cerebrosides. Fresh milk fat, as well as the fat-free portion of the milk, always contains small amounts of free fatty acids. (See also Chapter 7.) The quantity of such acids will vary with the treatment that the milk fat has received. The possibility of lipase action in this connection should not be overlooked. The acid degree is generally employed to express the level of free fatty acids in a fat. This term may be defined as the milliliters of normal alkali required to titrate 100 g. of fat to the phenolphthalein endpoint. It should be noted that the lower fatty acids, particularly butyric acid, have some degree of water solubility and that measurement of only those acids recovered with the milk fat may not be truly representative for milk as a whole.

Trace quantities of organic sulfur have been detected in milk fat (31). Although sufficient numbers of samples have not been analyzed quantitatively to present such data at this time, the element has been consistently observed qualitatively in the low melting portion of milk fat analyzed at the junior author's laboratory. When the low melting fraction is saponified and then acidified to liberate the free fatty acids, hydrogen sulfide becomes clearly evident both by odor and by detection with lead acetate paper.

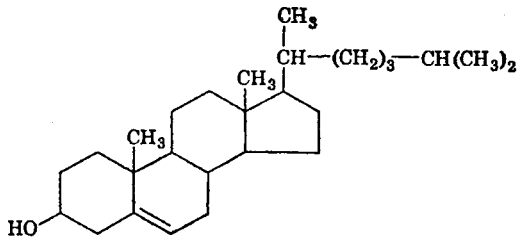
UNSAPONIFIABLE MATTER

In defining the unsaponifiable matter of milk fat, it may be advisable first to consider how this fraction is determined quantitatively. The principle involved in the determination concerns hydrolyzing a weighed amount of fat in an excess of approximately 0.5*N* alcoholic base by boiling for 1 hour. Under these conditions triglycerides and other ester materials are decomposed to the fatty acid salts, glycerol, and small amounts of other ester components. The resulting soap solution is diluted with water and extracted several times under specified conditions with ethyl ether. The ether extract, after some washing to remove impurities, is evaporated and dried to constant weight. This dry residue represents the unsaponifiable matter which for milk fat generally falls within the range of 0.30 to 0.45% by weight.

In defining the unsaponifiable matter in accordance with the above procedure, it must be remembered that boiling in alcoholic base for periods as long as an hour may accomplish reactions in the fat other than simple saponification. Most certainly anyone wishing to characterize the unsaponifiable matter in milk fat after such treatment would have to contend with the question as to whether the material was present in the native fat or is an artifact of the saponification. The possibility of some changes in the fatty acids during saponification also should not be overlooked. At the very least, some shifting (alkali isomerization) of double bonds in certain of the unsaturated acids can be anticipated. In most instances the components of the unsaponifiable matter of milk fat are postulated to be present on the basis of their structure and properties. However, these constituents are usually measured in the fat under more direct and mild conditions than those involved in the isolation of the unsaponifiable matter. Merely the sensitivity of many of these constituents to oxidation dictates that valid analytical results for them could not be expected after the rigorous and lengthy procedure for isolating unsaponifiable matter.

The unsaponifiable matter of milk fat has never been adequately characterized. Undoubtedly it is more complex than present information would indicate. The principal known components are cholesterol and related sterols, the fat soluble vitamins A, D, E, and K (see Chapter 13), traces of squalene, unidentified waxes, and an homologous series of *n*-alkyl methyl ketones containing odd numbers of carbons, C_3 to C_{15}.

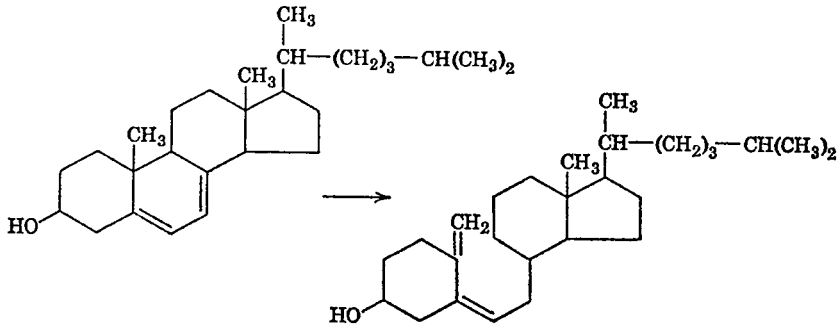
Sterols. The principal sterol of milk is cholesterol ($C_{27}H_{45}OH$), the formula for which follows:



The cholesterol content of milk ranges from approximately 0.25 to 0.4% by weight of the fat. This range is slightly below that of the unsaponifiable matter (0.3 to 0.45% by weight of the fat), which fact leads to the conclusion that the unsaponifiable matter of milk fat is largely cholesterol. Although such a conclusion may be valid, there are several matters from the literature which may be pertinent to this

point. Cholesterol is apparently disposed in milk in three different phases; namely, in true solution in the fat, as part of the fat globule membrane complex, and in complex formation with protein in the non-fat portion of the milk. Thus, values for cholesterol content clearly would be a function of the means by which it was recovered for analysis from milk. In addition, many of the methods which are used for the analysis of cholesterol in milk are not specific for this sterol alone. Until demonstrated to the contrary, it seems plausible to assume that cholesterol values may include plant and animal hormones as well as other sterols, all of which may be present in milk.

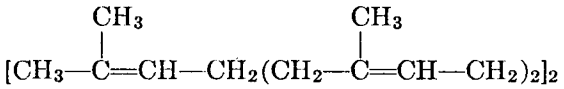
In addition to cholesterol, milk fat contains traces of lanosterol, vitamin D, and possibly 7-dehydrocholesterol. When milk is irradiated with ultraviolet light of suitable intensity the vitamin D activity is enhanced. Since, from a nutritional standpoint, this activity appears to resemble that produced by vitamin D_3, it is postulated that the precursor is 7-dehydrocholesterol and the resulting vitamin the activated form of this compound, as shown by the following equation.



Fat-soluble vitamins. The fat-soluble vitamins A, D, E, and K are constituents of the unsaponifiable matter of milk fat. Although the vast field of vitamin chemistry is beyond the scope of this text, several aspects of their relation to the chemistry of milk lipides seem worthy of at least brief mention. The tocopherol (vitamin E), and perhaps to a lesser degree the vitamin A and carotene, content of milk fat is correlated with its susceptibility to oxidative deterioration. To a considerable extent the levels of these vitamins in the milk fat are a function of the type and quality of roughages and supplements fed to the cow (16). Although all three of these materials are known to have antioxygenic properties, it appears that there are yet other substances, unidentified, associated with the milk fat which have a more profound bearing on its oxidative stability.

The observations of White et al. (34) that vitamin A and carotenoids concentrations in milk appear to vary directly with globule size are of interest since the implication may be drawn that these substances are disposed at the surface of the fat globules. Furthermore, this subject may have some bearing on the manner of milk fat synthesis. It should be pointed out that the evidence for a surface disposition of these substances is indirect and that by all of the various means used to separate membrane material from the milk fat proper, the carotene and vitamin A invariably are found with the fat. The diffusion of the carotenoids and vitamin A from an initial position at the surface may possibly explain this rather anomalous situation.

The hydrocarbon squalene ($C_{30}H_{50}$) is a component of the unsaponifiable fraction of milk fat. It has the following formula:

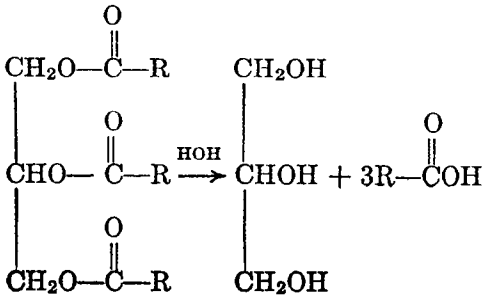


The presence of squalene in a fat appears to be related in some manner to vitamin A content. Squalene also is an intermediate in the biogenesis of cholesterol. Fish liver oils which are high in squalene are usually low in vitamin A and vice versa. The similarity in structure between squalene and the side chains of vitamin A and carotenoids is notable.

PROPERTIES OF MILK LIPIDES

THE INTACT FAT

All fats are primarily a complex mixture of glycerides. A triglyceride can be defined as an ester of glycerol and three moles of fatty acid. Like other esters, a triglyceride can be hydrolyzed under suitable conditions to yield its components, as shown in the following reaction.



R = Alkyl

A fat composed of triglyceride molecules containing only one fatty acid (all three R groups the same) would have to be a pure compound. Such fats do not exist except by synthesis. Rather, natural fats contain triglycerides composed of different fatty acids. The acids differ not only between glyceride molecules but within the same molecule as well. As discussed subsequently there appear to be literally thousands of different triglycerides in a medium such as milk fat. Completely adequate means of separating these various triglycerides for characterization and study have not been developed. However, much useful information has been obtained regarding the physical and chemical properties of native glycerides through study of fat constants (see following), the fatty acid composition, and properties of the more easily synthesized triglycerides.

Milk fat constants. For the purpose of characterization, certain well-known physical and chemical constants have been derived for the more common fats. These constants serve as an indication of the types of component fatty acids present in fats. They also enable the detection of fat adulteration qualitatively and, in some instances, quantitatively. Some of the more commonly used constants for milk fat are defined and discussed briefly in subsequent sections.

Refractive index. The basic principle in determining refractive index concerns the fact that the degree of bending of light waves passing through a liquid or transparent solid will be a characteristic for the particular liquid or solid. In the case of milk fat the constant may be determined readily with an Abbe refractometer. The reading is normally made at 40° C. The refractive index of milk fat generally ranges between 1.4538 and 1.4578. This value is low in comparison to that for other fats and oils mainly because of the greater number of saturated glycerides and short chain acids in milk fat.

Saponification number. The saponification number may be defined as the number of milligrams of KOH required to saponify one gram of fat. This value may range from 210 to 233 for milk fat and more often falls within the range of 225 to 230. In essence, this constant is an indication of the average molecular weight of the fatty acids present. With the exceptions of coconut and palm kernel oil, this constant for milk fat is well above those for other fats and oils.

Iodine number. The iodine number is the number of grams of iodine absorbed by 100 grams of fat under specified conditions. This constant is a measure of the unsaturated linkages present in a fat. The iodine number for milk fat falls within the range of 26 to 35, which is low in comparison to most other fats.

Reichert-Meissl number. This value is the number of milliliters of 0.1*N* alkali solution required to neutralize the volatile, soluble fatty acids distilled from 5 grams of fat under specified conditions. This constant for milk fat is quite significant since it is primarily a measure of butyric acid. The value for milk fat ranges between 17 and 35, which is well above that for all other fats and oils.

Polenske number. This value is the number of milliliters of 0.1*N* alkali solution required to neutralize the volatile and insoluble fatty acids distilled from 5 grams of fat under specified conditions. Whereas the Reichert-Meissl number is primarily a measure of butyric and caproic acid content, caprylic and capric acids, which are somewhat steam volatile but largely insoluble in water, are indicated mainly by the Polenske number. The Polenske number for milk fat ranges from 1.2 to 2.4.

Melting point. The term *melting point* which is used in connection with fats and oils is to some extent a misnomer. Unlike pure chemical compounds, fats and oils do not exhibit sharp melting points. Rather, as a result of the mixture of glycerides which is characteristic of the composition of all fats, they exhibit a melting range that may vary considerably depending upon the particular fat, and the way in which the melting is effected. As with the other fat constants, the melting range should be determined under specified conditions so that the only significant variable is the particular fat and so that results on various fats can be compared on a common basis. Melting ranges from 30° to 41° C. are reported in the literature for milk fat. These data are derived by placing a small quantity of the fat in a capillary tube, hardening the fat under refrigeration for a specified period of time followed by gradually raising the temperature of the sample suspended in a suitable melting point bath until a transparent condition of the sample is achieved.

Specific procedures for these and other milk fat constants have been published (32). Table 4 presents data on certain of the fat constants for several common fats.

Melting characteristics and acid distribution. For investigational purposes it is frequently necessary to fractionate milk fat into portions having various melting characteristics. For this purpose three principal methods have been employed; namely, low temperature crystallization from solvents such as acetone and hexane, direct crystallization of the melted fat without benefit of added solvent, and Winterization. The latter two methods differ only in that Winterization employs hydraulic expression of the crystallized fat instead of

Table 4. PHYSICAL AND CHEMICAL CONSTANTS OF SOME COMMON FATS

Fat	Melting Point, ° C.	Refractive Index at 40° C.	Iodine No.	Saponification No.	Reichert-Meissl No.	Polenske No.
Beef tallow	42–48	1.4566–1.4596	35–43	194–200	1	1
Cocoa butter	28–33	1.4537–1.4580	32–42	192–198	1	...
Coconut oil	20–28	1.4477–1.4495	6–10	245–262	6–8	15–20
Cottonseed oil	...	1.4696–1.4718*	103–112	192–196	1	...
Lard	36–45	1.4580–1.4620	50–80	193–200	1	1
Milk fat	30–41	1.4538–1.4578	26–35	210–233	17–35	1–3
Palm kernel oil	23–30	1.4492–1.4543	10–18	243–255	4–8	7–12
Peanut oil	...	1.4620–1.4653	88–98	186–194	1	...

* At 25° C.

vacuum filtration. Usually, the lower melting fractions for milk fat contain somewhat greater levels of unsaturated acids and the fat constants for these fractions reveal a lower saponification number, a higher iodine value, a higher refractive index, and, of course, a much lower melting range. The literature on this subject is rather conflicting and confusing. The differences in the fat constants of these fractions are not nearly so great as might be anticipated and the fractions that have been obtained are probably far from clear-cut since, in most instances, no efforts were made toward repeated crystallization of the fractions.

Many of the desirable attributes and defects of certain dairy products are related to the physical state of the fat or, more specifically, to the way crystallization of the fat has been induced. With the notable exception of work by King (15), much of the available information is in the realm of theory and speculation. There are, however, a few helpful guiding principles which can be offered. Three significant factors which affect the melting properties of a fat are: (*a*) the nature of the component acids, (*b*) the distribution of the acids in the glycerides, and (*c*) the polymorphic forms of the fat crystals. The shorter chain acids and unsaturated acids tend to depress the melting range, whereas the longer chain saturated acids raise the melting range. With respect to distribution of the acids, two types have been proposed, even and random. The theory of even distribution implies that the acids tend to be distributed equally among the triglyceride molecules. In other words, a fatty acid will not repeat in the same molecule until its level exceeds 33% of all the fatty acids present. It will not repeat more than twice unless its level exceeds 67%. Random

distribution proposes that a fatty acid will appear in a glyceride molecule to an extent depending on its level in the fat and on statistical probabilities regarding its distribution at that level. In general, vegetable fats tend to conform more closely to even distribution, whereas animal fats seem to exhibit a pattern closer to random distribution. Even distribution of the fatty acids makes for somewhat sharper melting characteristics in a fat, the rather wide melting range of milk fat suggesting closer conformance to random distribution of the acids. However, such a situation might be anticipated in milk fat, irrespective of distribution, because of the great variety of fatty acids which it contains.

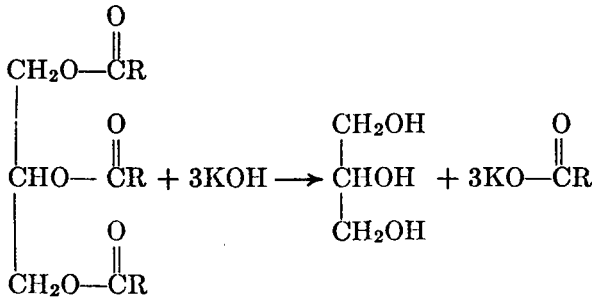
Many fats and glycerides crystallize in more than one form, and this characteristic is termed *polymorphism*. Some of these crystal forms are relatively stable; others are transient, passing from less to more stable states. Such factors as agitation, rates of heating and cooling, and storage period can have a modifying effect on the crystallization process. The relation of polymorphism to the physical state of the fat from the standpoint of plasticity and melting qualities is fairly obvious. The intricate relationship which may be involved regarding susceptibility of milk fat to churning and attack by lipase (see Chapters 6 and 9) is not so evident. X-ray diffraction studies indicate that the polymorphic forms of fat crystals result from differences in the orientation of the carbon chain axis with respect to the plane of the terminal groups.

THE FATTY ACIDS

Isolation and Purification

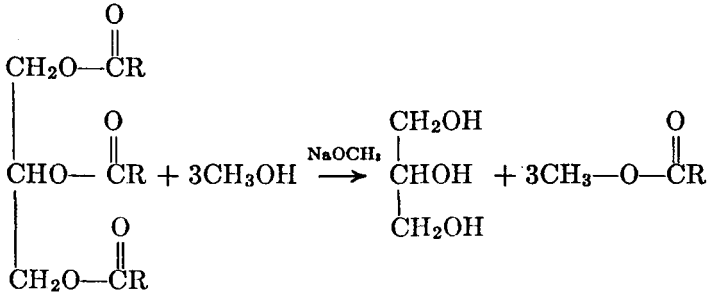
The major components of fats are the acids. In the case of milk fat, the fatty acids account for over 85% and the glycerol for approximately 12.5% of the weight. Glycerol is a non-varying component of all fats, whereas the fatty acids represent the significant variable. Consequently the chemistry of a particular fat depends primarily on its component acids.

Recovery of the fatty acids from a fat is accomplished by hydrolysis with base (saponification). Usually the reaction is carried out by refluxing the fat for several hours with the calculated amount of KOH dissolved in ethyl alcohol. The reaction may be expressed as follows:



As shown, the resultants of the reaction are one mole of glycerol and three moles of potassium salts of the acids (soaps). In order to secure the free acids, the soaps must be acidified, which step is usually accomplished by treating with mineral acid after removal of the alcohol.

For reasons mentioned later, it is a common practice to recover the fatty acids as their methyl esters. In this procedure the fat is refluxed with several times its volume of methanol in the presence of a suitable catalyst. Both acid and base type catalysts are used and perhaps the most efficient is sodium methoxide (9). The reaction for preparing methyl esters from triglycerides may be expressed as follows:



For fats in general there are a large number of procedures which may be used for separating the acids in a more or less pure state from the saponified or hydrolized fat. These include: fractional crystallization, fractional distillation, steam distillation, preparation and debromination of polybromides (unsaturated acids only), adsorption and partition chromatography, crystallization of urea complexes, and separation of lead salts.

Fractional crystallization. The purification of a material by crystallization from a solvent is an everyday technique used in the laboratory. Since it has a number of advantages over other types of fractionation and purification, it seems plausible that it would find

acceptance in application to fatty acids, as well as their methyl and glycerol esters. A fundamental requirement for crystallization from a solvent is the state of supersaturation, that is, more material must be dissolved than the solvent can actually hold at a given temperature. Ordinarily this is accomplished by adding the material to be dissolved (solute) to the solvent, heating the mixture until equilibrium or maximum solution is reached, and then removing excess solute by filtration. On cooling, the filtrate will give rise to crystals, the time required for crystallization and the yield of crystals being a function of a number of variables. Heating is used as a matter of convenience; however, under suitable conditions the same results can be achieved by dissolving the solute in the solvent at room temperature and then cooling the solution to lower temperatures. This modification of the technique has been rather essential in the application to lipides because of the dangers of decomposition inherent in the use of heat. By stepwise lowering of temperature it is possible to collect successive fractions which crystallize from the solvent. In practice, temperatures ranging from 20 to —70° C. and such solvents as acetone, petroleum ether, methyl alcohol, and 95% ethyl alcohol are employed. A general procedure which is useful for the separation of saturated from unsaturated fatty acids involves crystallization from acetone at —30° C. Under these conditions, saturated acids crystallize readily and the filtrate affords a concentrated fraction of the unsaturated acids.

One of the principal criticisms of fractional crystallization is that it yields only concentrated fractions rather than pure acids. However, it has been shown in a number of investigations that reasonably precise manipulations enable satisfactory results. It is also important to recognize that fractional solvent crystallization may be best used in conjunction with other purification techniques. For example, it may be employed either as a preliminary purification technique for methyl esters of fatty acids to be finally purified by distillation, or the distillation can be used as the preliminary purification and the fractional crystallization as the last step. The review by Brown (4) is appropriate for readers interested in the methods and literature in this field.

Fractional distillation. Distillation is a basic method of purifying liquids. It involves many modifications of apparatus and technique. Those interested in the various applications of distillation in the field of lipides are referred to the review by Weitkamp (33). Our concern here is with the separation of fatty acids. With respect to the milk fat acids, it is usually more satisfactory to prepare the methyl esters and fractionally distill them since they exhibit substantially lower boil-

ing points than the free acids (see Tables 5 and 6). Moreover, the methyl esters are more stable toward heat than the free acids, particularly the unsaturated acids. It will be evident from Table 6 that the methyl esters of saturated acids present in milk fat differ in boiling

Table 5. SOME PROPERTIES OF THE MAJOR SATURATED FATTY ACIDS OF MILK FAT

Name	Formula	Melting Point, ° C.	Boiling Point, ° C.	Solubility in H_2O, g./100 g., 20° C.	Refractive Index, n_D^{70}
Butyric	$CH_3(CH_2)_2COOH$	−8	163.5	∞	1.3991*
Caproic	$CH_3(CH_2)_4COOH$	−2	205	0.968	1.4170*
Caprylic	$CH_3(CH_2)_6COOH$	16	238	0.068	1.4280*
Capric	$CH_3(CH_2)_8COOH$	31.5	270	0.015	1.4169
Lauric	$CH_3(CH_2)_{10}COOH$	44	299	0.006	1.4230
Myristic	$CH_3(CH_2)_{12}COOH$	58	>300	0.002	1.4273
Palmitic	$CH_3(CH_2)_{14}COOH$	64	>300	0.001	1.4309
Stearic	$CH_3(CH_2)_{16}COOH$	69	>300	0.000	1.4337

* n_D^{20}.

Table 6. SOME PROPERTIES OF THE METHYL ESTERS OF THE MAJOR SATURATED FATTY ACIDS IN MILK FAT

Methyl Ester	Boiling Point, ° C.	Melting Point, ° C.	Refractive Index, n_D^t
Butyrate	102	...	1.3879^{20}
Caproate	150	...	1.4070^{15}
Caprylate	193	...	1.4069^{45}
Caprate	224	...	1.4161^{45}
Laurate	134*	5	1.4220^{45}
Myristate	162*	18.5	1.4281^{45}
Palmate	184*	30.5	1.4317^{45}
Stearate	204*	39	1.4346^{45}

* At 10 mm. of mercury pressure.

points, a fact that serves as the basis for separation by distillation. The efficiency of separating the esters is determined primarily by design and operation of the distillation equipment, especially the efficiency of the fractionating column. In this latter portion of the apparatus the actual separation of close boiling components occurs. Such factors as the height of the column, the material with which it is

packed, the way it is packed, and the quantity of material distilled through it per unit of time are basic factors in its efficiency of operation.

Steam distillation. Steam distillation is important particularly with respect to milk fat because of the volatile fatty acids which it contains. The relative amounts of the various volatile fatty acids of a fat are reflected in two well-known fat constants, the Reichert-Meissl and Polenske numbers. The former is essentially a measure of butyric and caproic acids content, whereas the latter measures primarily C_8 to C_{10} acids which are volatile with steam but insoluble in water. It must be remembered that steam distillation is not a precise means of separation. The conditions under which the distillation is performed may have a profound bearing on the numbers and quantities of the various fatty acids recovered. For example, small amounts of C_{12} to C_{18} fatty acids are usually recovered in the Polenske number distillation and a portion of the caprylic acid contributes to the Reichert-Meissl number.

Preparation and debromination of polybromides. A procedure which has been found very useful for separating unsaturated acids from saturated acids of about the same chain length and molecular weight concerns preparation and debromination of the polybromide derivatives of the unsaturated acids. A classic example is the separation of 9-decenoic acid from the C_{10} methyl ester fraction of milk fat acids by Bosworth and Brown (2). The C_{10} ester fraction was treated with bromine, in which case the unsaturated acid added bromine to its double bond to form the dibromide and the saturated esters remained unchanged. Addition of bromine raised the boiling point of the unsaturated acid ester substantially, enabling fractional distillation of the dibromide derivatives from the balance of the ester material. The purified bromide ester was then debrominated with zinc and hydrochloric acid in methanol to yield the methyl ester of the original unsaturated acid. The free acid was obtained by careful hydrolysis of the ester. The series of reactions involved may be represented as follows:

$$\begin{array}{l} CH_2{=}CH{-}(CH_2)_7{-}COOCH_3 \\ \qquad\downarrow Br_2 \\ CH_2Br{-}CHBr{-}(CH_2)_7{-}COOCH_3 \\ \qquad\downarrow \begin{matrix} Zn \\ HCl \end{matrix} \\ CH_2{=}CH{-}(CH_2)_7{-}COOCH_3 + ZnBr_2 \\ \qquad\downarrow HOH \\ CH_2{=}CH(CH_2)_7{-}COOH + CH_3OH \end{array}$$

This same procedure has been used very effectively in connection with the higher molecular weight unsaturated acids. The bromination-debromination procedure, together with disruptive oxidation, established the position of the double bonds in arachidonic acid (5, 8, 11, 14 eicosatetraenoic acid).

Chromatography. A great variety of methods for separating fatty acids has been offered by the rapidly expanding field of chromatography. The primary objective of chromatography is to resolve mixtures into pure components. With careful selection of conditions, it is possible by chromatography to separate any one compound from all others. The principle of separation depends upon the relative tendency of a substance to distribute itself between two phases, one which is stationary and the other which moves continuously. The mobile phases are ordinarily liquids or gases and the stationary phases, solids or solids coated with liquids. A great number of paper and column chromatographic methods have been devised for separating the fatty acids. Specific references to these may be found in Lederer and Lederer (18). Various applications of chromatography in the field of lipides have been discussed by Allen (1). A chromatographic method particularly useful in connection with milk fat is that designed by Keeney (14) for the quantitative measurement of butyric acid. This method employs an immobile phase of ethylene glycol, ammonia, and bromcresolgreen on a silicic acid support and a mobile phase of hexane containing a small quantity of butanol. Butyric acid moves down the column as an orange band on a green background and, after complete elution, the butyric acid fraction is measured quantitatively by titration with base.

Recent data indicate that for most efficient separation, especially of the lower molecular weight acids, vapor phase chromatography of the methyl esters is highly useful (13). With this procedure, efforts to separate the acids as such are confronted first with the problem of their high boiling point, and, second, their tendency to exist as dimers in the vapor phase. These difficulties are eliminated through use of the methyl esters. Since vapor phase chromatography works with milligram amounts or less of material, the procedure is not well suited to large-scale preparations.

Crystallization of urea-fatty acid complexes. Organic compounds constituted of unbranched carbon chains form inclusion compounds with urea. It appears that a lattice work of urea molecules forms along the axis of the carbon chain. These crystalline complexes can be formed with fatty acids or their esters by simple addition to

an alcoholic solution of urea. The urea adducts can be fractionally crystallized from solution by varying the temperature. This technique has been particularly useful in separating the polyunsaturated acids since their urea adducts are more soluble than those of the saturated acids. A further attractive feature of this technique is that inclusion of the unsaturated acids with urea appears to protect them from oxidation. The subject of urea adducts has been reviewed by Schlenk (26).

Separation of lead salts. A classical method for separating saturated from unsaturated fatty acids involves preparation of the lead salts and differentiating these on the basis of solubility in alcohol or ether. The insoluble lead salts arise mainly from solid acids (saturated) whereas the soluble lead salts come from the unsaturated (liquid) acids. The procedures involved in this separation are described by Hilditch (10). This type of separation is tedious and difficult. It appears to be giving way to some of the more precise and convenient techniques mentioned previously.

SATURATED FATTY ACIDS

It is generally stated that milk fat is composed of the even-numbered, straight chain fatty acids containing 4 to 18 carbons. From a gross compositional standpoint this is quite true, but it seems important to emphasize that such a statement substantially oversimplifies even our current knowledge of the subject. Evidence regarding other acids which are present in limited amounts is given in the section on composition of milk fat.

The saturated fatty acids of milk fat are distinctive for several reasons. The volatile low molecular weight members are highly significant in flavors and off-flavors of milk and its products. The lowest member, butyric acid, is unique to milk fat. The C_6 to C_{12} acids are found elsewhere mainly in coconut and palm oils. Although milk fat has a relatively low iodine number (see Table 4), it is fairly soft and low melting because of the depressing action which the low molecular weight saturated acids have on its melting range.

Because of their relative stability, the saturated fatty acids are less significant from a chemical standpoint than the unsaturated acids. Under normal conditions of processing and storage of dairy products, it is assumed that the carbon chain of the saturated milk fat acids is very stable, or perhaps analogous in stability to that of the saturated hydrocarbons. The principal reactive point in the molecule therefore

is the carboxyl group. This group, as discussed adequately in many organic chemistry texts, undergoes a great variety of reactions, which for the most part are not at issue here. Its properties, as an acid, to bind base and to undergo esterification with hydroxl groups are of most notable importance in the lipides field. Data on some physical properties of the major saturated fatty acids of milk fat and of their methyl esters are presented in Tables 5 and 6 respectively.

UNSATURATED FATTY ACIDS

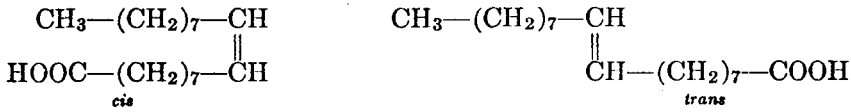
The unsaturated fatty acids play a rather profound role in the physical and chemical properties of milk fat. Of singular importance is their role in oxidative deterioration. Various terms are employed in the literature to specify unsaturation, such as olefinic, ethenoid, and ethylenic. The term *unsaturated* is used here for this purpose and signifies the presence of carbon to carbon double bonds. Numbers of such double bonds within a compound are indicated by the prefixes mono, di, tri, and poly insofar as possible.

Isomerism. One of the important characteristics of unsaturated fatty acids is their capacity to exist in a number of isomeric forms and to be converted rather easily from one of these forms into another. The significant factor about such isomerism is that each isomeric form represents a distinctly different compound which, although it may be similar to its other isomers in many respects, may show definite differences in physical and chemical properties. In addition, since unsaturated fatty acids are susceptible to autoxidative deterioration, the specific decomposition products which are formed may depend upon the particular isomeric unsaturated fatty acid involved. Further, since both the position of the double bond and the geometric configuration of groupings about the double bond afford possibilities of isomerism, the number of potential isomers with any given chain length acid is large. This is particularly true of polyunsaturated fatty acids.

In unsaturated compounds, position isomerism merely refers to alternative possibilities regarding the position of double bonds. For example, the monounsaturated normal C_{18} acid may have a double bond in any one of 16 positions in the carbon chain, and thus may exist as any one of 16 position isomers. Although all of these possibilities have the same molecular formula and, with the exception of the terminally unsaturated member, the same functional groups, they are all separate and distinct compounds. Chain branching also intro-

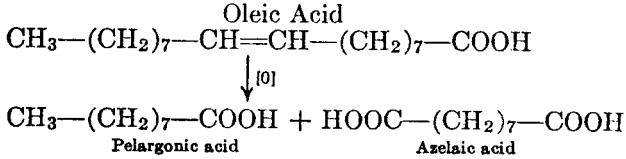
duces a concept of position isomerism. Although traces of branched chain unsaturated acids may occur in milk fat, this form of isomerism is not included in the present discussion.

The second type of isomerism which we wish to consider is that termed *geometric*. Simply stated, this refers to the configuration of substituents around the double-bonded carbon. An appropriate example of this type of isomerism may be illustrated by 9-octadecenoic acid, the *cis* form of which is oleic acid and the *trans* form, elaidic acid.



As indicated, the variations in structure which are possible by geometric isomerism are termed *cis* and *trans*. These isomers result from the fact that the electronic arrangement between double-bonded carbons is rigid and, unlike that of single-bonded carbons, is not free to rotate in space. Thus in the case of a *cis* isomer, the carbon chain is bent back upon itself, whereas in the *trans* configuration the two ends of the carbon chain are inclined in opposite directions from each other. It will be evident that in a diunsaturated fatty acid, such as 9,12-octadecadienoic, four different possibilities of *cis-trans* combination can exist. These are *cis*-9, *cis*-12, *trans*-9 *cis*-12, *cis*-9 *trans*-12, and *trans*-9 *trans*-12. With greater degree of unsaturation many additional geometric isomers are possible. In general, the more symmetrical *trans* form of an isomeric compound has the higher melting point. An example concerns the *cis* and *trans* 9-octadecenoic acids, more commonly called oleic and elaidic, melting point 14° and 51.5° C. respectively.

Since the physical and chemical properties and even the nutritive value of a fat may depend upon which isomeric unsaturated fatty acids it contains, means of locating the position of double bonds and their configuration are of considerable importance. The classical method for determining the position of double bonds is by disruptive oxidation of the unsaturated fatty acid and characterization of the decomposition products. For example, permanganate oxidation of oleic acid establishes the double bond to be in the 9 position by virtue of the formation of pelargonic and azelaic acids as decomposition products.



An additional aid in determining the position of double bonds in polyunsaturated fatty acids concerns the spectral properties of the acid in the ultraviolet region before and after treatment with strong alkali (alkali isomerization). Such treatment of linoleic acid, for example, produces a strong ultraviolet light absorption maximum in the vicinity of 230 mμ. This absorption, which is distinct in both magnitude and wavelength from that produced by the isolated 9, 12 position of the double bonds in the acid, results from a shifting of the bonds to a conjugated position as follows:

$$\overset{12}{\text{—CH}}\text{=}\overset{11}{\text{CH}}\text{—}\overset{10}{\text{CH}_2}\text{—}\overset{9}{\text{CH}}\text{=CH—}$$

$$\downarrow$$

$$\text{—CH=CH—CH=CH—CH}_2\text{—}$$

$$\text{or}$$

$$\text{—CH}_2\text{—CH=CH—CH=CH—}$$

As indicated, both 9, 11 and 10, 12 isomers are possible and are produced. Greater electronic resonance of the conjugated forms accounts for the change in light absorption. This phenomenon serves as the basis of quantitative measurement for certain of the unsaturated fatty acids (21). It has the advantage of easy distinction between the presence of conjugated and nonconjugated acids, both of which may be present in a fat either naturally or as a result of various treatments. The presence of conjugated triene and tetraene in unsaturated fatty acids, and fats as well, is revealed by absorption maxima at approximately 268 and 278 mμ (triene) and 316 mμ (tetraene). Figure 1 shows changes in the ultraviolet absorption maxima of anhydrous milk fat before and after alkali isomerization. These data make evident significant quantities of fatty acids containing 2 and 3 isolated double bonds, although there are indications of some conjugation in the unisomerized sample (see Table 3).

A treatment of the complicated methods and considerations involved in determining *cis-trans* isomerism, particularly as they relate to the polyunsaturated acids, is beyond the scope of this text. It is worthy of note, however, that infrared spectrophotometry has proven highly useful in this connection. The isolated *trans* double bond shows distinctive absorption at a wavelength of 10.36 μ. Tentative assignments for various *cis* and *trans* combinations have been made (22). The infrared spectrum of anhydrous milk fat, as shown in Fig. 2, reveals a small band at 10.36 μ indicative of some *trans* unsaturation in the medium. This point and the subject in general of unsaturated fatty

acids in milk fat have been investigated recently by Smith et al. (29). No doubt the *trans* unsaturation exhibited in the infrared spectrum of

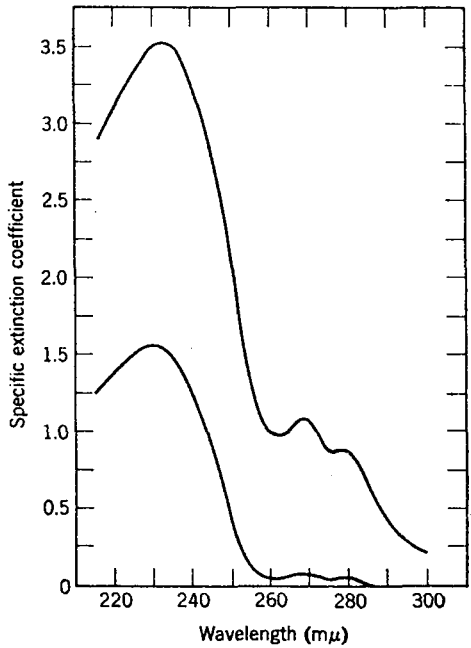


Fig. 1. Ultraviolet absorption spectrum of milk fat. Before alkali isomerization (lower); after alkali isomerization (upper).

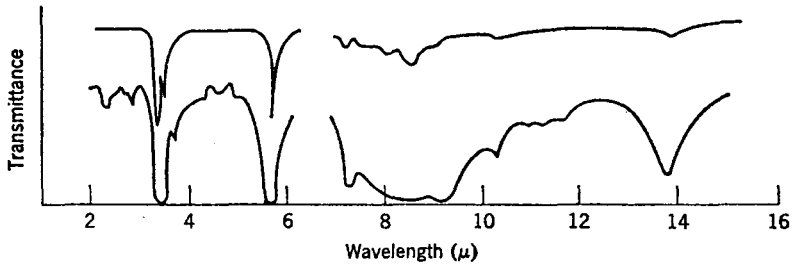


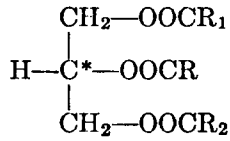
Fig. 2. Infrared spectra of milk fat in carbon tetrachloride, 0.5% solution (upper) and 10% solution (lower).

milk fat is due, at least in part, to vaccenic acid which is *trans*-11-octadecenoic acid.

Disregarding for the moment geometric isomerism, the general impression has been that 9-octadecenoic (oleic) acid, 9,12-octadecadienoic

(linoleic) acid, 9,12,15-octadecatrienoic (linolenic) acid, and 5,8,11,14-eicosatetraenoic (arachidonic) acid are the principal unsaturated acids of importance in milk fat. In addition, it has been thought that these acids are of an all-*cis* configuration. As mentioned previously, the extremely numerous possible combinations of these isomers make analysis for any one specific member difficult. Thus, for the moment it seems most plausible to express the levels of the polyunsaturated acids in milk fat in terms of conjugated and nonconjugated, as shown in Table 3, until this matter can be further clarified.

One additional form of isomerism that is possible with glycerides concerns the β-carbon of the glycerol, since under certain conditions this carbon may be asymmetric, that is, carry four different substituent groups. This is possible when the α and α' positions of the glycerol are esterified with two different fatty acids. The necessary configuration may be expressed:



*Asymmetric carbon

This type of isomerism, which normally promotes optical activity but apparently has little effect in the case of glycerides, is discussed also in relation to lactose (Chapter 3).

Oxidation. For the purposes of definition, lipides containing unsaturated fatty acids can be submitted to chemical oxidation by various agents or they can autoxidize, a more or less spontaneous and catalytic deterioration, owing to contact with atmospheric oxygen. Chemical oxidation is not at point in the present discussion beyond noting that such agents as ozone, potassium permanganate, and chromic acid will cleave the double bonds, and that certain less rigorous agents such as peracids give epoxy and dihydroxy acids. As mentioned previously, oxidizing agents which affect chain cleavage are important in establishing the position of double bonds. By far the overriding consideration of importance with milk fat oxidation is its tendency to occur spontaneously in the presence of air. This type of oxidation is particularly important with reference to flavor (Chapter 12). Here again we are confronted with a complex aspect of chemistry which has only begun to unfold in very recent years. The work of Farmer and Sutton in 1943 (8) established the profound significance of hydroperoxides and free radical mechanisms associated with them

in the autoxidation of fats. Although this initial work concerned methyloleate, data have been compiled to establish the broad applicability of their observations to lipide materials.

The reactions involved in hydroperoxide formation in a fat appear to proceed by a free radical mechanism. The initial step probably concerns removal of hydrogen from the methylene group adjacent to a double bond. The resulting radical may add oxygen to form a second type of free radical. The resulting peroxide-containing free radical may then react with another mole of unsaturated compound by abstracting hydrogen to form the hydroperoxide and a new free radical. The equations involved here may be expressed:

$$-CH_2-CH=CH- \rightarrow -\overset{*}{C}H-CH=CH- + H$$

$$-\overset{*}{C}H-CH=CH- + O_2 \rightarrow -CH(OO^*)-CH=CH-$$

$$-CH(OO^*)-CH=CH- + -CH_2-CH=CH- \rightarrow$$

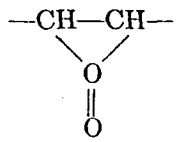
$$-CH(OOH)-CH=CH- + -\overset{*}{C}H-CH=CH-$$

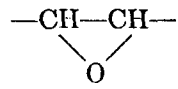
The chain reaction properties of this sequence are evident and probably account for the autocatalytic effects observed in autoxidation. Shifting of the double bond under these conditions has been observed, particularly for the 9, 12 arrangement in linoleic acid. In fact, hydroperoxide formation in fats and fat derivatives appears to be closely correlated with increase in diene conjugation. This shifting of double bonds leads naturally to a large number of alternative hydroperoxides from any given polyunsaturated compound. By the same token, a multiplicity of hydroperoxide decomposition products is possible, which may account for the variety of unsaturated carbonyl compounds that have been detected in autoxidized fats. That area of chemistry dealing with hydroperoxide decomposition is yet quite obscure. However, in aqueous solution, autoxidizing sodium linoleate shows some decomposition of monohydroperoxide before the maximum level is achieved. In this instance the addition of a second mole of oxygen is accompanied by destruction of diene conjugation. The observation of 2-enals (R—CH=CH—CHO) as decomposition products in a number of autoxidized fats suggests the worthiness of investigating their precise relation to hydroperoxide decomposition.

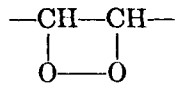
It has been mentioned elsewhere that under suitable conditions,

ascorbic acid promotes milk fat oxidation and oxidized flavor development (Chapter 12). In this phenomenon a ratio of reduced to dehydro form of the compound of approximately 1 : 1 is required. Under these conditions ascorbic acid affords a poised system that is ideal for both donation and acceptance of hydrogen, and it is clearly possible that such a system would serve as an aid to propagation of hydroperoxide formation.

Many other intermediate forms have been postulated in the autoxidation of lipides, including moloxides, epoxides, and cyclic peroxides.


Moloxide


Epoxide


Cyclic peroxide

The extent to which such intermediates may exist in autoxidizing lipides is not clear; however, the main course of the deterioration through the hydroperoxide structure seems firmly established.

Measurement of milk fat oxidation. In any food system, stability of the lipides towards autoxidation is extremely important. The fact that lipides are liable to attack by oxygen has created the need for measurement of the phenomenon as well as methods of prevention. The most direct approach to measurement of lipide oxidation is organoleptic analysis since, from a practical standpoint, fat oxidation has proceeded too far when flavor deterioration has reached the point of being objectionable. There are a variety of chemical methods which may be used to measure oxidative deterioration in lipides. One of the most useful so far as milk fat is concerned is the ferric thiocyanate peroxide test devised by Loftus Hills and Thiel (19). The procedure has recently been adapted to milk and a variety of fat-containing dairy products (24). The basis of the test involves the conversion of ferrous ion to the ferric state in the presence of ammonium thiocyanate to yield the red pigment ferric thiocyanate. Presumably it is the peroxide groupings in the fat which oxidize the ferrous ions and which are being measured by the method. Another method which has come into recent use is that employing 2-thiobarbituric acid (5). Under suitable conditions this reagent reacts with oxidizing lipides to form a red pigment, the density of which appears to be well correlated with the degree of flavor deterioration. Malonic dialdehyde (OHC—CH_2—CHO) has been shown to yield the same pigment as derived from oxidized lipides. This test has found wide application to many food items other than

dairy products. Some fundamental observations on the nature of the test have been published (23). In addition to these two techniques, the peroxide value as determined by Lea (17), the iodine value, and various procedures to analyze for the presence of carbonyl compounds have been employed for evaluating fat oxidation.

The peroxide value, by the method of Loftus Hills and Thiel, appears to be the best available means for following oxidative deterioration in milk and milk products. It has extreme sensitivity and appears to correlate well with flavor deterioration. It is important to realize that the level of peroxides in a product may be the net effect of total formation less those peroxides which may have decomposed. The iodine value, a measure of unsaturation, has found some use as an indicator of oxidation by virtue of the destruction of double bonds in the process. For example, studies of lipide oxidation in milk have revealed that the iodine number of the phospholipide fraction drops very significantly whereas the glyceride portion shows no change in iodine value. This fact is frequently cited as evidence that it is primarily the phospholipides rather than the glycerides which give rise to oxidized flavor in fluid milk. Since, in the initial stages of hydroperoxide formation, destruction of double bonds does not occur, the value of iodine number as a criterion of oxidative deterioration is debatable. For fats in general, the evidence that carbonyl compounds are formed during autoxidation and that these compounds are significant in the off-flavors produced is well established. Thus, it is plausible that methods for evaluating fat oxidation are based on detection of such compounds. However, from the standpoint of decomposition products, it seems that carbonyls have been studied extensively to the possible neglect of other compounds which may be formed.

Prevention of autoxidation. In general, the methods employed to prevent oxidized flavor in dairy products (see Chapter 12) are effective in preventing autoxidation of the lipides. These methods include avoidance of contact with certain trace metals, particularly copper and iron, and the use of various antioxygenic measures. Because of the considerable stability of milk fat toward autoxidation, compared with many other fats, the need for antioxidant addition has not been severe. A further consideration in this regard is that health regulations strongly limit the substances that may be added to dairy products.

Although the addition of antioxidants to milk and milk products has been shown effective in certain instances, some difficulties arise from their use. The development of atypical flavors, directly traceable to the antioxidant, is frequently encountered. With the exception of

anhydrous milk fat, effective distribution of the antioxidant also may be a problem. Since milk lipides are protected by an adsorbed membrane, the addition of an antioxidant to milk products may not accomplish disposition of the agent with the lipide phase, the site to be protected.

The precise mechanism whereby antioxidants prevent autoxidation of lipides has not been established. It appears that they interfere in some way with the chain reaction mechanism by which hydroperoxides are formed. Most natural and synthetic antioxidants are compounds containing two or more phenolic hydroxyl groups. The ortho and para configurations of the hydroxyls appear to be much more effective than the meta. Frequently, substances known as synergists are used in conjunction with antioxidants to enhance their effectiveness. These synergists seem to have a beneficial effect through a capacity to sequester metallic pro-oxidants. Citric, phosphoric, and other polycarboxylic acids are commonly employed as synergists.

Of the three antioxidants, propyl gallate, nordihydroguaiaretic acid (NDGA), and 6-palmitoyl-l-ascorbate (at levels of 0.01, 0.005, and 0.1% respectively), propyl gallate is most effective in preventing oxidative flavor deterioration in milk fat. NDGA performs slightly less satisfactorily and the ascorbic acid derivative performs little better than with no antioxidant added. None of these agents is effective in preventing the buttery-coconut-like flavor defect in milk fat, which is due to the formation of δ-decalactone. This flavor appears to be of widespread importance in a variety of stored dairy products (see Chapter 12).

Hydrogenation. Hydrogenation consists of the addition of hydrogen to double bonds. The process ordinarily requires, in addition to hydrogen under pressure, the use of a suitable catalyst. Special preparations of nickel, platinum, and palladium in various forms are among the more common catalysts. In a broader sense, hydrogenation may be defined to include addition of hydrogen to functional groups other than carbon to carbon double bonds. For example, triglycerides may be reduced to a mole of glycerol and 3 moles of fatty alcohol by hydrogenation in the presence of a copper-chromite catalyst.

Hydrogenation is an indispensible process in the field of vegetable fats and oils. It enables imparting to these oils suitable physical properties and storage stability required for shortening and margarine. The commercial hydrogenation of anhydrous milk fat may be accomplished without difficulty by the same techniques used for vegetable oils.* Such treatment of milk fat can produce alterations ranging

* Communication to the junior author from the Girdler Corp., Louisville, Ky.

from a very slight lowering of the iodine value to essentially complete saturation at iodine values <1. Products with such a low iodine value are high melting waxy materials which do not lend themselves well to incorporation in food products. In addition, they are considered to be poorly digestible. Milk fats hydrogenated to iodine values ranging from 12 to 18 show very satisfactory physical properties and, when suitably processed, excellent flavor stability during storage. However, there is no great incentive to hydrogenate milk fat. The fat is an expensive raw material at the outset. Hydrogenation and associated processes remove or destroy most of the fat soluble vitamins. The end product in many respects is on a par with hydrogenated vegetable oils with which, pricewise, it is in no position to compete. Further, in order to hydrogenate milk fat it must be removed from the native medium—milk or cream—dried, and treated. If the hydrogenated product is to be used as a stable form of milk fat in dry milk, for example, it must be re-emulsified in the fluid product before drying. There obviously is little feasibility in so much processing.

Investigations regarding the chemistry of milk fat often reveal the desirability of laboratory hydrogenation of the entire fat or fractions thereof. This can be accomplished in solutions in heptane (sulfur free) using spongy nickel catalyst at room temperature and approximately 50 lb. hydrogen pressure. Under these conditions, a product exhibiting iodine value below 0.5 can be achieved in 24 hours. For further information on the subject of hydrogenation, the interested reader should consult a suitable text in the field of vegetable oil processing (6).

Other reactions. Unsaturated fatty acids and fats which contain them undergo a number of additional types of reactions which are beyond the scope of our interest here. Such reactions include polymerization, alkali cleavage, Diels-Alder type addition, and the addition of halogen and hydrogen halides. The addition of halogen is in the order $F_2 > Cl_2 > Br_2 > I_2$. This order is reversed in the case of hydrogen halides.

PHOSPHOLIPIDES

Since phospholipides are found in all plant and animal tissue, it is not surprising to find small amounts of them in milk. This section deals with the isolation, structure, and properties of these materials. The cerebrosides are briefly considered also. Cerebrosides do not contain phosphorus and thus are not truly phospholipides, but they exhibit

some properties similar to those of the phospholipides and are conveniently discussed with the latter.

Isolation. The isolation and fractionation of crude phospholipides is based primarily on solvent extraction techniques. The use of fresh source materials is of the utmost importance. Phospholipides are generally subject to rapid deterioration in air. Some of them are readily hydrolyzed by relatively mild acid or alkaline conditions. Such hydrolysis can result in the cleavage of both the phosphate and fatty acid ester linkages. In general, the phospholipides from the fat globule membrane of milk may be isolated as follows: fresh raw milk is separated in the cold and the cream is washed repeatedly with cold

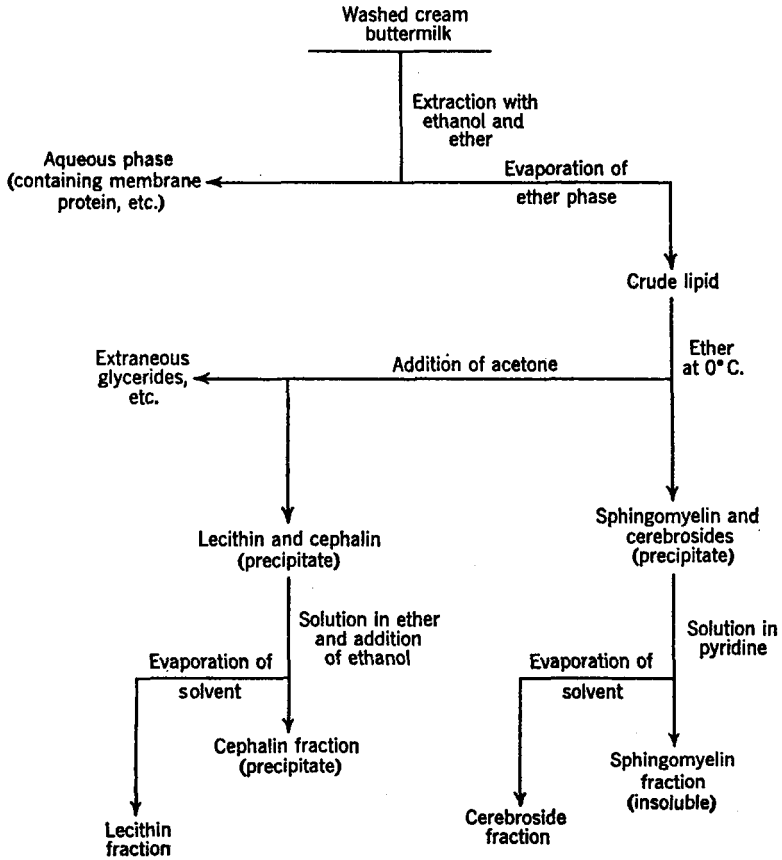
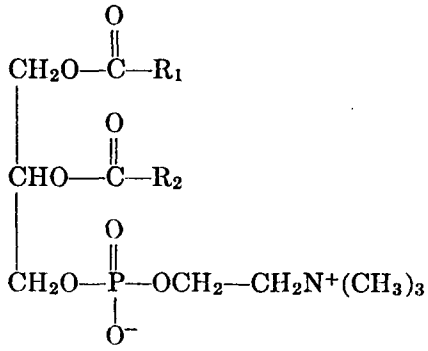


Fig. 3. A scheme for the fractionation of milk phospholipides and cerebrosides from fat globule membrane material.

distilled water. The washed cream is churned to butter and the resulting washed cream buttermilk is extracted with alcohol and ether. Phospholipides are precipitated from the concentrated extract by treatment with acetone. The mutual solubility of triglycerides and phospholipides in ethyl ether and the differential solubility of the two in acetone have served as the general basis for nearly all of the isolation work done on the milk phospholipides to date. Chromatographic separation is now coming to the fore (25). A compilation of literature dealing with this subject is given by King (15).

Limiting isolation techniques for the phospholipides to those substances that are soluble in ether and insoluble in acetone will exclude lipide material behaving in the manner of sphingomyelin and cerebrosides. Such material is classified as insoluble in both ethyl ether and acetone. A scheme which may be employed for the crude fractionation of the groups lecithin, cephalin, sphingomyelin, and cerebrosides is presented in Fig. 3. The crude individual isolates may be purified further by solvent extraction and chromatography.

Structure and properties. When a particular phospholipide is spoken of, it must be remembered that this may refer to a particular group of compounds, all having the same type of structure, or to one specific compound. The formula for lecithins as a group or type may be represented:

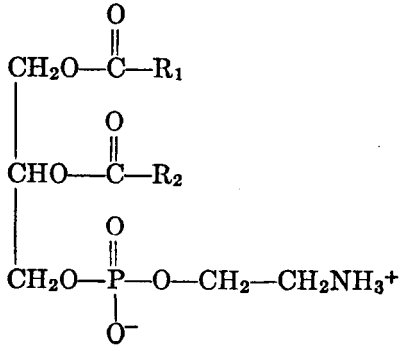


In this formula the α-glycerolphosphorylcholine portion of the structure is common to all members. However, the particular fatty acids, represented by R_1 and R_2, may vary, not only in chain length but also in isomerism, as discussed above. In our present discussion, the treatment will be limited mainly to consideration of specific phospholipides as groups rather than as individual compounds.

LECITHIN. Since this compound contains a strongly acidic phosphate group and a quaternary nitrogen atom of basic strength, the zwitter ion formula for its structure, as represented above, is usually given. The calculated value for the isoelectric point of lecithin is pH 7.5. Experimental observations on purified preparations have generally yielded figures in the vicinity of pH 6.7. Preparations that are contaminated with free fatty acids will, of course, exhibit lower values. Lecithin can be hydrolyzed with aqueous acids or alkalis to yield fatty acids, choline, and glycerol phosphoric acid. Generally, lecithin is thought of as containing one mole of oleic and one mole of stearic acid for the R groups in the above formula. However, it may contain many other long chain saturated or unsaturated acids. Naturally occurring lecithin fractions ordinarily are highly unsaturated and exhibit iodine numbers of 100 or greater. The compound, when freshly isolated as a "pure" preparation, is colorless and odorless. It rapidly autoxidizes to a brown material having the typical tallowy odor of oxidized lipides. This odor is presumably due to unsaturated carbonyl compounds.

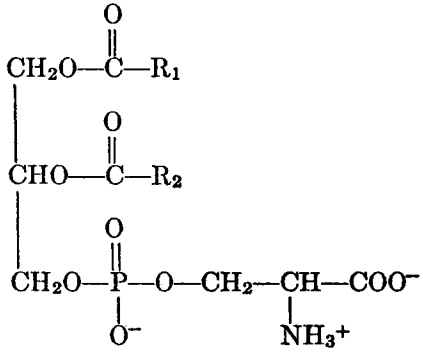
Lecithin is soluble in most organic solvents. Its insolubility in acetone affords the classic means of separating it from glyceride material. From the standpoint of inhibiting autoxidative deterioration, storage under acetone is advantageous. From a solubility standpoint, one of its most distinctive properties concerns its capacity to complex with various materials to alter their solubility and impart surface active properties.

CEPHALIN. The so-called cephalin fraction was originally thought to consist only of phosphatidyl ethanolamine but later was found to contain phosphatidyl serine as well. The structure of phosphatidyl ethanolamine may be represented as follows:



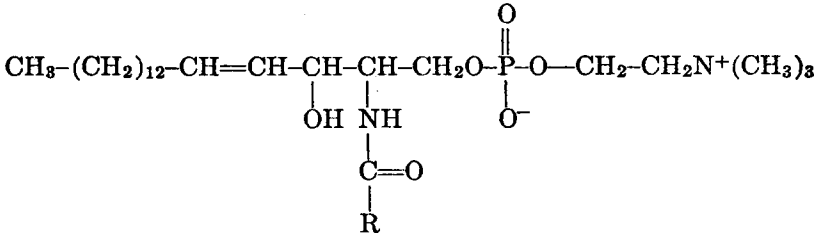
The ethanolamine group is more weakly basic than the choline of lecithin. At physiological pH, phosphatidyl ethanolamine probably exists in combination with various metal ions, since its isoelectric point should be relatively low. It is somewhat more soluble than lecithin toward the polar end of the solvent spectrum (i.e., in methanol, ethanol, glacial acetic acid, and moist ether). Like lecithin, it autoxidizes in air with darkening. It appears to be somewhat more stable toward hydrolysis at the ethanolamine-phosphate linkage than is lecithin at the choline-phosphate linkage.

Phosphatidyl serine has the following type structure:



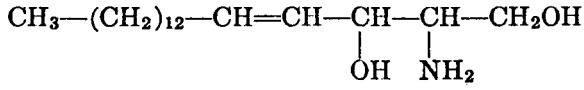
It is more stable toward autoxidation than is lecithin. It is soluble in the more non-polar solvents, but is insoluble in methyl and ethyl alcohol. As indicated by the assigned structure, phosphatidyl serine is a strong acid which is combined with sodium and postassium ions in the natural state.

SPHINGOMYELIN. Sphingomyelin is generally represented by the type formula



Hydrolysis yields sphingosine, choline, a fatty acid, and phosphoric acid. Regarding basic and acidic groups, sphingomyelin parallels leci-

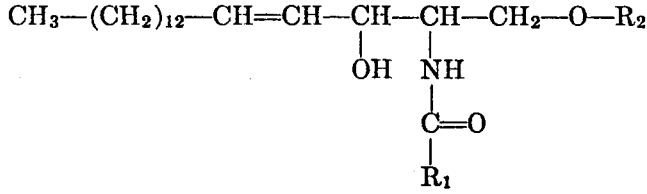
thin. The compound appears to be neutral with an isoelectric point of about pH 7. Sphingosine has the following formula:



The fatty acids associated with sphingomyelin as represented by the R group in this structure ordinarily fall in the C_{20} to C_{24} range.

In comparison to the other two types of phospholipides which have been described, sphingomyelin is much less susceptible to autoxidation and is relatively stable in air. Since it is more stable it would be expected to contain less unsaturation and lower iodine number than lecithin and cephalin. Iodine values of approximately 30 for this substance have been reported. It is soluble in benzene, glacial acetic acid, and warm ethanol. The reasonably pure preparations of sphingomyelin that have been recovered from animal tissue have exhibited very high melting points (209° C.).

CEREBROSIDES. Like sphingomyelin, the cerebrosides contain the organic base sphingosine (see previous section). Although they do not contain phosphorus, they are classified with sphingomyelin as sphingolipides. The cerebrosides are characterized by the presence of galactose in their structure. They also contain fatty acids of a unique nature with chain lengths in the vicinity of C_{24}. These appear to be connected through an amide linkage. The structure of cerebrosides may be represented by the following formula, in which R_1 represents the alkyl group of the fatty acid and R_2 the galactosyl radical:



The cerebrosides can be secured as white crystalline materials. They are essentially insoluble in ether and petroleum ether, but are soluble in hot alcohol, acetic acid, or pyridine. The cerebrosides are hydrolyzed by aqueous or alcoholic solutions of acids to sphingosine, fatty acid, and galactose, or their alcoholic derivatives.

BIOGENESIS OF MILK FAT

A complete consideration of milk fat synthesis involves not only the processes of the mammary gland but rumen physiology and general metabolism of the cow as well. The details of these subjects are beyond the bounds of this book, yet some consideration of milk fat synthesis seems in order. One publication (27) has attempted to correlate the theories and observations in this field into a single systematic explanation. The interested reader may also wish to consult the text by Espe and Smith (7) which contains appropriate information on the subject.

Much of the recent valuable work which has been done on the synthesis of milk fat has employed slices or homogenates of the mammary glands from various mammals, including those of rats, rabbits, sheep, goats, and cows. These tissue slices and homogenates have been incubated in substrates containing various radioactively labeled materials, particularly glucose and acetate. The degree to which the findings from one species are applicable to another is, of course, open to question. However, sufficient data seem to have been accumulated to indicate a general similarity, at least among the ruminants.

Glycerol for the synthesis of the milk fat triglycerides is derived primarily from glucose. The pathway involved is apparently the conventional breakdown of glucose to trioses and the immediate precursor is thought to be α-glycerol phosphate. It is a point of interest that this latter product by alternative mechanisms could be the raw material for both phospholipide and triglyceride synthesis. The fatty acids involved in the triglyceride synthesis may result from at least the following three sources: (*a*) ingested fat, (*b*) depot fats of the animal body, and (*c*) short chain acids, particularly acetate, resulting from rumen metabolism. Interest has centered mainly around the latter origin, since it appears to account for the variety of short chain acids found in milk fat.

The lower fatty acids of milk fat, and to some extent the longer chain acids, appear to be synthesized by a chain lengthening and hydrogenating mechanism which uses acetate and β-hydroxybutyrate as substrate and which involves coenzyme A for synthesis. The evidence for such a mechanism comes from classical studies with carbon 14-labeled acetate. When this material was fed to the cow, radioactivity was observed to be distributed in alternating carbons of the fatty acid chain. Moreover, it was concentrated primarily in the shorter chain

acids and to a lesser extent in some of the long chain acids. Evidence supporting this mechanism has come from a number of studies and seems firmly established. Recent studies along these lines also indicate that the cholesterol synthesized by the mammary gland also originates from acetate.

That group of monounsaturated acids found in milk fat, ranging in chain length from C_{10} to C_{16} with double bond in the 9 position, poses a problem of explanation. The uniformity regarding position of the double bond, which coincides with its position in oleic acid, may possibly indicate the latter acid as origin and chain reduction by 2 carbon units as the mechanism of formation.

Beyond the nature of the raw materials and whence they may be derived, little is known regarding the actual synthesis of the triglycerides. Presumably esterase-type enzymes are involved and it is notable that the esterification invariably goes to completion since the occurrence of more than trace amounts of mono- and diglycerides in nature is seldom if ever encountered. In fact, presence of these substances in fats is a rather reliable indication of decomposition. Once the triglyceride molecule is formed in the milk secreting cell, it may naturally tend to aggregate with like molecules since aqueous solution is impossible. The question arises as to what determines globule size. The two factors which appear to be primarily involved are the amount of available phospholipide and the rate of fat synthesis. It has been proposed, and calculations tend to support, that the amount of phospholipide available (for a monolayer) probably determines the amount of globule surface area which is possible. Assuming a given weight of fat, small globules would present a greater surface area than larger globules. Assuming the phospholipide supply to be constant during synthesis of milk fat, globule size becomes primarily a function of rate of synthesis. In any event, it is significant that phospholipides, carotene, and vitamin A all appear to vary with the surface area of the globules in milk. Since the phospholipides are surface active and are presumably dispersed uniformly over the surface of the fat globules, they may well have some bearing on the sealing-off process which determines globule size. Moreover, since fat synthesis represents creation of non-polar material from a polar phase, it is logical to assume that phospholipide, as interphase-active substance, may be of basic importance in the mechanism.

The extent to which phospholipides are synthesized in the mammary gland is not known, nor is their exact role or relation to milk fat synthesis understood. It is quite possible that the blood normally carries

sufficient of these substances to meet the need of milk secretion. It is also probable that one of their principal functions in milk is to maintain the fat in a finely emulsified state.

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CHAPTER

3 LACTOSE

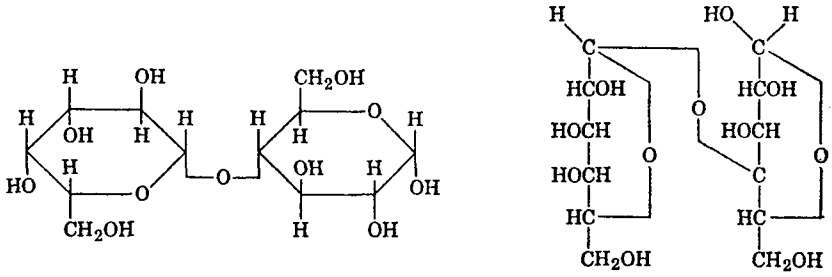
Lactose, milk sugar, is the carbohydrate of milk. Although trace amounts of glucose, galactose, and other sugars are present (see Chapter 7), lactose is the only sugar in milk in significant quantities. Moreover, in most mixed milks lactose is the solid constituent present in the greatest quantity. It generally ranges between 4.7 and 4.9%. It is true that in the milk of individual cows and in the milks of high fat-testing breeds, milk fat content may at times exceed the amount of lactose present. The most important factor affecting the level of lactose in milk is the condition of udder infection. Mastitis promotes an increased level of chlorides in the milk and depresses secretion of lactose. However, from an osmotic pressure standpoint, these changes are compensating and the osmotic pressures of normal and mastitic milk are generally the same. As suggested by these relationships, the lactose content of milk is inversely proportional to ash content.

Lactose has many aspects of significance in milk and milk products. It is a controlling factor of first importance in fermented and ripened dairy products, it contributes to the nutritive value of milk and milk products, it is related to the texture and solubility of certain stored dairy products, and it plays an essential role in the color and flavor of highly heated (caramelized) dairy products. In essence, milk would

not be milk without lactose. The milk of a few species of mammals may contain principal carbohydrates other than lactose and a few rare instances of lactose occurring in the plant kingdom have been reported. These instances, however, are isolated exceptions to the generally valid rules that lactose occurs nowhere other than in milk and that lactose is the characteristic sugar of milk.

PROPERTIES

Structure. The structural formula for lactose is as follows:



Haworth formula

Straight chain formula

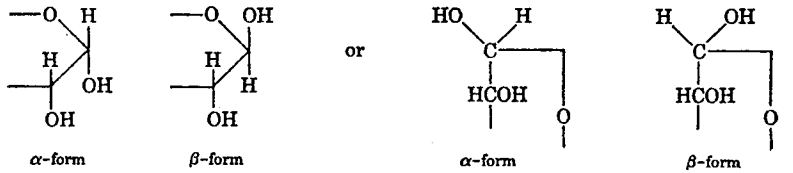
α-Lactose

The two presentations of the structure are made because both forms are in general use. The Haworth-type formula (left) is being used increasingly for all carbohydrates. It more nearly represents the actual spatial arrangement of the atoms. The straight chain formula (right) appears to have some advantage when demonstration of reactions is at point. In any event, no two-dimensional presentation is entirely adequate since the molecules are three-dimensional. In the above formulas the left-hand portion of the molecule is the galactosyl radical; the right-hand portion is glucose. The only difference between these two hexoses involves the substituents on carbon 4.

On acid or enzymatic hydrolysis, one mole of lactose yields one mole each of glucose and galactose, establishing it to be a disaccharide of the two hexoses. Oxidation with bromine water yields lactobionic acid which on hydrolysis produces galactose and gluconic acid, thus demonstrating that the linkage between the sugars is galactosidic, that is, combined through the reducing group of galactose, and that the free reducing group is that of the number one carbon of glucose. Additional evidence supporting this point can be obtained through hydrolysis of

lactose phenylosazone. This derivative on acid hydrolysis yields galactose and glucozone, indicating the bound condition of galactose and the reactive state of the glucose portion of the molecule. Methylation and hydrolysis experiments establish that there is 1–4 linkage between galactose and glucose and that the indicated hydroxyl groups are free and available to methylation. Existence of a β-galactosidic linkage between the two hexose units is established by the fact that β-galactosidase enzyme from almond emulsin, and not α-galactosidase, is capable of hydrolyzing the linkage. Additional support for the linkage is evident from the synthesis of lactose by a method which ordinarily gives β-galactosides. Thus, specifically, lactose is 4-(β-D-galactopyranosyl)-D-glucose. The reader may wish to consult the text by Pigman and Goepp (6) for a more detailed consideration of structure and nomenclature of sugars.

Physical forms. Lactose exists in two basic isomeric forms. These forms, designated α and β, are closely analogous to the isomeric α- and β-glucoses. The designations refer to the configuration of substituents on the number one carbon of the glucose moiety.



The β-form exhibits a specific rotation of $[\alpha]_D^{20} = +35.0°$ whereas the α-isomer shows a specific rotation of $[\alpha]_D^{20} = +89.4°$, both on the anhydrous weight basis. The α-form ordinarily is not met in the anhydrous state although it can be so prepared. It has a characteristic tendency to crystallize as the monohydrate, and in supersaturated solutions crystallizing below 93.5° C. it is this hydrate form of lactose which is deposited. Above 93.5° C., crystallization or drying of lactose solutions yields the β-anhydride, the other commonly encountered form of lactose. Careful drying of lactose monohydrate under vacuum above 65° C. yields the α-anhydride, which form is stable indefinitely in the absence of water. α-lactose anhydride is converted to its hydrate below 93.5° C. and to the β-anhydride above 93.5° C. All of these forms of lactose undergo mutarotation in aqueous solution, yielding a specific rotation of $[\alpha]_D^{20} = +55.4°$ (anhydrous basis) at equilibrium. The achievement of equilibrium at room temperature is a gradual process which in simple aqueous solution requires about 24 hours. This

equilibrium is obtained instantaneously in the presence of a trace of ammonia (pH 9 or higher). At 20° C. and rotational equilibrium, a lactose solution will be composed of 62.25% β-form and 37.75% α-form. Thus the equilibrium constant is 1.65 at this temperature. In all probability trace quantities of the free aldehyde form of lactose also exist in equilibrium solutions.

Depending upon the temperature and conditions of drying, the rapid removal of water from lactose solutions and from various dairy products can result in the formation of a lactose glass. These glasses are actually highly concentrated solutions containing various amounts of α- and β-lactose. The fact that such a glass has a vapor pressure which is not ordinarily in equilibrium with that of the air, tends to make it, and products containing it, very hygroscopic.

Solubility. The α- and β-forms of lactose show distinct differences in solubility. Whereas at 15° C., approximately 7 g. of α-monohydrate will dissolve initially in 100 g. of water, approximately 50 g. of β-lactose will dissolve in this amount of water. Thus, although in solutions of lactose under rotational equilibrium the β-form is present in the largest amount, the α-form, because of appreciably lower solubility, crystallizes out. Actually, this crystallization phenomenon involves a continuing shift; as the α-hydrate crystallizes out, some β-lactose shifts to the α-form in order to maintain equilibrium distribution. This process will continue indefinitely until crystallization ceases. As with most organic compounds, both α- and β-lactose exhibit increased solubility in water at elevated temperatures. The relationships are shown in Fig. 1.

Lactose is very slightly soluble in ethyl and certain other alcohols. It is insoluble in the more non-polar organic solvents such as ether, chloroform, benzene, and ligroin. A practical point, worthy of note in the laboratory preparation of lactose and certain of its derivatives, concerns the patience required in achieving crystallization. Whereas many types of organic compounds will deposit from supersaturated solutions in well-defined crystalline aggregates after periods of a few minutes or, at most, a few hours, lactose and its derivatives, and carbohydrates in general, ordinarily may require days and, at times, even months to accomplish satisfactory crystallization. One factor which is worth avoiding in this connection is the excessive viscosity of highly concentrated solutions. Rather than build up concentration to such a level it frequently is better to let the solution remain more dilute and afford it adequate time to crystallize.

The relative insolubility of lactose in water, as compared with most

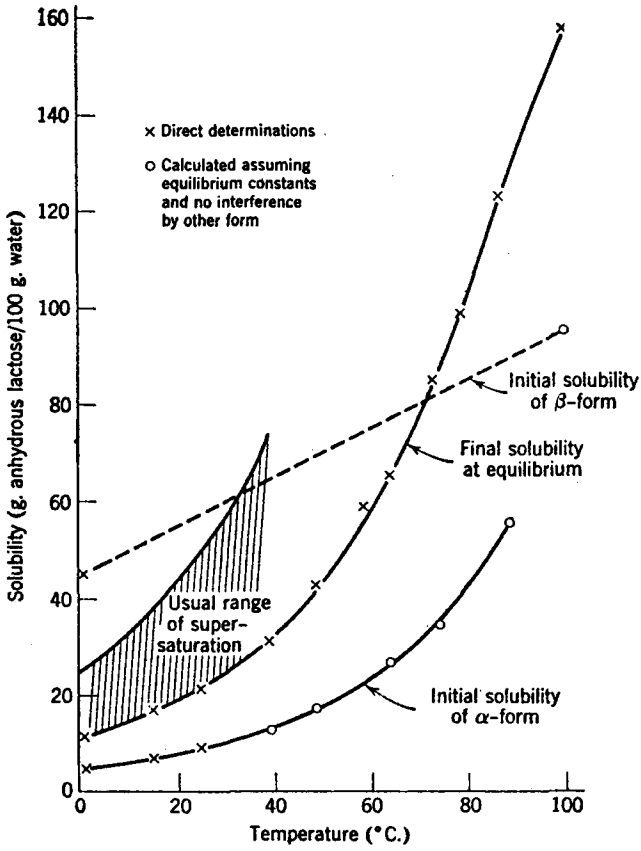


Fig. 1. Solubility of lactose in water. Data from Whittier (9).

other sugars, coupled with its capacity to form supersaturated solutions, is a matter of practical importance regarding certain dairy products. In water at 25° C. the final solubility of lactose is approximately 18%. Thus milk concentrated 3 : 1 approaches saturation when held at room temperature. Cooling of such concentrated milk or the addition of substantial quantities of sucrose renders it supersaturated and susceptible to crystallization of α-lactose hydrate. Such crystals, which not uncommonly deposit in a characteristic tomahawk shape, are very hard and, when they are permitted to grow in an undisturbed manner, achieve sufficient size to impart a distinct gritty or sandy sensation in the mouth.

Other properties. In the course of studies on lactose, many physical characteristics and properties have been observed. Some of

the more useful of these for the α- and β-forms are presented in the following table.

Table 1. SOME PHYSICAL PROPERTIES OF THE TWO COMMON FORMS OF LACTOSE

Property	α-Hydrate	β-Anhydride
Melting point*	202° C. (dec.)	252° C. (dec.)
Specific rotation† $[\alpha]_D^{20}$	+89.4°	+35°
Solubility (g./100 ml.)		
Water at 20° C.	8	55
Water at 100° C.	70	95
Specific gravity (20° C.)	1.54	1.59
Specific heat	0.299	0.285
Heat of combustion (cal./g.$^{-1}$)	3761.6	3932.7

* Values vary with rate of heating, α-hydrate loses H_2O (120° C.).

† Values on anhydrous basis, both forms mutarotate to +55.4°.

Both forms of lactose melt with decomposition. Determination of this constant by the capillary tube method clearly reveals this phenomenon. As the temperature is raised, the crystals turn from white to yellow to a light tan, which at the point of melting quickly turns to a dark brown, accompanied by the evolution of considerable gas.

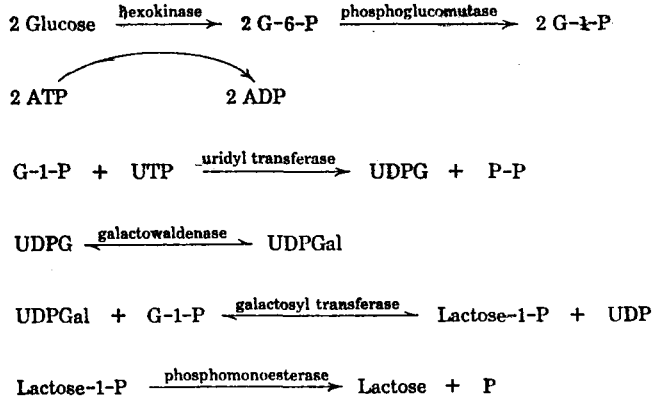
Lactose is only about one-fifth as sweet as sucrose. Enzymatic inversion of lactose to a syrup of glucose and galactose enhances the sweetness considerably. Lactose readily adsorbs odors and in recent years has enjoyed substantial use as an adsorbant for various types of compounds in chromatographic analysis. Lactose exhibits the properties of a very weak acid and shows a number of dissociation constants in the region of 1×10^{-12} to 1×10^{-14}.

SYNTHESIS

Lactose may be synthesized either chemically or enzymatically. Chemical synthesis can be accomplished through interaction of tetra-acetyl galactosyl chloride, sodium ethylate, and glucose. Several other multi-step syntheses of lactose, which have helped to prove its structure as well, are known. Because of the huge quantities of lactose which are available from cheese whey, chemical and in vitro enzymatic methods of syntheses are not of commercial importance.

However, the biogenesis of lactose may be of considerable importance from the standpoint of understanding the composition of milk

and the secretion mechanism. The in vitro enzymatic synthesis of lactose from glucose by mammary tissue is well established. In addition to the critical enzymes, glucose, uridine, and sources of phosphate are essential raw materials. Lactic acid also has been proposed as a precursor of lactose. The synthesis of lactose, as proposed by Gander and Boyer,* involves first the conversion of 2 moles of glucose to 2 moles of glucose-6-phosphate. This step is accomplished by hexokinase and the phosphate is furnished by the conversion of 2 moles of adenosine triphosphate (ATP) to adenosine diphosphate (ADP). The glucose-6-phosphate is then converted to 2 moles of glucose-1-phosphate by phosphoglucomutase. In the presence of uridine triphosphate the glucose-1-phosphate is transformed to uridine diphosphoglucose through action of uridyl transferase. The latter glucose derivative is converted to the corresponding galactose derivative by the enzyme galactowaldenase. Galactosyl transferase couples one mole each of uridine diphosphogalactose and glucose-1-phosphate to form lactose-1-phosphate. The lactose-1-phosphate is hydrolized to lactose by phosphomonoesterase. It is notable that a number of the sugar phosphates of this scheme, which follows, have been demonstrated as trace constituents of milk.



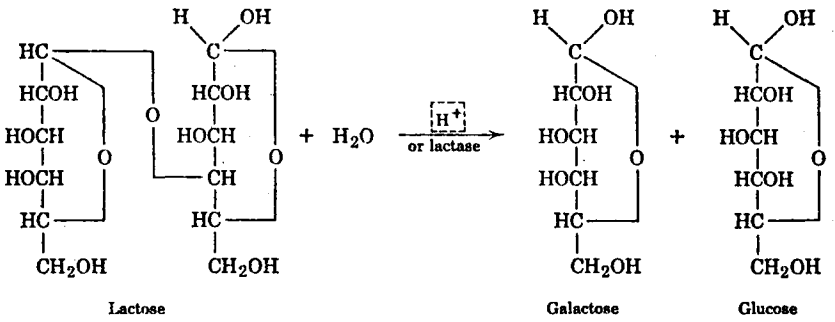
CHEMICAL REACTIONS

It is a well known fact that under rigorous reaction conditions, carbohydrates undergo extensive, complex chemical changes. With

* Personal communication, 1956.

certain reagents, this is true even under mild conditions. Although lactose is no exception to these general rules, much of its chemistry is quite straightforward and involves these four sites of attack: (*a*) the 1–4-linkage between the galactose and glucose units; (*b*) the reducing group of the glucose unit; (*c*) the hydroxyl groups of both the glucose and galactose units; and (*d*) the carbon to carbon bonds.

HYDROLYSIS. Hydrolysis of lactose to glucose and galactose may be effected satisfactorily by any of the three following agents: mineral acids, lactase enzyme preparations, and ion exchange resins.



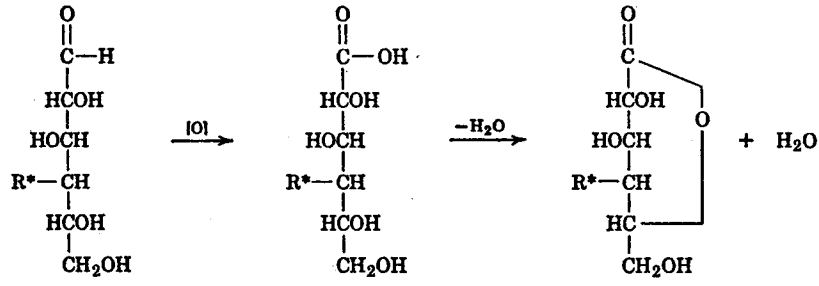
Organic acids, such as citric, which satisfactorily hydrolyze (invert) sucrose are largely without effect on lactose. Thus, it is necessary to resort to the stronger mineral acids, such as hydrochloric and sulfuric, for the hydrolysis of lactose. In general, higher temperatures and higher concentrations of acid favor more rapid hydrolysis. However, such conditions also favor undesirable side reactions such as discoloration, bitter and odorous substances, formation of furan compounds, and other sugar fragments. A 10% lactose solution adjusted to pH 1.2 with hydrochloric acid is essentially quantitatively hydrolyzed when held at 150° C. for one hour. At times, achieving such temperatures with aqueous solutions in the laboratory is not practical and it may be found more convenient to accomplish the hydrolysis of a 5% solution at 90° C. by treating it with 10 ml. of concentrated hydrochloric acid per 100 ml. of solution for 90 minutes.

There are at least three significant origins of lactase enzymes which may be used for the hydrolysis of lactose. These are: (*a*) certain species of yeasts, for example, *Torula cremoris;* (*b*) the intestinal mucosa of mammals, particularly that of the calf; and (*c*) β-galactosidase from almonds. Of these, the lactase preparations from yeast appear to be the most important. At least two commercial firms have manufactured such preparations in pilot scale quantities. However,

no large-scale uses for lactase have been developed to date. One commercially available lactase from yeast shows optimum activity between pH 6.0 and 6.5 and an incubation temperature of 45° C. Somewhat lower incubation temperature affords more prolonged activity of the enzyme. This lactase is inactivated by heating to 75° C. for 15 minutes. Enzyme concentrations between 1 and 5%, based on lactose, will hydrolyze about 7 times their weight of lactose per hour at 40° C. Hydrolysis proceeds rapidly to 50% of total and more slowly to 70%. Activity beyond 75% hydrolysis is very slow. Yeast lactase has been observed to act satisfactorily in whole milk, skimmilk, whole and skimmilk concentrates, sweetened condensed milk, and whey.

The hydrolysis of lactose in aqueous solution has been achieved satisfactorily with sulfonated polystyrene resins in the hydrogen form. Since sulfonic acids are strong acids, hydrolysis by them does not differ in principle from the action of mineral acids. However, it is claimed that discoloration of the lactose solution and certain other undesirable side reactions are avoided by ion exchange resin hydrolysis. This appears reasonable since under such conditions hydrolysis would be effected primarily at the surface of the resin.

Oxidation. The extent to which lactose may be oxidized will vary with the particular reagent, its concentration, and other conditions of the reaction. Thus, by selection of conditions it is possible to derive oxidation products from lactose, which range from relatively simple alteration of the reducing carbon in the glucose portion of the molecule to a carboxylic acid group, to complete degradation, with the end products being CO_2 and water. The conversion of lactose to lactobionic acid or, more properly, 4-(β-D-galactopyranosyl)-D-gluconic acid is one of the most significant reactions of lactose. The essence of this reaction is as follows:



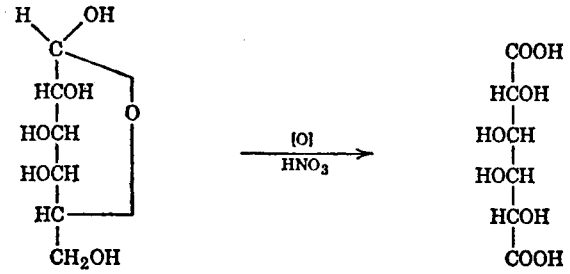
Lactose — Lactobionic acid — Lactone of lactobionic acid

R* = β-D-galactopyranosyl-

In methods for the quantitative measurement of lactose in which its reducing property serves as the basis of measurement, lactobionic acid is the resultant product. As indicated, such compounds as lactobionic acid have a profound tendency to form lactones through inner esterification with the hydroxyl group of the number 4 or 5 carbon.

Reagents that favor the complete oxidation of lactose to CO_2 and water are: acid or alkaline potassium permanganate; alkaline conditions with catalytic amounts of cerous hydroxide, ferrous sulfate, and sodium sulfite; and sunlight with zinc oxide serving as catalyst. The complex changes induced by alkali in the absence of oxidative catalyst are discussed under the section on dehydration and fragmentation. Complete oxidation of lactose to CO_2 and water also can be accomplished by biological oxidation. For this purpose a sewage sludge inoculum containing mixed bacterial and protozoal cultures is required together with abundant aeration.

A host of intermediate oxidation products from lactose are possible under various conditions. The oxidation of lactose with dilute nitric acid yields mucic acid, which is produced from the galactose portion of the molecule according to the following scheme:

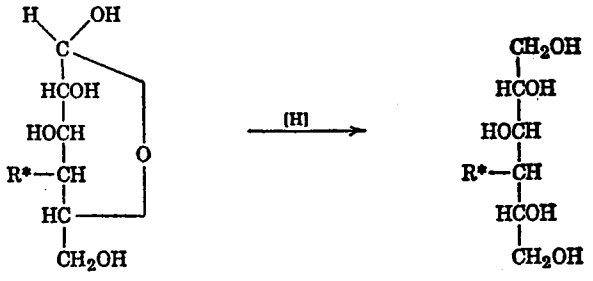


Galactose Mucic acid

Oxidation with concentrated or hot nitric acid leads to a number of short chain acids, such as tartaric, racemic, and oxalic. Gluconic and galactonic acids are obtainable from lactose under acidic oxidizing conditions. These could result either through hydrolysis of lactose, first to glucose and galactose followed by subsequent oxidation of the hexoses, or through formation of lactobionic acid followed by hydrolysis to gluconic acid and galactose, the latter then being oxidized to galactonic acid. Periodate oxidation yields formaldehyde, and reac-

tion of lactose with iodine under pressure yields formaldehyde, formic acid, and humic substances.

Reduction. Simple reduction of lactose yields lactositol or more specifically 4-(β-D-galactopyranosyl)-D-sorbitol. This reaction may be represented as follows:



Lactose

Lactositol

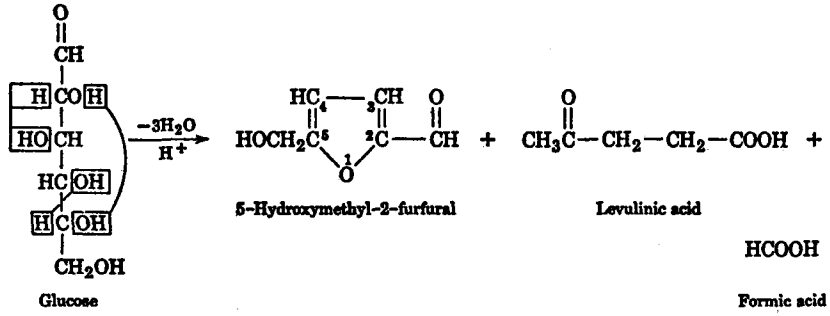
R* = β-D-galactopyranosyl-

Lactositol may be produced from lactose under several different reaction conditions. Perhaps of first importance is electrolytic reduction, since large-scale production of the compound could be effected by this means in the same manner that sorbitol is produced commercially from glucose. Using certain conditions of pressure and temperature it is possible to hydrogenate (reduce) lactose to lactositol. An 80% yield has been reported under such conditions, using a temperature of 145° C. and a pressure of approximately 120 atmospheres. When conditions favor hydrolysis of the lactose or lactositol, hexitols (dulcitol and sorbitol) are obtained. Under highly rigorous conditions of reduction, various other monohydric and polyhydric alcohols are obtained.

So far as is known there are no significant uses for lactositol. The compound is a hard crystalline solid melting at 146° C. In water it has a specific rotation of $[\alpha]_D^{25} + 14°$. Except for being slightly sweet, it is odorless and tasteless.

Dehydration and fragmentation. The decomposition of lactose in basic and acidic solutions under the influence of heat involves some unique chemistry. This phase of carbohydrate chemistry has attracted the interest of investigators for many years. In its applied aspects the study of sugar decomposition overlaps that area of chemistry that deals with the browning of foods. The browning of milk and the interaction of lactose with amino compounds is considered at length in Chapter 11.

The number of operative chemical mechanisms and end products in the decomposition of a sugar can be very large. The investigations of Nef have revealed that over 90 compounds are formed in the alkaline degradation of glucose. Comprehensive treatment of the matter is beyond the scope of this text. However, it seems of value to trace some of the possible pathways by which lactose can be degraded and to reveal some of the end products which may be formed. Under acid conditions as mentioned previously, lactose undergoes hydrolysis to glucose and galactose. Glucose, and probably galactose as well, can then undergo dehydration to hydroxymethylfurfural. Additional end products are levulinic and formic acids. Although the latter compounds can result from acid hydrolysis of hydroxymethylfurfural, it has not been established that the furan compound is an essential intermediate. Starting with glucose, this series of reactions may be represented as follows:

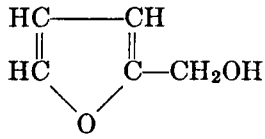


Hydroxymethylfurfural, when heated in the presence of acids, gives rise to a brown color. Unquestionably some of the color produced in heated acidic lactose solutions is formed in this manner. Although levulinic and formic acids and hydroxymethylfurfural are the only compounds which are well documented as occurring in the acid decomposition of lactose, there are no doubt others as yet unidentified. From the findings regarding glucose and from the knowledge of sugars in general, it may be deduced that lactose would exhibit greater stability under acidic than under alkaline conditions.

In the area of neutrality and under alkaline conditions the extent and mode of lactose decomposition is governed by buffer capacity of the medium, as well as by pH. At these pH values the principal decomposition products that are formed are relatively strong acids, formic acid in particular. The result is that lacking any buffer

capacity there is a fairly rapid shift to acidic conditions, which tends to make the sugar somewhat more stable towards further decomposition and tends to promote those products that are characteristic of acid decomposition.

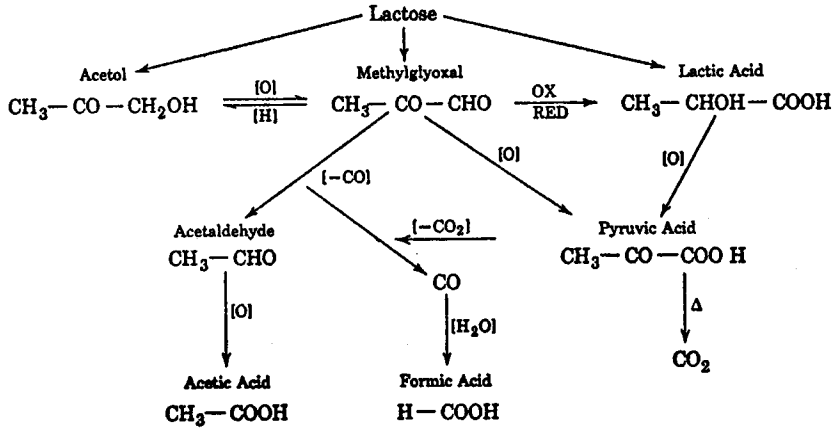
A number of compounds have been demonstrated in heated lactose solutions buffered in the vicinity of neutrality or weak alkalinity. These include hydroxymethylfurfural, furfuryl alcohol, acetol, and lactic, formic, and acetic acids. Although substantiating evidence is lacking, it might well be anticipated that levulinic, pyruvic, and saccharinic acids, methylglyoxal, acetaldehyde, and formaldehyde are produced from lactose under these conditions. These compounds have been demonstrated from glucose. In alkaline solutions of lactose, heat generation of hydroxymethylfurfural is inhibited. In a buffered solution at pH 6.6, only trace quantities of the compound are detectable. On the other hand, furfuryl alcohol



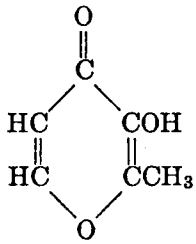
is not produced on heating a lactose solution buffered at pH 4.6. Its formation from lactose is rather unique and is favored by mild buffering with sodium carbonate. Although furfuryl alcohol can be produced from maltose and cellibiose, it apparently cannot be formed from sucrose or any of the monosaccharides. The mechanism of furfuryl alcohol formation from lactose is not known, but studies with lactose-1-C^{14} have suggested that it comes largely if not completely from the glucose portion of the molecule.

Acetol, CH_3-CO-CH_2OH, can be produced from most sugars by heating in aqueous solution. Its formation from lactose is favored by heating under weakly alkaline conditions. Whereas many of the tests for acetol do not satisfactorily differentiate it from methylglyoxal, CH_3-CO-CHO, the test with *o*-aminobenzaldehyde developed by Baudisch and Deuel is specific for the compound.

The means by which most of the lactose fragments are formed is not known. Studies with lactose-1-C^{14} have revealed that the major part of the formic acid produced is derived from the number one carbon of lactose. From available evidence regarding the decomposition of other reducing sugars, it is possible to formulate a scheme relating some of the smaller lactose fragments.



In a lactose solution containing casein, glycine, or small quantities of ethanolamine, it is possible to demonstrate the production of maltol



on heating. This compound is not generated from lactose in the absence of amino groups. Moreover, it is not formed from sucrose or hexoses, but can be produced from maltose or cellibiose. Maltol, melting point 160° C. with sublimation, exhibits the properties of a phenol, weakly acid and positive to the ferric chloride test with a reddish-purple color. It has a pleasant odor reminiscent of a combination of vanilla and burnt sugar.

The heating of lactose under anhydrous conditions results in the formation of dehydrated sugars known as glycosans. In addition to a lactosan, pyrolysis of lactose produces 1,6-anhydroglucopyranose and 1,6-anhydro-D-galactopyranose. Polymeric products of unknown constitution are also produced in large yields under such conditions. The fusion of lactose with alkali leads to the formation of pyrocatechol and succinic acid.

With the exception of hydrolysis, the heat decomposition of lactose generally can be explained on the basis of three mechanisms, as follows: (1) dehydration, (2) dealdolization, or the reverse of aldol condensation, and (3) internal oxidation-reduction rearrangements. Mechanism 1 accounts for formation of such types of compounds as

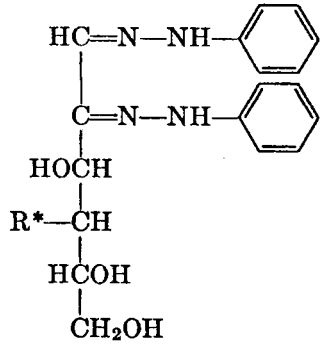
furans, pyrones, and glucosans; mechanism 2 reveals a probable means by which triose and other fragments may be formed; and mechanism 3 is required in accounting for the development of terminal methyl and certain other groups. Regarding polymeric substances, it is probable that dehydration and aldol condensation are primary mechanisms of formation.

Derivatives and identification. A substantial number of lactose derivatives are reported in the literature. To date few of these have proven to be of practical significance. However, several derivatives are important from the standpoint of characterizing lactose, and information concerning them is presented here.

The oxidation of lactose with nitric acid, resulting in the formation of mucic acid, is frequently employed as a criterion for identifying the sugar. With the exception of galactose, this acid is not produced by nitric acid oxidation of any other sugar. Mucic acid is soluble in about 300 parts of cold water and 60 parts of boiling water. This solubility characteristic, coupled with its tendency to deposit as well-defined crystals, makes mucic acid a useful identifying derivative. The melting point of the compound is variously reported between 200° and 255° C., depending upon the rate of heating. In addition, decomposition occurs during the melting. For these reasons, melting point data on the compound are not of much value. Mucic acid formation from lactose may be demonstrated as follows:

To 50 ml. of a 1% solution of lactose add 12 ml. of concentrated HNO_3. Evaporate this solution nearly to dryness on a steam bath and then add 10 ml. of water. After standing overnight at room temperature, a white crystalline precipitate of mucic acid is evident.

Probably the most useful derivative for identification of lactose is the phenylosazone. It has the following structural formula:



*R**=β-D-galactopyranosyl- .

A procedure for the preparation of lactose phenylosazone is as follows:

To 0.2 g. of lactose in a test tube add 0.4 g. of phenylhydrazine hydrochloride, 0.6 g. of $NaC_2H_3O_2$, and 4 ml. of distilled water. Mix these materials; stopper the tube with a one-holed cork or plug of cotton and heat in a boiling water bath for 20 minutes. Remove the tube from the water bath and set it aside to cool. The crystal habit of the osazone can be studied by pouring a little of the cooled reaction mixture on a watch glass, removing excess solvent with a blotter, and examining the crystals under the microscope. The crystals may also be recovered, recrystallized and dried for melting point determination.

The fact that, under the conditions of the above test, lactosazone will not precipitate from hot solution even after two hours is a distinguishing feature of the sugar. Maltose is the only other common sugar which behaves in a similar manner. The crystal habit of lactosazone is characterized as a furry rosette in its typical form which is distinctive from that of other osazones. Although the melting point of the derivative (200° C.) is similar to that of a number of other sugar osazones, it is helpful in differentiating it from some.

Another lactose derivative that is helpful for identification purposes is the octaacetate, m.p., 100° C. This derivative may be prepared as follows:

Lactose, 2 g., is added to 20 ml. of anhydrous pyridine. Acetic anhydride, 10 ml., is added with shaking, and the solution is boiled for 5 minutes under a reflux condenser. The cooled mixture is poured into 50 ml. of ice water. The derivative is removed by filtration and washed with cold 2% HCl, and then with water. Purification is accomplished by recrystallization from alcohol.

One of the most useful tools in the identification of sugars is paper chromatography. The multiplicity of methods in this field places it beyond the scope of this book. The interested reader is referred to the comprehensive treatment of the subject by Lederer and Lederer (3). One paper chromatographic method that is useful in the quantitative measurement of lactose is described briefly in the section of this chapter on measurement.

Most color reactions of sugars are not highly specific. However, one reaction of lactose is of sufficient value to warrant description here. When lactose is reacted with methylamine and NaOH, a carmine color

develops, Fearon's test for reducing disaccharides. The test may be applied to the detection of lactose in milk as follows:

To 4 ml. of water and 4 drops of milk add 3 or 4 drops of 5% aqueous methylamine hydrochloride. Boil the mixture for about 30 minutes and then add 3 to 5 drops of 20% NaOH. A yellow color forms which slowly changes to carmine, owing to the presence of lactose. This reaction is specific for reducing disaccharides. Glucose, galactose, fructose, sucrose, starch, and many sugar degradation products do not interfere.

In addition to the reactions, derivatives, and their properties that have been cited, the capacity of lactose to reduce Fehling's solution and the fact that it is not fermented by ordinary yeast also are helpful criteria of identification.

MEASUREMENT OF LACTOSE

There are perhaps more methods for the measurement of lactose than for any other milk constituent. This fact, among other things, testifies to the inadequacy of some of the methods. Several of these methods have been selected for discussion here, not because they are ideal, but because they are in general use to some extent; and for instructional purposes, they bring out certain analytical principles regarding lactose. The methods for consideration are: (*a*) polarimetric (saccharimetric), (*b*) copper reduction (Munson-Walker), (*c*) picric acid, (*d*) chloramine-T, and (*e*) chromatographic. Many modifications of these methods exist. Since a critique of methods is not of primary concern here, only one modification of each is presented.

Polarimetric Method

The presence of asymmetric carbon atoms in all the natural sugars makes them what is termed optically active or capable of rotating the vibrational plane of polarized light. The degree and direction of this rotation is a property of the particular sugar. Since it is a function of sugar concentration in aqueous solutions, measurement of the rotation can be employed as a method of analyzing for the sugar. For measuring this property of sugar solutions a polarimeter or saccharimeter is employed. Saccharimeters are polarimeters specially designed for determining the per cent of sugar directly. It is necessary to know at

the outset how much rotation will result from a known concentration of the particular sugar. This value is expressed in the physical constant, specific rotation, or $[\alpha]_D^{20}$, which is defined as the rotation in angular degrees of the plane of polarized monochromatic light that is produced by solution of the optically active substance having a concentration of 1 g. in 1 ml. of solution and polarized in a column 1 dm. long. The value employed for lactose is +55.4° on an anhydrous basis or +52.6° on the basis of lactose monohydrate. Ordinarily, the D line of the sodium spectrum ($\lambda = 589.3$ mμ) is used as the monochromatic light source. Since temperature is a critical variable, readings are usually made and reported at 20° C.

Specific rotation may be expressed as follows:

$$[\alpha]_D^{20^\circ} = 100a/lc$$

in which $[\alpha]_D^{20^\circ}$ = specific rotation at 20° C. referred to the D line of the spectrum,
a = observed angular rotation in degrees,
l = length of column of solution in decimeters,
c = concentration in grams per 100 ml.

In measuring the lactose content of milk, the medium must first be freed of protein and fat and a clear solution obtained. This is usually accomplished by treating with acid mercuric nitrate reagent and filtering. The filtrate is polarized in a polarimeter tube, yielding the value a in the equation. Assuming that a 200 mm. tube is used, the above equation for specific rotation can be simplified by inserting the constants for lactose and the length of the tube:

$$55.4 = 100a/2c$$

or by transposition, the grams of lactose per 100 ml. of filtrate (c) = $a/1.108$. In order to convert this value to per cent lactose, corrections for the volume of protein and fat removed must be made. It is worthy of note that the validity of a polarimetric method ordinarily rests on the assumption that only one optically active substance is present in significant amounts.

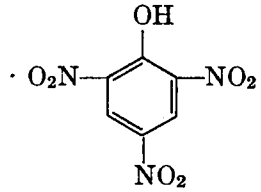
Munson-Walker Method

The basis of this method concerns the reduction of a cupric salt complex to Cu_2O by lactose. When heated together under specified conditions, the amount of oxide that precipitates from solution is

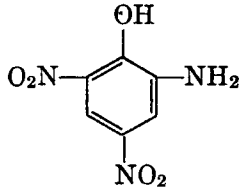
proportional to the lactose present. A weighed sample of milk is freed of protein and fat by treating with a precipitating agent and filtering. An aliquot of the clear filtrate is heated with the alkaline $CuSO_4$ reagents under exactly specific conditions. The tartrate in this reagent is used to keep $Cu(OH)_2$ from precipitating. The amount of Cu_2O precipitated is determined gravimetrically and the lactose equivalent to the quantity of oxide is read from Munson-Walker tables. The percentage of lactose can then be calculated from the weight of lactose found in the aliquot of the sample. This is strictly an empirical method since the amount of Cu_2O formed depends markedly on the reaction conditions. It is, however, capable of great precision if the prescribed method is followed.

Picric Acid Method

This method is based on the fact that reducing sugars in alkaline solution reduce yellow picric acid to mahogany red picramic acid.



Picric acid



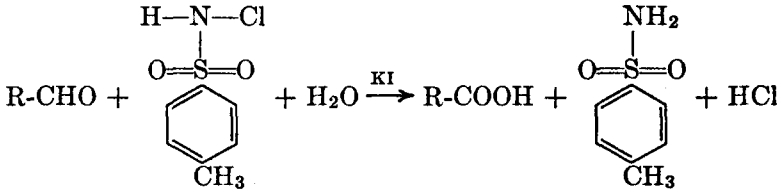
Picramic acid

Recent modifications of the method have adapted it to photoelectric colorimetry and the simultaneous determination of sucrose and lactose. In this method a weighed quantity of milk is diluted to a specified volume with saturated picric acid. The well mixed solution is filtered and a measured volume of the clear filtrate is mixed with Na_2CO_3 reagent in a sugar tube and placed in a boiling water bath for development of color. Optical density of developed color at 520 mμ is proportional to the lactose content. Since sucrose is readily inverted by heating in saturated picric acid solution, it is possible to determine both lactose and sucrose by making determinations before and after inversion (5).

Chloramine-T Method

As in other methods, protein and fat are removed from the milk with a precipitating agent, and to a measured amount of clear filtrate

a known quantity of chloramine-T reagent is added, together with KI solution. After standing for a specified period of time, the unreacted chloramine-T is measured by titration of liberated iodine with sodium thiosulfate solution. The difference in the amount of chloramine-T in a blank solution and that remaining in the sample analyzed is equivalent to the lactose in the sample. By means of an equation the percentage of lactose can be calculated. A particular advantage of this method is that no specialized laboratory instruments or equipment are required. The principle of this method also is based on the reducing property of lactose. It appears that the reagent, N-chloro-*p*-toluenesulfonamide (chloramine-T) acts as an oxidizing agent through its rather loosely bound chlorine in the following manner:



Chromatographic Method

The advantage of a chromatographic method for measuring lactose lies in its specificity. There are many conditions under which values for lactose derived from various methods are placed in doubt. For example, the measurement of lactose in heated milk products is complicated by both the destruction of lactose and the production of nonlactose-reducing substances. Under many circumstances, there is a possibility of lactose inversion to glucose and galactose. When lactase enzyme is present, not only does hydrolysis take place, but small quantities of polysaccharides are also produced. In instances where extensive fermentation of lactose has occurred, it is obviously difficult to arrive at valid figures for its concentration. Thus, it would seem that the most nearly perfect situation for measuring lactose under these problematical conditions would be a method that would isolate the lactose from all other spurious substances. Such conditions can best be achieved by chromatography. The method described here, that of Honer and Tuckey (2), is primarily a research method. Its complexity, coupled with the number of reagents, the specialized

equipment, and the lengthy time required to complete an analysis, makes it distinctly unsuited for routine work.

The method employs a descending paper partition technique. The solvent system is composed of ethyl acetate, pyridine, and water. After a specified flow time, a control strip (containing authentic lactose) is cut from the main sheet and the position of lactose spots on the rest of the paper is determined by developing the control strip with silver nitrate reagent. The lactose spots are then cut from the balance of the chromatogram and the amount of lactose in these spots determined by boiling them in alkaline ferricyanide reagent. The optical density of the blue ferricyanide color complex thus developed is measured in a spectrophotometer at 600 mμ. The amount of lactose is derived from a calibration curve, developed with known levels of lactose plotted against optical density of developed color.

To sum up the status of methods for measurement of lactose, there are many methods with many modifications and most of these have proven useful to some degree. However, there is still a definite need for a quick, simple, accurate method, specific for lactose under a wide variety of conditions.

Those methods which depend on the reducing power or optical activity of lactose are bound to have limitations since these are general properties possessed by many compounds. Although such compounds occur to a limited extent in freshly secreted milk (e.g., citric acid, ascorbic acid, glucose, and galactose), under conditions of heat treatment or fermentation, much confusion can result from lactose destruction and the formation of both reducing and optically active compounds.

MANUFACTURE AND USES OF LACTOSE

Manufacture. As with most articles of commerce, the quantity of lactose manufactured is determined in a large measure by demand. Prior to World War II, lactose production reached a stable level of approximately 7 million pounds annually in the United States. Penicillin production during World War II greatly increased the demand for lactose and production achieved a level of about 23 million pounds by 1946. During the postwar years lactose production has declined somewhat. There are three principal reasons for this: increased competition from lactose manufactured abroad, increased competition from

cheaper carbohydrate sources in the United States, and a lack of new large-scale uses for lactose. Regarding the latter, the need for intensive research appears to be urgent.

Potential production of lactose is very large. About 10 billion pounds of whey are an annual by-product of cheese and casein manufacture. This represents approximately one-half billion pounds of lactose, and only 4% of this amount is actually being manufactured into milk sugar. Unquestionably, the most important single limiting factor in the increased manufacture of lactose is competition from cheaper sources of carbohydrate. There are many products and applications in which lactose can be used to advantage; but, with very few exceptions, cane and corn sugar products, by-products of the paper making industry, and others, can do the job as well as lactose and more economically. The most plausible means of meeting this problem appears to be development of products whose merits depend upon the distinctive properties of lactose and its derivatives, and the direct use of whey, which is a more economical form of lactose than its various purified grades.

The manufacture of milk sugar from whey generally involves the following basic steps: (*a*) liming and heating of the whey, (*b*) filtration to remove precipitated proteins, (*c*) evaporation of the filtrate to obtain a concentrated lactose syrup, (*d*) cooling and crystallization of the syrup, (*e*) washing of the crystals, and (*f*) drying of the crystals. Further refinement of the product can be accomplished by recrystallization. Actually there are a very large number of methods and modifications of methods for the manufacture of lactose. These vary primarily from the above procedure in the means by which impurities are removed. In recent years there has been a definite trend towards the use of ion exchange resins in the manufacture of lactose. By one such method it is possible to obtain a lactose which is 98 to 99% pure, and the process thus yields a finished product of excellent quality. There has been increased interest recently in β-lactose because of its superior solubility over that of α-lactose hydrate. Crystallization of β-lactose is accomplished by concentrating or drying at temperatures above 93.5° C. However, the initial processes of purification and concentration are the same as for the α-hydrate. For detailed information on methods of manufacture, as well as uses, of lactose, the interested reader should refer to the comprehensive reviews of these subjects by Whittier (9) and Weisburg (8) and the text by Whittier and Webb (10).

Uses. The primary uses of lactose are: (*a*) as an ingredient in infant foods and special dietary products; (*b*) in the formulation and standardization of pharmaceuticals, tablets, and pills; (*c*) in the mold substrate for penicillin production; (*d*) in the production of caramel color; (*e*) as seed material to induce fine crystal formation in certain dairy products; (*f*) in the production of hydrolyzed lactose syrups; and (*g*) the preparation of certain lactose derivatives.

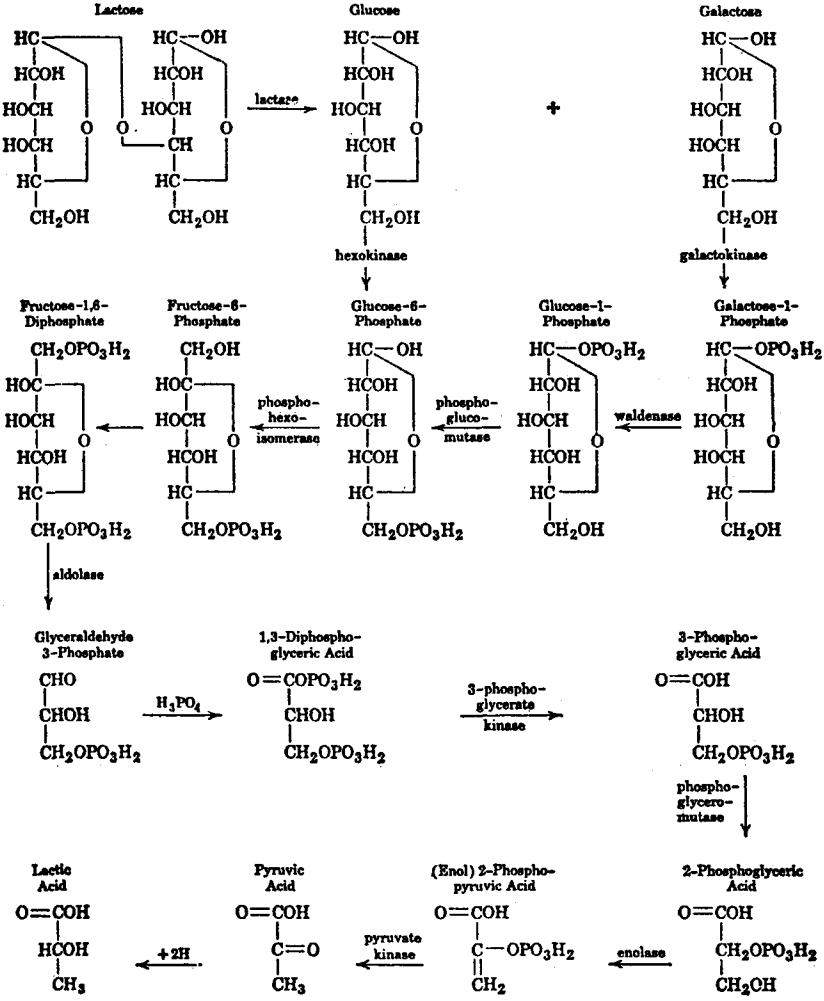
The manufacture of hydrolyzed lactose syrups is worthy of some additional comment. Although demand for such products does not appear to be significant at the present time, the hydrolysis of lactose does tend to increase its utility in some respects. Hydrolyzed lactose is sweeter; it is much more readily fermentable by a larger variety of microorganisms and it has much less tendency to crystallize as hard, gritty crystals. In instances where lactose tends to promote diarrhea, its preliminary hydrolysis, in part or in total, to glucose and galactose should be beneficial.

Certain lactose derivatives are manufactured on a commercial basis. Although producible from other carbohydrate sources, lactic acid has been manufactured from lactose by fermentation for a long number of years. Lactobionic acid, which can be produced from lactose by electrolytic oxidation, will form highly concentrated water solutions as its calcium salt. Such soluble forms of calcium are useful in the field of pharmacy. The most useful derivatives of lactose are obtained by direct fermentation of whey, which is more economical as a starting material than is lactose. Currently, butanol, acetone, lactic acid, alcohol, riboflavin, and vinegar are produced in limited quantities by fermentation of whey. A most promising fermentation application of lactose in whey involves the commercial production of yeast as a feed. Since protein is the most expensive basic ingredient in feedstuffs, it is clear that improving the protein content or potential of whey would enhance its value for feed purposes. Whey is relatively high in carbohydrate and low in protein. A typical analysis for cheese whey would be: water–93%, lactose–5%, protein–0.8%, ash–0.7%, and fat–0.4%. Moreover, lactose has distinct limitations regarding the amounts which can be fed to livestock and poultry. The conversion of this lactose, in the presence of simple forms of nitrogen, to yeast cells which are rich in protein has obvious advantages. A further attractive feature of the process is the rather ideal character of whey as a fermentation substrate.

FERMENTATION OF LACTOSE

The fermentation of lactose is worthy of careful consideration by those interested in the chemistry of milk and its products. This process is distinctive and of basic importance in the dairy field. The manufacture of cultured milks, and cheeses, the processing of certain by-products, the manufacture of lactic acid, the utilization of milk and milk products in the animal body, the estimation of quality in milk, and the differentiation and inhibition of certain microorganisms all rest on the phenomenon of lactose fermentation. Although variations in the mechanisms and products of lactose fermentation may be numerous, a consideration of the basic pathway from lactose to lactic acid is in order. Not all of the steps and conditions involved in this biochemistry are fully understood. Some facts and more plausible speculations in this connection follow.

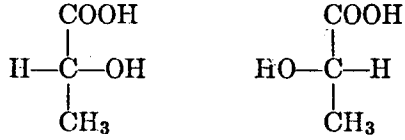
So far as is known, lactose is not used directly by any organism but is broken down first into glucose and galactose. It may be safely assumed that the lactase enzyme that accomplishes this hydrolysis is an endo enzyme, since unfermented lactose remains unchanged in the medium when the organisms are removed or cease activity. Current evidence indicates that microorganisms capable of fermenting lactose contain a waldenase-type enzyme which can convert galactose-1-phosphate into glucose-1-phosphate. It is probable that this mechanism accomplishes the isomerization of galactose into the glucose fermentation scheme. A scheme which accounts for the stepwise degradation of lactose to lactic acid is presented subsequently. It is an adaption to the so-called "Meyerhof-Embden system" and represents in part the major pathway for glucose breakdown in muscle, yeast, and many bacteria. It is not necessarily the only lactose breakdown system, nor is it necessarily applicable to all lactose-fermenting microorganisms. However, it appears that the main feature of lactose-fermenting organisms is their possession of a lactase rather than unique mechanisms of glucose metabolism. A fact which seems to suggest that there is more than one pathway to lactic acid is that microorganisms can produce various forms of this acid. The amount of lactic acid produced in a lactic fermentation may range from 75 to 95% of the total acidity. Volatile and other acids account for the remainder. These acids and related compounds are discussed under the subject of flavor in cultured dairy products (Chapter 12).



A Scheme for the Fermentation of Lactose to Lactic Acid

LACTIC ACID

Lactic acid, CH_3-CHOH-COOH, contains an asymmetric carbon atom, that is, the number 2 carbon to which four different groups are attached. Thus the acid occurs in D and L forms and as mixtures of the two.



D(−)lactic acid (m.p.52.8° C.)

L(+)lactic acid (m.p.52.8° C.)

Streptococcus lactis produces predominantly the D form. Others, including *Streptococcus citrovorus* and *paracitrovorus*, produce mainly L-lactic acid. The extent to which the various forms are produced is a function not only of the particular organisms, but also of cultural conditions and the presence of non-lactic producing contaminants as well. There are differences among microorganisms regarding capacity to produce lactic acid. In general, the maximum titratable acidity achieved in milk by Streptococci is in the range of 0.8 to 1.0%. However, Lactobacilli will reach levels of 1.5 to 2.0%. These latter levels of acidity are unsuitable in most cultured dairy products, but are sought in the commercial production of lactic acid. Hence we find various Streptococci to be the principal organisms in starters for butter, cheese, and buttermilk manufacture, and only limited use of Lactobacilli for such purposes. The difference between the two groups of organisms appears to be mainly a matter of acid tolerance. Neutralization of the substrate when acid production has leveled off will ordinarily stimulate further development of acid if additional lactose is available.

Lactic acid is a colorless, syrupy, slightly hygroscopic liquid. It is odorless and sour tasting. Thus the sourness of fermented dairy products results primarily from this acid, but the aroma of such products is due to other substances. Because of its tendency to form anhydrides and ester-type polymers, lactic acid cannot be prepared readily in pure form. It exhibits a boiling point of 122° C. at 15 mm. pressure, a melting point of 16.8° C. (D-L form) and specific gravity of approximately 1.25. It is miscible with water and alcohol, soluble in ether, and insoluble in the more non-polar solvents. Concentrated aqueous solutions of lactic acid contain appreciable amounts of anhy-

dride and lactide. However, the acid is substantially regenerated from such impurities in dilute aqueous solutions, especially in the presence of base or on heating. Lactic acid is essentially non-volatile with steam.

Reactions of both an acid and an alcohol are exhibited by lactic acid. It forms salts, esters, and amides in conventional manner at the carboxyl group. It gives the iodoform test (CH_3-CHOH-grouping). Acetylation of the hydroxyl group proceeds readily. Destructive distillation of lactic acid yields acetaldehyde and formic acid. Distinctive color tests for lactic acid include a red color in the resorcinol-sulfuric acid test and a yellow color with ferric chloride. The latter reaction is widely used in quantitative methods for lactic acid.

A review of properties and reactions of lactic acid is given by Fisher and Filachione (1).

NUTRITIVE VALUE OF LACTOSE

In many respects the nutritive role of lactose is unique. Like other carbohydrates, it serves as a source of energy and yields 4 calories per gram. However, lactose does not appear to be as effectively utilized for this purpose as most of the other dietary carbohydrates. Lactose passes through the stomach largely unchanged. Both lactose and galactose persist further along the alimentary tract than the other common sugars. The presence of lactose at these higher levels in the intestine results from its greater resistance to hydrolysis and its relatively slow rate of absorption. Important results of its presence there are the favoring of an acid type of fermentation which, in turn, favors better utilization of calcium and phosphorus. In addition, the lactic fermentation tends to discourage the growth of putrefactive organisms. In general, these properties of lactose are primarily important in the nourishment of the young.

Beyond these facts, the exact value of lactose nutritionally speaking is far from clear. Lactose has a tendency to produce diarrhea in animals when fed at elevated levels. This characteristic of lactose, as well as certain other toxic conditions which have been attributed to it, ordinarily varies with the species of animal and depends upon the feeding of extremely high levels of the sugar. In this connection, the adage comes to mind that eating too much of anything is not good for one. Actually the status of knowledge regarding the nutritive value of lactose is quite unsatisfactory, the results are conflicting, and analogies between experimental findings are not possible because of

wide variations in the test animals and experimental conditions that have been employed. There appears to be some evidence in support of the following additional properties for lactose: (*a*) Growth of certain species is more satisfactory when butterfat and lactose are fed together than when butterfat alone is fed as part of the diet. (*b*) Lactose may aid in intestinal synthesis of vitamins in certain species. (*c*) Lactose may have some value in reducing diets because of its less efficient utilization compared to other carbohydrates, its tendency to encourage gastrointestinal motility, and its property of discouraging fat deposition in the body. (*d*) Lactose appears to facilitate calcium utilization, possibly through formation of readily assimilable calcium lactate in the intestine. (*e*) In the young, galactose, derived from lactose, may be utilized in the synthesis of cerebrosides, important structural units in the brain, and the medullary sheaths of the nerves.

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CHAPTER

MILK PROTEINS 4

INTRODUCTION

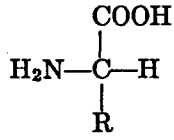
Proteins constitute an extremely important class of naturally occurring compounds that are essential to all living processes. They perform a variety of functions ranging from structure to reproduction in living organisms. Milk, the natural food of young mammals, contains a number of proteins that serve their nutritive requirements. Milk proteins also represent one of the greatest contributions of milk to human nutrition. Since the milk proteins are of such great importance and since some of them are unique products of the mammary gland, a great deal of study has been made of their secretion, composition, and properties. Their peculiarities pose some interesting problems and possibilities in the manufacture of dairy products. Much information has accumulated on the behavior of milk proteins in processing operations, but many phenomena remain unexplained. Naturally, progress in the knowledge of milk proteins has tended to follow the accumulation of new information and the development of new concepts in the general field of protein chemistry. Furthermore, a considerable part of the present knowledge of milk proteins has been discovered by protein chemists, not primarily interested in dairy problems, who have studied them as interesting proteins.

The objective of this chapter is to present the chemistry of the milk proteins within the framework of present-day concepts of protein chemistry. Consequently, it seems desirable to preface the discussion with a brief survey of the chemistry of proteins in general. Only the barest essentials are presented here. Those wishing to delve more deeply into protein chemistry are referred to the work entitled *The Proteins,* edited by Neurath and Bailey (34), which treats the subject exhaustively, or to the shorter treatises by Haurowitz (19), and by Fox and Foster (7). Some aspects of the chemistry of milk proteins have been covered in two reviews by McMeekin (28, 29). A summary and review of information on some of the minor proteins and enzymes of milk has been compiled by Whitney (56).

REVIEW OF PROTEINS IN GENERAL

Composition

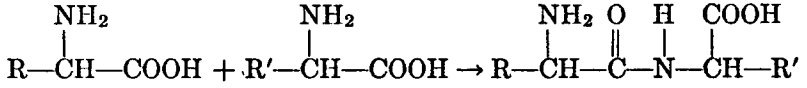
Proteins are polymers of certain L-α-amino acids* that are formed by living organisms. Some of them contain non-amino acid constituents as well, but the basic and overwhelmingly important units are amino acids. Of all the vast number of different α-amino acids that are conceivable or that could be produced in the laboratory, only 20 occur regularly in proteins. One of these, hydroxyproline, seems to be absent from milk proteins. It should be reemphasized that the amino acids found in proteins possess the common features that they are all "α" (i.e., that the amino group is on the carbon atom next to the carboxyl) and that they are all, with the exception of glycine which has no asymmetric carbon atom, of the L-configuration. The latter statement does not mean that they are all levorotatory; as a matter of fact, some of them are dextrorotatory. It merely means that the arrangement of groups on the asymmetric carbon is analogous to L-glyceraldehyde which serves as the stereochemical standard for the sugars and amino acids. The general formula for α-amino acids of the L-series is therefore:



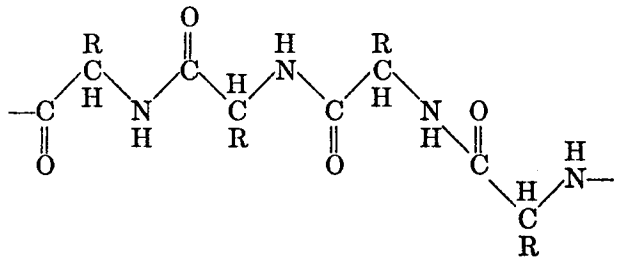
* Proline of course is a cyclic *imino* acid, but otherwise similar in structure to α-amino acids.

They differ in the R-group that they possess. The R-groups of the 19 amino acids found in milk proteins are given in Table 1. The chemistry of proteins is to a large degree concerned with the reactivity of these functional groups. They may be grouped into the general types of aliphatic hydrocarbon, alcohol, acid, amide, bases, thiol (or sulfhydryl), disulfide, thioether, aromatic, and heterocyclic, as is indicated in Table 1.

The fundamental linkage involved in proteins is the peptide linkage formed by reaction of an amino and a carboxyl group.

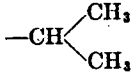
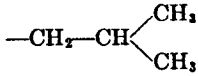
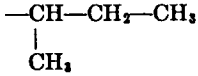
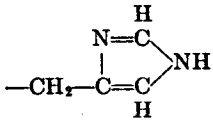
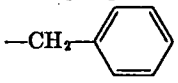
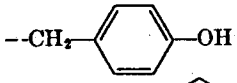
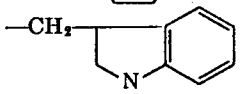
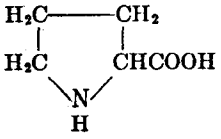


With difunctional units such as the amino acids, long chain polymers can be built up in this way. Thus the proteins consist of long chain polymers or polypeptides formed by a succession of peptide linkages and having the R groups attached along the chains. Such a polypeptide chain may be represented as follows:



For analysis of a protein for its constituent amino acids, it is hydrolyzed, usually with acid. Since tryptophan is destroyed by acid hydrolysis it must be determined separately. A variety of techniques are available for determining the individual amino acids in the protein hydrolysate. These include (*a*) chemical methods, which involve some characteristic reaction of the amino acid; (*b*) microbiological methods, depending on its ability to stimulate the growth of microorganisms; and (*c*) chromatographic methods based on its rate of migration on a chromatographic column or paper strip. These methods have been developed in the last few years to the point that many proteins have been analyzed completely for their amino acids.

Table 1. AMINO ACIDS OCCURRING IN MILK PROTEINS

Amino Acid	R Radical		Name of Reactive Group
	Formula	Classification	
Glycine	—H	Aliphatic hydrocarbon	...
Alanine	—CH_3	Aliphatic hydrocarbon	...
Valine		Aliphatic hydrocarbon	...
Leucine		Aliphatic hydrocarbon	...
Isoleucine		Aliphatic hydrocarbon	...
Serine	—CH_2OH	Alcohol	Hydroxyl
Threonine	—CH_2CH_2OH	Alcohol	Hydroxyl
Aspartic acid	—CH_2—COOH	Acid	Carboxyl
Asparagine	—CH_2—$CONH_2$	Acid amide	Amide
Glutamic acid	—CH_2—CH_2COOH	Acid	Carboxyl
Glutamine	—$CH_2CH_2CONH_2$	Acid amide	Amide
Arginine	—$CH_2CH_2CH_2$—NH—C(=NH)—NH_2	Basic	Guanidyl
Histidine		Basic heterocyclic	Imidazole
Lysine	—$CH_2CH_2CH_2CH_2NH_2$	Basic	Amino
Cysteine	—CH_2SH	Thiol	Thiol or sulfhydryl
Cystine	—CH_2S—SCH_2—	Disulfide	Disulfide
Methionine	—CH_2CH_2S—CH_3	Thioether	Thioether
Phenylalanine		Aromatic	Phenyl
Tyrosine		Aromatic	Phenol
Tryptophan		Aromatic-heterocyclic	Indole
Proline*		Heterocyclic	...

*Imino acid.

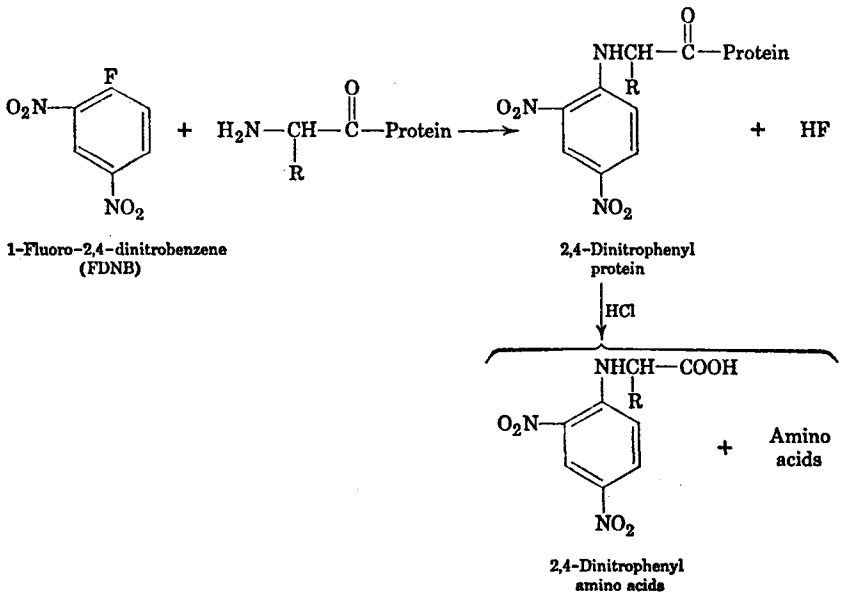
Some proteins contain materials other than amino acids, sometimes in large amounts. In some of these cases it appears that we are dealing with a mutual combination of a protein and some other substance. For example some lipoproteins appear to consist of protein and lipide portions which can be separated. In other cases the non-amino acid constituent represents an integral part of the protein structure, as for example with the phosphate groups esterified with threonine and serine in casein. In any event the proteins containing non-amino acid constituents may be lumped together as "conjugated proteins." Some of the important groups of such proteins are:

Name	Non-amino Acid Constituent
Lipoproteins	Lipide (e.g., phospholipide or carotenoid pigment)
Glycoproteins	Carbohydrate
Phosphoproteins	Phosphate
Metalloproteins	Metal such as iron or copper
Nucleoproteins	Nucleic acids

Structure

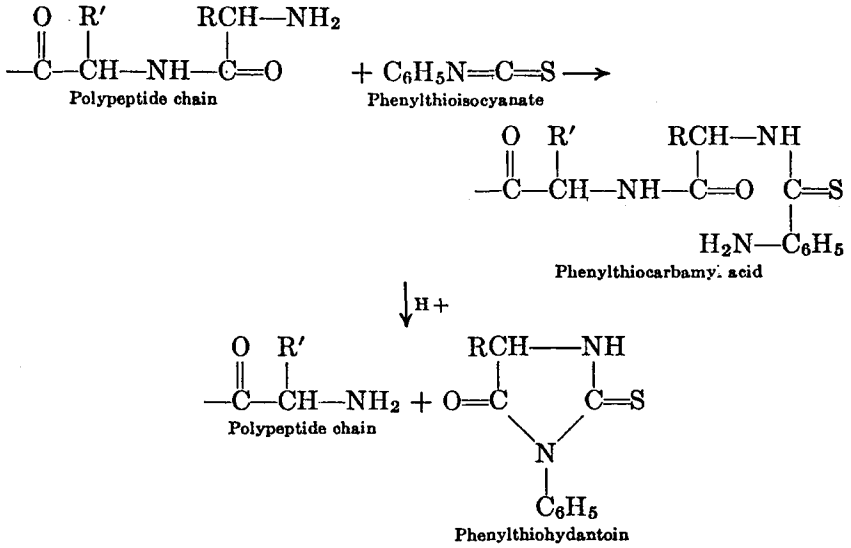
Obviously even the complete analysis of a protein for amino acids and other constituents only partially characterizes it. The sequence of amino acid residues in the polypeptide chains, the number of such chains per molecule, and the configuration (i.e., the arrangement of the molecule in space) still must be determined.

One of the most important developments in recent years has been in methods for determining the end groups of polypeptide chains (8). An unbranched polypeptide chain possesses one terminal amino group and one terminal carboxyl group. Since there is no evidence for the existence of branched chains in proteins, the determination of terminal groups is generally considered to reveal the number of polypeptide chains in the molecule. In general, the methods of end group analysis involve one of three principles: (*a*) labeling the end group by condensing it with a reagent that will withstand subsequent hydrolysis of the protein and that can be identified in the hydrolysate; (*b*) transforming the end group to some other group that can be identified in an hydrolysate, and (*c*) specifically detaching the end group. One of the most successful of these methods to date is the fluorodinitrobenzene method devised by Sanger (46) for determining the N-terminal residues. It involves the following reactions:



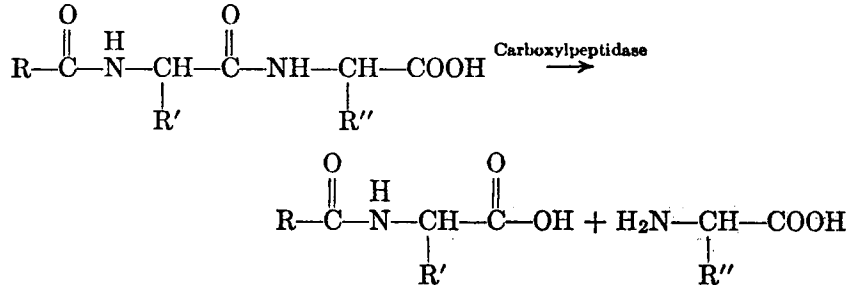
The FDNB reacts readily with the free amino groups of proteins in mildly alkaline conditions at room temperature and the dinitrophenyl group, once attached, is resistant to acid hydrolysis. Thus the DNP protein can be hydrolyzed and the terminal residue that had a free amino group in the original protein appears as a labeled DNP acid. The DNP amino acids are readily detected because they are bright yellow compounds. The FDNB also reacts with the imidazole group of histidine and the phenol group of tyrosine, but the derivatives are colorless. The sulfhydryl group of cysteine reacts to yield a colored compound which is hydrolyzed by acid. Of course, if histidine and tyrosine are present in the protein in terminal positions with the amino groups free, di-DNP-histidine and di-DNP-tyrosine will be formed. The ϵ-amino group of lysine residues also reacts with FDNB, and thus α-DNP-lysine, ϵ-DNP-lysine, and di-DNP-lysine are possible products. The hydrolysis products are usually separated by fractionation with an organic solvent, followed by a chromatographic separation, and finally in quantitative work they are estimated colorimetrically. This method has been applied successfully to identifying and determining the N-terminal residues of a large number of proteins. Furthermore, it can be applied to the peptides resulting from partial hydrolysis of proteins. Employing it in this manner, together with other techniques, Sanger determined the entire sequence of amino acids in insulin.

Another method, which is described by Edman (6), employs phenylthioisocyanate as a reagent to cleave the N-terminal amino acid while at the same time converting it to a readily identifiable derivative.



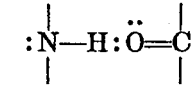
The phenylthiohydantoin can be extracted and identified. In theory at least, the process could be repeated over and over again giving a stepwise degradation of the protein and a determination of the complete sequence. This has never been accomplished with a protein however.

Although various chemical methods have been proposed for determining the C-terminal amino acid in a polypeptide chain, none has yet been developed that is generally applicable. The most satisfactory procedure at present employs an enzyme, carboxypeptidase, which preferentially splits off the C-terminal amino acid.



Actually, the rate of release of amino acids must be followed, since once the terminal acid is removed the next one is attacked, and so on. The amino acid released most rapidly is considered to be the C-terminal one.

Many proteins reveal more than one polypeptide chain since they yield more than one N-terminal or C-terminal group per molecule. In some cases the chains appear to be linked to each other through disulfide bonds (—S—S—) of cystine, the two ends of the cystine molecule being involved in peptide linkages in different chains. This disulfide bond is the only covalent or primary chemical bond other than the peptide linkage that is known with certainty to contribute to the structure of proteins. However, two kinds of non-covalent bonds are very important in maintaining the structure. These are salt linkages (i.e., the mutual attraction of positively and negatively charged groups) and hydrogen bonds, which may be represented thus:



Hydrogen bonds result from the attraction between the proton of an —OH, =NH, or $—NH_2$ group and an unshared electron pair of an atom such as the oxygen in a —C=O group. In proteins, hydrogen bonds are formed for the most part between the imino group of one peptide linkage and the carbonyl group of another.

There is extensive evidence that in the protein molecule the polypeptide chain is coiled and folded in a structure specific for each individual kind of protein. This structure is maintained by the secondary bonds, consisting of salt linkages and hydrogen bonds, and it persists in the protein in solution unless the protein is treated in such a way as to disrupt these linkages. The specific configuration of a protein apparently is imparted to it at the time of its formation by the organism. If this configuration is disrupted by treatment with an agent which is later removed, the protein does not revert to its original configuration and is said to be denatured.

In summary then proteins consist of large, highly specific molecules. Their properties are determined by the number, kind, and sequence of amino acid residues in the polypeptide chains and by the configuration of folding and coiling of these chains. Present knowledge of the overall composition of many proteins is nearly complete, but the sequence and configuration are virtually unknown for most proteins.

Criteria of Homogeneity

According to present ideas the molecules of a given protein are physically and chemically identical. In this respect they differ from polymers made by man in the laboratory which exhibit a range of values for any property such as molecular weight. A given protein often occurs in mixtures with other proteins and non-protein constituents and it is necessary to separate it in a pure state for characterization. In order to do this, certain criteria of purity must be set up. In general, the aim of much present-day protein research is to prepare homogeneous proteins that can be analyzed and characterized chemically. The methods available for following fractionation procedures and for determining the degree of homogeneity may be classified as chemical, biological, and physical.

Chemical methods are sometimes useful to follow the removal of an impurity during a protein fractionation procedure. Futhermore, if the protein being purified contains some distinctive group or element (such as copper, iron, or phosphate), chemical analyses may serve to follow fractionation. Obviously analysis for one of the 19 common amino acids is not of much value as a criterion of homogeneity since most proteins contain most or all of the acids.

Many proteins, such as enzymes, hormones, toxins and antibodies, possess specific biological activities which are useful in following their purification. Even those proteins that do not have such biological activity will usually cause the formation of antibodies when injected into an animal, and such antibodies can be used as reagents in tests for the presence and concentration of the antigen (i.e., the protein being sought).

Physical measurements are widely used to assess protein homogeneity. Protein molecules differ in size, shape, hydration, and charge, and these differences are reflected in differences in their sedimentation and diffusion rates, solubility, and electrophoretic behavior. Measurements of these properties are particularly valuable because they can reveal directly the nature and extent of any heterogeneity in a preparation.

Sedimentation behavior may be studied by centrifuging protein solutions in high-speed centrifuges. Apparatus for this purpose has been developed to a high state of perfection. Two alternative procedures are used: either the solution is centrifuged until the protein

molecules are in equilibrium and their distribution throughout the system is measured (sedimentation equilibrium); or sufficient centrifugal force is applied to cause them to sediment out and their rate of sedimentation is measured (sedimentation velocity). In either method the measurements are made by optical techniques while the material is being centrifuged. These methods classify the molecules with regard to size (and, to some extent, shape) and permit the detection of heterogeneity and the calculation of molecular weights.

The rate of diffusion of protein molecules when a protein solution is placed in contact with protein-free solvent is determined by the size and shape of the molecules. Here again techniques have been developed for following the rate of diffusion by optical measurements. This is another valuable tool for assessing homogeneity. Diffusion rates are needed for calculation of molecular weight by the sedimentation velocity method.

Solubility is a valuable criterion of homogeneity of a protein. If a pure substance is added in increasing amounts to a given amount of solvent, it all dissolves until the solution is saturated, whereupon no more dissolves regardless of how much excess is added. If, however, a mixture of two or more substances of differing solubility is added in increasing amounts to a given volume of solvent, a solid phase will appear when the solubility of the least soluble is exceeded, and more will dissolve until the solubility of the most soluble is reached. Thus, from plots of amount of material added versus amount dissolved in an appropriate solvent, it is possible to distinguish homogeneous proteins from mixtures.

Electrophoretic techniques classify protein molecules according to net charge. Proteins contain a number of ionizable groups from which protons are dissociated in specific zones of pH. The two most common of these groups are the carboxyl and amino groups, but others are the guanidyl groups of arginine, imidazole groups of histidine, and phosphate ester groups in phosphoproteins such as the caseins. Carboxyl groups dissociate as follows:

$$RCOOH \rightleftharpoons RCOO^- + H^+$$

Thus, when acid is added to the system the reaction goes to the left; when base is added, so that the concentration of hydrogen ions is decreased, it goes to the right. Amino groups ionize as follows:

$$RNH_3^+ \rightleftharpoons RNH_2 + H^+$$

The ratio of $RCOOH/RCOO^-$ or RNH_2/RNH_3^+ at any pH can be calculated from the pH and the dissociation constant.

$$pH = pK_a + \log \frac{[RCOO^-]}{[RCOOH]}$$

or

$$pH = pK_a + \log \frac{[RNH_2]}{[RNH_3^+]}$$

In these expressions pK_a is the reciprocal of the logarithm of the dissociation constant K_a which is defined as:

$$K_a = \frac{[RCOO^-][H^+]}{[RCOOH]} \quad \text{or} \quad K_a = \frac{[RNH_2][H^+]}{[RNH_3^+]}$$

At a pH equal to the pK_a, equal quantities of dissociated and undissociated forms of a group are present—that is, it is half dissociated. The pK_a values of the carboxyl groups are in the range pH 3.0–5.0, those of the amino groups in the range pH 7.5–10.5. Imidazole groups dissociate near neutrality having a pK_a in the neighborhood of 6.0 and the guanidyl groups have a pK_a of about 12. The phosphate groups of the caseins, if present as the monoester, have one pK_a at about 2.0, and another at about 6.5. If present as the diester, only the pK_a 2.0 is present.

Thus at any given pH, a protein will possess a net charge determined by its contents of the various ionizable groups. The net charge in turn determines the rate and direction at which the protein will migrate when placed in an electric field. A number of techniques are available for determining the rate of such migration. The cell for electrophoresis can be made to fit a microscope stage and the motion of quartz particles coated with the protein can be measured. This technique is useful for measuring the migration of a pure protein at a series of pH values and thus determining the isoelectric point. It is not applicable to separating or analyzing a mixture of proteins however. For this purpose the U-tube cell and technique of Tiselius is most useful. A boundary is established between a buffered solution of protein and its solvent. Upon application of an electrical field, the protein particles move away from the boundary.

A homogeneous protein will move at a single uniform rate at a given pH; a single boundary will persist between protein solution and buffer. In a protein mixture, on the other hand, each kind of molecule will move at its characteristic rate with the result that a number of boundaries will develop, each representing the disappearance of one kind of

molecule. The linear displacement of a boundary from the starting point, the time, and the voltage drop across the cell are used to calculate the mobility of the protein, which is expressed as centimeters per second per unit change in electrical field strength (volt/cm.) or cm.^{2} sec.$^{-1}$ volt^{-1}. The position of each boundary and the change in protein concentration across it are conveniently determined by the change in refractive index across the boundary. Precise equipment is available which optically records the rate of change of concentration versus distance of migration (see Fig. 1). These techniques of electrophoresis thus serve not only to assess homogeneity, but also as an analysis of mixtures for the proportions of individual proteins present. Another technique of electrophoresis involves the use of paper, starch, or other suitable supporting medium. The protein is applied as a spot or strip and the electrical field is applied to the ends of the supporting material. This method is especially useful for analyzing the homogeneity of very small amounts of protein.

Electrophoretic techniques are sometimes used as preparative methods for separating proteins.

Protein Classification and Nomenclature

In the earlier years of protein research, proteins were classified largely on the basis of rather empirical fractionation procedures. Thus, *albumins* were defined as proteins soluble in water and dilute salt solutions whereas the term *globulin* was applied to proteins insoluble in water but soluble in dilute salt solutions. Globulins were precipitated by saturation with magnesium sulfate or half saturation with ammonium sulfate. Similarly, various other classes of proteins were defined partly on the basis of solubility and partly on composition.

Modern techniques have shown that many of the preparations that had been considered individual proteins are in reality mixtures and that the rigid classification of albumins, globulins, etc., is not justifiable since individual proteins exhibit gradations from one category to another. The subject is introduced at this point only because the older nomenclature has tended to cling to the milk proteins with some resulting confusion. In presenting information on milk proteins current terminology of workers in the field will be used.

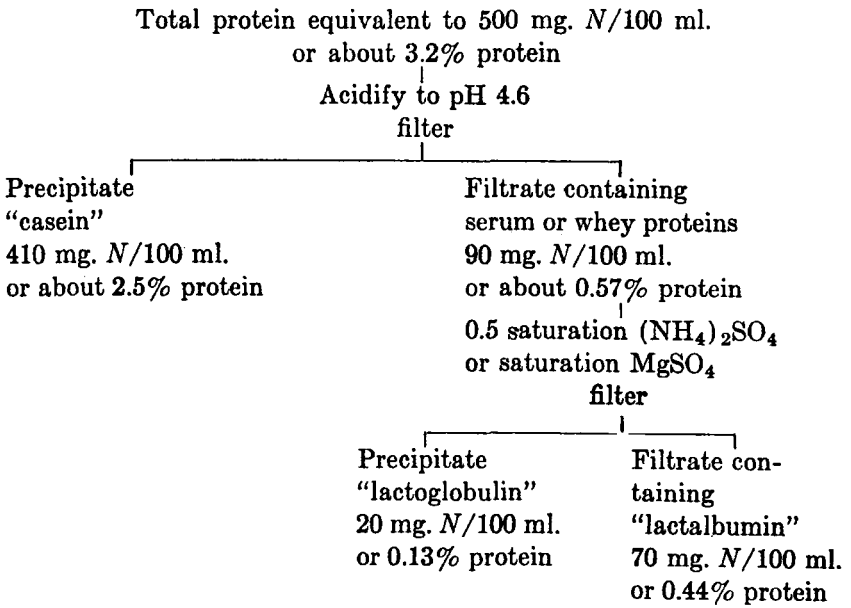
FRACTIONATION OF THE PROTEINS OF MILK

Milk contains a number of protein components which differ in composition and properties. For many years researchers have been at-

tempting to separate them into well-defined entities. The fractions obtained and the names applied to them have evolved gradually as more rigorous criteria of purity and new methods of separation have been developed. The following paragraphs give a brief survey of the fractionation procedures and the present status of the "individual" proteins that have been prepared from milk.

Classical Resolution and Nitrogen Distribution

Casein was early recognized as a distinct protein fraction because of the ease with which it coagulates in natural souring and with rennet. The protein remaining in solution after removal of the casein is called whey protein or milk serum protein. Originally it was considered a single entity, but about 1900 it was found that this mixture could be separated into two rather distinct fractions in half saturated ammonium sulfate or saturated magnesium sulfate solutions. The fraction insoluble in such solutions was designated "lactoglobulin," the soluble fraction "lactalbumin." The following diagram illustrates these methods of fractionation and the distribution of proteins among the three fractions:



N.B. The factor 6.38 is used to convert nitrogen to protein in the case of milk proteins.

Rowland (44) defined conditions and procedures for a scheme to analyze milk quantitatively for these fractions. Furthermore, he observed that if milk is heated, about 80% of the whey proteins are changed so that they precipitate with the casein when it is precipitated by acidification to pH 4.6. He considered that this 80% represents the lactoglobulin and lactalbumin and that the remaining 20% is a separate protein to which he applied the name "proteose-peptone." Proteoses and peptones are polymers of amino acids which are of lower molecular weight than proteins. They often are formed by partial hydrolytic degradation of proteins. They are usually not heat denaturable, hence it was easy for Rowland to reason by analogy that the proteins of milk which remain soluble in acid after heating are proteoses and peptones. The nature of the proteins in this fraction has not been clearly established. The issue is complicated by the fact that the fraction may consist in part of native proteins and in part of breakdown products resulting from heat treatment. Nevertheless the Rowland analytical scheme is often used, the results being presented in terms of casein N, albumin N, globulin N, proteose-peptone N, and non-protein N. These quantities are determined as follows:

Determinations:

I. Total N
II. Non-casein N (in filtrate from precipitation of casein at pH 4.6 with acetic acid-sodium acetate).
III. Non-protein N (in filtrate from precipitation of protein with 12% trichloracetic acid).
IV. Proteose-peptone N + non-protein N (in filtrate from precipitation of casein and denatured serum proteins at pH 4.6 from a boiled sample of the milk).
V. Globulin N (in precipitate from treatment of filtrate II with $MgSO_4$).

The actual distribution is somewhat as follows:

	Mg./100 ml.	Per Cent of Total N
Total N = I	540	100
Casein N = I–II	430	79.5
Albumin N = (II–IV)–V	43	8.0
Globulin N = V	19	3.5
Proteose-peptone N = IV–III	18	3.0
Non-protein N = III	30	5.5

There is considerable variation among milks in the nitrogen distribution determined in this way. Table 2 shows data obtained in a survey involving 81 samples of commercial bulked milk drawn from

Table 2. NITROGEN DISTRIBUTION IN 81 SAMPLES OF COMMERCIAL BULKED MILK

	Content		Average
	Range, mg./100 ml.	Mean, mg./100 ml.	Per Cent of Total N
Total N	482–770	566	100
Casein N	349–602	431	76.2
Albumin + globulin N	60–110	76	13.4
Proteose-peptone N	17–46	28	5.0
Non-protein N	23–42	31	5.5

Data of Harland et al. (18).

10 widely scattered plants in the United States in three different seasons of the year (18). Albumin and globulin were not determined separately in this survey.

Isolation and Fractionation of Casein

Casein exists in milk in the form of rather large colloidal particles containing the protein and also considerable quantities of calcium and phosphate and a little magnesium and citrate (see Chapter 10). These particles can be separated from milk by high-speed centrifugation, leaving the whey proteins and dissolved constituents in solution. They are commonly referred to as "calcium phosphocaseinate" or "calcium caseinate-phosphate." Centrifugation studies and examination with the electron microscope show them to be 30–300 millimicrons in diameter. It is not entirely clear whether they exhibit a continuous gradation of sizes or whether they exist in a series of discrete sizes.

Casein can be removed from milk in a number of ways besides high-speed centrifugation. As mentioned earlier, the fundamental definition of casein is operational—it is defined as that protein precipitated from skimmilk by acidification to pH 4.6 to 4.7. This is the basis for preparing "pure" whole casein for laboratory and industrial use, as well as for the manufacture of cottage cheese. The calcium and phosphate associated with casein in the original particles progressively dissolve as the pH is lowered until, at the isoelectric point of pH 4.6 to 4.7,

the casein is free of bound salts. For laboratory use the precipitate is purified of occluded materials by washing, redispersion, and reprecipitation. Commercial casein is usually merely washed. The character of the curd obtained depends on the temperature, larger clots being formed at higher temperature, and also on agitation. Thus, temperature and stirring are usually controlled to produce curd particles of convenient size for subsequent handling.

A number of different specific methods have been devised for preparing acid-precipitated casein for laboratory use. These differ in the kind and strength of acid, rate of adding acid, temperature, etc. All have the common objective of preparing a "pure" salt-free product. In some cases the product is dried with ethanol followed by ether and since one of the caseins (γ) is appreciably soluble in 50% ethanol some of it may be removed by this treatment. A method for preparing laboratory casein is given by Dunn (5), who also refers to other earlier methods. Procedures for commercial preparation of casein are summarized by Whittier and Webb (57).

A second important means of removing casein from milk is by rennet coagulation. The enzyme rennin, from the fourth stomach of young ruminants, has the ability to slightly change casein (see Chapter 10) so that it coagulates in the presence of divalent cations such as calcium. This process is, of course, that used in preparation of cheese curd. It involves the coagulation of the calcium caseinate-phosphate particles as such because the pH does not drop and colloidal calcium and phosphate are not dissolved. Thus, the product prepared by rennet coagulation has a high ash content as compared with acid-precipitated casein. It is preferred for use in casein plastics.

Casein can be precipitated by saturating milk with sodium chloride at room temperature. This procedure is not employed as a preparative method, but is sometimes used for analytical purposes. The entire calcium caseinate-phosphate particles are precipitated by this treatment. A small amount of the whey proteins is co-precipitated with the casein so that the saturated sodium chloride serum contains only about 80% as much protein as acid serum.

Since whole casein is relatively easy to prepare at low cost in large yields and a high state of purity, and since it has some important industrial uses, it has been studied to a considerable extent from a chemical standpoint. There is a wealth of information on its physical properties and on its organic reactions. This is well-summarized in a monograph edited by Sutermeister and Browne (52).

Previous to 1944 many attempts were made to fractionate casein with various solvents. This work definitely showed that casein is heterogeneous because fractions differing in composition, in solubility, or in rennet coagulability were obtained. One of these fractions, soluble in 50% alcohol and having a low phosphorus content (35), corresponds to the protein now known as γ-casein. With this exception, however, the various fractions obtained probably did not represent individual proteins and the various methods yielded different fractions.

Mellander (30) found that in an electrical field casein separates into three different components moving at different speeds. He called these α-, β-, and γ-casein in descending order of mobility. An electrophoretic pattern of casein showing the three fractions is shown in Fig. 1. This observation opened the way to use of electrophoresis as a criterion of purity, and the actual separation of casein into the three fractions soon followed. The fraction soluble in 50%

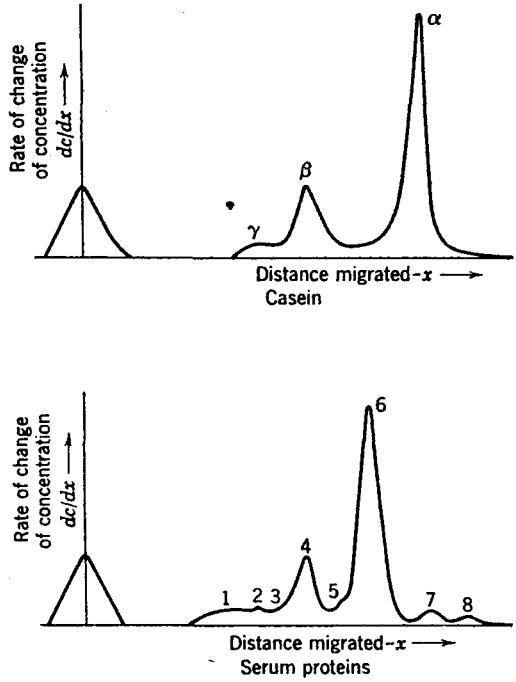


Fig. 1. Typical electrophoretic patterns of milk proteins at (pH 8.40, $\mu = 0.1$): 1. euglobulin (immune protein); 2. pseudoglobulin (immune protein); 3. unknown; 4. α-lactalbumin; 5. unknown; 6. β-lactoglobulin; 7. blood serum albumin; 8. unknown.

alcohol comprises on the average 3–4% of the total and corresponds to the γ-fraction observed by Mellander. The remainder of casein can be separated into two fairly homogeneous fractions by isoelectric precipitation (54). If the isoelectric point is approached very slowly from the acid side, a fraction preferentially precipitates at pH 4.2. This corresponds to the α-casein observed by Mellander. By repetition of the isoelectric precipitation it can be separated completely from the remaining β-casein. Other methods have been described for separating the α-, β-, and γ-caseins by fractional precipitation from alcohol solutions and urea solutions. All of these methods involve conditions which minimize interaction among the three caseins. Whole casein contains about 75% α-, 22% β-, and 3% γ-casein on the basis of electrophoretic analysis. Actual quantitative fractionations have never been attained.

Each of the caseins is fairly but not entirely homogeneous. For example, α-casein under some conditions separates on electrophoresis into two components moving at slightly different speeds. None of the caseins has been prepared in a crystalline state.

Resolution of Lactalbumin

Three relatively homogeneous proteins have been crystallized from the lactalbumin fraction. When a concentrated solution of lactalbumin is dialyzed exhaustively at pH 5.2 to remove the salt, a protein called β-lactoglobulin crystallizes out. This was first accomplished by Palmer (36) in 1934. β-Lactoglobulin accounts for about 50 to 60% of the total lactalbumin fraction. It is a globulin in that it is very insoluble in water and requires small amounts of salt to dissolve it. The prefix "β" used in the name identifies the protein with a protein designated "β" by Pedersen (39) in an ultracentrifuge sedimentation pattern of skimmilk. It also serves to distinguish this protein from the classical "lactoglobulin" fraction insoluble in half-saturated ammonium sulfate. β-Lactoglobulin has been studied a great deal by protein chemists. A method of preparing it is described by Larson and Jenness (23). It has been found to be not entirely homogeneous but to consist of two very similar proteins called β-lactoglobulin A and β-lactoglobulin B, which differ slightly from each other in solubility and electrophoretic mobility. There is evidence that some cows produce only A, some only B, and some both.

The mother liquor remaining after β-lactoglobulin has crystallized out can be fractionated further. An albumin that appears to be

identical to that in bovine blood can be crystallized after appropriate fractionation of the mother liquor with ammonium sulfate and ethanol (41). Specifically that fraction of the mother liquor precipitated between ammonium sulfate concentrations of 2.6*M* at pH 9.1 and 3.4*M* at pH 5.0 is refractionated with ethanol. The fraction precipitated at −5° C. between 33% ethanol, pH 5.3 and 40% ethanol, pH 4.8 is brought to crystallization by adding ammonium sulfate to a concentration above 2.6*M*. A small amount (0.03*M*) of sodium caprylate facilitates the crystallization. This bovine "blood" serum albumin comprises about 5 or 6% of the lactalbumin fraction of milk.

If the mother liquor from the crystallization of β-lactoglobulin is acidified to pH 4 and adjusted to 1.3*M* $(NH_4)_2SO_4$, a precipitate forms. From this precipitate another protein, which is called α-lactalbumin, can be crystallized (12). This comprises about 15 to 20% of the lactalbumin. The prefix "α" in this name was assigned because this protein corresponds to the component that Pedersen designated as "α" in an ultracentrifugal pattern. This protein is much less soluble in dilute salt solution than is β-lactoglobulin.

The three proteins β-lactoglobulin, blood serum albumin, and α-lactalbumin constitute the bulk of the lactalbumin fraction, but a number of other proteins appear to be present in small quantities.

Resolution of Lactoglobulin

Two fairly well defined components can be isolated from the lactoglobulin fraction (48). This is accomplished by refractionating lactoglobulin (originally precipitated at 0.5 saturation with ammonium sulfate), taking only that portion precipitated between 0.25 and 0.40 saturation with ammonium sulfate at pH 4.6. Upon dialysis this subfraction separates into two components, a precipitate which is called euglobulin (meaning true globulin) and a protein remaining in dispersion called pseudoglobulin (meaning false globulin). These proteins carry the immunological properties or antibodies of milk and are often called the "immune globulins." They each comprise about 5% of the total whey proteins. They are present in much higher concentration in colostrum than in normal milk.

Other Proteins

In addition to the proteins that have been mentioned, milk contains a number of other proteins each of which is present in small quantity.

All of these miscellaneous proteins together may amount to 5 or 6% of the total milk protein.

The proteins of the "proteose-peptone" fraction mentioned earlier have not been defined adequately. This fraction is prepared by acid precipitation of the casein and denatured albumin and globulin fractions from heated milk and concentration of the resulting serum. Such preparations exhibit at least three electrophoretically distinct components, but it is not known to what extent these represent artifacts produced by the heat treatment. Only by isolation of these materials from unheated milk will it be possible to be sure that they are actual original components.

A protein fraction different from any of the well-characterized milk proteins can be isolated from the surface of the fat globules where it exists in close association with phospholipides (32, 37). This fat globule "membrane" protein consists in part of the enzymes alkaline phosphatase and xanthine oxidase, but it is not known whether or not it contains any non-enzyme protein (see Chapter 9).

Various other enzymes occur in milk (see Chapter 6). Of these, peroxidase is probably present in the greatest quantity, representing as much as 1% of the total whey proteins. Peroxidase and xanthine oxidase have both been crystallized from milk.

Quantitative Variations of the Individual Proteins of Milk

Quantitative electrophoresis can be used to analyze for the proportions of individual proteins in a mixture. For the milk proteins, it is necessary to analyze the casein and whey proteins separately to avoid overlapping of the peaks. These analyses are usually made with the proteins dispersed in a buffer with a pH high above their isoelectric points in order to attain high mobilities and optimum separation of the components. A buffer consisting of diethyl barbituric acid and its sodium salt (Veronal) at pH 8.4–8.6 and an ionic strength of 0.1 is popular for this type of work. Figure 1 shows typical electrophoretic patterns obtained for casein and the serum proteins in this Veronal buffer. The several peaks in the patterns have been identified on the basis of mobility with individual proteins that have been isolated from milk. The percentage composition is determined by measurements of areas under the peaks. Thus, for example:

$$\text{Per cent } \alpha\text{-casein} = \frac{\text{Area of } \alpha\text{-casein peak}}{\text{Total area of casein pattern}} \times 100$$

The percentages obtained in this manner are subject to two reservations: (*a*) It is not absolutely certain that each electrophoretic peak arises solely from the protein to which it is ascribed; another unrecognized protein or proteins may contribute to it. (*b*) The ratios between peak areas may not be exactly the same as the ratios of proteins themselves because of interactions among proteins in the electrophoresis experiment. Nevertheless, the electrophoretic analysis is very useful for comparative purposes. Typical results obtained by measurements of patterns such as those in Fig. 1 are given in Table 3

Table 3. RANGE OF PROTEIN COMPOSITION OF COW SKIMMILK

Protein or Fraction	Rowland Nitrogen Distribution, g./100 ml.	Electrophoretic Analysis, g./100 ml.
Total Casein	2.2–3.4	
α-Casein		1.4–2.3
β-Casein		0.5–1.0
γ-Casein		0.06–0.24
Total Serum Proteins	0.40–0.80	
Albumin fraction		
β-Lactoglobulin		0.20–0.40
α-Lactalbumin		0.07–0.15
Blood serum albumin		0.02–0.05
Globulin fraction		
Immune globulins		0.05–0.11
Proteose-peptone	0.06–0.17	

Data of Larson et al (25, 43).

in comparison with typical results obtained by the Rowland procedure. Obviously the proportions of the individual proteins vary considerably from milk to milk. Some of this variability is associated with breed of cow, as may be seen in Table 4. Holstein milk is characteristically high in γ-casein and low in β-lactoglobulin. Ayrshire milk, on the other hand, tends to be low in γ-casein. In general, however, the variations in protein composition are greater among cows within a breed than between breeds.

Characteristic and important variations in protein composition occur during the progress of the lactation period, as may be seen in Table 5. The percentage of α-casein in the total casein is rather constant after the first few days. β-Casein is present in relatively low amounts at

Table 4. Breed Differences in the Composition of Milk Proteins

Breed	No. of Samples	Casein, g./100 ml.				Serum Proteins, g./100 ml.				
		Total	α	β	γ	Total	Immune Globulins	β-Lacto-globulin	α-Lac-talbumin	Blood Serum Albumin
Ayrshire	5	2.64	1.71	0.85	0.08	0.51	0.06	0.31	0.11	0.03
Brown Swiss	4	2.78	1.83	0.84	0.11	0.53	0.07	0.31	0.11	0.04
Guernsey	5	2.88	1.92	0.82	0.14	0.58	0.08	0.35	0.11	0.04
Holstein	6	2.38	1.58	0.60	0.20	0.56	0.09	0.30	0.13	0.04
Jersey	4	2.72	1.83	0.76	0.13	0.66	0.08	0.39	0.15	0.04

Data of Rolleri, Larson, and Touchberry (43).

Table 5. Changes in the Composition of the Specific Proteins of Casein and the Serum Proteins During the Lactation Period

Cow No.	Casein or Serum Protein Component	Approximate Day in Lactation Period (% of total casein or serum proteins)					
		0	30	100	200	300	330
C (Guernsey)	α-Casein	73	65	67	67	69	70
	β-Casein	19	30	30	30	27	27
	γ-Casein	8	5	3	3	4	3
	Immune globulins	70	11	12	13	15	19
	α-Lactalbumin	21	21	20	19	17	15
	β-Lactoglobulin	6	64	63	63	64	62
	Serum albumin	3	4	5	5	4	4
D (Holstein)	α-Casein	78	68	67	67	66	67
	β-Casein	14	27	27	26	26	20
	γ-Casein	8	5	6	7	8	13
	Immune globulins	82	15	15	18	18	25
	α-Lactalbumin	4	26	23	21	20	19
	β-Lactoglobulin	12	53	55	55	55	50
	Serum albumin	2	6	7	6	7	6

Data of Larson and Kendall (25).

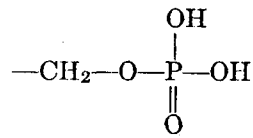
parturition, whereas the concentration of γ-casein is relatively high. Of course the most pronounced effect is the high concentration of the immune globulins in milk at parturition. Furthermore, the production of the immune proteins decreases less rapidly than that of the others near the end of lactation. Thus their relative concentration increases at that time.

COMPOSITION OF THE INDIVIDUAL MILK PROTEINS

Table 6 gives the nitrogen, phosphorus, sulfur, and amino acid contents of whole casein and of the eight milk proteins that have been isolated to date. The analyses reported for the blood serum albumin were made on a sample isolated from blood; the corresponding and apparently identical protein isolated from milk has not been so completely analyzed. Complete amino acid analyses are not available for the eu- and pseudo- globulins.

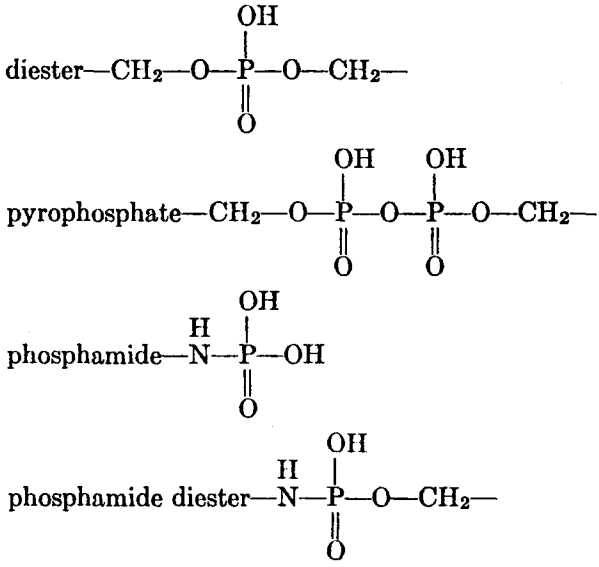
A factor of 6.38 is usually used to convert Kjeldahl nitrogen analyses of milk proteins to protein content. This corresponds to a nitrogen content of 15.65%, which, as shown in Table 6, is not far from the actual nitrogen content of the several milk proteins.

The distinguishing feature of α- and β-caseins is their phosphorus content. Casein has long been recognized as a phosphoprotein par excellence. γ-Casein has a low phosphorus content and the serum proteins are virtually or completely free of phosphorus. The phosphorus appears to be incorporated in the α- and β-caseins as esters of phosphoric acid with the hydroxy amino acids serine and threonine. The evidence for this view is that phosphate is readily released by mild alkaline hydrolysis, that titration curves exhibit an inflection characteristic of the second dissociable hydrogen of orthophosphoric acid (see Fig. 2), and that peptides containing serine, threonine, and phosphate can be isolated from casein by appropriate hydrolysis. The linkage most commonly considered is the simple monoester:



However, treatment of casein with enzymes specific for various types

of phosphate ester linkages indicates that the following may be present:



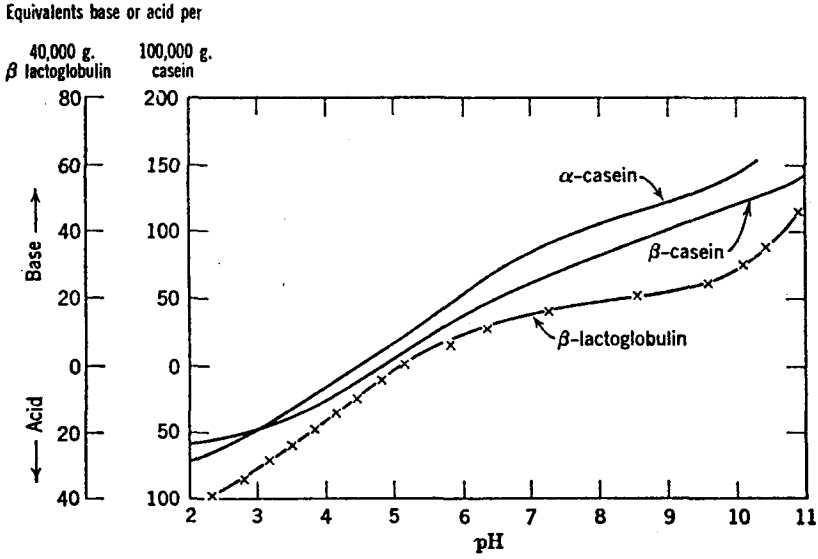


Fig. 2. Titration curves of α- and β-casein and β-lactoglobulin.

Table 6. COMPOSITION OF MILK PROTEINS

Content (g./100 g.) in

Constituent	Whole Casein	α-Casein	β-Casein	γ-Casein	β-Lactoglobulin	α-Lactalbumin	Blood Serum Albumin	Euglobulin	Pseudoglobulin	
									(a)	(b)
Nitrogen	15.63	15.53	15.33	15.40	15.60	15.86	16.07	16.05	15.29	15.9
Phosphorus	0.86	0.99	0.61	0.11	0.00	0.02	0.00	0.00	0 00	...
Sulfur	0.80	0.72	0.86	1.03	1.60	1.91	1.92	1.01	1.00	1.1
Amino N	0.93	0.99	0.72	0.67	1.24	...	...	...	...	...
Amide N	1.6	1.6	1.6	1.6	1.07	...	0.78	...	...	...
Hexose	...	...	...	...	...	...	...	2.93	2.96	...
Hexosamine	...	...	...	...	...	...	...	1.58	1.45	...
Glycine	2.7	2.8	2.4	1.5	1.4	3.2	1.8	...	...	...
Alanine	3.0	3.7	1.7	2.3	7.4	2.1	6.2	...	...	...
Valine	7.2	6.3	10.2	10.5	5.8	4.7	5.9	10.4	9.6	8.7
Leucine	9.2	7.9	11.6	12.0	15.6	11.5	12.3	10.4	9.6	8.5
Isoleucine	6.1	6.4	5.5	4.4	6.1	6.8	2.6	3.0	3.0	4.2
Proline	11.3	8.2	16.0	17.0	4.1	1.5	4.8	...	...	10.0
Phenylalanine	5.0	4.6	5.8	5.8	3.5	4.5	6.6	3.6	3.9	3.9
Cystine	0.34	0.43	0.0–0.1	0.0	2.3	6.4	5.7	3.3	3.0	...
Cysteine	0.0	0.0	0.0	0.0	1.1	0.0	0.3	0.0	0.0	...
Methionine	2.8	2.5	3.4	4.1	3.2	1.0	0.8	0.9	0.9	1.3
Tryptophan	1.7	2.2	0.83	1.2	1.9	7.0	0.7	2.4	2.7	3.2
Arginine	4.1	4.3	3.4	1.9	2.9	1.2	5.9	5.1	3.3	5.6
Histidine	3.1	2.9	3.1	3.7	1.6	2.9	4.0	2.0	2.1	2.3
Lysine	8.2	8.9	6.5	6.2	11.4	11.5	12.8	6.3	7.2	6.1
Aspartic acid	7.1	8.4	4.9	4.0	11.4	18.7	10.9	...	...	9.4
Glutamic acid	22.4	22.5	23.2	22.9	19.5	12.9	16.5	...	...	12.3
Serine	6.3	6.3	6.8	5.5	5.0	4.8	4.2	...	...	...
Threonine	4.9	4.9	5.1	4.4	5.8	5.5	5.8	10.6	10.3	9.0
Tyrosine	6.3	8.1	3.2	3.7	3.8	5.4	5.1	...	...	6.7

Sources of Data:

Whole casein and α and β casein—Gordon et al. (11).

γ-Casein—Gordon et al. (10).

β-Lactoglobulin—Brand et al. (2) [except alanine from Keston et al. (22), and isoleucine from Smith et al. (50)].

α-Lactalbumin—Gordon and Ziegler (13).

Blood serum albumin (from blood)—Tristram (53).

Euglobulin—Smith et al. (47, 49, 51). (Values for hexose, hexosamine, valine, leucine, phenylalanine, and tryptophan for a sample isolated from colostrum.)

Pseudoglobulin—(a)—(Milk)—Smith et al. (47, 49, 51).

(b)—(Colostrum)—Hansen et al. (15).

As a matter of fact, experiments by Perlmann (40) suggest that in α-casein 40% of the phosphate is bound as monoesters, 40% as phosphamide diester, and 20% as pyrophosphate. In β-casein a considerable portion of the phosphate appears to be present as diester. The presence of esterified phosphate and the manner in which it is incorporated in the α- and β-caseins are very important in determining the properties of these proteins.

Sulfur is present in the caseins in the form of the amino acid methionine plus a little cystine, but the serum proteins contain appreciable quantities of cystine in addition to methionine. β-Lactoglobulin and blood serum albumin also contain cysteine which is important because of its free sulfhydryl (—SH) group. Most of these groups in milk are in β-lactoglobulin since the blood serum albumin is present in such low concentration.

The milk proteins differ considerably from each other in amino acid content. However, they all contain appreciable amounts of a wide variety of amino acids, and in particular they all contain considerable quantities of the dietary essential amino acids (see Chapter 13 for information on the nutritive value of the milk proteins). The high tryptophan content of α-lactalbumin is very unusual; no other common protein contains such a high percentage of this amino acid. All of the milk proteins contain considerable quantities of aspartic and glutamic acids, part of the free carboxyl groups of which are present in the form of amides $-\overset{\overset{O}{\|}}{C}-NH_2$. These groups are split with the release of ammonia when the protein is hydrolyzed for analysis. Such ammonia is reported in Table 6 as amide N. The values reported for glutamic and aspartic acid include that present in the protein as amides. In general, only the total amide is known since the proportions of asparagine (the amide of aspartic acid) and glutamine (the amide of glutamic acid) are difficult to determine.

The immune globulins are distinguished by the presence of carbohydrate, containing 2.5 to 3.0% of hexose and about 1.5% of hexosamine.

The sequence of amino acids in the polypeptide chains of the milk proteins has not been determined very thoroughly. Both α- and β-caseins contain arginine and lysine as N-terminal groups. β-Lactoglobulin contains two N-terminal leucines and two C-terminal isoleucines per molecule. Bovine serum albumin (isolated from blood)

contains one N-terminal aspartic acid and one C-terminal alanine. α-Lactalbumin contains one N-terminal glutamic acid and one C-terminal leucine. These results suggest that the β-lactoglobulin molecule consists of two polypeptide chains having the same end groups. The molecules of bovine serum albumin and α-lactalbumin, on the other hand, apparently consist of single chains.

PHYSICAL PROPERTIES OF THE MILK PROTEINS

Size of Particles in Solution

Data on the molecular weights of some of the milk proteins are given in Table 7. β-Lactoglobulin and blood serum albumin each

Table 7. SOME PROPERTIES OF MILK PROTEINS

Property	α-Casein	β-Casein	γ-Casein	β-Lactoglobulin	α-Lactalbumin	Blood Serum Albumin	Euglobulin	Pseudoglobulin
Molecular wt.	?	24,100	?	35,500	16,000	65,000	about 180,000	about 180,000
Solubility, mg. *N*/100 ml.								
Water at 25° C. at pI	0.05	0.41	1.0	12.0				
50% alcohol at 25° C.	0.135	1.90	14.5					
0.02*M* NaCl at 25° C.	...	...	...	150.	...	...	...	...
0.05*M* NaCl at 25° C.	...	...	...	...	3.2			
Isoelectric point (pI)								
(*a*)	4.7	4.9	5.8	...	4.1–4.8	...	...	...
(*b*)	4.1	4.5	5.8–6.0	5.18	...	4.72	6.02	5.60
Mobility[c] at pH 8.4, $\mu = 0.1$	−6.75	−3.05	−2.01	−5.1	−4.2	−6.7	about −1.8	about −2.5
Optical Rotation $[\alpha]_D^{25}$	−90.5[d]	−125.2[d]	−131.9[d]	−30.4[e]	−60[f]	−49[e]	...	...
$E_{1cm.}^{1\%}$ at 280 mμ	10.25	4.75	5.0	9.3	...	6.6	3.9	13.0
Refractive index increment at 578 mμ				0.00186		0.00190		

(*a*) Point of minimum solubility.
(*b*) Point of no electrical migration.
(*c*) Values multiplied by 10^5.
(*d*) At pH 8.4, $\mu = 0.1$.
(*e*) At pH 4.8, $\mu = 0.1$.
(*f*) At pH 6.8, 8.3, and 9.7.

appear to be homogeneous with regard to particle size. Each has been studied by a number of methods. Their respective molecular weights are 35,500 and 65,000. The actual dimensions of the molecules are not known with as much certainty as the molecular weights, because it is difficult to determine the molecular shape and dimensions unambiguously apart from the degree of hydration. However, present evidence indicates that serum albumin (from blood) is a rod shaped molecule about 150 A. in length and 38 A. in diameter. (The unit A. signifies angstroms, A. = 10^{-8} cm.) These values at least give a general idea of the size and shape of the molecules of one of the proteins occurring in milk.

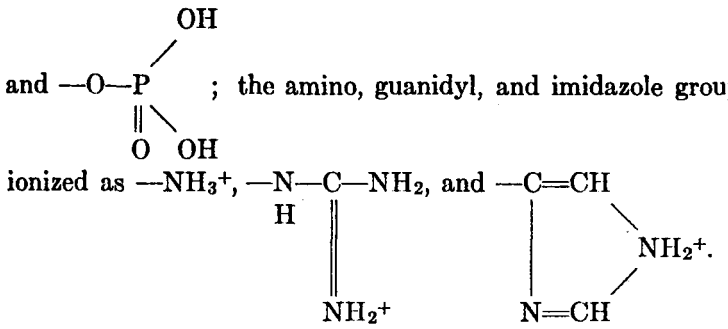
α-Lactalbumin, although not studied so thoroughly, appears to consist of homogeneous particles with a molecular weight of about 16,000. The euglobulin and pseudoglobulin fractions are not entirely homogeneous in particle size, but most of the particles of each have a molecular weight of about 180,000.

Determination of the size of casein particles in solution is complicated by the fact that the caseins exhibit a strong tendency to aggregate. The degree of aggregation depends on temperature, pH, and salt concentration. Thus it has been difficult to define and determine minimum molecular weights for the caseins, and consequently no values for α- and γ-caseins have been included in Table 7. β-Casein appears to have a molecular weight, below 15° C., of about 24,000. In milk, casein is present as very large particles, consisting of a complex of casein, calcium, and phosphate. (See Chapter 10.) Such particles have apparent molecular weights of several millions.

Electrical Properties

Table 8 gives a compilation of the principal ionizable groups of the caseins and of β-lactoglobulin as calculated from amino acid analyses. Since the molecular weights of the caseins are uncertain, the data are expressed as equivalents per 100,000 g. rather than as equivalents per mole.

Titration curves for the caseins and β-lactoglobulin are given in Fig. 2. Such curves depict the relation of pH to the amount of acid or base added to a solution of the protein. If one starts with a solution of protein at very acid pH (e.g., pH 1.0), its carboxyl groups and phosphates will be present in the undissociated state as —COOH



and —O—P(=O)(OH)—O— ; the amino, guanidyl, and imidazole groups will be ionized as $-NH_3^+$, $-NH-C(=NH_2^+)-NH_2$, and $-C=CH-NH_2^+-CH=N-$. The protein will have a strong net positive charge. If base is then added, the various groups will be neutralized at pH's in the neighborhood of their respective pK_a values (see Fig. 3). First the carboxyl and primary phosphate, then the secondary phosphate and imidazole, and finally the amino and guanidyl groups are neutralized. The shape of the titration curve and the amount of base consumed in the titration is, of course, determined by the amount and kind of ionizable groups.

The titration curves for the caseins and β-lactoglobulin differ principally in the greater consumption of base by the α- and β-caseins in

Table 8. IONIZABLE GROUPS IN α- AND β-CASEINS AND β-LACTOGLOBULIN

Group	pK_a	Equivalents per 100,000 g. α-Casein	β-Casein	β-Lactoglobulin
Cationic Groups				
Guanidyl (arginine)	12.5	25	20	18
Amino (lysine)	10.5	61	44	82
Imidazole (histidine)	6.1	19	20	10
Terminal α-amino	9.5	10	7	10
Total cationic		115	91	120
Anionic Groups				
Carboxyl (aspartic)	3.9	63	37	90
Carboxyl (glutamic)	4.3	153	158	140
Terminal α-carboxyl	2.0	10	7	10
Phosphoserine	1.4 6.4	64	40	0
Total		290	242	240
Less amide		114	114	80
Total anionic		176	128	160

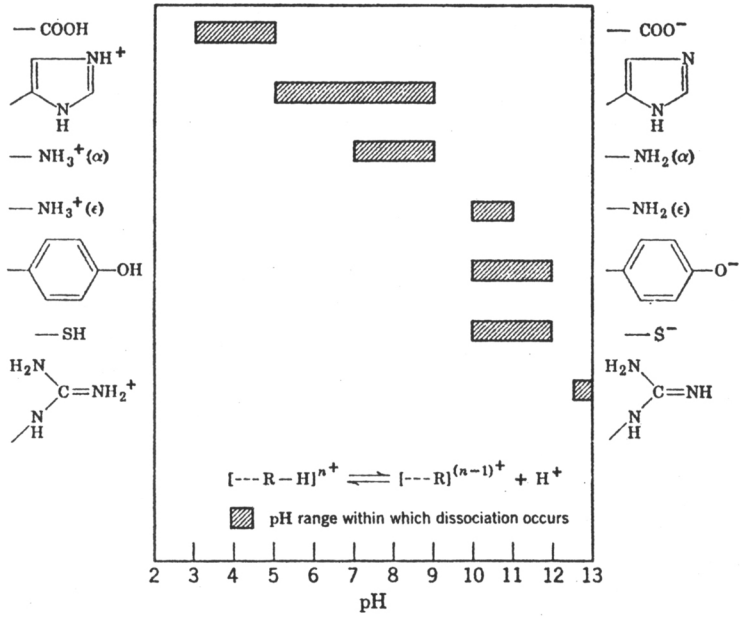


Fig. 3. Range of dissociation of various groups in proteins.

the neighborhood of pH 6 to 7, which is due to the neutralization of the secondary phosphate that these proteins contain. The titration curves are consistent with the numbers of ionizable groups in these proteins as determined by amino acid analyses. Obviously, the proteins contribute part of the conventional titratable acidity of fresh milk.

At one particular pH in the course of the titration the number of positively and negatively charged groups exactly equal each other, so that the particle has no net charge. This point is termed the isoelectric point, symbolized as *pI*. It is a very important property of the protein. At this point the particle does not migrate in an electric field. In general, also proteins exhibit minimum solubilities at their isoelectric points. Since proteins bind ions other than hydrogen and hydroxyl, the value of pI depends to some extent on the amount and kind of other ions in the system. The concentration of salts must always be specified in giving values for pI. Isoelectric point is conveniently determined by measuring the rate and direction of electrical migration of protein particles at various pH values and interpolating to the point of no migration.

The isoelectric point of whole casein is often given at pH 4.6. This is a value found using electrophoretic and solubility techniques. It is also the point used for precipitation of the casein from milk. In careful study in 1940, Moyer (33) measured the migration of casein particles microscopically at different pH's and salt concentrations. The isoelectric point varied with salt concentration, approaching a value of 4.51 at zero salt concentration. At an ionic strength of 0.1 the pI was 4.33. The individual casein components α, β, and γ have rather different isoelectric points, as may be seen in Table 7. The difference in isoelectric point of α- and β-caseins enabled their first separation. The isoelectric points of the other milk proteins are also given in Table 7.

Electrophoretic patterns of casein and the whey proteins at pH 8.6 and ionic strength of 0.1 in veronal buffer were presented in Fig. 1. The electrophoretic mobility of each component is given in Table 7.

Hydration and Solubility

The term *hydration* is used to refer to the binding of water by molecules of a dry or a dissolved substance. Proteins bind water rather avidly. As a matter of fact, they are the principal water binding components of many biological systems.

The fundamental mechanism of hydration is electrostatic attraction of the polar water molecules by ionized or polar groups in the protein. Not only the actual ionic groups mentioned previously but also the peptide bonds —CONH— are considered to be points of attachment for water molecules.

Dry proteins bind water to an extent determined by the temperature and the vapor pressure of water in the system. In general, a plot of grams of water absorbed versus relative vapor pressure is an S-shaped curve such as is shown in Fig. 4 for β-lactoglobulin. Such a curve shows that a small amount of water is bound very firmly and only liberated at very low vapor pressures. A larger amount is bound if the vapor pressure is increased and above a relative vapor pressure of 0.8 the amount bound increases very rapidly. The mechanism of water binding that produces a curve of this shape is not entirely understood, but it is apparent that some of the water molecules are held much more tenaciously than others. The binding of water by proteins in the dry state is a most important consideration in the manufacture and storage of dry milk products.

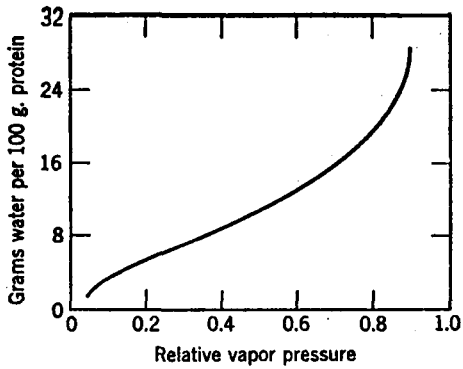


Fig. 4. Vapor pressure-moisture content curve for β-lactoglobulin. Data of Bull (4).

Hydration of proteins in solution is more difficult to determine accurately than hydration of dry proteins. It was formerly assumed that a sharp division could be made between "free" water and "bound" water in a protein solution and that the latter would not function as a solvent for added substances such as urea or glucose. Methods based on this assumption involved adding a definite weight of solute and determining its concentration in solution either chemically or by the lowering of freezing point or vapor pressure. However, such methods ignore the possibilities (*a*) that the added substance may compete with the protein for water molecules and thus reduce the original hydration, and (*b*) that the added substance may itself be bound to protein. Thus, methods of this kind are discredited at present.

Some information on the extent of hydration of proteins in solution can be gained by measuring the density of the dry protein and of protein solutions. Another approach is to determine the water content of protein crystals. Such methods indicate that the amount of water "bound" by proteins in solution is of the order of 0.3–0.4 g. per gram of dry protein.

Proteins vary greatly in their solubility in water. Some dissolve readily in pure water, others require a certain concentration of salts, still others dissolve in the presence of ethanol, whereas some are very insoluble in any mild aqueous solvent. Proteins were formerly classified partly on the basis of these differences in solubility, but such schemes are rather artificial and are gradually fading from use.

Proteins dissolve in water under conditions where their attraction for water is greater than the attraction between protein molecules. It is not possible at present to predict the solubility of a protein from its

composition although the composition and configuration certainly are the ultimate factors determining solubility. Present information on solubility of proteins has developed largely empirically.

The solubility of proteins depends to a great extent on the pH and the concentration of salts in the system. They exhibit a minimum solubility at the isoelectric point in the absence of salts because under such conditions the mutual attraction of positive and negative charges of adjacent molecules is greatest. As the pH is raised or lowered from the isoelectric point a net excess of positive or negative charges develops. These are capable of attracting water which in turn causes the protein to dissolve. Similarly, the addition of low concentrations of neutral salts increases the charge on proteins and thus increases their solubility. This phenomenon is known as "salting-in." On the other hand, in high concentrations of salt the salt ions compete with the protein for water molecules and cause a decrease in solubility. This is called "salting-out" and is widely used as a means of fractionating protein mixtures by precipitation. Ammonium sulfate is often used for this purpose because of its very high solubility. The addition of organic solvents such as ethanol to the system depresses the charge on protein particles because it lowers the dielectric constant of the medium and thus depresses the solubility of protein. Methods of fractionation based on the use of ethanol as a precipitant have become popular in recent years.

α- and β-Caseins are outstanding examples of proteins whose solubility depends primarily on pH and very little on salt concentration. They are very insoluble at the isoelectric point (Table 7) but dissolve readily on either side of it. As already noted, they can be separated because α-casein is less soluble at pH 4.2 and 2° C., and β-casein is less soluble at pH 4.9 and 20° C. Casein binds calcium and other bivalent cations very tenaciously at pH values alkaline to the isoelectric point. This causes aggregation and lowering of solubility. γ-Casein, of course, is appreciably soluble in 50% ethanol solution.

β-Lactoglobulin is very slightly soluble at its isoelectric point in the complete absence of salt, but is solubilized by either small concentrations of neutral salt or by changes in pH. These effects are illustrated admirably in Fig. 5. β-Lactoglobulin is prepared in the pure state by adjusting the pH to its isoelectric point and dialyzing free of salt, whereupon it crystallizes. α-Lactalbumin is much less soluble in dilute salt solution near the isoelectric point than is β-lactoglobulin (see Table 7). So far it has not proved possible to crystallize it at its isoelectric point in the manner of crystallization of β-lactoglobulin.

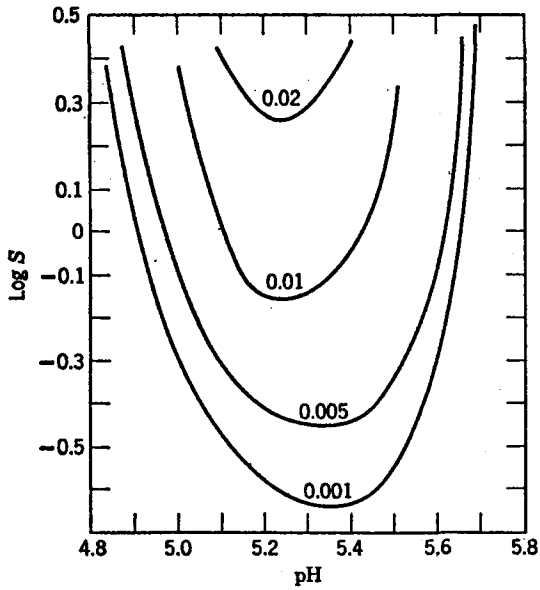


Fig. 5. The influence of pH and salt concentration on the solubility of β-lactoglobulin. Value on curve designates ionic strength. Solubility (S) expressed in grams per liter. Data of Grönwall (14).

Rather it is crystallized at pH 6.6 by adding ammonium sulfate to reduce the solubility. The crystals formed contain the protein and ammonium sulfate. Blood serum albumin is too soluble in water to exhibit a salting-in with added salt. However, such an effect can be demonstrated in aqueous ethanol solutions in which it is less soluble than in water. It can be crystallized from concentrated ammonium sulfate solution. This crystallization is facilitated if a little sodium caprylate is added, which presumably forms a complex with the protein. The immune globulins are less soluble than the other proteins of whey and are salted out at lower concentrations of ammonium sulfate. Upon dialysis one fraction precipitates. It is called euglobulin because it conforms to the classical definition of globulins as proteins insoluble in water. The fraction remaining in solution is called pseudoglobulin.

Optical Properties

The absorption of ultraviolet light with a wavelength of 280 mμ is a characteristic of proteins that depends on their content of the aromatic

amino acids, tyrosine, tryptophan, and phenylalanine. It is conveniently expressed in terms of $E^{1\%}_{1\text{ cm.}}$, which is defined as follows:

$$E^{1\%}_{1\text{ cm.}} = \log \frac{I_0/I}{pl}$$

where I_0 and I = intensity of light transmitted by solvent and solution respectively,
p = concentration of solute (%),
l = length of light path in centimeters.

Values are given in Table 7 for $E^{1\%}_{1\text{ cm.}}$ at 280 mμ for the several milk proteins. In experiments involving a single protein or a constant mixture it is often convenient to determine protein concentrations by measurements of light absorption at 280 mμ.

Optical rotation is also an important property of proteins and one in which they differ widely. This phenomenon results from the presence of asymmetric carbon atoms which are present in all of the amino acid constituents of proteins except glycine. It is expressed in terms of specific rotation.

$$[\alpha]^T_\lambda = \frac{100\alpha}{Cl} = \frac{100\alpha}{C'pl}$$

where α = angle of rotation of the polarized beam,
C = concentration of solute in grams/100 ml. solution,
C' = concentration of solute in grams/100 g. solution,
p = density of solution,
l = length of light path in decimeters,
T = temperature,
λ = wavelength of incident polarized light.

Monochromatic light from the yellow or D line of the sodium spectrum having a wavelength of 589.3 mμ is most often used in this work although sometimes the green line of the mercury spectrum with wavelength of 546.1 mμ is used.

Although the optical rotation of proteins depends fundamentally on their asymmetric carbon atoms, the arrangement and configuration of these atoms in the molecule in relation to other atoms and groups determines the actual value of the optical rotation. Specific rotations of proteins are always negative and for globular proteins the values of $[\alpha]^{25}_D$ are usually within the range of $-30°$ to $-60°$. Many proteins have virtually constant specific rotations over the range of pH from 5 to 10 but β-lactoglobulin is distinctive in that its specific rotation

increases markedly as the pH is raised over this region. Denaturation, which involves an uncoiling and unfolding of the unique structure of these proteins, produces marked increases in optical rotation. Measurement of this property is a sensitive means of following denaturation. The caseins have high specific rotations resembling those of the denatured globular proteins. The specific rotations of the caseins are not affected by "denaturing" treatments. Values of specific optical rotation of the individual milk proteins are given in Table 7.

The refractive index is a measure of the bending of a ray of light entering a solution. It is defined as

$$n = \frac{\sin i}{\sin r}$$

where n = refractive index,
i = angle of incidence,
r = angle of refraction.

The refractive index of protein solutions increases linearly with concentration. The difference between the refractive index of a 1% protein solution and its solvent is called specific refractive increment.

$$k = \frac{n - n_0}{c}$$

where k = specific refractive increment,
n = refractive index of protein solution,
n_0 = refractive index of solvent,
c = concentration of protein in grams/100 ml.

Values for k obtained at a wavelength of 578 mμ for blood serum albumin and β-lactoglobulin are given in Table 7. Most proteins except lipoproteins have specific refractive increments in this range. Lipoproteins have lower values. Refractive index measurements afford a quick and easy method of determining protein concentrations if both n and n_0 can be determined.

CHEMICAL REACTIONS OF THE MILK PROTEINS

Proteins, possessing a wide variety of reactive side chain groups, are potentially capable of participating in a considerable number of reactions. A great deal of study has been devoted to some of these chem-

ical reactions. Some of the objectives of such studies have been (*a*) production of useful derivatives through chemical modification, (*b*) elucidation of the structure of the protein, and (*c*) analysis for reactive groups.

When proteins are treated with a reactant under conditions involving no denaturation, it is frequently found that the reaction proceeds sluggishly or incompletely compared to the same reaction with an amino acid. Observations of this kind are explained on the basis that the coiled and folded structure of the protein offers steric hindrance to the reaction. In other words, the reactant is hindered in its approach to the reactive group by the structure of the protein. Such observations formed the basis for the original ideas that the protein exists in a coiled and folded structure which opens up upon denaturation.

The reactions described in the following paragraphs are those that seem to be of major significance insofar as the milk proteins and dairy problems are concerned. No effort is made to distinguish between reactions or conditions that involve denaturation and those that do not. As a matter of fact, hydrolysis, which involves degradation of the protein, is included.

Oxidation

Proteins are oxidized by many different reagents and to degrees determined by the severity of the conditions employed. The sulfhydryl group is the most susceptible to oxidation, being oxidized by relatively mild conditions. There has been a great deal of interest in oxidative reactions as analytical tools for measuring the sulfhydryl groups liberated by denaturing treatments. The goal has been to devise methods involving a definite stoichiometric reaction with —SH groups which are also specific in that they do not react with any other group in the protein. Few, if any, reagents and methods fulfill these requirements. The oxidizing agents ferricyanide, porphyridin, and O-iodosobenzoate seem to most nearly produce a stoichiometric oxidation of sulfhydryl groups to disulfides. Others that are not so satisfactory are iodine, cystine, tetrathionate, and 2,6-dichlorophenol-indophenol. In general, oxidative methods alone should not be relied upon for determining sulfhydryl groups. They should be checked by other procedures.

More drastic oxidation of the protein is produced by such reagents as hydrogen peroxide, oxygen, bromine, permanganate, dichromate, perbenzoate, and periodate. The phenol and indole groups of the tyrosine

and tryptophan residues respectively seem to be the next most susceptible after the sulfhydryl groups. Disulfide groups are oxidatively cleaved by strong oxidants such as performic acid with the formation of two sulfonic acid, $—SO_3H$, groups. This technique may be useful in separating the polypeptide chains of a protein for further analytical work.

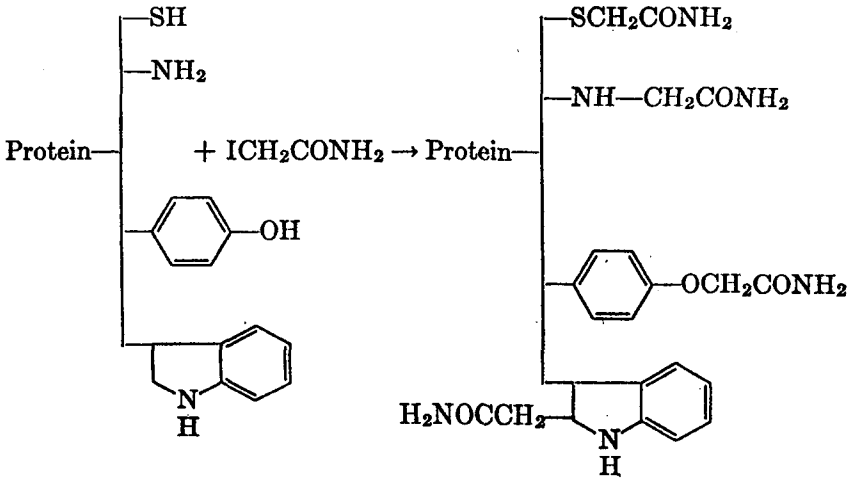
Reduction

The disulfide groups of proteins are reducible to sulfhydryls by treatment with low molecular weight sulfhydryl-containing compounds, such as cysteine, glutathione, thioglycolic acid, mercaptans, mercaptoethanol, hydrogen sulfide, or sodium sulfide. This reaction proceeds readily at room temperature and neutral pH. It can be used to analyze for disulfide groups, to modify proteins, and activate enzymes.

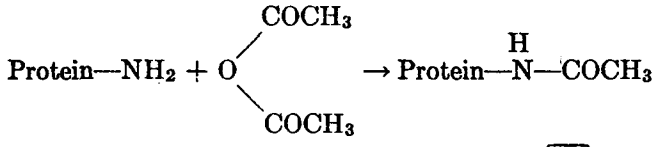
More drastic reduction of proteins with lithium aluminum hydride, $LiAlH_4$, reduces the free carboxyl groups in the protein to alcohol groups.

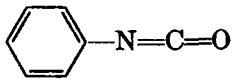
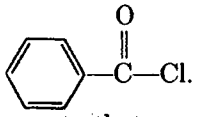
Alkylation, Arylation, and Acylation

Alkyl, aryl, and acyl groups can be introduced into a number of positions in proteins. For example, iodoacetamide reacts to form alkyl derivatives with sulfhydryl, amino, phenolic, and indolyl groups. Thus:



Similarly, fluorodinitrobenzene can be used to introduce aryl groups. The amino groups can be acetylated with acetic anhydride:



and can also be substituted with phenyl isocyanate  or benzoyl chloride  . These are only a few examples of large numbers of reagents that can be used to modify proteins. Such reagents are often used to block protein groups. For example, the participation of amino groups in the binding of water by casein was demonstrated by an experiment in which the amino groups were blocked by benzoylation.

Reaction with Formaldehyde

Formaldehyde reacts with primary amino groups, amide groups, and guanidyl groups. It does not react with secondary amide groups (peptide linkage). The initial rapid, reversible reactions seem to be:

$$RNH_2 + HCHO \rightleftharpoons RNHCH_2OH$$

and

$$RNHCH_2OH + HCHO \rightleftharpoons RN(CH_2OH)_2$$

This reaction is important in protein chemistry and dairy chemistry because it furnishes the basis for the well-known formol titration. It will be remembered that amino groups exist in an equilibrium between charged and uncharged forms.

$$RCH_2NH_3^+ \rightleftharpoons H^+ + RCH_2NH_2$$

Apparently formaldehyde reacts only with the uncharged form, with the result that the reaction goes completely to the right and the hydrogen liberated can be titrated. In other words, the products of the formaldehyde-amino group reaction are much weaker bases (show less tendency to hold protons) than the original amino groups. This reaction affords a means of determining amino groups in a protein. It has been proposed as a means for determining the protein content of milk. The formaldehyde titration can also be used to follow the course of proteolysis, since amino groups are liberated in this process.

In addition to the rapid primary reactions shown above, further slower reactions occur involving cross linking by means of methylene (—CH_2—) bridges. These may involve two amino groups or an amino group on the one hand and an amide, guanidyl, indole, phenol, or imidiazole group on the other. These reactions are essentially irreversible. The cross linkages established so modify and strengthen the protein structure that its solubility is greatly reduced and it becomes very resistant to swelling in water. This reaction is the basis for the manufacture of casein plastics and fibers.

The reaction between protein amino groups and aldehydes is a general one. It occurs between proteins and aldehydic sugars in many food products with the production of brown-colored compounds. The chemistry of these reactions insofar as milk products are concerned is summarized in Chapter 11.

Deamination with Nitrous Acid

This well-known reaction proceeds as follows:

$$RCH_2NH_2 + HONO \rightarrow RCH_2OH + N_2\uparrow + H_2O$$

The α-amino groups at the ends of polypeptide chains react rapidly, the ϵ-amino groups of lysine more slowly and the guanidyl groups of arginine very slowly indeed. This reaction is the basis of the Van Slyke method for determining amino groups. The nitrogen evolved is collected and measured either volumetrically or manometrically. This is a useful method for following the course of hydrolysis as amino groups are liberated or of reactions (such as the "browning" reaction)

Table 9. AMINO GROUPS AND LYSINE CONTENT OF α- AND β-CASEIN AND β-LACTOGLOBULIN

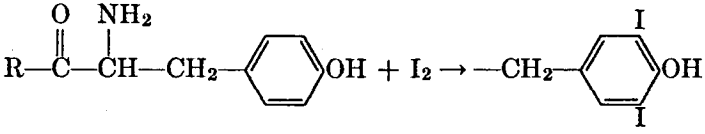
Protein	Amino Nitrogen as Per Cent	Amino Nitrogen as Groups per 100,000 g.	Lysine Groups per 100,000 g.
α-Casein	0.99	71	61
β-Casein	0.72	51	44
β-Lactoglobulin	1.24	92	82

in which amino groups participate and disappear. The amino nitrogen contents of some of the milk proteins determined by this method are given in Table 9, in comparison with the lysine content determined by independent analysis. The difference between amino nitrogen and ly-

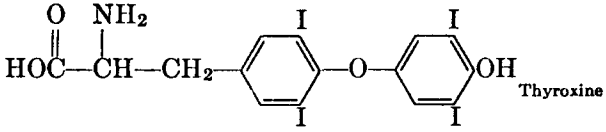
sine is taken to represent α-amino groups at the ends of the polypeptide chains in the protein molecule.

Iodination

The halogens replace hydrogen on the tyrosine rings of proteins. The principal interest is in iodination since proteins so treated have the activity of the thyroid hormone, thyroxine. It has even been possible to isolate thyroxine itself from iodinated casein. The tyrosine ring is first iodinated in the 3 and 5 positions.



The chemistry of the next step by which thyroxine is formed has not been explained.



Apparently the thyroxine is not set free as such but is still incorporated into the protein by a peptide linkage.

Color Tests

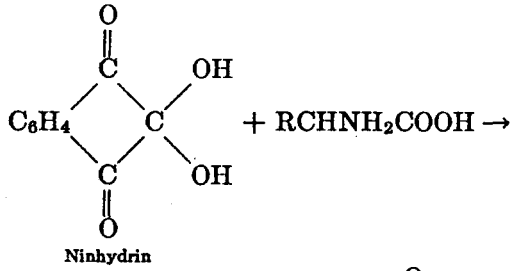
The reactive groups in proteins give a number of reactions well known for the development of characteristic colors. These are conveniently grouped as "color tests." They have frequently been used for qualitative detection in proteins of the particular group which they represent. Some of them are also useful for quantitative analysis for one or more of the following purposes: (*a*) determination of total protein, (*b*) following the "freeing" of groups by denaturation, and (*c*) determining the extent of hydrolysis.

Some of the more common and widely used of the protein color tests are described in the following paragraphs. Only methods that can be applied to intact (including denatured) proteins are included. Those that are applicable only to free amino acids are excluded.

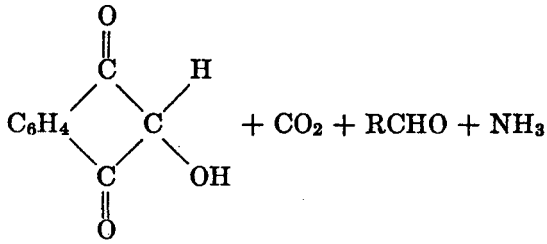
Biuret test. The biuret reaction involves adding a solution of copper sulfate to a strongly alkaline solution of the protein. A purple-violet color results. This test is characteristic of the peptide linkage,

being given by any compound containing two —CO—NH— groups separated by a carbon or a nitrogen atom. Thus it is given by biuret, NH_2—CO—NH—CO—NH_2, from which it takes its name. Short chain polypeptides give a pinkish violet color, longer ones including proteins a more purple hue. The amino acid histidine gives a pink color. Although originally designed for qualitative detection of proteins, the biuret method has come into wide use as a quantitative method of general applicability and moderate accuracy. It has the advantage that the color, which depends on the peptide linkage, does not vary greatly in intensity from protein to protein. Several procedures based on this method have been suggested for determining milk proteins, but it is not in general use in dairy research.

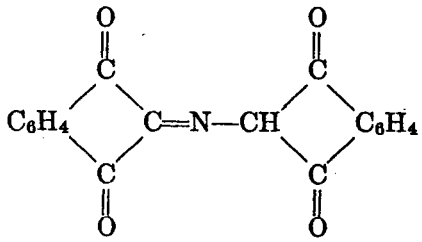
Ninhydrin test. Ninhydrin, triketo-hydrindene hydrate, is a general reagent for α-amino acids with which it reacts as follows:



Ninhydrin

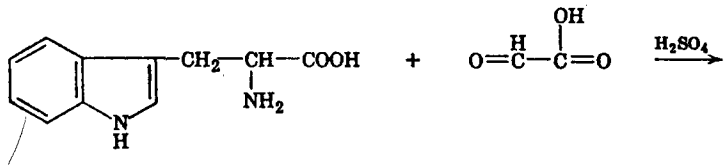


The monoalcohol resulting from reduction of ninhydrin condenses with another molecule of ninhydrin and one of ammonia to form a purple dye:

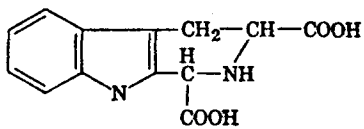


Various analytical methods for amino acids are based on determining the CO_2, the NH_3, the specific aldehyde, or the color formed. Ninhydrin is frequently employed as a spray reagent to detect amino acids on paper chromatograms. Proteins give the color best in an alkaline medium which can be produced by including a little pyridine in the reaction mixture. A very satisfactory scheme is to heat 1 ml. each of protein solution, 10% pyridine solution, and 1% ninhydrin solution together in a boiling water bath for about 20 minutes.

Tests for indolyl groups of tryptophan. Violet to blue colors develop when a mixture of protein and an aldehyde is layered over concentrated sulfuric acid. A number of tests based on this principle have been suggested; all depend on the presence of the indolyl group of tryptophan which reacts as follows (using glyoxylic acid as an example of an aldehyde):



Tryptophan Glyoxylic acid



This particular test with glyoxylic acid is known as the Hopkins-Cole test. A similar test was at one time recommended for detection of formaldehyde that had been added as a preservative to milk, the formaldehyde reacting with indolyl groups of the milk proteins to give a color.

Millon test for phenol groups.. A red color develops when a protein is heated with a solution of mercurous nitrate containing oxides of nitrogen or nitrous acid. Such a solution, called Millon's reagent, can be prepared by dissolving mercury in concentrated nitric acid. The reaction is specific for phenol groups.

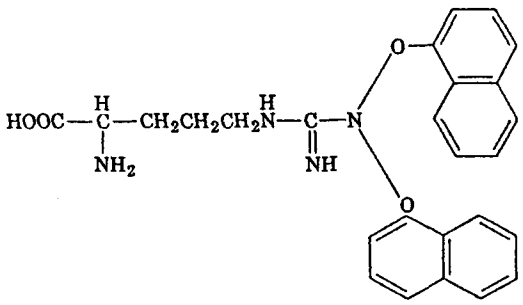
This reaction is used to analyze for tyrosine in protein hydrolysates and to detect the presence of tyrosine in proteins.

Xanthoproteic test. The well-known yellow color that forms when a protein is treated with concentrated nitric acid is due to nitration

of the phenol and indolyl groups. It has not been used to any extent for quantitative analysis.

Folin test for indolyl and phenol groups. A phosphomolybdotungstic acid reagent designed by Folin for phenol has been widely used for detection and analysis of indolyl and phenol groups in proteins. A characteristic blue color is formed when a protein solution is warmed with this reagent. This method is much used for quantitative determination of total protein, although it is obvious that the intensity of the color will vary from protein to protein because the content of tyrosine and tryptophan varies. The method is also useful for following the liberation of free tyrosine and tryptophan during protein hydrolysis. It has been employed to some extent for detecting bacterial and enzymatic degradation of proteins in milk and particularly in cheese.

Sakaguchi test. Arginine and other guanidyl derivatives react with hypobromite and α-naphthol to give a red colored product, probably of the following structure:



This is a very specific and sensitive test.

Nitroprusside test. Sodium nitroprusside, $Na_2Fe(CN)_5NO$, reacts with compounds containing sulfhydryl groups to produce an intensely red but somewhat unstable color. Sulfhydryl-containing proteins such as β-lactoglobulin give this test. In general, the —SH groups must be free and accessible and most —SH containing proteins require denaturation before they will give the test. It has been used a great deal in studies of denaturation, both for qualitative tests and as an end point indicator. Its use for detecting changes in heated milk has been discussed by Patton and Josephson (38).

Hydrolysis

Proteins are frequently hydrolyzed either completely or partially for analytical purposes. Thus quantitative analyses for the constituent amino acids of a protein usually necessitate a complete hydrolysis. Information on particular sequences of amino acids within the protein may be secured by partial hydrolysis. In the dairy field, protein hydrolysis is of interest not only from the analytical standpoint but also because of its tremendous importance in determining the body, texture, and flavor of cheese.

The common agents which hydrolyze proteins are acids, bases, and enzymes. The reaction is the same in any case:

$$RCONHR' \xrightarrow{+H_2O} RCOOH + R'NH_2$$

Strong acids such as 6N HCl or H_2SO_4 and a refluxing period of 12 to 48 hours are most frequently used for total hydrolysis. Hydrochloric acid can be removed by distillation, and sulfuric acid by precipitation as barium sulfate. Acid hydrolysis has the advantage that it does not racemize the amino acids (i.e., convert them into their optical isomers —in this case the D-amino acids). It does, however, cause destruction of tryptophan. This amino acid combines with small amounts of aldehydes produced during the hydrolysis to yield a dark brown substance called humin. The reaction probably is similar to that involved in the various tests for tryptophan, such as the Hopkins-Cole test. Serine and threonine as well as cystine and cysteine suffer decomposition to a small degree in acid hydrolysis.

Alkaline hydrolysis avoids destruction of tryptophan but it produces racemization and deamination and is not used to any extent.

Proteolytic enzymes (proteases) are seldom if ever used for total hydrolysis of proteins for analytical purposes. They are, however, useful for partial hydrolysis. For example, enzymatic hydrolysis has proved valuable in the isolation of various phosphopeptides from casein which have been of aid in understanding the manner in which the phosphate groups are incorporated into casein. Proteases have one advantage not possessed by acids or bases and that is specificity. A given protease attacks only certain specific peptide linkages. An illustration of this has already been mentioned. Carboxypeptidase attacks only the peptide linkage adjacent to a free carboxyl group. Thus it can be used to liberate the C-terminal groups for identification. Milk contains one protease having maximum activity at about pH 9.0 (see Chapter 6). It is not of any great importance in dairy products.

Proteases of microbiological origin, however, may be of tremendous importance in causing undesirable degradation of milk proteins. On the other hand, a limited breakdown with proteolytic enzymes has been suggested as a means of producing soft-curd milk. The ripening of all types of cured cheeses involves enzymatic hydrolysis of the protein. As the protein is degraded the characteristic mellowing of body and development of flavor occurs. A great deal of work has been done on following the rate of proteolysis in cheese by analysis for specific amino acids liberated. The ultimate objectives of such work is to determine which end products contribute to flavor and to devise accelerated ripening procedures. Much needs to be done in this field.

The rate and extent of hydrolysis of proteins can be determined by measurements of free carboxyl and amino groups by titration procedures, by the ninhydrin reaction, or (in the case of amino groups) by the Van Slyke nitrous acid method. Another procedure is to separate the reaction mixture into "protein" and "non-protein" by precipitating the former with an agent such as trichloroacetic acid and determining total nitrogen or phenol and indolyl groups (Folin method) in the non-protein fraction. Analysis for specific amino acids in a water extract is also valuable, particularly in cheese work.

DENATURATION OF MILK PROTEINS

One of the most characteristic properties of proteins is denaturation. Anyone who works with proteins soon becomes aware of the profound changes produced in solubility when proteins are heated or treated with alcohol. These are examples of denaturation.

It was pointed out previously that the molecules of globular proteins seem to consist of polypeptide chains coiled and folded in a manner specific for each protein. The molecules are maintained in their specific configuration by salt linkages and hydrogen bonds. The present concept of denaturation is that it involves the breaking of these bonds, allowing the protein molecule to unfold and uncoil from the specific structure it had originally into a more random configuration. When the agent responsible for denaturation is removed, the molecule may tend to refold and recoil but usually cannot regain its original structure. Thus it is usually irreversibly changed. No reactions involving primary covalent bonds (such as peptide linkages) occur during denaturation proper, but the unfolding of the molecule often exposes groups which may undergo chemical reaction (oxidation of sulfhydryl groups by atmospheric oxygen, for example).

Denaturation may be brought about by such physical agents as heat, sound waves, surface forces, pressure, ultraviolet irradiation, and ionizing radiations. It may also be produced by treatment with organic solvents such as alcohol and acetone and by such solutes as urea, guanidine, and ionic detergents. Exposure to conditions of very high or very low pH also causes denaturation. Proteins are generally stable at room temperature over a pH range from 4 to 8 and some are stable over a wider range.

Among the manifestations of denaturation are decreased solubility, loss of biological activity, increased reactivity of constituent groups, changes in molecular size and shape, increase in optical rotatory power, and loss of crystallizability.

In dairy chemistry the principal interest in protein denaturation centers around the effects of heat. The whey or serum proteins are considerably affected by heat treatment. Among manifestations of heat denaturation of these proteins are decrease in solubility at pH 4.7 or in concentrated salt solutions and increase in activity of various groups of which the sulfhydryl group has been most thoroughly studied. This topic is discussed more fully in Chapter 11.

Among the milk proteins, the caseins are often said to be "undenaturable," meaning that they themselves are not altered in the range of heat treatments (60° to 100° C. for times up to 5 hours) that denature other proteins. Apparently the caseins exist in more or less uncoiled, random structures rather than in tightly coiled specific structures and consequently are not susceptible to heat denaturation of the type exhibited by the serum proteins. Drastic heat treatments, such as autoclaving at 120° C., do cause a decrease in solubility of whole casein, but this is accompanied by a splitting out of the esterified phosphate groups and degradation as manifested by the appearance of non-protein nitrogen. Such changes are sometimes referred to as denaturation, but certainly they do not come within the definition of denaturation as an intramolecular change in configuration involving no hydrolysis of primary covalent bonds. No denaturation within the sense of that definition has ever been demonstrated for casein. Consequently the term should be avoided in reference to casein.

IMMUNOLOGICAL PROPERTIES OF MILK PROTEINS

It is well known that injection of a protein foreign to an animal (an antigen) causes the formation of specific antibodies for that protein. This is the principle involved in immunity. The proteins (and car-

bohydrates) of a given disease-producing organism act as antigens stimulating the formation of antibodies specific against itself. These antibodies circulate in the blood and are available to combat subsequent invasion by the same organism that caused their production. The fundamental mechanism involved is combination of antigen and antibody through specific interacting sites. In vitro the technique of agglutination of a cellular antigen can be employed to demonstrate the presence in a biological fluid of antibodies (agglutinins) specific for that antigen. Likewise, precipitation of a soluble (i.e., molecularly dispersed) antigen is a valuable technique for qualitative and quantitative determination of specific antibodies (precipitins). Since precipitins possess a high degree of specificity they are valuable as reagents for differentiating between proteins, particularly species-specific proteins, such as the caseins of different species of animals. The technique is to produce antibodies to each casein being examined by injecting it into an experimental animal such as a guinea pig or a rabbit and then to test the ability of each specific antiserum to precipitate each of the caseins. Experiments like this have shown that the caseins of the cow and goat are closely related because the antiserum against one shows considerable power to precipitate the other (9). On the other hand, the caseins of the rat, rabbit, and human being differ antigenically from each other and from those of the cow and goat. This technique has also been used to demonstrate the identity of serum albumin isolated from bovine blood with the blood serum albumin isolated from milk.

Milk itself contains antibodies formed by the cow against antigenic proteins and organisms with which she has come in contact. These antibodies undoubtedly gain access to the milk from the blood, but it is not entirely settled whether they pass unchanged into the milk or are altered slightly by the mammary gland. They are found in the immune globulin fraction, which has already been described, and identified in the electrophoretic pattern of the whey proteins. Actually, active antibodies may constitute only a portion of the total immune globulin fraction.

The concentration of the immune globulin fraction is very much greater in colostrum than in normal milk. Colostrum is the means by which the cow transmits passive immunity (i.e., preformed antibodies) to the calf. In some animals, antibodies are transferred from mother to fetus across the placental membranes, but this does not occur in ruminants and the calf is born without antibodies in its blood. If it is fed colostrum during the first day of life, the antibodies are able to

pass from the digestive tract into the blood stream to give the calf passive immunity. Subsequently, however, the calf loses the ability to transfer the antibodies from the intestine to the blood stream. Feeding colostrum after the calf is twenty-four hours old is ineffective in the acquisition of passive immunity. This change in the calf may be due to the secretion of proteolytic enzymes in the alimentary tract or to a change in permeability of the intestine.

The whole subject of the immune proteins of milk has been reviewed by Smith (48). Although much work has been done in this field, much remains to be learned about the specific properties of the antibody proteins.

SUMMARY OF METHODS FOR DETERMINING PROTEIN CONTENT

In previous sections mention has been made of various properties of proteins that can be used as the basis of analytical methods. It is the purpose of this section briefly to summarize such methods and to point out some of their peculiarities. The problem under consideration is the determination of the total amount of protein in milk, a milk product, or a milk fraction. We are not concerned at this point with identification of the kind of protein present.

The determination of the protein content of materials in which proteins occur in mixtures with other biological materials is not simple. Usually some means of separating protein from such materials must be found. For milk, trichloroacetic acid at a concentration of about 12.5% is commonly used as a protein precipitant for analytical purposes. It appears to precipitate all of the proteins, but some complications arise because the fat globules are entrapped in the protein precipitate. Of course, the fat can be removed by centrifugation and analyses performed on the skimmilk, but the globules carry a certain amount of protein adsorbed on their surface which is not accounted for by analyses on skimmilk. Furthermore, phospholipides and cerebrosides which contain nitrogen are precipitated with the proteins by trichloroacetic acid, thus complicating the determination of protein by nitrogen analyses.

Physical Methods

Direct weighing. Since the ultimate objective of protein analyses is to determine the weight of protein in a given quantity of material,

it might seem that separation of the protein and direct weighing would be the simplest method to use. Nevertheless, the difficulties of removing lipides, salts, other solutes, and water completely so complicate the procedure that it is not generally employed for routine analyses. Obviously, however, direct weighing is the ultimate standard for all other methods. For example, the factor for converting nitrogen to protein was arrived at originally by preparing a sample of pure protein and determining the ratio between nitrogen and dry weight.

Volume measurements. Methods based on precipitating protein, centrifuging in a calibrated tube, and measuring its volume, have often been suggested. The Hart method for casein is of this type. Fat is extracted with chloroform and the casein precipitated with acetic acid, centrifuged, and measured. In general, it is difficult to achieve satisfactory reproducibility with such methods, particularly between laboratories. The conditions of precipitating and centrifuging are extremely critical and must be standardized very rigidly. Methods based on volume measurements of proteins are not used to any extent in the dairy industry at present except for assessing the amount of insoluble material in milk powders (solubility index).

Turbidimetric methods. It is sometimes convenient to form a suspension of insoluble protein and to estimate the protein content by optical methods. Either light transmittance or light scattering can be measured. Methods employing the former principle are called turbidimetric, those using the latter, nephelometric. In recent years a turbidimetric method devised by Harland and Ashworth (16) has been used to a considerable extent for determining the concentration of undenatured serum proteins in heated and dry skimmilks. In this method the casein and denatured serum proteins are precipitated by saturation with sodium chloride. The resulting filtrate is then diluted and acidified and its light transmittance determined. The protein content is determined from a calibration curve in which per cent transmittance is plotted against protein nitrogen content of the filtrate (determined by Kjeldahl). Methods of this kind are rapid, require little sample, and are conveniently adaptable to photometers and colorimeters found in most laboratories. Nevertheless, turbidimetric methods are not of the highest accuracy and precision because light absorption in turbid systems depends not only on the amount of dispersed material present but also on its degree of dispersion. The degree of dispersion depends on time of standing after development of turbidity, pH, salt concentration, method and rate of precipitation, and concen-

tration of material. Only by the most rigid standardization of all of the details of such methods can they be employed at all successfully.

Refractive index measurements. As previously pointed out most proteins have a refractive index increment of about 0.0018, meaning that one g. of protein dissolved in 100 ml. of solution increases the refractive index by that amount. With an accurate refractometer such as the Zeiss dipping refractometer, which has a sensitivity of ± 0.00003, it is possible to determine protein concentrations satisfactorily. Of course, the refractive index of the solvent must be determined as well as that of the protein solution. Temperature must be carefully controlled since most proteins exhibit a considerable temperature coefficient for refractive index increment. A method based on this principle has been devised by Brereton and Sharp (2) for determining casein concentrations. The casein is isolated by acid precipitation, washed and dissolved in alkali, and the refractive index determined.

Absorption of ultraviolet radiation. The absorption of ultraviolet radiation at wavelengths in the neighborhood of 280 mμ, due to tyrosine, tryptophan, and phenylalanine residues in the protein, can be used as a method for determining protein content. This method is particularly valuable for a known pure protein whose extinction coefficient ($E^{1\%}_{1\,cm.}$) is known. It may also be useful as an approximate measure of the total protein content of a mixture if an average extinction coefficient can be assumed to apply. Obviously the extinction coefficients of proteins vary with their contents of tyrosine, tryptophan, and phenylalanine. The presence of materials other than proteins that absorb at this wavelength would seriously limit the method, but the common salts and other solutes do not absorb radiation at 280 mμ. Methods based on this principle have not been used to any extent for routine determinations of milk proteins.

Chemical Methods

Determination of nitrogen. By all odds the most widely used method for determining protein content is the Kjeldahl procedure for nitrogen. It is natural that this should be so since nitrogen is a characteristic element in proteins.

This method involves the oxidation of the sample with sulfuric acid and a catalyst. Carbon and hydrogen are oxidized to CO_2 and H_2O, and reduced forms of nitrogen (such as $—NH_2$ and $=NH$) are retained in the digest as ammonium ions. The digest may be made alkaline

and the ammonia distilled off, collected, and titrated, or it may be determined colorimetrically directly in the digest by means of Nessler's reagent.

Although it is widely accepted and used, the Kjeldahl method suffers from some rather serious difficulties. In the first place, there is the problem of separating protein from other nitrogenous materials. In milk about 5% of the total nitrogen is in the form of low molecular weight non-protein nitrogenous materials. The protein can be separated from these by precipitation with trichloroacetic acid. This precipitation, however, does not separate protein from nitrogen-containing lipides. Since the amount of lipide nitrogen is small, comprising only about 0.2% of the total milk nitrogen, it is usually neglected (i.e., included as protein nitrogen).

A second difficulty with nitrogen methods for determining protein arises from variation in the nitrogen content of various proteins. Proteins vary in nitrogen content from 14 to 19% and thus a single universal conversion factor cannot be used. Fortunately the principal milk proteins exhibit much less variation (see Table 6) ranging from 15.3 to 16.0%. An average factor of 6.38 (corresponding to 15.65% nitrogen) is commonly used for milk proteins to convert nitrogen to protein. In precise work on an individual milk protein the correct factor for that protein should be used.

A third group of difficulties concerns the Kjeldahl method itself. These center chiefly around the problem of digestion which must be complete and involve no loss of nitrogen. A great many different modifications of the original Kjeldahl procedure have been suggested to accelerate the digestion while still attaining complete digestion. Copper, mercury, or selenium are used as catalysts and frequently Na_2SO_4 or K_2SO_4 is added to elevate the boiling point during digestion. Although there is some confusion in the literature on the various modifications of the Kjeldahl procedure, the methods listed in Table 10 yield satisfactory results for milk and milk fractions.

The use of boric acid to receive the ammonia as it is distilled off (as in methods 2 and 3) is an advantage in that only one reagent, the standard acid for titrating, need be standardized and measured accurately. The methods of Menefee and Overman (31) and Larson and Jenness (24) are particularly adapted to use with the Rowland method of fractionation of the milk proteins (see p. 114).

Formol titration. Considerable use has been made of titration with formaldehyde as a means of determining protein content in milk and milk fractions. Such titrations have been of particular interest as

Table 10. MODIFIED KJELDAHL PROCEDURES FOR MILK PROTEINS

Method	Size of Milk Sample	Catalyst	Method of Collecting NH_3	Method of Determining NH_3
1. AOAC(1)	5 g.	HgO	Distillation into standard HCl	Titration with NaOH
2. Menefee and Overman (31)	1 g. (20/100 of 5 g.)	HgO and Na_2SO_4	Steam distillation into boric acid	Titration with HCl
3. Larson and Jenness (24)	1 or 2 ml.	SeOCl	Steam distillation into boric acid	Titration with HCl
4. Hetrick and Whitney (20)	0.025 g. (12.5/500 of 1 g.)	K_2SO_4 and H_2O_2	Colorimetric determination in digest with Nessler's reagent.	

rapid methods of determining the casein content of milk for cheese-making. All such methods involve titrating a sample of milk to the end point of an indicator such as phenolphthalein, adding a solution of formaldehyde, and titrating the acid liberated to the same end point. The amount of alkali used in the second titration is a measure of the amino groups that were originally present and combined with the formaldehyde.

One difficulty with the formol titration for milk analyses is that the milk proteins differ in amino nitrogen content and, since their proportions also vary, the factor relating formol titer to protein content is not constant. Furthermore, the end point is rather difficult to judge accurately. One worker, Pyne (42), sought to improve the titration by adding potassium oxalate to precipitate calcium. Considerably higher formol titers are obtained when potassium oxalate is used; the reason for this effect is not entirely clear. Some workers advocate the use of potassium oxalate and others do not.

The formol titration certainly has much to recommend it from the standpoint of rapidity and convenience. Its overall accuracy is probably of the order of $\pm 5\%$. Detailed directions for formol titrations of milk are given by McDowall and McDowell (27) and by Pyne.

Biuret method. In recent years the biuret reaction has been increasingly applied on a quantitative basis as a means of estimating protein content. It has the advantage that proteins do not differ

widely in the intensity of color produced. The lactose of milk interferes with the test because it complexes with copper and also reduces copper. Thus if milk is to be tested by the biuret method, the proteins must either be separated from the lactose by precipitation and washing (e.g., with trichloroacetic acid), or correction must be made for the effects of lactose (21, 45). The biuret method has been suggested as a means for determining casein and also for determining the undenatured serum proteins remaining after precipitating casein and denatured serum proteins with sodium chloride.

Folin method. The Folin method for phenol and indole groups has been applied in dairy chemistry principally for determination of tyrosine and tryptophan liberated by proteolysis. It has also been suggested as a means of determining total milk protein and various fractions. Its value for such purposes is very limited because of the large differences in the contents of tyrosine and tryptophan in the various proteins.

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CHAPTER 5 MILK SALTS

INTRODUCTION

The salts of milk include those constituents except hydrogen ions and hydroxyl ions that are present as ions or in equilibrium with ions. A large part of the salt constituents of milk are mineral materials including metals and inorganic acid radicals. In addition, some organic acid radicals are present. In a sense, also, the milk proteins constitute part of the salt system. They carry both positively and negatively charged groups and can form salts with positive and negative ions. At the pH of milk the proteins carry a net negative charge and thus form predominately salts with cations.

A distinction is usually made between the major salt constituents of milk and the trace elements. The former category includes potassium, sodium, magnesium, calcium, phosphate, citrate, chloride, sulfate, and bicarbonate. The latter group includes all other mineral and salt compounds. Obviously this distinction is made on the basis of concentration and not necessarily on the basis of importance in dairy processes and dairy problems.

The milk salts are important in three principal areas of dairy chemistry. In the first place, some of the salt constituents, particularly

calcium and phosphate, are of tremendous importance in nutrition. Milk is an excellent nutritional source of these materials. Secondly, the physical state and physical stability of the milk proteins, particularly the caseinate, is very dependent on the composition of the salt system. Thus, problems arising in heat coagulation of evaporated milk and in rennet coagulation of milk stem largely from variations in salt composition. In the third place, certain metallic elements in milk, particularly copper and iron, catalyze oxidation of milk lipides, which leads to undesirable flavors.

SALTS VERSUS ASH

Ashing or incineration of milk destroys the organic compounds, leaving the mineral constituents behind. Most tabulations of the gross composition of milk include a figure for milk ash of approximately 0.7%. Actually, the ash does not truly represent the salt composition of milk for the following reasons: (*a*) Organic radicals, such as citrate, are destroyed by the incineration procedure. (*b*) Phosphorus and sulfur of the milk proteins and lipides appear in the ash. The organic sulfur is present in a non-salt form and the phosphorus occurs in a sort of "semi-salt" form having part of its potentially ionizable groups involved in ester linkage with hydroxyl groups of the proteins or lipides. (*c*) Carbonates appearing in milk ash arise in part from carbon dioxide produced by decomposition of the organic materials. (*d*) Oxidation during incineration results in the formation of oxides of the metals. (*e*) Some salt materials such as sodium chloride and carbonates may be lost by volatilization, but this complication can be avoided (at least for sodium and chloride) by properly regulating the temperature to not over 600° C. It is evident that the salt combinations and equilibria in milk cannot be deduced from a study of ash alone. The ash "content" gives a rough idea of the total mineral content and, of course, ashing is a useful technique for getting rid of organic matter as a prelude to analysis for many of the mineral elements.

THE SALT COMPOSITION OF MILK

In presenting the composition of the milk salts, some consideration has to be given to the mode of expressing concentrations. Formerly, the vogue was to express the concentration of metallic elements and of

phosphates as the corresponding oxides. Since they certainly do not exist in the form of oxides in milk, the modern trend is to express their concentration in terms of the elements. This manner of expression will be followed in this chapter. Furthermore, the concentration of citrates will be presented in terms of the equivalent amount of citric acid, lactates as lactic acid, carbonates as CO_2, and inorganic sulfur as $SO_4^=$. For the most part, concentrations will be expressed in terms of milligrams per hundred milliliters, as milliequivalents per liter, or as percentage.

Normal Range of Composition

Although the ash content is relatively constant at about 0.7% of milk, the concentrations of the several salts vary considerably. Studies of the variation in concentration are much more extensive for the calcium, phosphate, and chloride than for the other components of the salt system. These components are determined relatively easily, and calcium and phosphate at least are very important from a nutritional standpoint. On the other hand, data on variation in content of sodium and potassium are relatively scarce because these elements have been relatively difficult to determine and because milk is not relied on as a source for them. Table 1 presents a compilation of the usual variations in the contents of the major salt constituents and an "average figure," which may be taken as representing normal milk. The salt composition of blood plasma is presented for comparison. The latter is much higher in sodium and chloride and a little higher in magnesium, but much lower in the other constituents.

Factors Associated with Variations in Salt Composition

A number of factors have been implicated as influencing the salt composition of milk. These may be enumerated as breed and individuality of cow, stage of lactation, feed, infection of the udder, season of the year, and bacterial action. Unfortunately, there are insufficient satisfactory comparative and systematic data on the effects of the various factors. Thus, the effects of each cannot be evaluated precisely. The data on the citrate content of milk are especially unsatisfactory. The whole subject of salt composition and factors affecting it was reviewed in 1931 by Allen (1). Some additional data have been accumulated since 1931, but in general this has not changed the conclusions that Allen drew at that time.

Table 1. VARIATION IN CONTENT OF MILK SALT CONSTITUENTS AND COMPARISON WITH SALT COMPOSITION OF BLOOD PLASMA

Constituent	Milk Plasma (skimmilk)			Bovine Blood Plasma, mg./100 ml.
	Average Content, mg./100 ml.	Usual Range, mg./100 ml.	Extremes Reported,* mg./100 ml.	
Sodium	50	35–60	11–115	330
Potassium	145	135–155	115–200	20
Calcium	120	100–140	65–264	10
Magnesium	13	10–15	2–23	25
Phosphorus (total)	95	75–110	47–144	...
Phosphorus (inorganic)	75	...	...	5
Chloride	100	80–140	54–242	350
Sulfate	10	...	...	...
Carbonate (as CO_2)	20	...	...	...
Citrate (as citric)	175	...	...	4

* Undoubtedly includes abnormal milks.

Breed and individuality of cow. Available information certainly indicates that there are breed and individual differences in the salt composition of milk. Unfortunately, the studies that have been made are insufficient in number and insufficiently systematic to enable us precisely to define the extent of these differences. Milk from Jersey cows is usually higher in calcium and phosphorus content than milk from Holstein cows. This difference undoubtedly reflects, in part, the greater concentration of casein in the Jersey milk. Casein contains phosphorus as an integral part of the molecule and binds calcium very tenaciously. On the other hand, Holstein milk is generally higher in chloride content than milk of the other breeds. The results of two rather thorough-going studies, each dealing with a single breed, are given in Table 2. These give a fairly complete picture of the salt composition of Holstein and Brown Swiss milk respectively, and of the variations that may be expected.

The salt composition of milk apparently is determined by genetic factors. This idea gains support from an experiment conducted by Hansson (9) in Sweden with three pairs of identical twins. His results

Table 2. SALT CONSTITUENTS IN MILKS OF HOLSTEIN AND BROWN SWISS CATTLE

	Content in mg./100 ml.			
	Holstein*		Brown Swiss†	
Constituent	Mean	S.D.‡	Mean	S.D.‡
Calcium	111.7	11.7	123.1	10.2
Magnesium	13.1	3.0	13.5	2.49
Sodium	57.6	18.4	43.8	7.25
Potassium	139.2	17.2	138.1	17.1
Phosphorus	95.1	9.0	108.3	14.1
Chloride	107.7	21.6	84.9	7.7

* One hundred thirty-four 28-day composite samples from 12 individual Holstein cows throughout one lactation period. Data of Black and Voris (3).

† Four hundred eighty-nine composites, each representing a single milking of a herd of Brown Swiss cows. Thirty-nine herds were involved and were sampled on one day each month throughout a year. Data of Overman et al. (11).

‡ Standard deviation.

indicated little difference within the twin pairs in calcium and phosphorus contents of the milks, but a greater difference between the pairs.

Stage of lactation. The salt composition of milk varies markedly during the lactation period, the greatest changes occurring at the beginning and end of the period. It is well known that the "ash content" of colostrum is higher than that of normal milk. This is due to a higher content of calcium, phosphate, magnesium, chloride, and sodium. The concentration of these elements rapidly decreases toward the normal value with successive milkings. However, the potassium content of colostrum is lower than that of normal milk and increases rapidly to a normal value. The data of Garrett and Overman (8) in Table 3 indicate the magnitude of the changes observed with two cows. The data of Ellenberger, Newlander, and Jones (7) clearly indicate that the calcium and phosphate contents of milk fall during the first five weeks of lactation. These data were obtained with a large number of cows and are shown in Fig. 1. They also indicate a marked rise in calcium and phosphate contents during the last 15 weeks of lactation. Somewhat similar trends have been found for the chloride content of milk. This is illustrated by data of Sharp and Struble (12) shown in Fig. 2.

Table 3. MINERAL COMPOSITION OF COLOSTRUM AND TRANSITION TO NORMAL MILK

Time after Calving	Sodium, %	Potassium, %	Calcium, %	Magnesium, %	Phosphorus, %	Chlorine, %
			Holstein Cow			
At parturition	0.074	0.137	0.256	0.037	0.235	0.118
6 Hours	.061	.128	.196	.027	.178	.118
12 Hours	.051	.132	.154	.014	.146	.101
24 Hours	.050	.145	.150	.013	.137	.102
2 Days	.049	.139	.148	.013	.127	.098
3 Days	.065	.146	.176	.013	.176	.099
11 Days	.036	.153	.130	.011	.113	...
			Ayrshire Cow			
At parturition	0.079	0.125	0.206	0.034	0.192	0.122
6 Hours	.050	.152	.154	.012	.123	.117
12 Hours	.072	.140	.142	.019	.142	.121
24 Hours	.065	.154	.124	.013	.129	.117
2 Days	.058	.171	.137	.014	.137	.100
3 Days	.056	.163	.131	.015	.125	.096
10 Days	.047	.152	.120	.011	.110	.068

Data of Garrett and Overman (8).

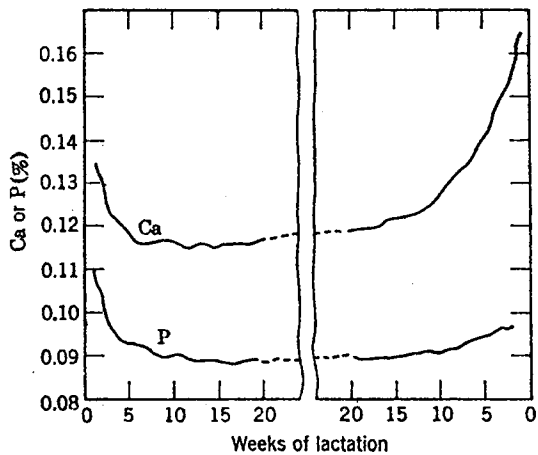


Fig. 1. Calcium and phosphorus contents of milk during first 20 weeks and last 20 weeks of lactation. Based on 900 composites representing 45 lactations. Data of Ellenberger et al. (7).

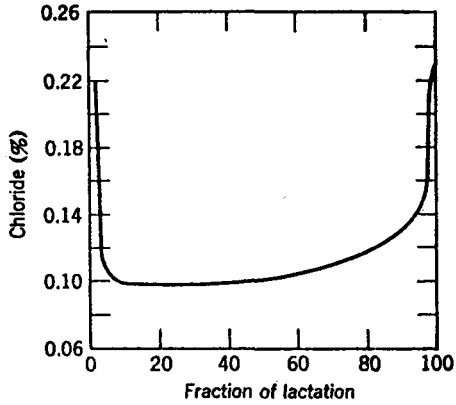


Fig. 2. Variation of chloride content of milk throughout lactation. Data of Sharp and Struble (12).

Feed. The effects of feed on the salt composition of milk are small. As a matter of fact, there has been some controversy as to whether feed has any influence at all on salt composition. The elements calcium and phosphorus have been most studied in this connection. In some experiments the effects of including large excesses of calcium and phosphate in the ration have been studied, and in other cases the effects of underfeeding with calcium and phosphate have been evaluated. It appears that the cow uses the skeleton as a "buffer mechanism" to serve as a reserve of calcium and phosphate. When extra amounts of these elements are fed to the cow, they are accumulated in the skeleton, and when insufficient amounts are included in the ration the cow draws on the skeletal reserves for these elements for milk. Some workers have noted no effects of feeding large excesses of calcium and phosphate. Others have noted small increases in the concentration of the elements in the milk. There is some difference of opinion, likewise, as to whether feed influences the citrate content of milk. Certainly it is known and will be pointed out later that seasonal variations occur in citrate content, but it is not established with certainty that they are due to differences in feeding.

Infection of the udder. It is well known that the chloride content of milk increases markedly in cases of udder infection, known as mastitis. The chloride content of most normal milks will fall below 0.12%. In mastitis, however, this increases to an extent dependent upon the severity of the infection and may attain values as high as 0.3%. Variations in sodium content very closely parallel

those in chloride. On the other hand, as the content of sodium and chloride increases, that of potassium decreases. These relations are demonstrated admirably in a study by Barry and Rowland (2) and are shown in Fig. 3.

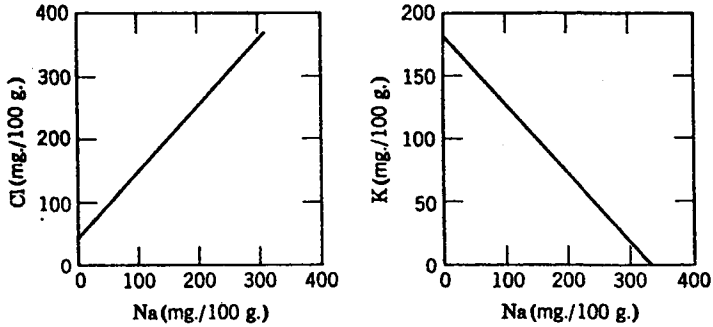


Fig. 3. Relations between sodium, potassium, and chloride in milk (including mastitic milks). Data of Barry and Rowland (2).

The milk from cows suffering from severe mastitis frequently shows evidence of direct passage of blood into the milk as indicated by the presence of high concentrations of sodium and chloride, blood enzymes, and red blood cells.

Seasonal variations. It is well established that salt composition of milk varies with the season, particularly in the north temperate region. The calcium and phosphate contents decline to a low in late summer, that is, in July, August, and September. The magnitude of this effect is not great, as is shown in Fig. 4, taken from the work

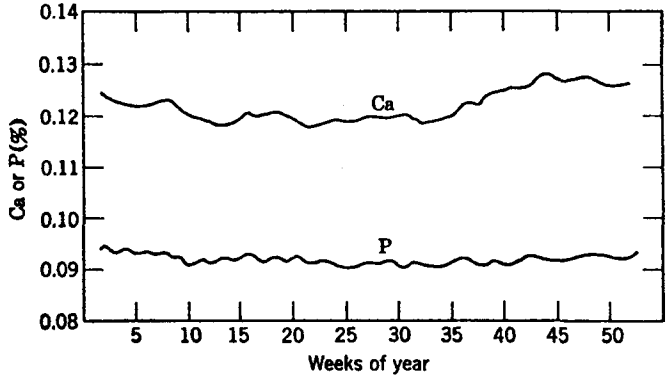


Fig. 4. Seasonal variation in calcium and phosphorus contents of milk. Data of Ellenberger et al. (7).

of Ellenberger et al. (7). A study of the composition of Brown Swiss milk by Overman et al. (11) also corroborates this variation. Overman's study also showed no significant seasonal variations in the sodium and potassium contents, and a scarcely significant variation in chloride content.

A number of groups of workers have studied seasonal variations in citrate content of milk. A seasonal variation with a low in the early winter months of November, December, and January has usually been found. This effect is shown in Fig. 5.

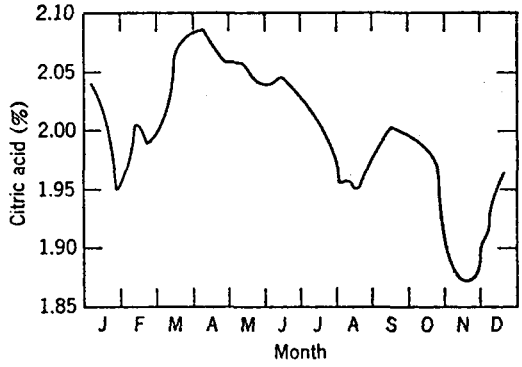


Fig. 5. Seasonal variation in citrate (citric acid) content of non-fat dry milk. Data of Heinemann (10).

The actual causative agents for the seasonal variations that have been noted in calcium, phosphate, and citrate contents of milk are not known. Both feed and variation in stage of lactation have been suggested as causes. As yet, the observed variations cannot be definitely assigned to any particular cause.

Bacterial action. The action of bacteria in milk alters the salt composition in two principal manners. In the first place, the production of acids from sugar by bacteria introduces additional anions into the system. The principal anion that thus arises is lactate, although other acids are also sometimes produced. Not only does the production of acid increase the total salt concentration of the milk, but also the reduction in pH results in a considerable shift in the salt equilibrium, as will be discussed later. The second effect of bacterial action on salts in milk is a decrease in the concentration of citrate by organisms that utilize this material. The possibility of bacterial action must be constantly kept in mind and must be avoided in comparative studies of salt composition of milk.

Trace Elements

A large number of elements have been detected in trace amounts in normal milk. These include a long list of metals, the metalloids arsenic, boron and silicon, and the halogens fluorine, bromine, and iodine. Our knowledge of these materials in milk is rather scanty. For many of them satisfactory quantitative data are lacking on the specific forms and compounds in which they occur.

Metals and metalloids are often detected qualitatively by spectrographic methods utilizing the specific wave bands of light emitted when the atoms are excited in an electric arc. A number of such studies of milk ash have revealed the presence of the elements listed in Table 4. This technique does not indicate the presence of the important element cobalt, which has been detected by other means. Furthermore, although titanium and vanadium are usually found in spectrographic analyses of milk ash, some workers believe that they are contaminants of the electrodes rather than true constituents of the milk itself. It must constantly be borne in mind that a trace element may gain entrance to milk either through the cow or by subsequent contamination. For some elements the level in the milk depends on the level in the feed. Metal utensils and equipment are especially important sources of some elements, such as copper, iron, nickel, and zinc. As a matter of fact, corrosion is the only source of nickel in milk since this metal is absent from milk as it comes from the udder, even though the cow ingests nickel in the ration. On the other hand, aluminum and tin surfaces are extremely resistant to corrosion by milk. Also, certain alloys such as stainless steels are very resistant to corrosion even though the individual metals of which they are composed are corrodible. Investigators of physiological problems are interested primarily in the trace elements secreted by the cow into the milk, but research on dairy manufacturing problems must take account of both the natural and the artificial sources of these elements.

Quantitative analyses have been made for some of the metallic elements that have been detected spectrographically. Obviously quantitative research on materials present in small traces is beset with many problems relating to the precision and accuracy of the analytical methods employed. Table 4 gives a general idea of the order of magnitude of the concentrations of those metals for which data are available. Insofar as possible, data obtained on milk as it comes from the cow have been segregated from those secured on commercial milk.

Table 4. TRACE ELEMENTS IN MILK

Metals and Metalloids	Spectroscopic Identification	Content in Milk Directly from Cow		Content in Commercial Milk, μg./l
		No Supplement in Ration, μg./l	Ration Supplemented with Soluble Salt of Element, μg./l	
Aluminum	+	150–1000	400–1400	
Arsenic	−	30–60		
Barium	+			
Boron	+	100–400	500–1500	
Chromium	±			
Cobalt	−	0.2–1.4	0.9–5.9	
Copper	+	30–170		50–200
Iron	+	about 300		100–600
Lead	±			
Lithium	+			
Manganese	+	12–35	18–130	
Molybdenum	+	20–150	200–700	
Nickel	−	0	0	0–100
Rubidium	+			
Silicon	±	870–2270		
Silver	±			
Strontium	+			
Tin	±			
Titanium	±			
Vanadium	±			
Zinc	+	1000–6000	3500–7000	
Halogens				
F				100–300
Br		180–250		
I		10–80	up to 2000	100–2000

Copper and iron have been studied more thoroughly than the other trace metals because of their importance in oxidative defects of milk lipides. Both of these elements occur to a large extent in the form of complexes with protein. Iron is known to be a constituent of the enzymes xanthine oxidase, peroxidase, and catalase, all of which occur in milk. Both copper and iron are bound to some extent by the fat globule membrane complex. Even when copper and iron are added to milk they are bound in some way, presumably by proteins, because only a small portion of the total is dialyzable.

PHYSICAL EQUILIBRIA AMONG THE MILK SALTS

Partition between Dissolved and Colloidal Phases

The content of certain salts in milk is greater than can be maintained in solution, that is, the solubility is exceeded. The excess of these salts is present not in true solution, but rather in colloidal particles containing casein, calcium, magnesium phosphate, and citrate. The chemistry of these particles is dealt with in Chapter 10.

Various methods of study have been used to separate the dissolved from the colloidal phases in milk in order to study the partition of salts between them. Of course, the distinction between the states of solution and colloidal dispersion results from arbitrarily drawing the line at some point of particle size defined by the operations used for separation. Obviously, any method used for this purpose must not alter the equilibrium between the dissolved and colloidal states. The method itself must not cause any transfer of dissolved constituents to the colloidal state or vice versa. Four principal methods used for this purpose are pressure ultrafiltration, equilibrium dialysis, high speed centrifugation, and rennin or papain coagulation. Pressure ultrafiltration involves forcing some of the dissolved phase of a sample of milk through a fine-pored filter by means of pressure. Clay, porcelain, collodion, and cellophane filters have been used for this purpose. The pore size of such filters must be small enough to prevent the passage of any smaller colloidal particles. Equilibrium dialysis involves dialyzing a small amount of water against a large amount of milk until equilibrium is established. This can be conveniently done with cellophane membranes. The material then within the sac at the completion of equilibrium dialysis represents a sample of the dissolved phase of milk. It is essential in using this technique that the amount of water be kept small in comparison to the amount of milk to avoid any undue dilution. High-speed centrifugation can be used to sediment the colloidal particles and leave the dissolved phase as a supernatant. Very much the same sort of separation can be obtained by treating milk with a small amount of rennin or papain, which coagulates the colloidal caseinate particles. The sera obtained by centrifuging or by rennin or papain coagulation contain the whey proteins or serum proteins of milk. On the other hand, the sera obtained by ultrafiltration or dialysis do not contain these serum proteins. All four methods of partition of the system give reasonably concordant results on the

distribution of salts between the two phases. Table 5 gives some data on the partition of calcium, magnesium, phosphate, and citrate as determined by rennin coagulation. In general, about a third of the

Table 5. DISTRIBUTION OF SALTS BETWEEN DISSOLVED AND COLLOIDAL STATE IN RAW MILK

	Total	Dissolved	Colloidal
	(mg./100 ml. of milk)		
Calcium	132.1	51.8	80.3
Magnesium	10.8	7.9	2.9
Total phosphorus	95.8	36.3	59.6
Citrate (as citric acid)	156.6	141.6	15.0

Data of Verma and Sommer (15). Average of 15 samples. Phases separated by rennet coagulation. Colloidal salts calculated by difference.

calcium, a third of the phosphate, 75% of the magnesium, and 90% of the citrate of milk are present in the dissolved state.

Confusion sometimes rises with regard to the phosphorus-containing compounds of milk. Actually, there are five classes of such compounds, the inorganic salts and organic esters in solution, and lipide, protein, and colloidal inorganic phosphates in colloidal dispersion. Figure 6 gives a picture of the distribution of the phosphorus of milk among these five different classes of compounds, and indicates the manner in which they are separated by such treatments as precipitation of the proteins with trichloroacetic acid, dialysis, and lipide extraction.

It is important to remember that the colloidal salts are in equilibrium with the dissolved salts. Various treatments of milk may cause transfer of salts from one phase to the other. For example, if milk is dialyzed by a long, continuous process involving repeated changes of the dialyzing water, much of the colloidal salt can be brought into solution and dialyzed away.

Equilibria among Salts and Ions in Solution

It is not difficult to decide that the principal dissolved salt constituents of milk consist of phosphate, citrate, chloride, sulfate, bicarbonate, sodium, potassium, magnesium, and calcium. The determination of the kinds of ion species present and the concentration of each

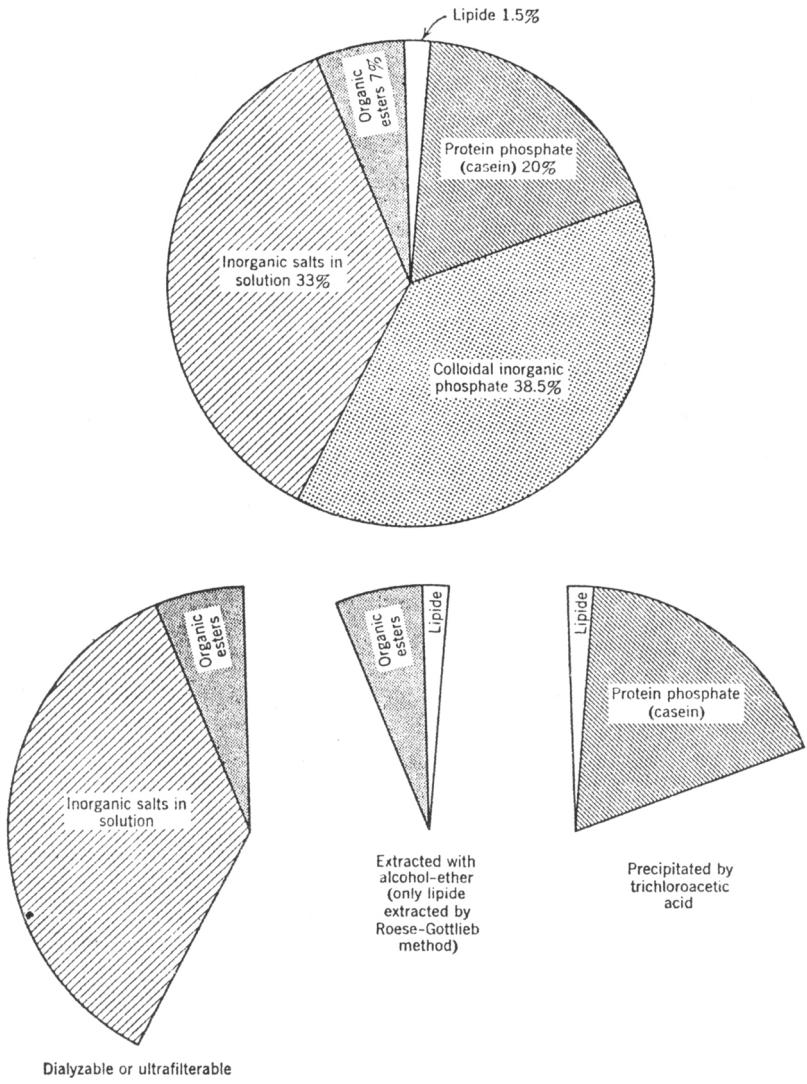


Fig. 6. Phosphate compounds in milk.

present a big problem. In fact, it is such a big problem that it has not yet been solved completely, but the following considerations enable us to solve it partially.

1. The sodium and potassium are not known to enter into any combinations with the other milk constituents at the pH of milk (pH 6.6). Consequently they may be regarded as entirely present as the cations Na^+ and K^+.

2. The chloride and sulfate are anions of such strong acids that they may be considered as entirely present as the free ions Cl^- and $SO_4^=$ at the pH of milk.

3. The salts of the weak acids (phosphates, citrates, and carbonates) are distributed among various ionic forms. A first approximation of these distributions can be arrived at by calculation according to the Henderson-Hasselbalch equation:

$$\text{pH} = pK_a + \log\frac{[\text{salt}]}{[\text{acid}]}$$

From the known pH and the dissociation constants (pK_a's of the acids) the ratios of the ionic forms can be calculated. A note of caution should be injected here; the dissociation constants vary considerably with the total ionic concentration and, consequently, there is some question as to the exact values of the dissociation constants to employ in making the calculations for milk.

The dissociation constants of the three acids in question are of the following orders of magnitude:

Acid	pK_1	pK_2	pK_3
Citric	3.08	4.74	5.40
Phosphoric	1.96	7.12	12.32
Carbonic	6.37	10.25	...

If these values are used in the Henderson-Hasselbalch equation the following ratios are obtained at pH 6.6:

		Conclusion
$\frac{\text{Citrate}^-}{\text{Citric acid}} = 3{,}300$		No appreciable amount of free citric acid or monocitrate is present. Tricitrate and dicitrate in a ratio of 16:1 are the predominant ions.
$\frac{\text{Citrate}^=}{\text{Citrate}^-} = 72.-$		
$\frac{\text{Citrate}^{\equiv}}{\text{Citrate}^=} = 16.-$		

$\frac{H_2PO_4^-}{H_3PO_4}$	= 43,600	Monophosphate and diphosphate are the predominant forms.
$\frac{HPO_4^=}{H_2PO_4^-}$	= 0.30	
$\frac{PO_4^{\equiv}}{HPO_4^=}$	= 0.000,002	
$\frac{HCO_3^-}{H_2CO_3}$	= 1.7	Bicarbonates in equilibrium with $H_2CO_3(CO_2 + H_2O)$ are the predominant forms.
$\frac{CO_3^=}{HCO_3^-}$	= 0.0002	

However, if the phosphate is distributed as above between $H_2PO_4^-$ and $HPO_4^=$, the citrate between citrate$^=$ and citrate$^{\equiv}$, the carbonate between H_2CO_3 and HCO_3^-, and all of the anions are added up (including Cl^- and $SO_4^=$), there is a deficiency of anions to account for all the cations Na^+, K^+, Ca^{++}, and Mg^{++}, considering dissolved calcium and magnesium to be completely in the ionic form. Even inclusion of the anionic groups of the proteins does not provide enough anions. This apparent discrepancy is explained by the following consideration:

4. There is extensive evidence that calcium and magnesium form soluble complex ions with citrates, phosphates, and bicarbonates. Thus, for example, in the case of citrate (using the symbol © for citrate) the following equilibria apply:

$$H©^= \rightleftharpoons ©^{\equiv} + H^+$$
$$©^{\equiv} + Ca^{++} \rightleftharpoons Ca©^-$$
$$Ca©^- + H^+ \rightleftharpoons CaH©$$
$$2Ca©^- + Ca^{++} \rightleftharpoons Ca_3©_2$$

A considerable portion of the calcium is held in the form of complex soluble ions such as $Ca©^-$. Analogous complex ions are formed with phosphate ($CaPO_4^-$) and with bicarbonate ($CaHCO_3^+$). Furthermore, magnesium doubtlessly behaves in an analogous manner in forming such ions. Studies of these equilibria and complexes have been made only with pure solutions, but they would definitely be expected to exist at the pH of milk. The formation of such complex ions has the effect of reducing the concentration of calcium and magnesium ions Ca^{++} and Mg^{++} in the solution and of taking care of the apparent discrepancy between cations and anions mentioned in the previous section. Figure 7 presents these equilibria graphically, including the equilibrium

between dissolved and colloidal phases. Only the calcium salts are included but, as previously mentioned, a similar scheme would apply to the magnesium salts.

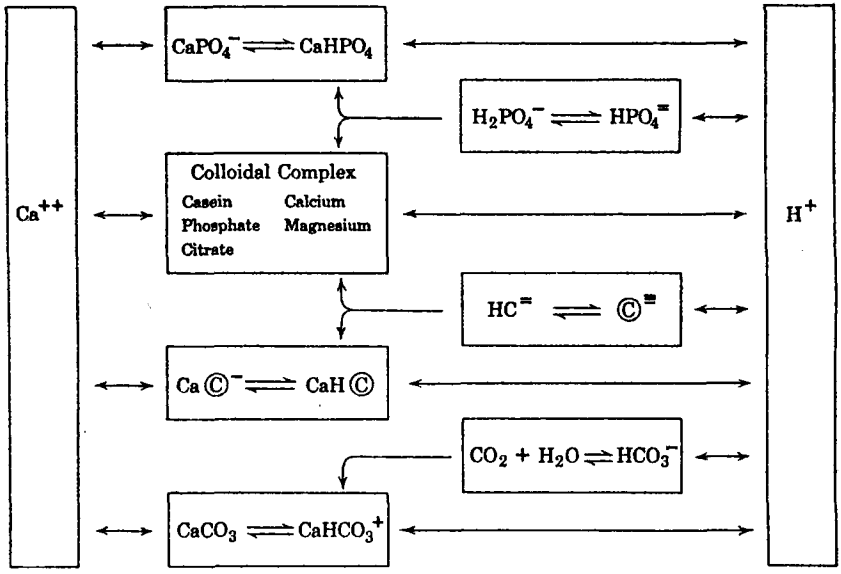


Fig. 7. Equilibrium among milk salts.

It is difficult to determine the concentration of each of the several species of ions in milk. It is one thing to postulate the existence of certain ions on the basis of general principles, but quite another to determine the concentration of each. Up to the present, the following items can be determined:

1. Hydrogen ion concentration, that is, pH.
2. Colloidal calcium, magnesium, phosphate, and citrate.
3. Dissolved or diffusible calcium, magnesium, phosphate, and citrate.
4. Total carbonate as CO_2.
5. Calcium and magnesium ion concentrations.

Calcium (and magnesium) ions and hydrogen ions occupy key positions in the scheme depicted in Fig. 7. Hydrogen ion activity is determined readily enough. A method for measuring calcium ions

was long sought. Of various methods proposed, two have been developed recently which give considerable promise. These are:

1. The Murexide method of Smeets (13). This depends on the formation of a complex between calcium and a dye called murexide:

$$Ca^{++} + M \rightleftharpoons CaM$$

The calcium-dye complex, CaM, has a maximum absorption of light at a wavelength of 480 mμ; the dye itself absorbs maximally at 520 mμ. The extent of formation of CaM can be determined by measuring the absorption at 480 mμ or, better yet, the difference between absorption at 520 mμ and 480 mμ. The calcium ion concentration of an unknown solution can be calculated by comparing its light absorption with those of appropriate standard solutions. This method can be applied to milk ultrafiltrates or equilibrium dialysates.

2. The ion exchange equilibration method of Christianson, Jenness, and Coulter (5), and van Kreveld and van Minnen (14). This depends on equilibration of skimmilk with a cation exchange resin. The amounts of calcium and magnesium bound by the resin at equilibrium are proportional to the calcium and magnesium ion concentrations in the milk. By comparison with appropriate standard solutions, the calcium and magnesium ion concentrations can be calculated.

The data that have been obtained with these methods indicate that the amount of calcium present as Ca^{++} is only about 8 to 10 mg. per 100 ml. out of a total of 125 mg. per 100 ml. Similarly, only about 2 mg. of magnesium out of a total of 13 mg. per 100 ml. is present as ions.

In a sense the system may be said to be "buffered" with respect to calcium and magnesium ions. If the concentration of these ions is diminished by any treatment, the supply tends to be replenished from the reserve of soluble and colloidal complexes. Conversely, any calcium or magnesium salts added to milk will be distributed between the ionic form and the complexes.

Effects of Various Treatments on Salt Equilibria

Effect of temperature. The equilibria among the various forms of salts and ions in the milk undoubtedly vary a great deal with temperature. Shifts in the balance among the various forms occur as milk is subjected to various cooling and heating treatments after it is withdrawn from the cow at 37° C. There is not much information on

these shifts in equilibria, however, since very little work has been done in determining the ionic balance in milks at different temperatures.

Since milk is essentially saturated with calcium phosphate and since the solubility of calcium phosphate decreases as the temperature is raised, it is not surprising that these materials are transferred from the dissolved to the colloidal phase with a rise in temperature. This has been demonstrated adequately. It would be expected, on the other hand, that lowering the temperature below that at which the milk is drawn from the cow would cause a transfer of calcium and phosphate from the colloidal particles to the dissolved state. This facet of the problem has not been studied adequately. Not only does variation in temperature affect the distribution of materials between the dissolved and colloidal state but also it would be expected to affect the balance among the various ionic forms, because the dissociation constants of the various complexes depend very markedly on temperature. Very little has been done in an experimental way to determine the extent of the changes that occur. It would be valuable to define the equilibria existing in hot milk particularly under the heating conditions that cause destabilization of the proteins.

Some of the effects of heat treatment of milk on the salts are irreversible, or at least very sluggishly reversible. These effects are discussed more fully in Chapter 11 under the effects of heat treatment on milk.

Effects of variation in acidity. The addition of acid to milk either directly or indirectly by bacterial action or by heat treatment causes pronounced shifts in the salt equilibria. It can be shown, for example, that as the pH is decreased by adding acid the calcium and phosphate are withdrawn from the colloidal particles until at about pH 5.2 all of the calcium and phosphate is in the dissolved state. Likewise, the proportion of calcium that is in the ionic form increases as the pH is decreased. A glance at Fig. 7 will indicate that a shift in the hydrogen ion concentration in the system will shift the equilibria among the salts throughout the system.

Effects of variation in CO_2 content. Milk as secreted by the cow contains about 20 mg. of CO_2 per 100 ml., or about 10% by volume. This gas is rapidly lost from milk, owing to the low content in the air. The loss is essentially irreversible under ordinary conditions of handling. Ordinary commercial milk may contain about half of the original CO_2, that is, about 10 mg. per 100 ml. The loss of CO_2 from milk is accelerated by heating and by agitation. It would be antic-

ipated from the diagram in Fig. 7 that the removal of CO_2 would affect the balance in the rest of the system. Such effects could be determined uncomplicated by any effects of heat simply by removing the CO_2 by vacuum treatment without heating. Virtually the entire carbonate system can be removed from milk in this way, with a resulting decrease in titratable acidity and increase in pH. It would be expected that the removal of CO_2 and the consequent rise in pH would be reflected in a shift in calcium phosphate from the dissolved to the colloidal state and probably also in a shift of calcium ion activity. Such effects have actually never been demonstrated, however.

The effects of concentration. As milk is concentrated, there is a tendency for calcium phosphate and calcium citrate to accumulate in the colloidal particles because the solubility is exceeded. As these materials are insolubilized, hydrogen ions are liberated, lowering the pH. The net result is an increase in concentration of citrate and phosphate in both the dissolved and colloidal states. Table 6 (see p. 180) illustrates this effect with skimmilk concentrated 2:1 and 3:1.

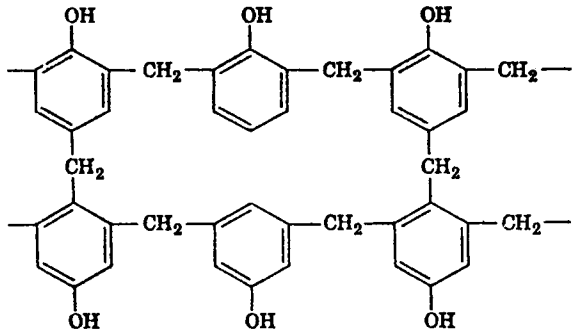
The ionic conditions in concentrated milk are so different from those in unconcentrated milk that it is often not possible to predict from the behavior of unconcentrated milk exactly how concentrated milk will behave. This is particularly true with the heat coagulation of concentrated milk.

The effect of sequestering agents and ion exchangers. It is sometimes desirable to treat milk in such a way as to alter its ionic balance in order to stabilize it or otherwise improve its utility for a particular purpose. The best known example of such a treatment is the addition of phosphate or citrate to stabilize milk against subsequent heat coagulation. This practice is common in the evaporated milk industry. Additions of phosphate or citrate result in the binding of more of the calcium in the form of soluble complexes and decreasing the activity of the calcium ions. Soluble reagents which thus tie up a particular ion are called sequestering agents and are said to sequester that ion. One such reagent which has come into prominence is ethylenediamine tetraacetate. This is a very powerful sequestering agent, particularly for divalent and polyvalent cations. It has been used in research and analytical studies of milk, but present legal restrictions prevent its actual use as a sequestering agent in the evaporated milk field.

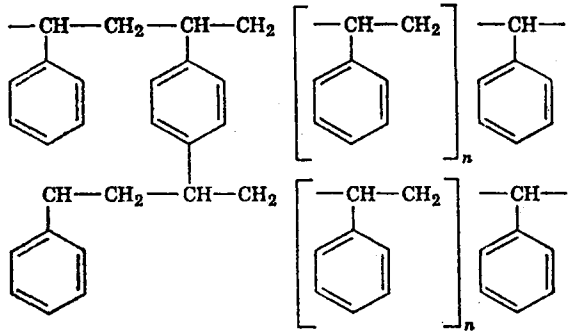
In recent years there has been a marked development of insoluble ionized materials, which can exchange one ion for another. Some of

these are naturally occurring materials such as various silicates, but even more prominent than these are various artificial resins.

These resins are synthetic polymers containing either acid or basic ionizable groups. They are usually either phenol-formaldehyde polymers:



or styrene-divinyl benzene copolymers:

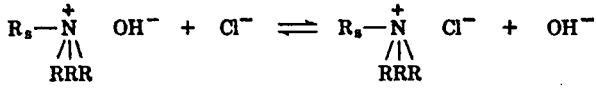


In any event, the active ionic centers are substituted on the benzene rings of the resin structures. The groups commonly employed for cation exchanges are sulfonate ($-SO_3H$) and carboxyl ($-COOH$). For anion exchanges the active groups are amino ($-NH_2$, $-NHR$, $-NR_2$) or quaternary ammonium ($-NR_3^+$) groups.

A cation exchange on a sulfonic resin would proceed as follows (using sodium and potassium as examples)

$$R_s-SO_3^- Na^+ + K^+ \rightleftharpoons R_s-SO_3^- K^+ + Na^+$$

Similarly an anion exchange on a quaternary ammonium resin would be (using chloride and hydroxide as examples)



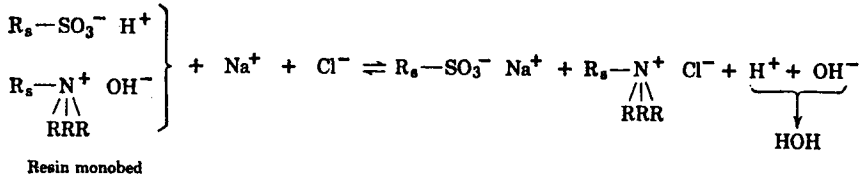
The extent of exchange in any experiment depends on the relative concentrations of the ions being exchanged and on their relative affinity for the resin. A resin can, of course, be put completely in the form of the salt of any given ion by treating it with an overwhelming concentration of that ion. A number of monographs, such as that edited by Calmon (4), give details of the principles and techniques involved in the use of ion exchangers for various purposes.

Milk can be treated with an ion exchanger in a batchwise fashion with the exchanger being filtered off following the exchange. More convenient, however, for large-scale continuous operation is the column technique in which the resin is placed in a column and the milk is allowed to flow through it.

One practical application of ion exchange techniques to dairy technology is in removing a portion of the calcium in order to stabilize the casein. This has been suggested for stabilizing evaporated milk and also for preventing "feather" formation in dry creams to be used in coffee. For removing calcium the resin can be prepared in the sodium state or in the mixed sodium-potassium state. Since exchange of calcium for the alkali metals usually results in some increase in pH, it is necessary to acidify to maintain the pH constant. This acidification can be conveniently accomplished by treating the milk with the resin in the hydrogen state. It is convenient, in fact, to make a mixture of a sodium resin and a hydrogen resin and to treat the milk with this mixture. Thus the removal of calcium can be accomplished with no change in pH. Another practical application of ion exchange to milk is in the production of low-sodium milk, which is required for certain diets, employing the resin in the potassium-calcium-magnesium form and running the milk through it. Under these conditions the sodium is exchanged for potassium, calcium, and magnesium, and the ratio of the latter three can be kept constant.

Simultaneous removal of both anions and cations from milk can be accomplished by so-called "monobed resin" treatments. A resin monobed consists of a mixture of a cation exchange resin in the hydrogen form and an anion exchange resin in the hydroxyl form. When a salt-containing solution is passed through such a mixture of resins

both anions and cations are absorbed by the resins and hydrogen and hydroxyl ions are released, which immediately combine to form water. These effects are illustrated by the following equations, using sodium chloride as an example:



Resin monobed

By such treatments milk can be deionized to any extent desirable in a single pass through a resin bed or by batchwise treatment with a mixture of the two resins.

Ion exchange and sequestration techniques may find increased use in the future preparation of various dairy products and milk derivatives. They also are useful in analytical procedures in solving research problems in the laboratory.

Table 6. EFFECT OF CONCENTRATION OF SKIMMILK ON pH AND DISTRIBUTION OF PHOSPHATE AND CITRATE

	Unconcentrated Skim	Concentrated Skim	
		I	II
Solids (per cent)	8.9	17.2	25.6
Colloidal caseinate phosphate (per cent)	2.8	5.5	8.4
pH	6.7	6.4	6.2
Citrate (as citric acid)			
Dissolved (mg./100 ml. water)	135	270	402
Colloidal (per cent of colloidal phase)	0.44	0.82	1.07
Inorganic phosphate			
Dissolved (mg./100 ml. water)	32	59	89
Colloidal (per cent of colloidal phase)	1.22	1.34	1.36

Data of Eilers and Korff (6). Samples I and II vacuum concentrated at temperatures not exceeding 50° C. Phases separated by rennet coagulation.

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CHAPTER 6 MILK ENZYMES

INTRODUCTION

Enzymes are organic catalysts elaborated by living cells that catalyze many of the reactions involved in their functions. All known enzymes are proteins; some of them contain non-protein moieties called prosthetic groups which are essential to their catalytic activity, others appear to consist entirely of proteins. Aside from their specific catalytic activity, the chemistry of enzymes is that of proteins in general. Most enzymes exhibit a high degree of specificity acting only on certain compounds (substrates) or linkages. Enzyme activity usually depends strongly on pH, exhibiting a rather sharp optimum. Since enzymes are proteins it is not surprising that they are inactivated by exposure to elevated temperatures, which denature proteins. Individual enzymes differ a great deal in their stability to heat treatment. The chemistry of the enzymes is treated exhaustively in a series of monographs by Sumner and Myrbäck (28). The characteristics of some of the milk enzymes as proteins have been summarized by Whitney (31).

Normal milk contains a number of enzymes. Apparently these are constituents and products of the mammary tissue that gain entrance

to the milk accidentally or unavoidably during the secretory process. So far as is known, the enzymes of milk do not participate in its principal function—the nutrition of the young. Some of the milk enzymes, such as lipase(s) and protease, act upon substrates present in milk, others only upon substrates foreign to milk. The milk enzymes constitute a very small portion of the total protein of milk; nevertheless their characteristic catalytic activities make it possible to establish very precise analytical techniques for estimating them.

This chapter deals only with those enzymes which are natural to milk, that is, those that are products of the mammary gland. It does not deal with enzymes of bacterial origin, which may gain entrance to the milk either before or after it is drawn from the udder. In some cases it has proved difficult to ascertain whether or not an observed enzymatic activity was due to a natural enzyme or to bacterial action. Bactericidal and bacteriostatic agents or short incubation times are used to prevent interference by bacteria when measuring enzymatic activity.

The principal enzymes natural to milk are catalase, peroxidase, xanthine oxidase, alkaline phosphatase, acid phosphatase, amylases, protease, lipases, and aldolase. A number of other enzymes occur in minor amounts and unconfirmed reports have been made of still others. The milk enzymes are of considerable importance in certain aspects of milk technology. The lipases, for example, are notorious for the production of hydrolytic rancidity in milk and dairy products. On the other hand, the heat lability of certain enzymes furnishes the basis for important tests for the extent of heat to which a sample of milk may have been subjected. Thus, the destruction of alkaline phosphatase is widely used as a test for the adequacy of pasteurization.

Naturally it is of interest to isolate and characterize the milk enzymes. Two of them, peroxidase and xanthine oxidase, have been isolated in crystalline form. A third, alkaline phosphatase, has been highly purified although it has not as yet been crystallized. Not much progress has been made in isolating the others.

CATALASE

Catalase catalyzes the decomposition of hydrogen peroxide as follows:

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2$$

Catalase activity may be determined by either a gasometric measurement of the oxygen produced or by a titrimetric determination of the hydrogen peroxide remaining after a given period of reaction. The latter can be conveniently accomplished by adding an iodide solution which is oxidized to iodine by the hydrogen peroxide. The iodine can be titrated with thiosulfate. A gasometric method for milk is given in the Laboratory Manual of the Milk Industry Foundation (2) and titrimetric methods are described by Anderson and Macwalter (4) and by Roberts (24).

Catalases occur in a number of different tissues, being particularly prominent in the liver, erythrocytes, and kidney of animals. They are also found in plants. Several crystalline catalases have been prepared, but, since the catalase content of milk is rather low compared with other biological sources, not much progress has been made in concentrating and purifying the enzyme found in milk.

It is known that the catalase content of milk varies considerably among cows and with the feed that the cow consumes. The catalase content is especially high in colostrum and in milk from mastitic udders. Furthermore, it tends to parallel the leucocyte count. It increases with the multiplication of bacteria in milk. In general, normal milk contains sufficient catalase to liberate 5 to 20 ml. of oxygen per 100 ml. of milk in 2 hours at 25° C.

Little is known about the manner in which catalase is distributed among the milk constituents. Statements are made in the literature that upon running whole milk through a separator the catalase activity tends to be concentrated in both cream and the separator slime. This would indicate that it is carried by both the fat globules and the leucocytes. Other statements indicate that catalase is precipitated with the casein under some conditions. The experimental basis for these statements is meager and more work is needed to clarify the situation.

Since milk catalase has never been isolated in a pure state, nothing whatever is known of its composition. All of the catalases that have been isolated, however, are hematoproteins containing iron in the form of a porphyrin. Presumably milk catalase is also of this type. The optimum reaction conditions for milk catalase have not been defined very precisely. Activity occurs over a wide range of pH but some inhibition occurs in strongly acid solutions. In general, catalase activity of milk is determined at its own pH of 6.5 to 6.6, no buffers being added. The determination of optimum temperature is complicated by the fact that the substrate, hydrogen peroxide, inactivates the enzyme, particularly at higher temperatures. Thus, the apparent

optimum temperature will be lower the higher the concentration of substrate used. Several procedures for determining catalase activity employ 10 to 15 ml. of milk and 5 ml. of 1% H_2O_2 or a final concentration of 0.25% H_2O_2. Under these conditions the apparent temperature optimum is at 20 to 25° C., and for comparative purposes it is sufficiently accurate to make the assay at "room temperature."

There is not much information on the inactivation of milk catalase by heat. Results of one group of workers (7), given in Fig. 1, indicate that the enzyme is rather stable to heat. Another worker (24) found 67 to 89% (average 84%) of the catalase activity destroyed by pasteurization of whole milk at 62° C. for 30 minutes. There are some reports that catalase activity is restored or regenerated upon holding milk after it has been heated at minimum conditions required for inactivation of the enzyme.

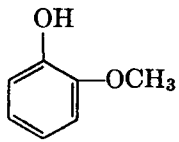
The determination of catalase has been suggested as a diagnostic tool for mastitic milk. It is not especially precise for this purpose, however, and is not recommended by most workers in the field.

PEROXIDASE

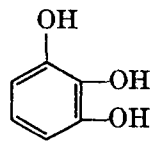
Peroxidase catalyzes the following reaction:

$$H_2O_2 + HA \xrightarrow{\text{peroxidase}} 2H_2O + A$$

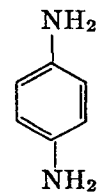
where HA is an oxidizable substance or hydrogen donor. A long list of materials serves as hydrogen donors and are oxidized in this reaction. These include (*a*) aromatic amines such as aniline and *p*-phenylenediamine; (*b*) phenols such as phenol, guaiacol, hydroquinone, and pyrogallol; (*c*) aromatic acids such as benzoic, salicylic, and gallic acids; (*d*) leuco dyes such as leucophenolphthalein; and (*e*) a number of miscellaneous materials, including tyrosine, tryptophan, ascorbic acid, iodide, and nitrite. Oxidation of a number of these substrates can be used as the basis of quantitative methods for determining peroxidase. The most widely used for milk have been colorimetric procedures depending on the oxidation of guaiacol, pyrogallol, or *p*-phenylenediamine to highly colored products.



Guaiacol



Pyrogallol



p-Phenylenediamine

A qualitative test, called Storch's test, involving the oxidation of *p*-phenylenediamine has been used extensively for determining whether or not any peroxidase remains in heated milk. A quantitative method employing this same reaction has been devised by Aurand and coworkers (5).

Peroxidase occurs in a number of different sources in nature, including the roots of the horseradish, the sap of the fig tree, and the leucocytes of blood. Its content in milk is high in relation to the other enzymes; it may represent as much as 1% of the total serum proteins of milk. As a matter of fact, milk is one of the best sources of this enzyme in nature. Peroxidases have been crystallized from horseradish, leucocytes, and milk. The peroxidase content of milk varies somewhat among cows and from day to day, but in general is much more constant than the catalase or xanthine oxidase contents.

On the fractionation of milk proteins with ammonium sulfate, the peroxidase follows the "lactalbumin" fraction. It can be further purified from this fraction by adsorption on tricalcium phosphate and step-wise elution with buffer. The final step in purification is actual crystallization (22). The enzyme crystallized from milk has a nitrogen content of 15.56%, iron content of 0.069%, molecular weight of 82,000, and an isoionic point of pH 9.6. The specific nature of the prosthetic group containing the iron is not known.

The optimum pH for activity of milk peroxidase has been reported as pH 6.8 (5). The optimum temperature for this enzyme is not mentioned in the literature but assays are generally made at room temperature. Heat inactivation of peroxidase in milk has been studied to some extent. It is a rather stable enzyme to heat as may be seen in Fig. 1. Unfortunately, results of different workers do not agree exactly. Milk peroxidase apears to be regenerated after initially disappearing when milk is treated with certain high-temperature, short-time heat treatments. Obviously this enzyme, which is so resistant to destruction by heat, is a valuable indicator for high-temperature heat treatments of milk. Another possible use for peroxidase is as a detector for hydrogen peroxide that may have been added to milk as a preservative.

XANTHINE OXIDASE

Xanthine oxidase catalyzes the following reaction;

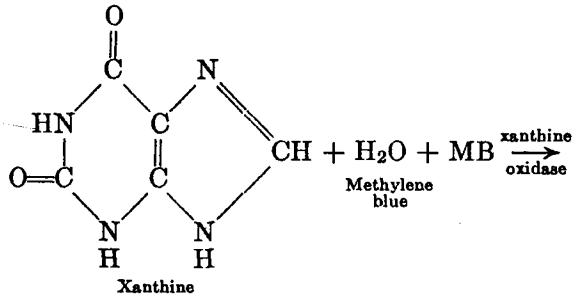
$$\text{Reduced substrate} + \text{Hydrogen acceptor} \xrightarrow[\text{oxidase}]{\text{xanthine}} \text{Oxidized substrate} + \text{Reduced form of acceptor}$$

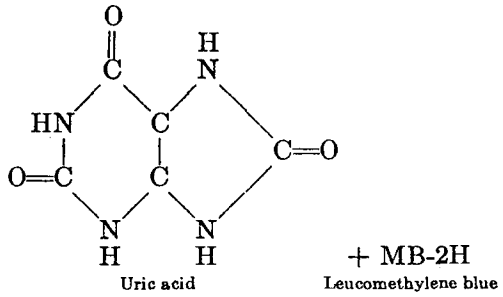
A variety of substrates are oxidized by this enzyme including aldehydes, oxypurines such as hypoxanthine and xanthine, pterins such as xanthopterin, and reduced diphosphopyridine nucleotide (DPN). Likewise, a number of materials can serve as hydrogen acceptors in the reaction. These include molecular oxygen, methylene blue, cytochrome C, ferricyanide, nitrate, and quinone.

For example, with an aldehyde as substrate and oxygen as the acceptor the reaction is as follows:

$$\underset{\text{Aldehyde}}{RCHO} + H_2O + O_2 \xrightarrow[\text{oxidase}]{\text{xanthine}} \underset{\text{Carboxylic acid}}{RCOOH} + H_2O_2$$

With xanthine as substrate and methylene blue as acceptor it is as follows:





In several classical methods for determining xanthine oxidase, methylene blue is employed as the hydrogen acceptor and the time required to reduce it to the colorless leuco-form is determined. Oxygen must be excluded from the reaction mixture because it spontaneously reoxidizes the leucomethylene blue. Thus, such tests must be performed in evacuated vessels. Another method is to perform the reaction in manometric apparatus and measure the uptake of oxygen. Recently two new methods have been proposed for determining xanthine oxidase

in milk. One, suggested by Zittle and co-workers (32), employs xanthine as substrate and a dye, triphenyl tetrazolium chloride, as hydrogen acceptor, whose red reduced form is stable to oxygen and can be extracted from the reaction mixture with toluene for measurement of color intensity. The other method, developed by Kuramoto and co-workers (15), makes use of vanillin as substrate and oxygen as hydrogen acceptor. The acid produced, vanillic acid, forms a blue compound with a reagent called 2,6-dibromoquinone chloroimide (BQC). This compound can be extracted with butanol and measured colorimetrically. The latter method is very simple in that no precautions for removing oxygen are necessary.

Xanthine oxidase is a prominent enzyme in milk. It was discovered by Schardinger in 1902 when he observed that methylene blue is decolorized by formaldehyde in the presence of fresh milk. Morgan, in 1922, demonstrated the presence of an enzyme in milk that catalyzes the oxidation of hypoxanthine and xanthine. The question then arose as to whether the enzyme observed by Schardinger and the xanthine oxidase described by Morgan are identical. After much work it was finally settled that a single enzyme is responsible for these various effects. Later it was found that reduced DPN and also pterins are oxidized by this enzyme. Xanthine oxidases are also found in liver and in bacteria, but milk is by far the best source of the enzyme for the research worker in enzyme chemistry.

The xanthine oxidase content of milk varies from cow to cow and increases gradually during the course of lactation. It is associated with the fat globules and thus the content increases during the course of the milking procedure. Geographical differences have been noted in the xanthine oxidase content of milk produced in different localities; apparently they reflect the content of available molybdenum in the herbage consumed by the cow.

Xanthine oxidase is bound to the fat globules and follows the fat upon separation of milk. Actually, a group of French workers (23) has demonstrated that the enzyme is tightly bound to the fat globules as the milk is secreted by the cow, but that it is released from them and rendered more active by treatments such as cooling, heating, agitation, and ultrasonic waves, and by various chemical agents such as detergents. Morton (17) has observed that this enzyme, along with alkaline phosphatase, occurs in small particles (microsomes), which contain protein and lipides and which are attached to the fat globules under some conditions and are released into the plasma under others (see Chapter 9). It is thus evident that the distribution of xanthine

oxidase between fat globules and plasma will vary with the treatment that the milk has received.

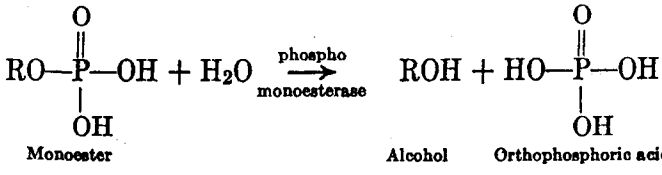
In general, attempts to concentrate and purify the enzyme have started with cream or buttermilk as the source. Fractionation with salts and adsorption on calcium phosphate or alumina columns followed by elution with phosphate buffer have been used to achieve high purification of the enzyme. Recently crystallization of the enzyme has been reported (6), but it is not entirely certain that the crystalline material consists entirely of the enzyme.

The enzyme consists of a protein, a flavin, and the metals molybdenum and iron. The molar ratio of iron to molybdenum to flavin was 8 : 1 : 2 in one highly purified preparation and 8 : 1.4 : 2 in one crystalline preparation. The protein is of high molecular weight (300,000 to 400,000).

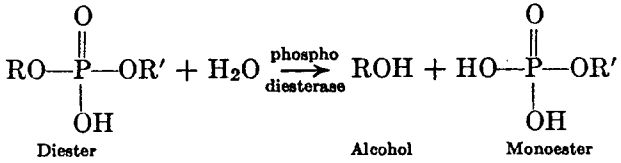
The activity of xanthine oxidase is uniform within the pH range of 6 to 9 but falls off outside of this range. It is inhibited by cyanide, arsenite, hydrogen peroxide, and *p*-chloromercuribenzoate. Not much information is available on the effect of temperature on xanthine oxidase activity in milk. The situation is complicated by the fact that heat treatments to about 65° C. seem to activate the enzyme (when activity is subsequently determined at 37° C., for example). Higher temperatures have both activating and inactivating effects. A temperature of 37° C. is commonly employed for assay purposes. The inactivation of xanthine oxidase by high temperatures is plotted in Fig. 1. It is moderately stable to heat, being more resistant than alkaline phosphatase but less so than peroxidase.

PHOSPHATASES

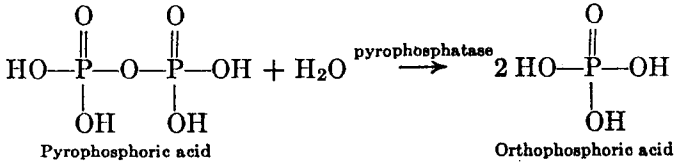
Phosphatases are enzymes that catalyze the hydrolysis of phosphate esters. There are a number of kinds of phosphatases differing in the specific substrates that they will attack and in their optimum pH and temperature requirements. Three important classes of phosphatases are monoesterases, diesterases and pyrophosphatases. Monoesterases catalyze the hydrolysis of monoesters as follows:



Diesterases catalyze the hydrolysis of diesters:



Pyrophosphatases catalyze the hydrolysis of pyrophosphates:



There is evidence that representatives of all three of these classes of phosphatases occur in milk, but by far the most work has been done on one of them, an alkaline monoesterase having a high optimum pH. The next most work has been done on an acid monoesterase having a low optimum pH.

A number of techniques are used to determine the phosphatase activity of milk or dairy products. The alkaline and acid phosphatases can be determined by methods differing only in the buffer employed to control the pH during incubation. Some early methods for alkaline phosphatase employed glycerol phosphate as substrate and the orthophosphate liberated was determined colorimetrically. Later disodium phenylphosphate was introduced as the substrate and the hydrolysis followed by estimation of the phenol liberated. This substrate has continued to be the most widely used. Results are often expressed in terms of micrograms of phenol liberated (1). In recent years two other substrates have been suggested, namely, phenolphthaleinphosphate and *p*-nitrophenylphosphate. Both of these are colorless but both yield colored products upon hydrolysis. They are thus very suitable for colorimetric analysis.

The alkaline phosphatase content of milk is rather variable; in one study (10) a variation of nearly 40-fold was found among individual milkings and a nearly 2-fold variation among bulked milks taken at different seasons of the year. Phosphatase activity per unit of milk seems to be inversely correlated to milk yield, reaching a minimum in 1 or 2 weeks after parturition and rising gradually to a maximum in about 25 weeks. It does not appear to be related to breed or feed of the cow or to fat content of the milk. The alkaline phosphatase of

milk is associated with the fat globules. According to Morton (17) it occurs in small particles called microsomes, which also contain xanthine oxidase (see Chapter 9). These particles are adsorbed on the surface of the fat globules. The microsomes also contain lipides which are very difficult to remove from the enzymes and proteins. Morton (18), however, found that butanol very effectively breaks the forces between the protein and lipide, setting the protein free. The highest purification of the enzyme has been attained by this butanol treatment followed by adsorption on specific adsorbants. It has been highly purified, compared to the original milk, but has not as yet been prepared in crystalline form.

Limited information (19) on the acid phosphatase indicates that it does not associate itself with the fat globules but rather is found in the skimmilk. It has not been concentrated to any significant extent. A comparison of the properties of the two principal milk phosphatases is given in Table 1 and their susceptibility to heat is given in Fig. 1.

Table 1. Comparison of Properties of Alkaline and Acid Phosphatases of Milk

Property	Acid Phosphatase	Alkaline Phosphatase
pH optimum	4.0	9.65
Inactivating heat treatment	88° C.–30 min.	62° C.–30 min.
Effect of light	Strongly inhibiting	?
Effect of F^-	Strongly inhibiting	Nil
Effect of Mg^{++}	Nil	Activating
Effect of Mn^{++}	Strongly activating	Activating
Effect of I_2	Not known	Inhibiting
Effect of Cysteine	Not known	Inhibiting
Effect of Be^{++}	Not known	Inhibiting
Effect of Zn^{++}	Not known	Inhibiting

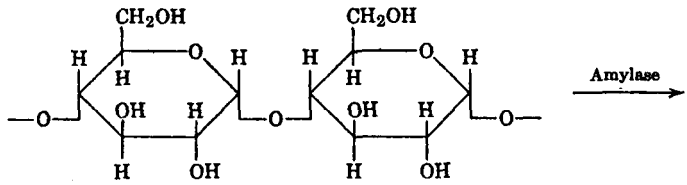
Obviously they are quite different. The acid phosphatase is the most heat stable milk enzyme yet described.

The alkaline phosphatase has come to be used as the method of preference for determining whether milk has been pasteurized adequately. It is inactivated by treatments that are sufficient to destroy such resistant pathogens as *M. tuberculosis*, but that are insufficient to appreciably damage the creaming ability of milk. A great deal of study has been devoted to the definition of the precise conditions of heat treatment which inactivate the alkaline phosphatase and to devising

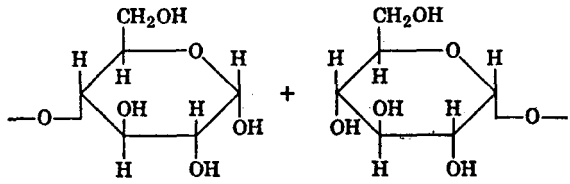
more precise and convenient methods for assessing the amount of phosphatases remaining after pasteurization (14).

AMYLASES

Amylases are enzymes which hydrolyze the α-1-4-D-glucosidic linkages in starch and glycogen as follows:



Section of amylose chain in starch



New terminal groups of chains

Starch and glycogen are high molecular weight polymers of D-glucose in which the glucose molecules are linked wholly or partly as illustrated above.

Two principal kinds of amylases called α- and β- are recognized. α-Amylases preferentially attack central α-1,4-D-glucosidic linkages in the starch molecule. This causes a rapid decrease in viscosity (liquefaction) of starch pastes with a slow production of free sugar (saccharification). α-Amylases are found in plants and microorganisms and in the pancreas, blood, urine, and saliva of animals. β-Amylases degrade starch by splitting off maltose (a disaccharide of glucose) from the ends of the polymer chains. Thus, in contrast to α-amylases, they cause a rapid accumulation of free sugar (saccharification) with a slow decrease in viscosity (liquefaction). β-Amylases have been positively identified only in plants. Amylase activity can be determined by incubating the enzyme source with a starch paste and measuring the decrease in viscosity, increase in free sugar, and loss of iodine staining power. The liquefaction or decrease in viscosity is

especially characteristic of α-amylase. Both α- and β-amylases produce free sugar and both lead to loss of iodine staining power.

α-Amylase has been reported in normal cow's milk by a number of workers (26). It has been studied rather intensively in the senior author's laboratory (9). Not much is known about variations in α-amylase content of milk except that it varies from cow to cow and is high in milks from mastitic udders. Upon separation of milk, α-amylase is found in the skimmilk, and upon fractionation of the proteins it is concentrated into the lactoglobulin fraction of the serum proteins. It can be further concentrated by dispersing the lactoglobulin in a solution containing 15% ethylene glycol and 29% ethanol and adding rice starch. The enzyme is preferentially adsorbed on the rice starch under these conditions. It can be recovered by eluting from the rice starch with water. In this way considerable purification can be achieved. Nevertheless, since the α-amylase content of milk is very low not much progress has been made in completely purifying the enzyme.

Milk α-amylase has an optimum pH at 7.4 and an optimum temperature at 44° C. It is rather unstable to heat treatment, being inactivated by heating at 45° to 52° C. for 30 minutes. Thus it is of no value as an index of pasteurization efficiency. Temperatures of 30 to 35° C. are suitable for assays of this enzyme. Milk α-amylase requires both calcium and chloride ions for activity, exhibiting no activity in the absence of these ions. On the other hand, it is inhibited in the presence of iodine.

There is some evidence for the presence of a β-amylase in milk, but the data on this point are not sufficiently clear-cut at present to justify including it as a normal milk constituent.

ESTERASES AND LIPASES

Esterases catalyze the hydrolysis of esters:

$$\underset{\text{Ester}}{R{-}\overset{\overset{O}{\|}}{C}{-}OR'} + H_2O \xrightarrow{\text{esterase}} \underset{\text{Acid}}{R{-}\overset{\overset{O}{\|}}{C}{-}OH} + \underset{\text{Alcohol}}{R'OH}$$

Many esterases have the ability to hydrolyze a great variety of esters composed of various aliphatic and aromatic acids and alcohols. However, some differences in specificity exist. Certain esterases exhibit rather marked preference for particular esters. Those that prefer-

entially hydrolyze the triglycerides are often called lipases. Esterases and lipases are widely distributed in nature, being found in microorganisms, the seeds of higher plants, and in many animal tissues, particularly in liver and pancreas.

Milk exhibits considerable esterase and lipase activity. It contains enzymes which catalyze the hydrolysis of many different esters and fats. Obviously the principal interest in these enzymes centers about their attack on their naturally occurring substrate in milk—the fat globules. Since milk fat contains a relatively high proportion of the short chain volatile fatty acids, lipolytic activity produces flavors that are undesirable in fluid milk or cream but are very desirable in certain types of cheese. (See Chapter 12.) Milk lipases and their action have been reviewed by Herrington (11).

The determination of water insoluble fatty acids (WIA) produced by hydrolysis of fat, both by natural enzymes and by bacteria, is useful for assessing the past history of butter. Since water soluble acids are largely lost in the buttermilk upon churning, a high content of WIA and a low content of free butyric acid in butter imply that extensive decomposition occurred in the cream before churning. A standard method for determining WIA is available (2).

Although milk apparently always contains lipase enzyme(s) it does not usually undergo lipolysis spontaneously. Certain activation treatments are necessary before the enzyme will attack the fat globules. Some of these treatments are homogenization, shaking, and various temperature manipulations, such as cooling to 5° C., then warming to 30° C., and finally cooling again to 5° C. These treatments apparently do not produce actual activation of the enzyme(s) but rather facilitate its attack on the fat globules, either by promoting adsorption of the enzyme on the globules or by altering the orientation of the adsorbed fat globule membrane. The hydrolysis of simple esters added to milk requires no such activation procedures.

It is common knowledge that the milk of cows late in their lactation period often develops hydrolytic rancidity spontaneously with no activating treatment. It is not known definitely whether this spontaneous lipolysis is the result of the presence of a different lipase enzyme or of a difference in the state of the fat globules and their surfaces in such milk, although recent work by Tarassuk and Frankel (29) indicates that the former is the case.

A number of methods are available for determining esterase and lipase activity. The classical one is titration of the liberated acids. Another involves the use of an ester substrate, such as *p*-nitrophenyl-

butyrate, which upon hydrolysis yields a colored compound that may be determined colorimetrically. Still another method depends on the reduction of surface tension of the medium by the liberated acids and/or mono- and diglycerides. Quantitative determinations of the hydrolysis of milk fat by milk lipase are beset by some serious difficulties. Direct titration of the fatty acids liberated is not accurate, because the contribution of the fatty acids to the titer is overshadowed by that of the buffer salts of milk and because any lactic acid produced by bacteria would complicate the results. Many efforts have been directed to separating the fat from milk by extraction or by churning and titrating it. Difficulties are encountered in securing quantitative recovery of the liberated fatty acids since certain of them (and particularly those that contribute most to rancid flavor) are water soluble and go partially into the aqueous phase when the fat is removed. It is often tacitly assumed that the intensity of rancid flavor parallels the free fatty acid titer. This will be the case only if all ester linkages in the fat are attacked with equal ease and if all of the acids liberated are titrated. It is not known whether or not the various linkages are hydrolyzed at the same rate. It would be interesting to have information on this point. On the other hand, much effort has been directed toward improving the quantitative recovery of the fatty acids liberated so that they may be included in the titration.

Not much progress has been made in characterizing the lipases and esterases in milk. Attempts have been made to resolve the question of whether one enzyme or several different enzymes are present by study of the effects of pH, temperature, various substrates, and inactivators on the hydrolysis. There is general agreement from a large number of experiments that a principal pH optimum for lipase and esterase activity occurs at a pH of 8.5–9.0 (differing somewhat with substrate). Two other lesser activity "peaks" have been observed at pH 5.4 and 6.3 (for the hydrolysis of tributyrin). There is also evidence of activity peaks at 6.5–7 and at pH 7.9. Since individual enzymes usually exhibit a rather sharp pH optimum, these data suggest a multiplicity of enzymes. The remainder of the discussion deals with the enzyme(s) having an optimum pH 8.5 to 9.0. The optimum temperature for these enzymes lies at about 37° C. This temperature is usually employed for incubation in quantitative work.

The enzymes in milk are capable of hydrolyzing a wide variety of esters, but the rates of hydrolysis are not parallel. For example, in a study by Frankel and Tarassuk (8), ten lots of mixed milk were examined for activity on four different substrates, milk fat, tributyrin,

methyl butyrate, and Tween 20. The differences among these milks in rate of hydrolysis of milk fat were not proportional to the differences in rate of hydrolysis of the other substrates. The hydrolysis of these various substrates exhibited different pH optima (milk fat 8.8–9.1, tributyrin 8.7–8.9, methyl butyrate 8.0, and Tween 20 8.8). Such findings again suggest the presence of several enzymes. Furthermore, it is evident that the activity upon a foreign substrate such as tributyrin cannot be taken as an index of the ability of a milk to develop hydrolytic rancidity.

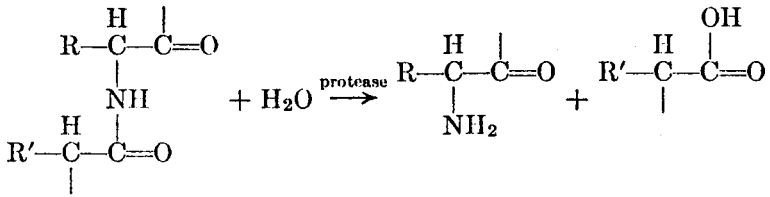
Milk lipases and esterases are inactivated by a variety of reagents and treatments. Some of these are: aging, heat treatment, exposure to sunlight, hydrogen peroxide, copper, formaldehyde, and trypsin. Any given one of these treatments reduces the activity toward different substrates to a different degree, again pointing to a multiple enzyme system. The enzyme(s) responsible for hydrolyzing milk fat is more resistant to these treatments than are those that hydrolyze simple esters. The lipase activity of milk decreases rather rapidly on holding after milking. Half of the activity (on milk fat) may be lost in 3 hours at 37° C. or 48 hours at 0 to 5° C. The enzymes are protected by the presence of fat globules because the rate of loss of activity is greater in skimmilk than in whole milk. It appears to involve oxidation because it can be prevented by deaerating and storing under nitrogen. Further evidence that lipase is susceptible to oxidative inactivation is that hydrogen peroxide and copper are strong inactivators. Milk lipases are rather labile to heat treatment. Figure 1 shows that the lipase (as measured by ability to depress surface tension on incubation of milk for 44 to 48 hours at 40° F.) is inactivated by heat treatments less drastic than pasteurization. Exposure to 50° C. for 15 minutes may reduce the lipase activity by one-half. Formaldehyde is a powerful inhibitor of milk lipases, probably acting in part by competing with the substrate for reaction with the enzyme. There have been statements made that various lipase enzymes in milk can be differentiated inasmuch as some are susceptible to formaldehyde whereas others are stable to it. At present, however, it seems that resistance to formaldehyde does not provide a clear-cut basis for distinguishing among lipases. Milk lipases are very readily inactivated by exposure to sunlight.

The lipases of milk are found largely in the skimmilk. Thus extensive lipolysis will occur if homogenized pasteurized cream is mixed with raw skimmilk. There is evidence that the enzyme(s) must be adsorbed on the fat globules in order to produce hydrolysis. Tarassuk and Frankel (29) have obtained evidence that milk, as freshly drawn, con-

tains two distinct lipases in the plasma. One of these, the lipase of normal milk or plasma lipase, is associated with the caseinate and the other, the naturally active or membrane lipase, is preferentially and irreversibly adsorbed on the fat-globule membrane when milk is cooled. The ratios of these two enzymes differ among milks. Certain milks containing high concentrations of the membrane lipase develop rancidity merely by cooling. On the other hand, certain activation treatments are necessary to effect transfer of the plasma lipase from the caseinate to the fat globules and thus to induce lipolysis.

PROTEASES

Proteases attack the peptide linkages which form the backbone of proteins.



They thus degrade the structure of proteins. A number of different kinds of proteases are known. They differ widely in their pH optima and in their specificity for individual substrates. Some of the best known proteases are the pepsin of the stomach (pH optimum 1.5–2.5), trypsin of the intestine (pH optimum 7–8), and papain from the latex of the papaya tree (pH optimum 5–5.5).

Several methods are available for measuring proteolytic activity (see Chapter 4). The carboxyl or amino groups may be determined as they are liberated, degradation products may be determined by nitrogen analyses of trichloroacetic acid filtrates, or the liberation of certain amino acids such as tyrosine and tryptophan may be measured by colorimetric analysis. The decrease in viscosity, and in some cases loss of turbidity, of a protein sol may also be used to assess proteolysis.

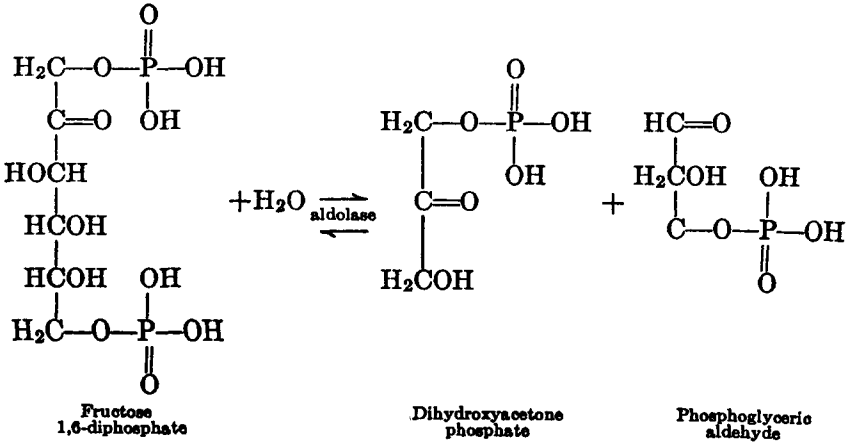
Milk contains at least one protease. In some older literature and texts the protease of milk was referred to as "galactase," which is a very confusing misnomer. Demonstration of the activity of this enzyme uncomplicated by bacterial action is not simple, particularly since incubation periods of several days are necessary with some methods of analysis. Nevertheless, the presence of a non-bacterial protease

appears to be well established. Significant proteolysis occurs on incubation of cows' milk at 37° C., even when the bacterial counts are negligible or bacteria are inhibited by antiseptics or antibiotics. Proteolytic activity is concentrated in the casein when the latter is precipitated from skimmilk. Warner and Polis (30) studied this enzyme somewhat after initially observing decreases in the viscosity of casein pastes on standing at high pH. The enzyme exhibits optimum activity at pH 8.5. Warner and Polis were not able to separate it from casein, but did concentrate it about 150-fold from the hydrolysis products after it had degraded casein extensively. Obviously in experiments on the properties of casein the presence of this enzyme must be reckoned with.

Storrs and Hull (27) demonstrated a small liberation of tyrosine (and tryptophan) in cows' milk incubated at 37° C. for times up to 6 hours, even when bacteria counts were very low or bacteria were inhibited by antibiotics. Human milk, incubated under the same conditions, exhibited much higher activity, but this does not necessarily mean that human milk contains more protease. The pH of human milk (about 7.0) is higher than that of cows' milk, and the higher activity may merely result from incubation at a pH nearer the optimum for the enzyme. Valid comparisons of activity could only be made at the optimum pH.

ALDOLASE

Aldolase catalyzes the hydrolysis of fructose 1,6-diphosphate into dihydroxyacetone phosphate and phosphoglyceric aldehyde.



It occurs in most cells and is especially prominent in muscle and in yeast. It is an important enzyme in the sequence of reactions involved in the metabolism of carbohydrates. Aldolase activity can be measured by adding 2,4-dinitrophenylhydrazine, which forms colored derivatives of the triose phosphates produced in the reaction.

Milk exhibits significant aldolase activity of about the same order of magnitude as blood serum. Most of our information on milk aldolase is contained in one paper by Polis and Shmukler (21). The aldolase in 100 ml. of whole milk will split about 12 micromoles of 1,6-fructose diphosphate at 37° C. in 1 hour. The enzyme is associated with the fat globules and upon separation is concentrated in the cream layer. It has been concentrated about 50-fold by fractionating the proteins with ammonium sulfate; it precipitates between 2.4 and 2.8*M* ammonium sulfate. Aldolase is rather unstable in milk, the activity decreasing rapidly at 37° and 45° C. It is more stable in the purified and concentrated state, however.

CARBONIC ANHYDRASE

Carbonic anhydrase catalyzes the hydration of carbon dioxide and the dehydration of carbonic acid:

$$CO_2 + H_2O \underset{\text{anhydrase}}{\overset{\text{carbonic}}{\rightleftharpoons}} H_2CO_3$$

This reaction proceeds at an appreciable rate in the absence of catalysts, but is accelerated by a number of catalysts of which carbonic anhydrase is one. This enzyme is a zinc-containing protein. It is found in various plant and animal tissues and is particularly prominent in red blood cells. Carbonic anhydrase can be determined either by measurement of CO_2 evolved from a carbonate solution or determination of the time required for the pH to drop sufficiently to change the color of an indicator when CO_2 is added to a carbonate solution. Employing a method of the latter type, Alfonso and Bertrán (3) demonstrated the presence of rather weak carbonic anhydrase activity in the milk of cows, goats, and sheep. Nothing further is known about this enzyme in milk.

MISCELLANEOUS ENZYMES

Alfonso and Bertrán (3) reported the presence of weak rhodanese activity in 10 out of 20 samples of cows' milk examined. Goat and

sheep milks exhibited somewhat greater activities. Rhodanese catalyzes the conversion (and hence the detoxification) of cyanide into thiocyanate:

$$\underset{\text{Cyanide}}{CN^-} + \underset{\substack{\text{Thio-}\\\text{sulfate}}}{S_2O_3^=} \xrightarrow{\text{rhodanese}} \underset{\substack{\text{Thiocyanate}\\\text{(rhodanide)}}}{CNS^-} + \underset{\text{Sulfite}}{SO_3^=}$$

Some texts mention the presence in milk of an enzyme called salolase, which hydrolyzes phenyl salicylate (salol) to phenol and salicylic acid. Evidence for the existence of a specific esterase of this kind is not clear and in any event it seems to be of little importance.

A number of texts and reviews state that lactase, an enzyme hydrolyzing lactose to glucose and galactose, is present in milk. However, the original work cited does not fully substantiate the statement, and it may be concluded that the presence of lactase in milk has not been demonstrated.

SUMMARY OF HEAT INACTIVATION OF MILK ENZYMES

Undoubtedly the most important property of milk enzymes, in relation to dairy processing, is their lability to heat. Heat treatment is essential to inactivate lipase and the activities of various other enzymes can be employed as indices of the extent of heat treatment that a sample of unknown history has received. In the foregoing discussions of the individual enzymes, reference has been made to their inactivation by heat. Information for several enzymes is summarized in Fig. 1. The times and temperatures required for a specified degree of inactivation are plotted against each other. The plots are similar to those presented in Chapter 11, showing the denaturation of proteins. A straight line relation exists between the temperature and the logarithm of time. Obviously the individual enzymes differ very markedly in stability. Lipase is the most easily inactivated of those that have been studied systematically, being completely inactivated by conditions somewhat below those required for pasteurization. Aldolase and α-amylase are apparently even more labile. Alkaline phosphatase is inactivated by "pasteurizing conditions," and the other enzymes, ranging up to the very heat-resistant acid phosphatase, require more drastic treatments. The data in Fig. 1 extend only up to about 100° C. and down to a few seconds. In recent years there has been considerable interest in high-temperature-short-time heat treatments ranging up

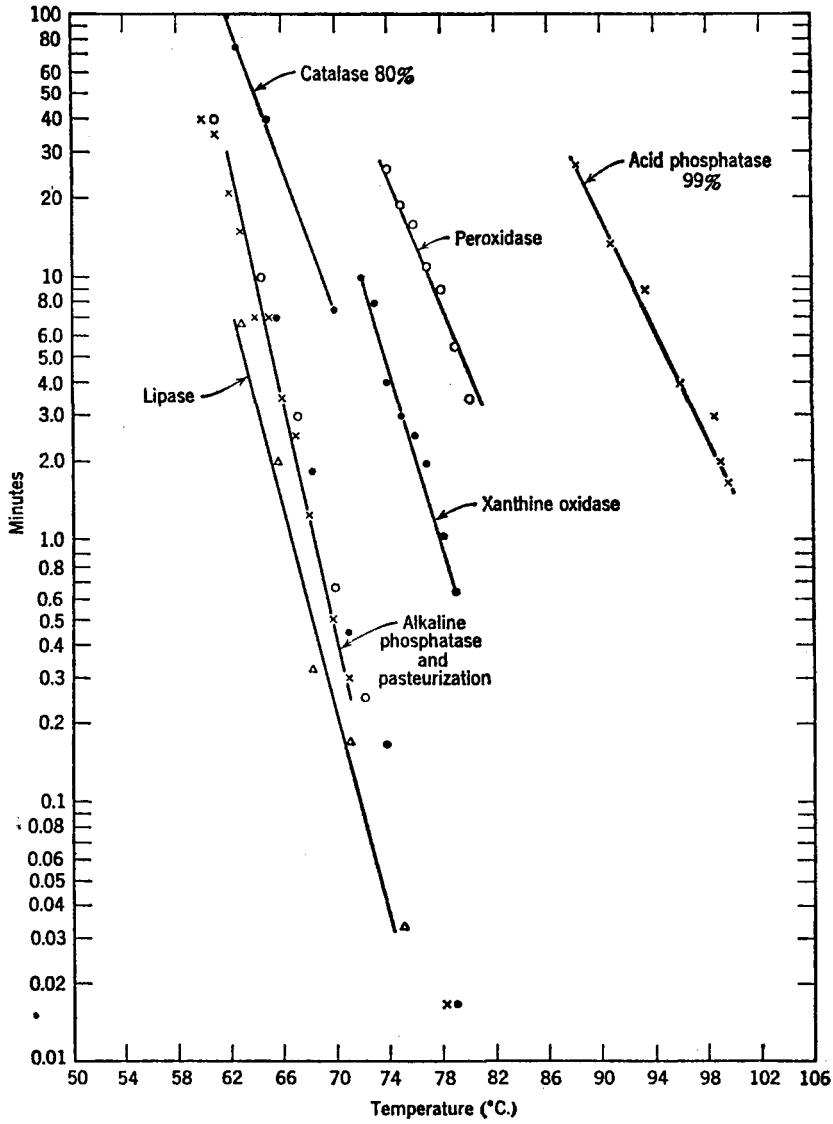


Fig. 1. Time-temperature relationships for inactivation of milk enzymes. References: lipase–Hetrick and Tracy (13); alkaline phosphatase–Hetrick and Tracy (12), Sanders and Sager (25), and Lear and Foster (16); acid phosphatase–Mullen (19); catalase–Burstein and Frum (7); peroxidase–Pien (20); xanthine oxidase–Pien (20).

above 150° C. with times of 1 second or less. Such treatments are particularly effective for destroying organisms with a minimum production of off-color and flavor. A peculiar "reactivation" of milk enzymes sometimes occurs, particularly after they have been inactivated by high-temperature-short-time heat treatments. Alkaline phosphatase, for example, may be completely inactivated when milk is tested immediately after heating. If allowed to stand at 30° to 40° C., however, the activity gradually returns. The activity thus obtained is only a small fraction of that of the original raw product, but it is sufficient to indicate that the milk has not been pasteurized. Reactivation poses a very serious problem in the use of the phosphatase test for the control of pasteurization by high-temperature-short-time heat treatments. The phenomenon of reactivation apparently is not due to the production of new enzyme by bacteria growing in the milk after heating. The mechanism of the effect is not known but may involve a transformation of the enzyme itself to an intermediate state from which it can revert to the active state, or a reversible reaction of some other constituents (such as a metal ion) required for activity. Reactivation has been observed with catalase and peroxidase, as well as with alkaline phosphatase.

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CHAPTER 7

MISCELLANEOUS SUBSTANCES

An introductory consideration of milk is usually concerned with its gross composition. In gross, milk contains water, milk fat, casein, whey proteins, lactose, and ash. Such a characterization of milk is inadequate in a number of respects. It oversimplifies a very complex medium. For example, probabilities suggest that milk fat alone must contain thousands of specific triglycerides. In addition, milk contains many substances in very low concentrations which frequently are very important. These are sometimes referred to as minor constituents, but it should be understood that the term *minor* refers to low concentration rather than lack of importance in dairy problems. One example which will suffice to illustrate the point is citric acid. This constituent (0.2% by weight in milk) is directly involved in the development of flavor and aroma of cultured dairy products. In the form of its salts it has a very significant bearing on the stability of milk proteins to heat treatment and freezing. The present chapter has been included because of this importance of the minor substances and since a knowledge of them contributes to a more complete understanding of milk.

For the most part, little is known about the minor constituents of milk. The means by which they are formed, the levels in which they occur, the factors that influence their content in milk, and their biological function, if any, in milk are not well established. In general, the main pathway of these substances appears to be from the blood, into the mammary gland and into the milk in unaltered form. This

is in direct contrast to such constituents as milk fat, the caseins, β-lactoglobulin, α-lactalbumin, and lactose, which are all products of gland synthesis. Another way in which minor substances may be produced in milk is by decomposition. Milk may be properly viewed as a dynamic biological fluid. Many of its constituents are quite unstable. In view of the fact that milk offers such an excellent medium for growth of microorganisms, it can be expected that the metabolic products resulting from the activity of such organisms may constitute a portion of the minor substances.

Milk also contains a number of enzymes. The bulk of evidence indicates that for various reasons these enzymes are relatively inactive in milk. This does not mean that they are completely inactive and that they do not bring about any change in the milk. Thus, it becomes clear that the minor substances in milk may vary considerably, that age and handling of the milk are important factors in this matter, and that establishing a minor substance as a true and consistent constituent of milk involves certain difficulties. Since a substance in cow's blood may find its way into the milk, it is evident that the diet of the cow, the metabolic activities in her body, and the general state of her health may profoundly influence the minor substances in milk.

A number of factors have conspired to make the information on the minor substances of milk much less adequate than that on the major constituents. Trace compounds in many instances have not been revealed or properly appreciated until fairly recent years. From a quantitative standpoint they have proved difficult to detect and measure. Furthermore, man naturally has been concerned with those constituents of milk that are quantitatively significant since they contribute the bulk of the food value to milk and its products. As a consequence of these facts, the investigations that have been conducted on the minor substances in milk are limited in number. Therefore, much of the data, particularly that regarding quantities present in milk, must be considered tentative and subject to confirmation or revision. It seems probable that application of the highly sensitive methods that are currently available will reveal many additional trace compounds and will afford means of carefully studying factors affecting their levels in milk.

CARBOHYDRATES

It has been known for some time that in addition to lactose, milk contains trace quantities of glucose. With the recent advent of paper chromatography it has been possible to show that milk contains galac-

tose and several other sugars as well (2, 12, 24). The glucose content of milk averages about 7.5 mg. per 100 ml. Galactose is present in lesser concentration and the limited data available indicate a level of approximately 2 mg. per 100 ml. of milk. The origin and significance of the two hexoses is not known. The possibility that they may arise through natural lactase activity in the milk or the metabolic activity of bacteria is a consideration. Natural lactase activity seems unlikely since milk does not show a loss of lactose and an increase in glucose and galactose on standing in a properly preserved condition. It seems more plausible that these sugars are merely residual in the metabolic and secretory activity of the mammary gland.

In addition to glucose and galactose, there are substantial indications of sugars which move more rapidly and less rapidly than lactose on paper chromatograms. The identification of these sugars has not been accomplished, but it is evident that some of them are polysaccharides and some may be sugar phosphates. In this connection, it is of interest that milk contains a so-called *Lactobacillus bifidus* factor (9). This factor favors the establishment and growth of *L. bifidus* in the alimentary tract of infants. The indications are that cow's milk contains about one-fiftieth the potency for this factor found in human milk. The factor is carbohydrate in nature and N-acetylglucosamine is an integral part of its structure. There are a number of naturally occurring combinations of hexoses and N-acetylglucosamine which show *L. bifidus* factor activity.

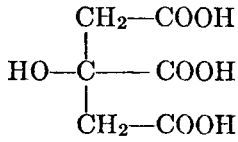
When milk is exhaustively dialyzed in the presence of preservatives, a point is reached at which the Molisch test for carbohydrates becomes no weaker. The color reaction becomes faint but it never can be rendered negative. Such observations, as well as certain reports in the literature, indicate that milk may contain small quantities of glycoproteins (22). The glycoproteins are to be distinguished from the lactose-protein products that result when milk is heated or dried under certain conditions.

ACIDS

From the standpoint of acid-base relationships, milk exhibits a rather complex system. Normally its pH is slightly on the acid side (6.6), and it will bind base to a titratable acidity of about 0.16%, calculated as lactic acid, when phenolphthalein is used as an indicator. The buffer nature of the milk system is shown by its reaction to litmus, which is amphoteric, that is, it will change both shades of litmus. It is clear that such a system must contain acids, primarily in the form

of salts with cations. It is of value to consider the acidic substances in milk, not only because they are part of the buffer system but because they are important in many instances in their own right. Excluding from consideration for the moment the fact that the acidic substances in milk are largely bound by the various cations, amino groups, and other nitrogenous base groupings, the types of compounds contributing to the acidity of milk may be classed as follows: carboxylic acids, free carboxyl groups of the proteins, enolic substances, carbon dioxide, free fatty acids, phosphoric and sulfuric esters, and inorganic phosphates.

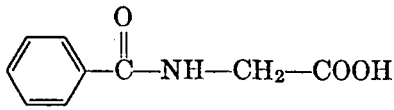
Carboxylic acids. Citric acid occurs in milk to the extent of 0.2% on an average. Excluding the fatty acids, it is the carboxylic acid that occurs in milk in the greatest quantity. Citric acid is synthesized by the mammary gland from pyruvic acid. The formula for citric acid is as follows:



In addition to its important role in the buffer substrate of milk together with the phosphates, citric acid is of primary significance in milk and milk products because: (*a*) it forms complexes with calcium and magnesium, which contribute to the stability of milk proteins toward flocculation by heating and freezing; and (*b*) it is a precursor of flavor and aroma compounds in cultured dairy products.

Although there are a number of suitable methods for determining citric acid in milk and its products, the classical method is based on its oxidation by potassium permanganate to acetonedicarboxylic acid. This acid is then treated with bromine to form pentabromoacetone, which is measured gravimetrically and from which weight the amount of citric acid can be calculated (1).

Another acid of importance in milk is hippuric acid or benzoylglycine.

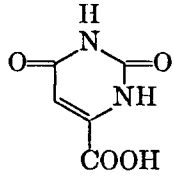


The animal body disposes of benzoic acid, which is toxic, by uniting it with glycine to form hippuric acid. This product is readily eliminated in the urine, and in herbivorous animals a small but significant amount

of the acid finds its way into the milk. It is reasonable to expect that the hippuric acid content of milk would vary with the nature of the feed. Feed containing high levels of substances metabolizable to benzoic acid would normally by expected to yield high levels of hippuric acid in the milk and urine. One study of samples secured between the period of July to January revealed a variation of from 3.1 to 6.4 mg. of the acid per 100 ml. of skimmilk with an average of 5.1 mg. per 100 ml. In the analysis of milk for nitrogen distribution, hippuric acid would contribute to the non-protein nitrogen content.

One method (18) of measuring the hippuric acid content of milk is based on continuous ether extraction of tryptic-digested, acidified skimmilk and measurement of extracted nitrogen by the Kjeldahl procedure. There is currently available a very satisfactory paper chromatographic method for hippuric acid (7). A solvent system composed of butanol–acetic acid–water (4:1:1) is used to move the sample. The paper is sprayed with a reagent composed of dimethylaminobenzaldehyde and acetic anhydride. The paper is then heated under specific conditions and areas containing hippuric acid develop a bright orange color, owing to the formation of an azlactone. The orange area is eluted from the paper with methanol and its optical density determined at 460 mμ. Through use of a calibration curve the amount of hippuric acid equivalent to any given color density can be determined.

Another little known acid in milk is orotic acid.



This acid occurs in milk to the extent of about 8 mg. per 100 ml. The concentration varies with the species, with individual animals, and with the stage of lactation. A study of the orotic acid content in the milk of cows, goats, ewes, mares, sows, rats, and women has revealed that the concentration is very low in sows, rats and women. Shortly after parturition, the concentration in cow's milk reaches four to five times the level normally found and then gradually decreases during the lactation period. The origin and significance of orotic acid in cow's milk is not known. The principal biological role of the acid appears to be as an intermediate in the synthesis of pentose nucleic acid pyrimidines. Orotic acid is an essential growth factor for *L. bulgaricus* 09. Although a paper chromatographic method (14) for meas-

uring orotic acid has been reported, microbiological assay has proved to be a satisfactory method of measuring the substance to date (10).

The isolation of orotic acid from fresh skimmilk is briefly summarized as follows: the casein is acid precipitated from fresh skimmilk and removed by filtration. The resulting whey is treated with live steam to a temperature of 93° C. and allowed to settle overnight, toluene being used as a preservative. The supernatant from the heat coagulated whey is adjusted to pH 3 and the orotic acid removed from it by adsorption on Norit A. The acid is fractionally eluted from the adsorbent with NaOH, concentrated, and precipitated as the sodium salt.

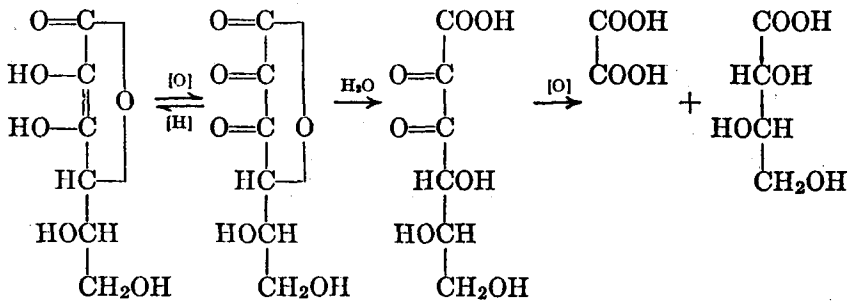
In addition to the acids mentioned, there also may be trace quantities of lactic and pyruvic acids in normal milk. Evidence concerning the presence of these acids is not well documented. They have not been recovered quantitatively from normal milk and conclusively identified. However, qualitative tests on the acid fraction recovered from milk indicate their presence in trace amounts. These trace levels well may be residual from certain synthetic processes of the mammary gland. It is also possible that such acids may originate from bacterial or enzymatic activity following secretion of the milk.

Recent studies have shown that milk may contain varying amounts of free fatty acids (23). The exact amounts and identity of such acids that occur in milk are not known. Their specific determination as a group depends upon extraction of the milk with non-polar solvents and determining the base binding power of such extracts. The values obtained vary rather widely and a noteworthy factor is that in a complete milking, the first milk drawn contains the highest level of free fatty acids, the mid-milk is intermediate, and the last drawn milk is lowest in these acids. The presence of lipase in milk is obviously a consideration in connection with its free fatty acids content. We might assume that such acids are residual in the synthesis of milk fat. However, available evidence indicates that in this process the fatty acids do not exist in free form, but are built up and synthesized into glycerides through an acetyl-coenzyme A mechanism.

Enolic substances. Another group of acidic substances in milk are the phenolic and enolic compounds. These compounds are characterized by the grouping $=\overset{|}{C}—OH$. Although such compounds have definite base binding capacity and exhibit acidity towards litmus, they are not as strong acids as the carboxylic type. Generally they are distinguished from true acids by virtue of their volatility and extractability from dilute sodium carbonate solution. They also are dis-

tinctive in that they give various colors when reacted with dilute ferric chloride reagent. The enolic structure is known to occur in the amino acid tyrosine, ascorbic acid, and uric acid. The exact state of the phenolic hydroxyl group of tyrosine, which amino acid is conjugated in the various proteins of milk, has not been definitely established. Until recently it has been assumed that this hydroxyl group is free; however, recent evidence suggests that it is combined by hydrogen bonding to various other groups in the protein. Certain enzymes, such as rennin and trypsin, are capable of breaking this linkage. In any event, this group must be considered as actually or potentially capable of binding base in milk and milk products.

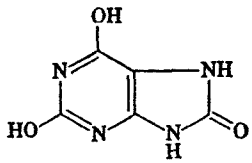
Ascorbic acid is a typical example of an ene-diol or reductone type of acid, —C(OH)=C(OH)—; it is a relatively strong acid and is sour to taste. It is significant in milk and milk products for a number of reasons. Its nutritional role is of course well established, but perhaps of equal significance with reference to milk are its properties as an oxidation-reduction-type compound and its role in the development of oxidative off-flavors in milk (discussed elsewhere). Ascorbic acid is deposited in milk in its reduced form during the secretory process. However the compound affords an excellent example of how various substances may be formed in milk by the decomposition of unstable precursors. A scheme for the decomposition of ascorbic acid as it o· ·urs in milk on standing in the presence of oxygen is as follows:



The first formed product, dehydroascorbic acid, can be converted back to reduced ascorbic acid under strong reducing conditions, that is, treatment with H_2S. The trend in stored milk is toward decomposition to diketogulonic acid (a compound which has no vitamin C activity). This change results from the instability of the dehydroascorbic acid at the normal pH values of milk. Cleavage of diketogulonic acid to oxalic and threonic acids does not occur readily in milk

since the reaction requires heat and an abundance of oxygen. The point here is that none of these acids, with the exception of reduced ascorbic acid, is present in fresh milk but, on standing, the presence of all four of them in milk is quite possible.

A third instance of the enolic hydroxyl group in milk is that from uric acid.



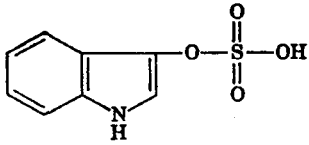
This acid forms the chief end product of the nitrogenous metabolism of birds and certain reptiles. It also is found in small amounts in the urine of most animals. As with most of the other components of the non-protein nitrogen fraction, uric acid finds its way into milk directly from the blood. The uric acid content of mixed milks averages about 2.3 mg. per 100 ml. Variations of from 1.13 to 3.69 mg. per 100 ml. of milk have been noted in individual milk samples. Like other purines, uric acid gives the murexide test. This consists in heating the substance with concentrated nitric acid, cooling, and adding ammonium hydroxide to an alkaline reaction. A violet color results. The measurement of uric acid in milk (20) is accomplished by freeing the milk of protein and fat (tungstic acid is commonly used). An aliquot of the filtrate is treated with sodium cyanide and arsenophosphotungstic acid reagents. After clarifying the reaction mixture, the amount of developed color is measured at a wavelength of 520 mμ. The uric acid content is derived by comparison with color from standard uric acid solutions and correction for dilution factors.

Phosphoric and sulfuric esters. A group of substances important in milk from the standpoint of acidity are those which contain phosphoric or sulfuric acid in esterified form. The most notable example of this is casein, which is distinctive as a type of protein because it is conjugated with phosphoric acid. The sensitivity of casein to certain cations, its well-defined isoelectric point behavior, its characteristic action on treatment with rennin, and its rather substantial base-binding power are primary resultants of the phosphoric acid in its constitution. Of course, the acid phosphate salts of milk serum are significant contributors to the acidity of milk. The phosphates of protein-free milk serum, the organic phosphate combined with the caseins, and the phosphorus involved in the structure of milk phospholipides do not account for all the phosphorus in milk. Small amounts of phosphorus

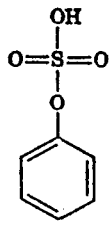
apparently are bound either physically or chemically with some of the other milk constituents as discussed subsequently.

Graham and Kay have made a rather systematic study of phosphorus distribution in milk. Their observations indicate that the acid-soluble ester phosphorus averages about 10 mg. (as P) per 100 cc. of milk and ranges between 7 and 21 mg. per 100 cc. It may be of value to compare these data with their findings (averages) for total P, 103.7 mg.; inorganic P, 67.6 mg.; acid insoluble P (casein and lipide P), 24.8 mg.; and lipide P, 6.5 mg. per 100 cc. of milk. Some additional information concerning the specific components of the Graham and Kay soluble ester phosphorus fraction is available. McGeown and Malpress have revealed the following sugar phosphates and their approximate concentrations in milk: galactose-1-phosphate, 1 mg./l. and glucose-1-phosphate, 1 mg./l. These workers also detected another glucose phosphate and a lactose phosphate (in which the positions of the phosphates were not established) and phosphopyruvic acid. The concentration of the latter three substances in milk were shown to be less than 0.1 mg./l. in each case. Nucleic acids also are known to occur in milk (13, 15, 17). Mandel and Bieth have reported 15 to 17 mg./l. of ribonucleic acid phosphorus in milk. Morton has reported the presence of nucleic acids associated with the lipoprotein particles of cow milk. Thus, nucleic acid phosphorus should be found in both the acid soluble and acid insoluble phosphorus fractions of milk.

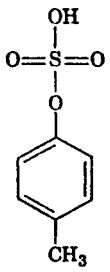
Other acid esters, presumably present in milk, are the sulfuric esters. The most notable example of this type is indican.* This compound is one of a group of sulfate esters which normally occur together in the urine of man and certain animals (11). The related compounds are the sulfate esters of phenol and *p*-cresol. Structural formulas for these compounds are as follows:



Indoxyl sulfuric acid (Indican)



Phenol sulfuric acid



p-Cresol sulfuric acid

*The term *indican*, used in another sense not of concern here, applies to the glycoside, indoxyl-3-glucoside, which occurs in the indigo plants of the orient.

As with hippuric acid, the primary metabolic role of these sulfate esters is as detoxication products. Phenolic compounds even in low concentration are quite toxic to the animal body. The metabolism of the amino acids tyrosine and tryptophan in the intestine leads to varying amounts of these phenolic compounds. Putrefactive conditions favor their production. In order to detoxify these compounds and promote their rapid elimination from the body, they are conjugated with sulfuric acid and eliminated, primarily in the urine. The only member of this group whose presence has been demonstrated in milk is indican (21). An average of 120 μg. and a range of 28 to 220 μg. of this compound per 100 ml. of milk have been reported. Indican is usually determined by oxidation and condensation to form the blue dye, indigo, or similar substances, followed by extraction with chloroform and photometric estimation of the color produced in terms of a standard. The presence and significance of indican and related sulfate esters in milk appear to be worthy of much more extensive investigation than they have been granted to date. Not only does the presence of such compounds in milk throw light on metabolic activity in the true stomach and rumen of the cow, but it also may be of significance regarding antioxidant properties of milk and the development of disinfectant-type off-flavors in milk.

NON-PROTEIN NITROGENOUS SUBSTANCES

When the proteins and fat of milk have been removed by precipitation with trichloroacetic acid and filtration, the filtrate contains a group of substances that are determined as non-protein nitrogen. Some accounting of this nitrogen may be made by specific determinations for creatin, creatinine, urea, ammonia, uric acid, and α-amino nitrogen (amino acids). This leaves a small quantity of nitrogen unaccounted for, and some of this is composed of the nitrogen from hippuric and orotic acids and indican, among other substances. A notable resemblance exists between the compounds in the non-protein nitrogen fraction of milk and those in the urine of the cow. Moreover, it is clear that these compounds for the most part are the end products of nitrogen metabolism in the cow's body. Thus it would seem that the bulk of them come directly from the blood of the animal, and that their levels in milk will be determined largely by factors influencing protein metabolism in the animal. For example, it has been established that the non-protein nitrogen content of milk increases with increasing protein content of the feed.

The non-protein nitrogen content of milk has been studied rather extensively by Shahani and Sommer. A summary of their findings is presented in the following table.

NON-PROTEIN NITROGEN COMPONENTS IN MILK

Component	Nitrogen per 100 ml., mg.		
	Low	High	Average
Total non-protein N	18.1	28.7	23.8
Ammonia N	0.17	1.19	0.67
Urea N	6.54	10.85	8.38
Creatinine*	0.19	0.65	0.49
Creatine*	3.55	4.51	3.93
Uric acid*	1.55	2.70	2.28
α-Amino N	2.20	5.18	3.74
Unaccounted N	5.63	14.45	8.81

* Reported as such.

The value for unaccounted nitrogen in the above table includes nitrogen contributed by hippuric acid, orotic acid, and indican. Assuming average levels of 5.1, 8.0, and 0.12 mg. for these compounds respectively, per 100 ml. of milk, they would account for approximately 1.8 mg. of nitrogen. Using the average figure of 8.8 mg. for unaccounted nitrogen, this leaves 7 mg. per 100 ml. of milk still unaccounted for.

A particular value of the non-protein nitrogen determination in milk is that it reflects changes induced in the protein-nitrogen fractions. Such changes are of interest in connection with various types of processing and proteolytic activity of enzymes and microorganisms. In the study by Shahani and Sommer, normal pasteurization, that is, 143° F. for 30 minutes, was found to be without effect on the nitrogen distribution in milk. However, pasteurization at 155° F. for 30 minutes coupled with homogenization at 2000 p.s.i. pressure increased the non-protein and α-amino nitrogen content. An increase in non-protein nitrogen also accompanies storage changes in evaporated milk.

The α-amino nitrogen fraction of the non-protein nitrogen is actually a class of compounds rather than any one specific compound. The determination is made by the classic Van Slyke method. The nitrogen value for this fraction is probably derived largely from a few amino acids and peptides that are present as such in the milk serum. The point regarding origin of the amino acids is open to debate since it

is always possible that they may result from decomposition of milk proteins. However, two different investigations (3, 25) have found essentially the same group of amino acids present. Since other substances appear to come directly from the cow's blood, it is logical that trace amounts of amino acids also might enter milk in this fashion. Those amino acids which have been noted qualitatively in trace amounts in fresh milk are: alanine, glutamic acid, glycine, leucines, and valine. The presence of aspartic acid and serine also have been reported.

OTHER MINOR SUBSTANCES

There are many other minor substances that may be present in milk either normally, occasionally, or very infrequently. For convenience of treatment these will be grouped together. They include low-molecular weight carbonyl compounds, gases, cellular material, foreign material, and decomposition products. It is well known that milk contains small and rather variable amounts of acetone and the related substances. Recent evidence has shown that acetaldehyde is a consistent component of milk in trace quantities. The probability that pyruvic acid, CH_3-CO-COOH, a carbonyl acid, is present in milk has been mentioned previously.

The gases normally present in milk are carbon dioxide, oxygen, and nitrogen. Milk fresh from the udder contains approximately 6.6, 0.1, and 1.2% of these gases by volume respectively. It may be readily deduced that the amount of these gases in milk would vary considerably with such factors as agitation, heat, and vacuum treatments.

A certain amount of cellular material is always present in milk. Such material is made up of bacterial cells and leucocytes as well as fragments of epithelial tissue and cells from the udder. The presence of leucocytes in milk is of practical significance in that it can create a sediment problem in homogenized milk. Clarification of the milk prior to homogenization is an effective means of combatting this problem. Excessively high numbers of leucocytes in milk is a good indication of udder infection.

It is obvious that the types of foreign material that may be found in milk are more or less infinite. Feed fragments that can fall from the coat of the animal or settle in the milk from the barn air are commonly found on microscopic examination of separator bowl residues. Although manure may be found in milk, reports of its incidence there

are somewhat overdone. Plant cellular material showing evidence of digestion of cell contents with cell walls still intact are rather good evidence of manure contamination. Foreign materials in milk, of recent prominence, are antibiotics, herbicides, insecticides, and disinfectants.

It seems of value to reemphasize the fact that many minor substances that may be detected in milk can result from decomposition of the various major milk components. Such decomposition products include free fatty acids, amino acids, glucose and galactose, and lactic acid, as well as various other fermentation products.

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CHAPTER 8

PHYSICAL PROPERTIES OF MILK

This chapter deals with some of the important physical properties of milk and their relation to dairy problems. Each physical property of milk is, of course, a resultant determined by the contributions of its constituents. Obviously, then, physical properties vary with composition. Furthermore, the processes to which milk is subjected in manufacturing and utilization induce changes in the constituents that are reflected in physical properties. It is the aim of this chapter to outline the physical principles involved, to define the contributions of constituents to each property, and to describe the effects of processing treatments. Those interested in a more basic discussion of physical principles of biochemical systems should consult the books by Bull (7) and Gortner (18).

ACIDITY AND PH RELATIONSHIPS

General Principles

The acid-base equilibria in a system such as milk arise from the presence of substances (acids) that liberate protons and of those that

combine with them (bases). In general terms an acid consists of a base combined with a proton:

$$\text{Acid} \rightleftharpoons \text{base} + \text{proton}$$

An acid and its corresponding base are said to form a *conjugate pair.* Acids and bases differ in their affinity for protons. An acid is said to be strong if it loses its proton readily, and a base is considered strong if it combines readily with a proton. In any given conjugate pair, therefore, if the acid is strong the base is weak, and vice versa. Actually, acids do not simply dissociate as indicated above, but react with another conjugate pair. For example, a weak acid (HA) reacts with water as follows:

$$HA + HOH \rightleftharpoons H_3O^+ + A^-$$

This forms a new acid, the hydrogen or hydronium ion H_3O^+, and a new base, the anion A^-. The proton or hydrogen ion is not free, but rather hydrated as the hydronium ion H_3O^+. Such hydrated protons are usually referred to as "hydrogen ions," however, and this terminology will be used in this chapter.

A base, ammonia, ionizes in water as follows:

$$NH_3 + H_2O \rightleftharpoons NH_4^+ + OH^-$$

Water itself dissociates to a slight extent:

$$HOH + HOH \rightleftharpoons H_3O^+ + OH^-$$

The extent of this dissociation can be expressed precisely by the dissociation constant, K_a:

$$K_a = \frac{a_{H_3O^+} \times a_{OH^-}}{a_{H_2O}}$$

where $a_{H_3O^+}$, a_{OH^-}, and a_{H_2O} are the activities of the hydrogen ions, hydroxyl ions, and undissociated water molecules respectively. Activity is essentially the concentration in moles per liter corrected by a factor to account for interionic attractions. It is the effective concentration. Since the activity of the undissociated water is overwhelmingly greater than that of the hydrogen and hydroxyl ions we may assume that it is constant and write

$$a_{H_3O^+} \times a_{OH^-} = K_w$$

where K_w is a constant, called the ionization constant, which includes both the dissociation constant and the activity of water. The numerical value for K_w varies with temperature, as may be seen in Table 1.

Table 1. VARIATION IN IONIZATION CONSTANT AND pH OF WATER WITH TEMPERATURE

Temperature ° C.	$K_w \times 10^{14}$	pH
0	0.114	7.47
10	0.292	7.27
20	0.681	7.08
25	1.008	7.00
30	1.469	6.92
40	2.919	6.77
50	5.474	6.63
60	9.614	6.55

The extent of dissociation increases as the temperature is raised. At 25° C. the ionization constant is approximately 1×10^{-14}. Since the hydrogen and hydroxyl ions are present in equal concentrations and since the activities in dilute solutions are approximately equal to concentrations, it follows that the concentration of each ion is the square root of 1×10^{-14} or 1×10^{-7} moles per liter. In other words, a liter of pure water contains one ten-millionth (0.0000001) equivalent per liter of hydrogen ions and the same concentration of hydroxyl ions.

The equation given above for ionization constant indicates clearly that in all aqueous solutions the product of the hydrogen and hydroxyl ion activities is a constant, that is, K_w. Thus, if an acidic substance that liberates protons is dissolved in water, the hydrogen ion concentration is increased and that of the hydroxyl ions is decreased, but their product remains constant at a value of 1×10^{-14} at 25° C. This fact makes possible the use of a very simple and useful scale for the expression of the hydrogen and hydroxyl ion activities in aqueous solutions. In biological systems we deal with very low concentrations of hydrogen and hydroxyl ions. For example, in normal milk the hydrogen ion activity is approximately 2×10^{-7} equivalents per liter or 0.0000002. Either of these modes of expression is rather awkward to handle. Sorenson, in 1909, made an important contribution to the subject by suggesting that the concentration be expressed in terms of a number, pH, which he defined as follows:

$$\text{pH} = -\log C_{H^+} = \log \frac{1}{C_{H^+}}$$

where C_{H^+} = hydrogen ion concentration. This definition was later modified to

$$pH = -\log a_{H_3O^+} = \log \frac{1}{a_{H_3O^+}}$$

where $a_{H_3O^+}$ is the activity of the hydrogen ion.

For milk with a hydrogen ion activity of 2×10^{-7} moles per liter:

$$pH = \log \frac{1}{0.0000002} = \log 5{,}000{,}000 = 6.70$$

Needless to say, an analogous expression can be written for hydroxyl ions

$$pOH = \log \frac{1}{a_{OH^-}}$$

Furthermore, it is easy to see that

$$pH + pOH = pK_w = 14$$

Thus, in aqueous solutions at 25° C. the sum of pH and pOH is always 14. In pure water, both pH and pOH have a value of 7. Since the sum of pH and pOH is constant, either one alone defines the status of the system and pH is used almost to the exclusion of pOH. A 0.1*N* solution of HCl will have a pH of approximately 1, and a 0.1*N* solution of NaOH, a pOH of approximately 1 and a pH of about 13.

Two important facts need to be remembered about the pH scale. In the first place it is logarithmic; a change of one pH unit denotes a tenfold change in concentration of hydrogen ions. Secondly, the scale is inverse with relation to hydrogen ion concentration; higher pH's mean lower concentrations of hydrogen ions, and vice versa.

The affinity of acids and bases for protons may be expressed in terms of titration curves and dissociation constants. The former is a plot of pH versus the amount of acid or base consumed in a titration. Typical titration curves for a strong acid, HCl, and a weak one, lactic acid, are shown in Fig. 1. The essential difference is that hydrochloric acid yields its protons at a low pH (high proton concentration), and lactic acid liberates its protons only gradually and at a higher pH. The dissociation of lactic acid can be written as follows:

$$HL + H_2O \rightleftharpoons H_3O^+ + L^-$$

where L = lactate, that is, $CH_3CHOHCOO^-$.

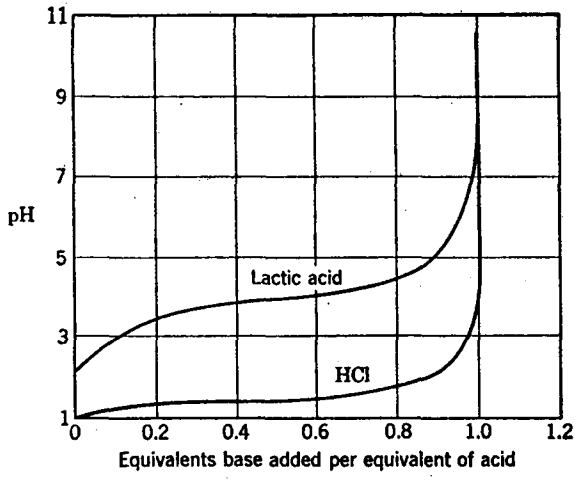


Fig. 1. Titration curves for 0.1*N* HCl and 0.1*N* lactic acid.

The dissociation constant is accordingly (neglecting the water as being constant):

$$K_a = \frac{a_{H_3O^+} \times a_{L^-}}{a_{HL}} = 1.39 \times 10^{-4}$$

rearranging:

$$a_{H_3O^+} = K_a \times \frac{a_{HL}}{a_{L^-}}$$

Taking negative logarithms of both sides of the equation:

$$\text{pH} = pK_a + \log \frac{a_{L^-}}{a_{HL}}$$

The pK_a or $\log 1/K_a$ is for lactic acid:

$$\log \frac{1}{1.39 \times 10^{-4}} = 3.86$$

It will be noted that when the acid is half neutralized, that is, when $a_{L^-}/a_{HL} = 1$, $\log a_{L^-}/a_{HL} = 0$, and consequently $\text{pH} = pK_a$. Thus the half neutralization point occurs at pH 3.86. Furthermore, it can be calculated from this equation that the acid is 10% neutralized at pH 2.86 and 90% neutralized at pH 4.86.

Lactic acid and its anion lactate form a typical buffer system. Buffers are defined as materials that resist change in pH. They characteristically consist of a weak acid and its salt or a weak base and

its salt. The basis of buffer action is readily discernible from the titration curve of lactic acid in Fig. 1. Near the middle of the neutralization range the pH is very insensitive to additions of acid or base. Additions of hydrogen ions to a mixture of lactic acid and lactate merely form a little lactic acid at the expense of lactate. Addition of hydroxyl ions produces the opposite effect. The buffering action of a weak acid is really only effective over a pH range extending 1 pH unit on either side of its pK_a, in the case of lactic acid from pH 2.86 to pH 4.86. Maximum buffering occurs at the pH equal to the pK_a.

Hydrogen Ion Concentration of Milk

The pH of normal fresh cow's milk ordinarily falls between 6.5 and 6.7. Values higher than 6.7 usually denote mastitic conditions in the udder, and those below 6.5 indicate the presence of colostrum or of bacterial deterioration. It must be remembered that although the range of pH 6.5–6.7 appears small, we are dealing with a logarithmic scale and the difference in actual hydrogen ion activity is considerable.

pH	$a_{H_3O^+}$
6.5	0.316×10^{-6} mole/l.
6.7	0.200×10^{-6} mole/l.

$$\frac{0.316-0.200}{0.200} \times 100 = 58\%$$

The pH of milk can be measured by a number of methods. Formerly, methods were proposed employing indicator dyes that change color at specific pH's. The opacity of milk makes the use of such dyes directly in milk of little utility. Of course, indicator papers impregnated with such dyes are of some use for rough approximations. By far the most satisfactory method of determining pH of milk is the electrometric procedure with the glass electrode. For precise work this method is vastly superior to all others. The glass electrode consists of a bulb of glass, part of which is of a special composition sensitive to hydrogen ions. Within the bulb is a chloride buffer to maintain a constant high hydrogen ion activity. A silver-silver chloride electrode dips into this buffer solution. When the glass bulb is immersed in a solution containing hydrogen ions, an electrical potential is set up across the glass membrane, owing to the difference in hydrogen ion concentration on the two sides. This potential is measured against

a standard saturated calomel electrode with a vacuum tube voltmeter. Most instruments are calibrated to read directly in terms of pH.

The Buffering of Milk

Fresh milk acts as a complex buffer because of its content of carbon dioxide, proteins, phosphate, citrate, and a number of minor constituents. Bacterial action introduces lactate and other organic anions as additional buffers. Titration methods have been widely used to assess increases in acidity due to the action of bacteria. Thus, interest has centered in the buffer behavior of milk over the pH range from the curdling point at about pH 4.8 to the commonly used phenolphthalein end point at about pH 8.3. Titration of fresh milk from pH 6.6 to pH 8.3 requires 13 to 20 ml. of 0.1*N* NaOH per 100 ml. (1.3 to 2.0 meq. per 100 ml.). Most fresh milk samples fall in the range of 1.5 to 1.8 meq. per 100 ml. In the United States, titration results are often converted to the equivalent percentage of lactic acid.

Per cent lactic acid = 0.09 × meq. per 100 ml. This practice undoubtedly arose from the use of titration to follow the activities of bacteria. Actually, fresh milk contains practically no lactic acid, and even in sour milk the original buffering systems of the milk play a significant role. There is no essential reason for expressing the titratable acidity of milk as percentage of lactic acid. European practice avoids this conversion; titratable acidity is left in terms of titer, that is, milliliters of alkali per given volume of milk. Table 2 gives

Table 2. VARIATIONS IN TITRATABLE ACIDITY OF MILK

Breed	No. of Samples	Titratable Acidity	
		Range, % lactic acid	Mean, % lactic acid
Ayrshire	229	0.08–0.24	0.160 ± 0.005
Holstein	297	0.10–0.28	0.161 ± 0.004
Guernsey	153	0.12–0.30	0.172 ± 0.005
Jersey	132	0.10–0.24	0.179 ± 0.006

Data of Caulfield and Riddell (8).

data on the titratable acidity of milk from cows of four breeds. The factors involved in the titration of milk are described and discussed by Sommer (35).

A typical titration curve for milk covering the range pH 5.5 to 9 is given in Fig. 2. It is immediately evident that the amount of acid

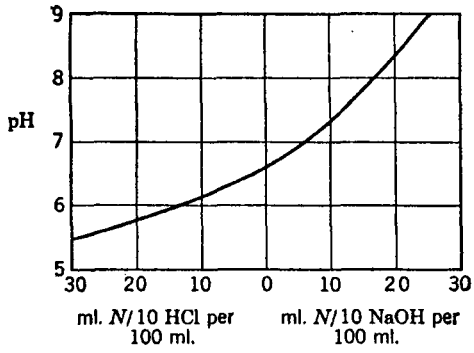


Fig. 2. Titration curve of milk.

or base required to shift the pH by a given increment varies considerably over the course of the curve. The fact that the curve is steeper in the region around pH 8.3, where phenolphthalein changes color, makes the end point with that indicator rather sensitive; only a small increment of alkali is necessary to produce the end point. The variations in buffer capacity over the titration range can be expressed more exactly in terms of "buffer index," $dB/d\text{pH}$, which is defined as the number of equivalents of acid or base required to shift the pH of a liter of solution by one unit.

$$\frac{dB}{d\text{pH}} = \frac{\Delta E}{\Delta\text{pH}}$$

when ΔpH is made infinitesimally small,
and where ΔE = equivalents required per liter between pH_1 and pH_2.

$$\Delta\text{pH} = \text{pH}_2 - \text{pH}_1$$

In other words, the buffer index, $dB/d\text{pH}$, is the slope of the titration curve at any point. The steeper the curve (considering pH as abscissa, and equivalents as ordinate) the greater is the buffer index. In the plot in Fig. 2 the curve is flatter the greater the buffer index because equivalents are plotted as abscissa and pH as ordinate. A plot of buffer index against pH yields a buffer intensity curve, as shown in Fig. 3. The pH of maximum buffering, that is, the "peak" in the graph, is characteristic of the kind of buffer. A given buffer exhibits maximum buffering at the pH of its pK_a. The height of the peak is proportional to the concentration of buffer. The curves in Fig. 3 show that Jersey milk has a much greater buffering capacity than Holstein milk, a fact due to its higher concentration of buffer salts.

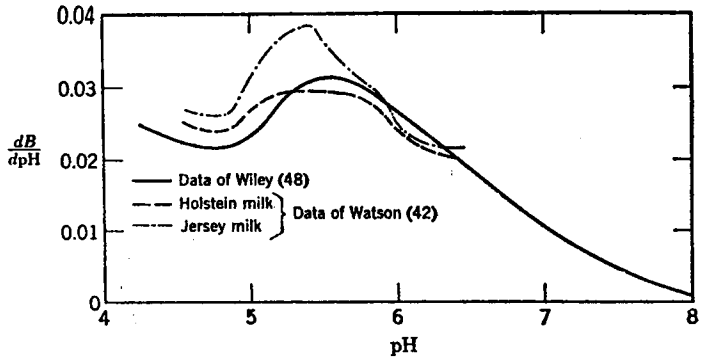


Fig. 3. Buffer intensity curves for milk.

Constituents of Milk Responsible for Buffer Action

Carbon dioxide. Milk as secreted by the cow contains about 20 mg. of CO_2 per 100 ml., or about 10% by volume. This gas is rapidly lost from milk owing to the low content in the air. The loss is for all practical purposes irreversible since milk is seldom exposed to atmospheres high in CO_2. The loss is accelerated by heating, agitation, or vacuum treatment. Commercial unpasteurized milk may contain about one-half of its original CO_2.

Of course carbon dioxide behaves like an acid in solution, being hydrated to carbonic acid, H_2CO_3. The data in Table 3 indicate

Table 3. Effect of Variations in Carbon Dioxide on the pH and Titratable Acidity of Milk

Treatment	CO_2, mg./l.	Acidity, % lactic	pH
Sample 1			
Untreated	108	0.150	6.48
Decarbonated	nil	0.133	6.60
Carbonated	240	0.185	6.30
Sample 2			
Untreated	110	0.150	6.50
Decarbonated	nil	0.130	6.62
Carbonated	180	0.170	6.40

Data of Foschini (17).

that when CO_2 is either removed from or added to milk the titratable acidity changes by approximately an equivalent amount.

$$1\%\ CO_2 \text{ by volume} = 2 \text{ mg. } CO_2/100 \text{ ml.} = 0.045 \text{ meq./100 ml.}$$
$$= 0.00405\% \text{ lactic acid}$$

Carbonic acid is treated as a monoprotic acid in this calculation since only one of its hydrogens is titrated in the pH range 6.6–8.3. The pK_a's of H_2CO_3 are 6.37 and 10.25. The CO_2 in milk as drawn accounts for a titer between pH 6.6 and 8.3 of about 0.5 meq./100 ml. out of a total of 1.3 to 2.0. Loss of CO_2 after milking reduces the contribution of this constituent to only 0.1 to 0.2 meq./100 ml. in ordinary commercial milk.

Proteins. The titration curves of casein and β-lactoglobulin are given in Chapter 4. The ionizable groups in the proteins exert considerable buffer action in the range from pH 4.6 to 8.3, in which we are normally interested. From curves such as these, and also from the difference between the titers of skimmilk and rennet whey, it can be calculated that the 2.5% casein usually present in milk contributes a titer of about 0.8 meq./100 ml. between pH 6.6 and 8.3. The whey proteins contribute about 0.1 to 0.2 meq. over the same range. In the titration of sour milk from pH 4.6 to 8.3, casein is responsible for about 2.5 meq. per 100 ml. and whey proteins for 0.7 to 0.8 meq. per 100 ml.

Phosphate. Orthophosphoric acid is a triprotic acid with three separate buffering ranges. The titration curve in Fig. 4 illustrates this fact. The three pK_a's of phosphoric acid are respectively 1.96, 7.12, and 12.32. Obviously, in milk we are primarily concerned with the second dissociable hydrogen ion. Phosphoric acid, moreover, exhibits a rather different titration curve if calcium is present during the titration. This peculiarity arises from the precipitation of insoluble calcium phosphate as the pH is raised. The titration curve in the presence of calcium is identical to that obtained in its absence until the first hydrogen is neutralized. As more alkali is added slowly the pH remains near 6 and insoluble calcium phosphate precipitates until all three hydrogens are neutralized. In essence this means that in the presence of calcium the third hydrogen of phosphoric acid is titrated in the neighborhood of pH 6. Considering the precipitation of tricalcium phosphate:

$$3Ca^{++} + 2H_2PO_4^- \rightarrow Ca_3(PO_4)_2 \downarrow + 2H^+$$
$$2H^+ + 2OH^- \rightarrow 2H_2O$$

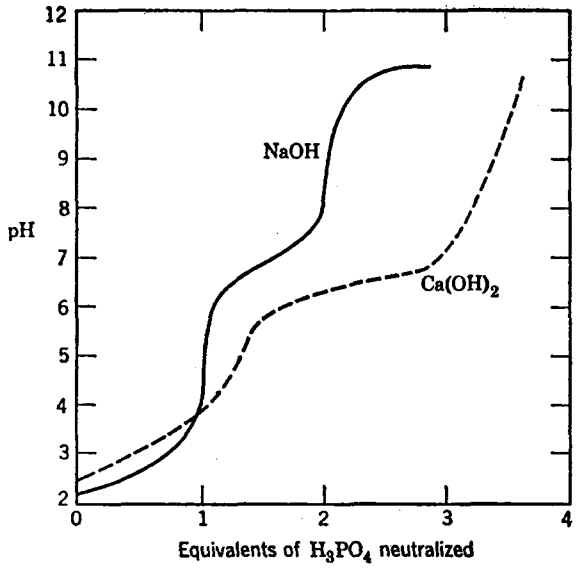


Fig. 4. Titration curves of phosphoric acid with NaOH and $Ca(OH)_2$.

This equation is undoubtedly an oversimplification of the actual process, but it illustrates the principle involved. The titer in the presence of calcium is greater as the time allowed to elapse after addition of each increment of alkali is made longer. This is because the precipitation of calcium phosphate is not instantaneous but requires some time. Thus if the titration is made quickly, the titration curve will not differ much from that obtained in the absence of calcium, but fading will occur at the end point because of continued precipitation of calcium phosphate. Unquestionably the precipitation of calcium phosphate during titration is one of the most important factors involved in the titration of milk. Milk ordinarily contains about 1 meq. of dissolved inorganic phosphate per 100 ml. At pH 6.6, about 0.6 meq. is in the form of $H_2PO_4^-$ ions, which are titrated to $HPO_4^=$ between pH 6.6 and 8.3. Thus the inorganic phosphate should account for a titer about 0.6 meq. per 100 ml. Since calcium is present somewhat more alkali is required. At first glance it might seem that the size of the calcium effect could be determined by adding oxalate to precipitate the calcium out of solution before titrating. However, this procedure introduces another complication because it disrupts the colloidal calcium phosphate of the caseinate particles (see Chapter 10) by precipi-

tating its calcium and setting its phosphate free as $PO_4^=$ ions which partially neutralize the other buffers of the milk. The true size of the calcium effect on the titration of milk can be determined by adding oxalate to rennet whey, which contains no colloidal calcium phosphate. When this is done the titer of the whey to the phenolphthalein end point is reduced by about 0.4 meq. per 100 ml. In summary we can say that the phosphate of milk contributes about 0.6 + 0.4 = 1.0 meq. per 100 ml. to the titratable acidity as ordinarily determined.

Citrate. Citric acid is triprotic with pK_a's of 3.08, 4.74, and 5.40. Obviously, in milk at pH 6.6 it exists predominantly as the trivalent citrate ion and contributes very little buffering capacity to the titration of fresh milk between pH 6.6 and 8.3. Probably not over 0.1 meq. per 100 ml. can be attributed to citrate. Citrate contributes little even to the titratable acidity of sour milk, because in the presence of calcium it is completely neutralized below pH 4.8 since it forms slightly dissociated calcium citrate complexes (see Chapter 5). Citrate does, however, exert one very important effect on the titratable acidity of milk. Its ability to complex with calcium tends to delay the precipitation of calcium phosphate during the titration and to reduce the calcium effect mentioned in a previous paragraph. This effect can be demonstrated readily with simple solutions of phosphate, calcium, and citrate. It is unlikely, however, that differences in titratable acidity among milks can be attributed to variations in citrate content because the latter is rather constant.

Lactate. As already mentioned, lactic acid is a monoprotic acid with a pK_a of 3.86. Even in sour milk at pH 4.86 it will exist to the extent of 90% in the form of lactate ion. Thus titration of sour milk does not involve neutralization of lactic acid itself to any great degree. The effect of formation of lactic acid in milk by bacterial fermentation of the lactose is to titrate the other buffers—principally phosphate and protein—to a lower pH. For example

$$CH_3CHOHCOOH + HPO_4^= \rightarrow H_2PO_4^- + CH_3CHOHCOO^-$$

These then are titrated back to their original pH and beyond when the actual titration with alkali is conducted. Acidification by the development of lactic acid also dissolves the colloidal calcium phosphate associated with the caseinate particles. This also increases the titer, not only by the addition of more titratable phosphate but also by increasing the size of the calcium effect.

Miscellaneous constituents. A number of acidic materials are present in milk in small amounts (see Chapter 7). These make a small contribution to the buffering capacity of milk.

Factors Affecting the pH and Titratable Acidity of Milk

Dilution and concentration. The general effect of diluting milk is to raise the pH and lower the titratable acidity. Concentration has just the opposite effects. Figure 5 shows a plot of pH versus concentration of milk solids-not-fat. This graph is a composite representing

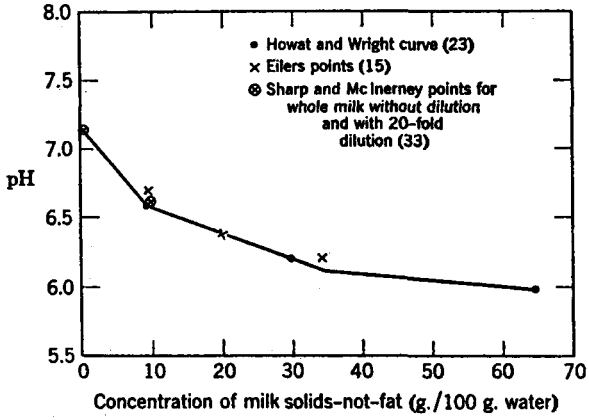


Fig. 5. Relation of pH to concentration of milk solids.

the results of several workers, some dealing with concentrated solutions of non-fat dry milk, others with concentrated skimmilk, and others with dilutions of milk. The effect of dilution on titratable acidity has also been studied considerably, largely because certain workers have felt that the phenolphthalein end point would be more readily detected in diluted milk. Sommer and Menos (36), for example, reported values of 0.172, 0.149, and 0.110% lactic acid for dilutions of nil, 1 + 1, and 1 + 9, respectively. McDowall and McDowell (27) have very pertinently pointed out that this effect is due in part to a change in the pH of the phenolphthalein end point as the milk is diluted. It seems to be influenced by the concentration of salts and proteins in the solution being titrated. McDowall and McDowell found the phenolphthalein end point to occur at pH 8.17 in undiluted milk, at pH 7.99–8.14 in milk diluted twofold, and at pH

7.69–7.99 in milk diluted tenfold. Not all of the effect of dilution on titratable acidity is due to depression of the phenolphthalein end point, however. When milk at various dilutions is titrated to a constant pH of 8.2 with a glass electrode pH meter, the titratable acidity still declines as much as 0.03% (as lactic acid) on tenfold dilution.

The effects of dilution and concentration are undoubtedly due to shifts in distribution of calcium and phosphate between the dissolved and colloidal states (see Chapter 5). Upon concentration, dissolved calcium and phosphate are shifted to the colloidal state with the release of hydrogen ions. Dilution produces the opposite effect. The lowered titratable acidity in diluted milk is due to a decreased rate of precipitation of calcium phosphate during the titration. Fading of the phenolphthalein end point is a greater problem in diluted than in undiluted milk. Fading is apparently due to slow continued precipitation of calcium phosphate.

Concentration of indicator. When phenolphthalein is relied on for detection of the end point, minimum concentration of indicator is necessary to insure constant titration results. The basis for this situation is that the indicator is a weak acid, the ratio of whose red and colorless forms is determined by the pH. The end point is detected by a certain concentration of the red form. If too little total indicator is present the pH must be increased in order to put enough of it in the red form to give the proper pink tinge. Thus the titer is higher if insufficient indicator is used. In general, 0.5 ml. of 1% phenolphthalein per 9 ml. of milk is in the range where concentration of indicator is not critical.

Effect of heat treatment. The effects of heat treatment on the acidity of milk are mentioned in Chapter 11. Essentially, the heat induces three changes that affect the acidity. The loss of CO_2 causes a slight decrease in titer and increase in pH. The transfer of calcium and phosphate to the colloidal state produces a slight increase in titer and decrease in pH. This change is slowly reversed after heating. Finally, drastic heating causes the production of acids by the degradation of lactose.

Summary of titration methods. From the foregoing paragraphs it is obvious that the titration of milk will yield reproducible results only if the procedure is defined precisely. Table 4 summarizes some of the principal methods used in the United States and in Europe. The method should be specified when results are reported.

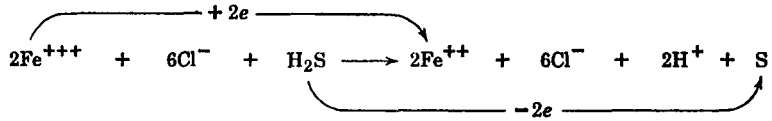
Table 4. SUMMARY OF PROCEDURES USED FOR DETERMINING TITRATABLE ACIDITY OF MILK

	U.S. MIF (2)	U.S. AOAC (1)	England British Standard (13)	Europe Dornic Method (41)	Europe Soxhlet-Henkel Method (41)	Europe Thorner's Method (5)	Netherlands Standard Method (3)
Sample	9 or 18 g.	20 ml. or 20 g.	10 ml.	10 ml.	50 ml.	10 ml.	10 ml.
Dilution	1:1	2:1	none	none	none	2:1	none
Phenol-phthalein	0.5 ml., 1% in 35% alcohol	2 ml., 1% in alcohol	1 ml., 0.5% in 50% alcohol	1 drop, 1% in alcohol; or 2 drops, 2% in alcohol	2 ml., 2% in alcohol	5 drops, 5% in alcohol	0.5 ml., 2% in 70% alcohol
Alkali	0.1*N* NaOH	0.1*N* NaOH	*N*/9 NaOH	*N*/9 NaOH	*N*/4 NaOH	*N*/10 NaOH	*N*/10 NaOH
End point	First definite pink	First persistent pink	Pink matching rosaniline standard	Light rose	Light rose		Pink matching fuchsin standard
Expression of results	Per cent "lactic acid"	Per cent "lactic acid" or ml. 0.1*N* NaOH/100 g.	g. "lactic acid" per 100 ml.	°D = tenths of a ml. *N*/9 NaOH per 10 ml.	°S.H. = ml. *N*/4 NaOH per 100 ml.	°Th. = ml. *N*/10 NaOH per 100 ml.	°N = tenths of a ml. of 0.1*N* NaOH per 10 ml.

OXIDATION-REDUCTION POTENTIAL

Definition

In the case of organic materials, oxidation may be defined as the uptake of oxygen or the loss of hydrogen. In the same manner, reduction may be defined as the process of losing oxygen or gaining hydrogen. However, in a broader sense the processes of oxidation and reduction are not necessarily limited to the gain or loss of oxygen and hydrogen. In ionic systems it can be demonstrated that the phenomenon may involve simply the loss or gain of electrons. The following equation, involving ferric chloride and hydrogen sulfide, demonstrates this point:



In this equation two electrons are gained by the ferric ion in being reduced to ferrous ion, whereas the hydrogen sulfide loses two electrons in being oxidized to elemental sulfur. Therefore, oxidation can be defined as the loss of electrons and reduction as the gain in electrons. An associated fact which can be deduced in this connection is that every oxidation is accompanied by a reduction, and vice versa.

In the field of chemistry, compounds frequently are characterized as oxidizing agents or reducing agents. It will be clear from the above that this refers (depending upon whether an ionic or organic substrate is involved) to the relative ease with which the compound gains or loses electrons, gains or loses oxygen, and gains or loses hydrogen.

The relative tendency of a substance in solution to yield or take on electrons can be measured if a standard substance that will donate or accept electrons can be inserted in the system to establish a difference in potential. In practice, the potential difference created by a platinum electrode in a solution of an oxidant or reductant is measured by completing a circuit through a calomel half-cell and a potentiometer. The voltage measured under these conditions reflects the oxidizing or reducing capacity of the solution. This potential is called the oxidation reduction potential or the redox potential. It is designated by the symbol "E_h." Positive potential, which involves loss of electrons from the platinum electrode, is indicative of oxidizing properties, whereas

negative potential, which involves gain of electrons at the platinum electrode, reveals reducing capacity.

In addition to potential, oxidation reduction systems also have a quality known as poise. This term, which is closely analogous to buffer capacity in pH and acidity equilibria, refers to the resistance that a system offers to change in oxidation-reduction potential when treated with oxidizing or reducing agents. The analogy between the proton transfers involved in acidity and the electron transfers that occur in oxidation-reduction is emphasized by the following equations for the E_h of any single system.

$$E_h = E_o + 0.06 \log \frac{\text{Ox}}{\text{Red}} \qquad \text{for 1 electron transfer}$$

and

$$E_h = E_o + 0.03 \log \frac{\text{Ox}}{\text{Red}} \qquad \text{for 2 electron transfers}$$

where E_h = oxidation reduction potential,
E_o = standard oxidation reduction potential (i.e., the potential when Ox/Red = 1),
Ox = concentration of oxidized form,
Red = concentration of reduced form.

These equations are obviously similar to that for buffer action. The amount of poising depends on the total concentration of the system and the ratio of oxidized to reduced form. It is maximal when Ox/Red = 1, that is, at the standard oxidation-reduction potential, E_o. The E_o is a characteristic of the system in the same way that pK_a is a characteristic of an acid. It is used to compare the relative oxidizing or reducing abilities of one system with another.

Methods of Measuring Oxidation-Reduction Potential

Basically there are two methods for measuring the E_h of a system. These are electrometric and colorimetric. Of these two the electrometric is much more reliable. Many dyes owe their color to an unstable state of their electrons. The gain or loss of electrons frequently induces change in color of these dyes and the change in color occurs at an E_h characteristic for each dye. Thus, some of them are particularly suited as so-called E_h indicators. They have proved particularly useful in studying E_h in living tissues where electrometric determinations are difficult or impossible to make. The relative merits of this proce-

dure, together with values for various dyes as E_h indicators, are presented by Gortner (18).

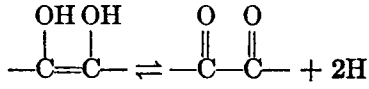
The E_h of milk has usually been determined in most investigations with the aid of a platinum electrode, calomel half-cell, and a sensitive potentiometer. In order to enhance the sensitivity in this type of circuit, which is connected by means of KCl-agar bridges, various amplifiers and galvanometers frequently have been used. This type of instrumentation yields data on potential but it tells nothing regarding the poise of the system. For this and other reasons, Michaelis (28) recommends potentiometric titration with either an oxidizing or reducing agent. Readers interested in details of such procedures should consult his treatment of the subject.

The measurement of E_h in biological systems such as milk is not always accomplished easily. Difficulty often is encountered in reproducing results between electrodes and in achieving a steady potential. Frequently, careful cleaning of electrodes in hot mineral acid solution, evacuation of the system, or purging it briefly with oxygen-free nitrogen and allowing several hours for a steady state to be reached will overcome difficulties in securing reasonable and reproducible results.

The Oxidation-Reduction Potential of Milk

The E_h of milk normally falls within the range of +0.2 to +0.3 volt. The values are more commonly found to fall between +0.23 and +0.25 volt. Data of Harland et al. (20) indicate that the E_h of fresh unheated milk is largely the result of its oxygen content. By deaerating with nitrogen, these workers observed a decrease of over 0.4 volt in the E_h of unheated milk. These findings corroborate earlier observations of Saal and Heukelom (15).

The actual poise of the E_h system in milk has received very little study. However, it seems probable that the principal poising material under normal oxygen tension may be ascorbic acid. The reductone structure of this compound affords one stage in its oxidation-reduction equilibrium. This may be represented as follows:



Investigations of the redox system in milk generally have been limited in number and scope. Nearly all measurements have been based on the electrometric method employing platinum electrodes and

calomel half-cell. The literature to approximately 1940 on the E_h of milk, with special reference to the effects of heat, has been reviewed by Gould and Sommer (19). Their comprehensive study of this matter, together with that of Josephson and Doan (25), has shown very clearly the relation between a lowering of the potential by heat treatment, the onset of cooked flavor, and the appearance of sulfhydryl reducing substances in milk. This change in redox potential on heating is shown in Fig. 6. The effects of heat treatment and drying on the

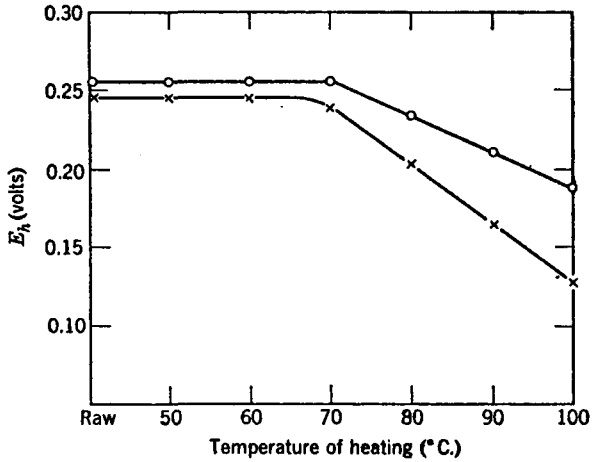


Fig. 6. The influence of heat on the oxidation-reduction potential of milk: o — o, no holding period. x — x, holding period 30 minutes. Data by Gould and Sommer (19).

various kinds of reducing substances and the E_h of milk have been evaluated more recently by Harland et al. (20). In general, they found that removal of oxygen prior to heating and/or the use of HTST preheating tended to yield maximum quantities of the various reducing substances, to lower E_h values in both the fluid milk and dried milk made from it, and to produce dried milks of superior oxidative stability. They noted further that E_h's of both fluid milk and freshly reconstituted dry milk were a function of their ascorbic acid content.

In addition to heat treatment, bacterial activity and contamination with trace metals, particularly copper, influence the E_h of milk. In all probability bacteria accomplish a lowering of the potential through use of available oxygen in the medium as well as by elaborating reducing substances in the course of their metabolism. The well-known

methylene blue test of milk is based on this phenomenon. The potential at which methylene blue exists half in the colored and half in the colorless, leuco form (E_o at 30° C.), is + 0.24 volt. It is not uncommon for bacteria in the actively growing phase to carry the redox potential of milk to negative values. Of course, under these conditions methylene blue would be completely decolorized.

Cupric ion is a very strong electron acceptor and a potent oxidizing agent. As a consequence, contamination of milk with copper tends to raise the E_h. The initial phases of this change involves the destruction of reduced ascorbic acid.

In considering the E_h of milk it is well to remember that hydrogen ion concentration may be a related factor. In many cases, reducing substances exist as anions (Red^-) and are capable of accepting hydrogen ions ($Red^- + H^+ \rightleftharpoons H\ Red$), thus preventing the anions from contributing to E_h. Since milk is not a known system and the precise nature of the substances contributing to its E_h have not been established, it is not possible to derive a satisfactory correction factor for the effect of pH on the oxidation-reduction potential of milk. In general, the following equation holds for the relation between E_h and pH of univalent systems of known composition:

$$E_h = E_1 - 0.06\ pH$$

E_h is the potential corrected for pH, and E_1 is the oxidation-reduction potential at a particular pH. In bivalent systems, such as quinone-hydroquinone, the correction factor —0.03 pH is used.

Apparently the difficulties involved in securing reliable data on the oxidation-reduction potential of milk have discouraged research on the subject. Milk is a delicate biological system that is synthesized in large measure by oxidation-reduction processes. Moreover, two very significant considerations in practical milk handling involve the oxidation-reduction potential. These are bacterial activity and changes in flavor. The relation of the E_h to oxidized and cooked flavors has been discussed here and in Chapter 12. It seems probable that the complex relations between trace metal contamination, ascorbic acid destruction, oxidized flavor, and lipide oxidation could be greatly clarified through titrimetric studies of the redox system in milk.

DENSITY AND SPECIFIC GRAVITY

General Considerations

Density is the physical property used to compare masses of different substances or of a given substance under different conditions. Density is weight per unit volume:

$$\text{Density} = \text{weight/volume}$$

Obviously the numerical value of density depends on the units of weight and volume used in calculating it. Thus it has been found convenient to express density in reference to some standard substance. Water is commonly used as the reference substance. The dimensionless constant thus obtained is called specific gravity:

$$\text{Specific gravity} = \text{density of substance/density of water}$$

Since the density of any substance including water varies with the temperature, it is necessary to specify the temperature when reporting densities or specific gravities. Any temperature can be used, but in practice certain standard temperatures have become established by long usage. In the metric system the density of water is 0.999973 g. per $cm.^3$ at 3.98° C. For most purposes, it can be said to be 1.0000 at 4° C. When the specific gravity of a substance is referred to water at 4° C., it is numerically equal to the density of that substance in the metric system. In dairy chemistry the specific gravity of milk has usually been reported at 60° F./60° F. (meaning density at 60° F. divided by that of water at 60° F.) or at 20° C./20° C.

Methods of Measurement

The density and specific gravity of milk may be determined by either measuring the weight of a known volume or the volume of a known weight. The weight of a known volume may be determined either with a pycnometer or with a hydrostatic balance. A pycnometer is a vessel that can be filled with liquid at a given temperature and weighed. The volume contained at any temperature can be calculated by dividing the weight of water contained by the density of water at that temperature. Then:

$$\text{Density of milk at } t^\circ = \frac{\text{weight of milk contained at } t^\circ}{\text{volume of pycnometer at } t^\circ}$$

If specific gravity is sought, it can be calculated simply as follows:

Specific gravity of milk at $t°$

$$\text{relative to water at } t° = \frac{\text{weight of milk contained at } t°}{\text{weight of water contained at } t°}$$

Strictly speaking, the weights of both milk and water should be corrected for the buoyancy of the air and expressed as weights *in vacuo*. In practice, in dairy chemistry this correction is seldom made, both weights being taken in air.

The hydrostatic balance operates on the basis of Archimedes' principle, namely, that a body immersed in a liquid is buoyed up by a force equal to the weight of the liquid displaced. A plummet which can be suspended from the arm of a balance is used. The density can be computed as follows:

$$\text{Density of milk} = \frac{A - W_m}{V}$$

where A = absolute mass of the plummet, determined by weighing in air and correcting for buoyancy of air,
W_m = weight of plummet immersed in milk at $t°$,
V = volume of plummet at $t°$, determined by weighing plummet immersed in water.

Here again specific gravity can be calculated simply as follows:

Specific gravity of milk at $t°$

$$\text{relative to water at } t° = \frac{\text{loss of weight in milk at } t°}{\text{loss of weight in water at } t°}$$

A plummet may be used with an analytical balance, or for somewhat less accurate work special balances called Westphal balances are available.

In the measurement of density and specific gravity by the determination of the volume of a known weight, a float called a lactometer is employed. The principle involved is that a floating object sinks until it has displaced a weight of fluid equal to its own weight. The volume of displaced fluid is greater the smaller the density of the fluid. Lactometers are constructed of a large glass float with a slender glass stem carrying a scale on which small increments of displaced volume can be read. The scale is actually calibrated, not in terms of volume but in a function of either density or specific gravity. The two principal types of lactometers are the Quevenne and the New York Board of

Health. The Quevenne is calibrated in degrees which bear the following relation to specific gravity:

$$^{\circ}Q = 1000S - 1000$$

where $^{\circ}Q$ = Quevenne degrees,
S = specific gravity at 60° F./60° F.

Thus, a specific gravity of 1.032 is equivalent to 32° Q. The New York Board of Health scale is related to specific gravity as follows:

$$^{\circ}\text{N.B.H.} = \frac{1000S - 1000}{0.29}$$

It was derived by assigning a value of 0 to water and 100 to milk with specific gravity of 1.029 (considered as the minimum).

$$^{\circ}\text{N.B.H.} \times 0.29 = {}^{\circ}Q$$

Both of these types of lactometers are calibrated for specific gravity at 60° F./60° F.

Specific Gravity of Milk

An extreme range of 1.0135 to 1.0510 has been reported for the specific gravity at 60° F./60° F. of fresh whole milk from individual cows. Usually the range for normal milk is much narrower than this. Table 5 gives results from milk from cows of various breeds. The

Table 5. SPECIFIC GRAVITY OF MILK FROM COWS OF VARIOUS BREEDS

Breed	No. of Cows	No. of Samples	Specific Gravity at 20° C./20° C. Range	Mean	Standard Deviation
Ayrshire	14	208	1.0231–1.0357	1.0317	0.0022
Brown Swiss	17	428	1.0270–1.0366	1.0318	0.0016
Guernsey	16	321	1.0274–1.0398	1.0336	0.0018
Holstein	19	268	1.0268–1.0385	1.0324	0.0018
Jersey	15	199	1.0240–1.0369	1.0330	0.0024

Data of Overman, et al. (29).

range for mixed herd milk seldom lies outside the range of 1.030 to 1.035. An average value often quoted is 1.0320 or 1.0325.

The density of milk varies inversely with the temperature. Primarily this effect represents a change in the density of water, which is

the major constituent of milk. However, Whitaker, Sherman, and Sharp (44) showed (see Fig. 7) that the density of skimmilk relative to that of water declines slightly as the temperature is raised, particularly in the range from 5° to 40° C. Consequently the specific gravity (referred in every case to water at the same temperature) declines slightly over this range. This phenomenon may result from a slight change in the amount of water bound by the protein. Lactose seems not to be involved since the specific gravity of a 5% lactose solution remains constant over this temperature range.

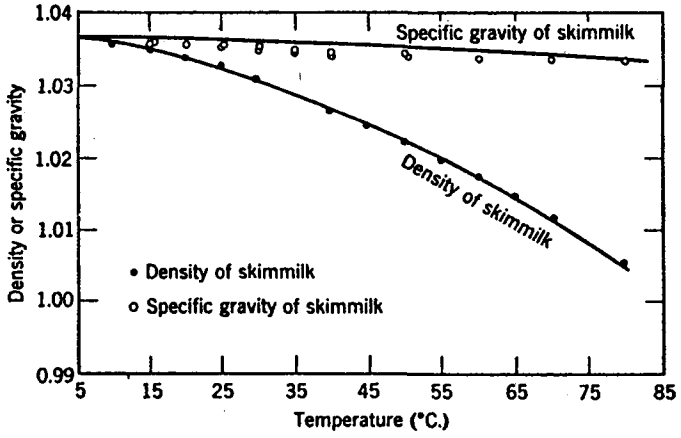


Fig. 7. Relation of density and specific gravity of skimmilk to temperature. Data of Whitaker, Sherman, and Sharp (44).

The coefficient of expansion of water increases with temperature. It is, for example, of the order of 0.00015 ml./ml./° C. in the range 10° to 20° C. and 0.00055 ml./ml./° C. in the range 60° to 70° C. The coefficient of expansion of milk fat is considerably greater than that of water, and relatively constant; over the temperature range of 0° to 60° C. it is about 0.0008 ml./ml./° C. Milk fat expands by about 0.045 ml./ml. when it melts. Therefore, the density of whole milk changes more than that of skimmilk for a given change in temperature, and the change is greater the higher the fat content. Complications occur when density is measured in the temperature range in which the fat is partially solid and partially liquid. It has long been known that the specific gravity of milk increases slowly when it is held after milking. This effect continues after the gas bubbles have escaped and

thus is not due to loss of entrapped gas. At 15° C. the increase may continue for one or two days. At 5° C. or lower the increase is completed in about 6 hours. It amounts to about 0.001. This increase in specific gravity is known as Recknagel's phenomenon, after the man who observed it in 1883. Although Recknagel himself ascribed it to a change in hydration of casein, it has subsequently been shown to arise from slow solidification of the fat. In Chapter 9 it is pointed out that fat solidification is a slow process and that the degree of solidification depends not only on the temperature but also on the previous temperature history of the sample. It is hardly rational to determine specific gravity at room temperature (15° C., 20° C., or 25° C.) where the fat may be solid to an unknown extent. This situation is well illustrated by the data in Table 6. When the measurement was made at 30° C. the fat was all or nearly all liquid and nearly identical values were obtained in the warmed and cooled samples. At 15° C., however, a much lower value was obtained for the sample cooled to temperature (more liquid fat) than in that warmed to temperature (more solid fat). A recent recommendation is to measure density at 102° F. (38.9° C.) in order to insure that all of the fat is in a single state.

Water has long been known to exhibit a maximum density at about 4 C., but the temperature of maximum density of milk has been in doubt. Recent work (45) has demonstrated that pasteurized homogenized whole milk has a maximum density at about —5.20° C. Of course, it is in a supercooled state at this temperature.

Relation of Specific Gravity to Composition

It is obvious that the specific gravity of milk is the resultant of the specific gravities of its constituents.

If S = specific gravity of milk,
N = specific gravity of mixture of solids-not-fat,
M = specific gravity of fat,
T = percentage of total solids by weight,
F = percentage of fat by weight,
$T - F$ = percentage of solids-not-fat by weight,
$100 - T$ = percentage of water by weight,

then
$$S = \frac{F + (T - F) + (100 - T)}{\frac{F}{M} + \frac{(T - F)}{N} + \frac{100 - T}{1}}$$

or $$S = \frac{100MN}{NF + M(T - F) + MN(100 - T)}$$

This is a general equation which can be solved for any one of the three factors, specific gravity, fat, or total solids, if the other two are known and if values for M and N are known and constant. It is the basis for a number of equations for calculating the total solids from the fat content and the specific gravity. When rearranged and solved for T, it becomes:

$$T = \frac{N - M}{M(N - 1)} F + \frac{N}{N - 1}\left(\frac{100S - 100}{S}\right)$$

Fleischmann substituted values of 0.93 for M and 1.6007 for N, which gives the following:

$$T = 1.2F + 2.665\left(\frac{100S - 100}{S}\right)$$

Then in terms of lactometer degrees, $L = 1000S - 1000$,

$$T = 1.2F + 2.665\frac{0.1L + 100 - 100}{S}$$

or $$T = 1.2F + 0.2665\frac{L}{S}$$

Babcock simplified this equation still further and wrote the following very well-known equation:

$$T = 1.2F + 0.25L$$

A great many different equations of this general type have been suggested by various workers since the time of Fleischmann and Babcock. Many of the modifications have involved simply substituting different coefficients and adding or subtracting arbitrary constants in an effort to make the equations fit a series of experimentally determined values. Nevertheless, it has been a rather common observation that any given equation is applicable only to the data from which it was derived and does not fit other data.

One major difficulty with the use of these equations for calculating the total solids content is that the specific gravity of the fat is not a constant particularly at temperatures of measurement where it is partially

solid and partially liquid, and where the Recknagel phenomenon may be occurring (see Table 6). Variations in specific gravity of the solids-not-fat are small and probably do not materially influence the accuracy of the equations. Sharp and Hart (32) recommended measuring the

Table 6. EFFECT OF TEMPERATURE ADJUSTMENTS ON SPECIFIC GRAVITY OF MILK

Treatment	Specific Gravity at 15°/15° C. or 30°/30° C.
Held 24 hr. at 2° C.; warmed to 15° C.	1.03236
Held ½ min. at 45° C., cooled to 15° C.	1.03134
Difference	0.00102
Held 24 hr. at 2° C.; warmed to 30° C.	1.03008
Held ½ min. at 45° C.; cooled to 30° C.	1.02998
Difference	0.00010

Data of Sharp and Hart (32).

specific gravity after heating the milk to 45° C. for 30 seconds and cooling to 30° C. This insures that the fat is entirely liquid. On the basis of their data for the specific gravity of fat and solids-not-fat at 30° C. they derived the following formula:

$$T = 1.2537F + 0.2680L/S$$

Herrington (21) very pertinently pointed out that the Sharp and Hart equation was derived for and applies to values of L and S obtained at 30° C./30° C. If the Quevenne lactometer which is calibrated at 60° F./60° F. is used, then allowance must be made for the difference in density of water at 30° C. and 60° F. and for the expansion of the glass between 60° F. and 30° C. When this is done the equation becomes:

$$T = 1.2537F + \frac{268(Q + 3)}{Q + 1000}$$

A lactometer for reading at 102° F. has been proposed recently (43). At this temperature the equation is:

$$T = 1.33F + \frac{273L}{L + 1000} - 0.40$$

It appears that the methods involving measurement of specific gravity at 30° C. or 102° F. and the appropriate formula give reasonably satisfactory estimations of the percentage of total solids.

Specific Gravity of Dairy Products

The specific gravity of cream decreases regularly with increase in fat content. Data for specific gravity at 35° C./4° C. are given in Fig. 8 for creams up to 40% fat. Skimmilk has a specific gravity of 1.0320 to 1.0365 at 15° C./15° C.

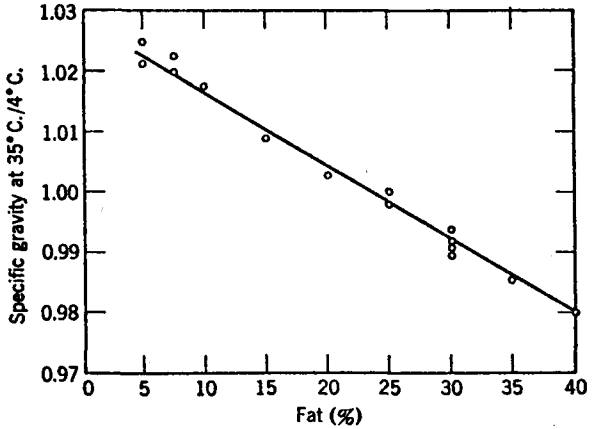


Fig. 8. Specific gravity of milk and cream as a function of fat content. Data of Bearce (6).

The removal of water in the manufacture of concentrated milk products raises the specific gravity. In fact, specific gravity measurements are universally employed to control the composition of such products. Baumé hydrometers are widely used for this purpose. The Baumé reading is related to specific gravity as follows:

$$\text{Specific gravity at } 60° \text{ F.}/60° \text{ F.} = \frac{145}{145 - \text{Bé}}$$

where Bé = Baumé scale reading at 60° F.

Because the high viscosity of some of the concentrated milk products makes readings at 60° F. uncertain, the Baumé reading is often taken at 120° F. Table 7 gives information on composition and specific gravity of some standard concentrated products.

Table 7. SPECIFIC GRAVITY AND COMPOSITION OF CONCENTRATED MILK PRODUCTS

Product	Fat, %	Solids Not Fat, %	Sucrose, %	Total Solids, %	Specific Gravity At 60° F.	Specific Gravity At 120° F.	Baumé Reading At 60° F.	Baumé Reading At 120° F.
Evaporated whole milk	7.9	18.0	...	25.9	1.0662	.0518	9.0	7.14
Sweetened condensed whole milk	8.0	20.0	44.5	72.5	...	1.288	...	32.3
Sweetened condensed skim-milk	0.5	27.5	42	70	...	1.325	...	35.7

VISCOSITY

The viscosity of various fluid dairy products and, perhaps to a lesser extent, of milk itself is of practical importance. In many instances "richness" in the eyes of the consumer is indicated by the viscosity of a product. On the other hand, consumer taste in the matter of viscosity may show great variation. For example, some sections of the United States prefer a highly viscous chocolate milk, whereas others desire a relatively "thin" product. Viscosity is also very meaningful in a practical way to the processor. In the case of evaporated milk, an optimum viscosity lies between the extremes of gelation and a thinness that permits rising of the fat. In ice cream mix, undue viscosity results in losses of product because of adherence to equipment. The viscosity of table cream and buttermilk are obvious considerations in their acceptance by the consuming public. These limited examples suffice to establish the importance of viscosity in milk processing.

Definition and Principles

Viscosity has been defined by some simply as the resistance of liquids to flow or pour. More specifically, it depends on internal friction within a liquid and the relation between kinetic motion and free surface. Viscosity may be measured in three ways: (*a*) by time of flow under a fixed pressure such as with an Ostwald pipette, (*b*) by measuring the force required to move two layers of liquid past each

other as with a MacMichael viscosimeter, and (*c*) by measuring the fall of a ball through a column of liquid as is accomplished with falling-ball viscosimeters.

Viscosity may be measured in absolute or relative terms. The absolute unit of measurement is the poise,* named after Poiseuille. The centipoise is also commonly used and represents one one-hundredth of a poise. Relative viscosity is ordinarily measured in terms of the rate of flow of a liquid. This may be stated in terms of volume during a fixed period of time, or time for a fixed volume to flow under specified conditions. The absolute viscosity of water at 20° C. is equal to 1.005 centipoises. A useful definition of the centipoise then is that viscosity exhibited by water at 20° C.

In addition to viscosity, such terms as *apparent viscosity* and *plasticity* also are used in conjunction with milk and its products. Apparent viscosity is ordinarily met in concentrated fluid milk products, particularly in ice cream mix. It refers to a thickened condition of the product which can be dispelled by agitation. It results from the formation of gel structure in the medium. Plasticity is ordinarily differentiated from viscosity on the basis of the force necessary to cause flow. Liquids of substantial fluidity start to flow and continue to do so by the driving force of their own weight. However, certain relatively non-fluid substances will only start to flow after application of external pressure. These substances are said to exhibit plastic flow. Curves denoting the differences between these types of flow are shown in Fig. 9. Curve *A* shows true viscous flow which is a straight line function of the force applied; curve *B* shows the effect of apparent viscosity on viscous flow in which the internal structure is being broken down in the process; and curve *C* illustrates plastic flow in which an initial outside force, represented by *f* and known as yield value, is required to initiate flow.

Viscosity of Milk

The viscosity of milk is a complex property. Extensive studies have indicated that normal milk falls within the range of 1.5 to 2.0 centipoises at 20° C. However, close agreement between investigators for absolute viscosity of milk has not been achieved. This problem has

* It is the force of one dyne acting on an area of one square centimeter between two parallel planes one centimeter apart, to produce a difference in flow rate between the planes of one centimeter per second.

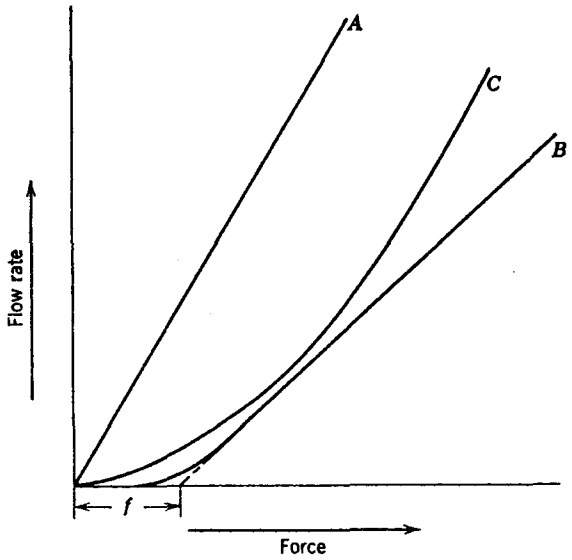


Fig. 9. Flow curves for substances exhibiting viscosity (*A*), plasticity (*B*), and apparent viscosity (*C*).

been carefully evaluated by Cox (11), who has concluded that viscosity of whole milk cannot usefully be regarded as independent of the experimental circumstances in which the observations are made. Various reasons for these difficulties may become evident in the following discussion of factors affecting the viscosity of milk.

In normal fluid milk the following factors appear to influence the viscosity: state and concentration of the protein, state and concentration of the fat, temperature of milk, and age of the milk. As shown in Table 8, the viscosity of a 5% lactose solution at a given temperature is not much greater than that of water, but skimmilk has an appreciable viscosity approaching that of whole milk. The difference in viscosity between 5% lactose and skimmilk indicates the role of importance played by the milk proteins. Figure 10 also reveals the role of various milk fractions in the viscosity of skimmilk at various temperatures. These data confirm those of Table 8 and show the relatively small contributions by lactose, the whey proteins, and milk salts, and the substantial contribution by casein alone (difference between curves for skimmilk and rennet whey). The increase in viscosity that results from increasing total solids of skimmilk by concentration is illustrated in Fig. 11.

Table 8. THE VISCOSITY OF MILK AND RELATED MEDIA AT VARIOUS TEMPERATURES

Temperature, ° C.	Whole Milk*	Skim-milk†	5% Lactose†	Water†
5	3.254	3.96	1.76	1.519
10	2.809	2.47	1.50	1.308
15	2.463	2.10	...	1.140
20	2.127	1.79	1.15	1.005
25	1.857	1.54	1.03	0.894
30	1.640	1.33	0.91	0.801
35	...	1.17	...	0.723
40	...	1.04	0.74	0.656
45	...	0.93	...	0.599
50	...	0.85	0.62	0.549
55	...	0.77	...	0.506
60	...	0.71	0.52	0.469
65	...	0.66	...	0.436
70	...	0.62	0.45	0.406
75	...	0.59	...	0.380
80	...	0.57	0.39	0.357

* Data by Soxhlet (37).
† Data by Whitaker et al. (44).

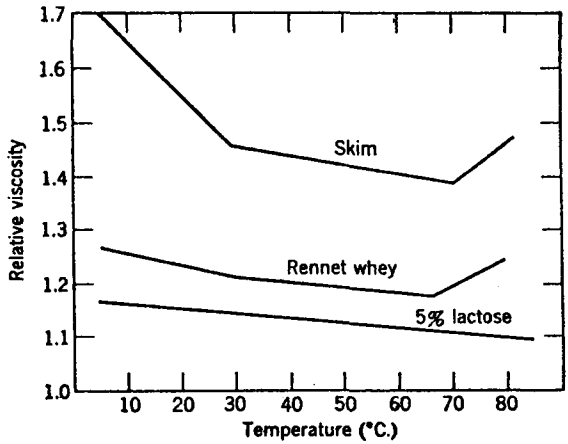


Fig. 10. Viscosity of skimmilk, rennet whey, and 5% lactose relative to that of water at various temperatures. Data of Eilers et al. (15).

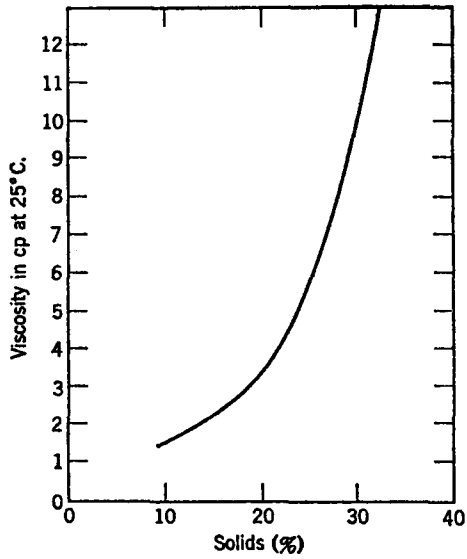


Fig. 11. Effect of concentration of skimmilk on viscosity. Data of Eilers (15).

The tendency of milk to increase in viscosity upon heating as it approaches the point of coagulation of the proteins is a well-known fact (see Fig. 10) and is the basis for producing high viscosity in superheated condensed milk. Conditions and treatments that affect the stability of casein are very significant in the viscosity of milk. Some such factors are acidity, salt balance, heat treatment, and the action of various enzymes and bacteria.

The effect of milk fat on the viscosity of whole milk does not appear to be as great as that of casein. It is significant, however, and will depend on such factors as the amount of fat, the size of the globules, and the extent of clustering of the globules. These factors all influence the degree of internal friction that suspended particles contribute during flow. Clustering of the globules is probably the most important, since numerous large irregular clusters afford the greatest internal friction. The importance of the clustering phenomenon (see Chapter 9) in the viscosity of cream is confirming evidence.

Much of the interest in the contribution of milk fat to viscosity has centered in studies of homogenized milk. This subject has been reviewed by Trout (40), and the effects of homogenization on the viscosity of milk has been studied more recently by Whitnah et al. (46, 47). Although the results of these studies on homogenized milk have

yielded conflicting opinions regarding the importance of milk fat, the bulk of evidence indicates that homogenization does increase the viscosity of milk, and that as pressure is increased viscosity increases in a linear manner. Increases in the amount of fat surface, the amount of protein bound by the fat particles, and the degree of clustering and clumping of the fat are undoubtedly factors contributing to the increased viscosity of milk upon homogenization. The level of fat in the milk undergoing homogenization and the temperature of homogenization are critical factors in this connection. Viscosity to an undesirable degree can be promoted by homogenizing milks of relatively high fat content (5 to 6%) after cooling (to 80° F.).

The viscosity of pasteurized milk, both homogenized and unhomogenized, increases with age. The increase as noted by Whitnah et al. (46) for a sample of commercially homogenized (2,500 p.s.i.) milk was observed to be a linear function of the logarithm of storage time, Fig. 12. The cause of this increase is not known with certainty, but recovery from the loss of viscosity resulting from pasteurization probably contributes. Fat clustering, equilibration of the milk salts, and enzymes and bacterial activity also may be important in this phenomenon.

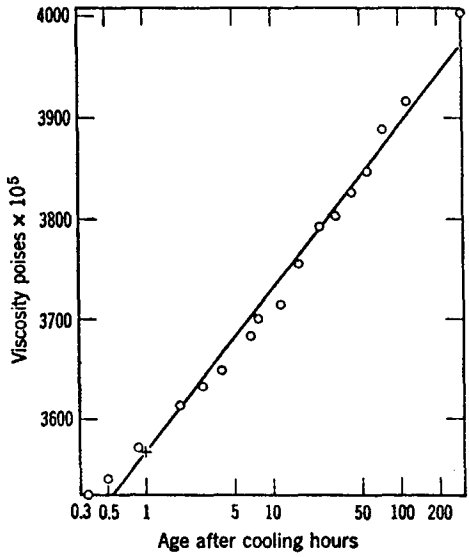


Fig. 12. Changes in viscosity of homogenized milk with age. Samples stored and measured at 4° C. Data of Whitnah et al. (46).

SURFACE TENSION

General Principles

At an interface between liquid and gas, such as water and air, the molecules of liquid in the surface are subject to forces of attraction sideways and downward but not upward. Molecules in the interior of the liquid are acted on by mutual attractive forces in all directions. The cohesion or tension of molecules in the surface, resulting from the imbalance of forces acting on them, causes the surface to act as though covered with a film or skin. This phenomenon is called surface tension. Surface tension often is classically demonstrated by carefully placing a needle on the surface of water. Although the needle is of much greater density than the water and will rapidly sink to the bottom once it has penetrated through the surface at any point, careful horizontal positioning will enable the force of surface tension to keep the needle afloat. Surface tension is usually expressed in dynes per centimeter (dynes/cm.), which is an expression of the force acting along a linear component of surface. Surface tension is the intensity factor in surface energy whereas area is the capacity factor, thus, surface energy equals surface tension times area. By virtue of this fact, it can be seen that extremely fine emulsions may have a great degree of surface energy because of their large surface area. The physical chemistry of surfaces is a field of study that has undergone tremendous development in recent years as a result of the work of Langmuir and others. Surface and interfacial tension, adsorption at an interface, the formation of emulsions, and the energy relations involved are all surface phenomena of very great importance in milk and its products. These subjects are treated incidentally at various points in this text in connection with such subjects as the fat globule membrane and creaming phenomena. Students of dairy chemistry are urged to consult more detailed treatment of the subject, such as that given by Gortner et al. (18) and Bull (7).

Measurement. There are several methods of measuring surface tension, including those based on the following principles: (*a*) the force required to pull a metal ring free from the surface of a liquid; (*b*) the number of drops formed when a given amount of liquid is allowed to fall from a pipette; and (*c*) the pressure required to force a bubble through a nozzle immersed in the liquid. The first of these methods is by far the one most commonly used for milk and fluid dairy

products. For this purpose, variations of an instrument known as the DuNouy tensiometer are used. In measuring the surface tension of milk, the critical discussion of this matter presented by Herrington (22) is pertinent. Reproducible results on a given sample of milk are difficult to secure, not only between laboratories but in the same laboratory. Briefly summarized, Herrington's discussion points up the following as critical variables in surface tension measurement: the temperature history of the milk, age of the milk, time required for measurement, and correction factors required for individual instruments.

The surface tension of milk. The surface tension of milk is influenced by a number of factors and it is difficult to specify with any certainty an average value. Values ordinarily fall within the extremes of 40 to 60 dynes/cm. The mid-point of this range probably represents a reasonable approximation of the average value as determined for milk at 20° C. This may be compared with water which has a surface tension of 72.75 dynes/cm. at that temperature. The surface tension of water is to some degree an upper limit, since there are many substances which when added to water will lower its surface tension, but very few which will increase the value appreciably. It is a well-known law that substances tend to concentrate in the surface in proportion to (*a*) their ability to depress surface tension, and (*b*) their concentration. Salts and sugars do not tend to accumulate in the surface because they do not lower the surface tension. The important surface tension depressants of milk are the proteins and lipides. In the senior author's laboratory, the surface tensions of a number of fractions all from a single original lot of milk were as follows: rennet whey, 51–52; skimmilk, 52–52.5; whole milk, 46–47.5; 25% cream, 42–45; and sweet cream buttermilk, 39–40 dynes per cm. The proteins of whey are effective depressants. Although casein alone is capable of reducing surface tension, its presence in skimmilk does not further reduce it below the level attained with the whey proteins alone. The fat globules in milk and cream (as well as traces of free fat on the surface) serve to reduce surface tension significantly below that of skimmilk. The fat globule membrane materials released from the fat globules during churning are exceedingly surface active and are responsible for the very low surface tension of buttermilk.

The surface tension of water varies with temperature from 75.6 at 0° C. to 66.2 at 60° C. Milk, skimmilk, and cream exhibit a change of comparable magnitude with temperature.

The surface tension of milk is influenced also by fat content, lipolysis, and the addition of water. Data by Dunkley (14), shown

in Fig. 13, illustrate the effects of age, fat content, and lipolysis in lowering the surface tension of milk. The fatty acids released by lipolysis are very effective depressants of surface tension.

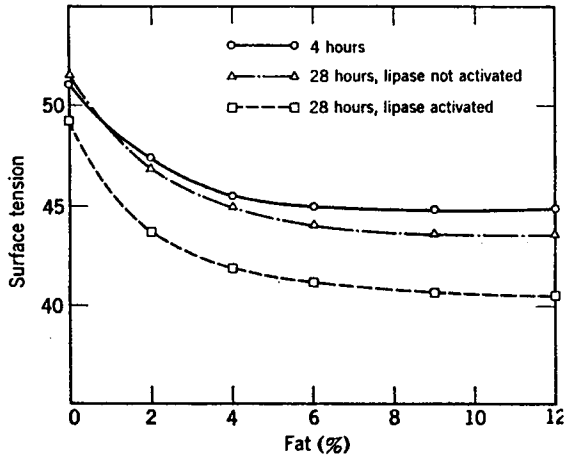


Fig. 13. The effect of fat contents, age, and lipase activity on the surface tension of milk (dynes/centimeter, 20° C.). Data of Dunkley (14).

The fact that lipase action results in a lowering of surface tension has led to some use of this analysis as an indicator of lipolysis. However, the fact that surface tension of milk is influenced by so many additional factors and that reproducible surface tension values are very difficult to obtain has favored measurement of free fat acidity for the purpose. Furthermore, as pointed out by Herrington (22), butyric acid is principally responsible for the rancid flavor resulting from lipase action, but this acid has very little effect on the surface tension of milk. It seems probable that acids with longer chain lengths, particularly caprylic and capric, and possibly mono- and diglycerides are responsible for lowering of surface tension in this instance.

REFRACTIVE INDEX

General Principles

When light passes at an oblique angle from a less dense medium such as air into a more dense medium such as water, it is bent or

refracted. The magnitude of the bending, expressed as a ratio of the sines of the angles of incidence and refraction of the light, is the refractive index. It is designated by the letter n.

$$n = \frac{\sin i}{\sin r}$$

where i = angle of incidence,
r = angle of refraction.

The refractive index of a pure liquid is a characteristic constant for that liquid, under specified conditions of temperature and wavelength of light. The refractive index of water at 20° C. with the D line of the sodium spectrum (589.3 mμ) is $n_D^{20} = 1.33299$. The refractive index of liquids containing dissolved material, such as milk, is increased over that of water to an extent determined by the number and kind of molecules of dissolved material. Thus it is evident that measurement of refractive index can be useful in determining the total solids content of foods. Since food components that are not in solution have little or no effect on refractive index, the term *total soluble solids* is frequently used in the food industry to denote the type of food solids that can be measured by refractive index. One of the most important uses of refractometry in this connection is in the analysis of syrups, jams, and jellies for sugar content.

Two types of refractometers, the Abbé and the immersion, are commonly used. The design and operation of these instruments is discussed at length in most texts on food analysis (39, 49). Both types have been used in investigations of milk and milk products, but because of its greater sensitivity within an appropriate narrow range the immersion refractometer has been most generally employed for milk (13).

The refractive index of milk. Since milk contains material which ranges in dispersion from that truly dissolved to colloidal material which is opaque to the transmission of light, a problem is posed in the determination of refractive index. Efforts to observe refraction on the medium as such are hampered by the effects of colloidal material in rendering the line dividing the fields in the refractometer indistinct. Therefore, the fat at least must be removed. Observations on the nonfat phase of milk yield values for refractive index generally falling within the range $n_D^{20} = 1.3440$ to 1.3480. In order to secure sharper division of the two fields within the refractometer, various means have been employed to secure a clear serum through the removal of the casein. These include natural souring and addition of such coagulants

as acetic acid and copper sulfate solution, among others. It will be clear that the particular agent used has considerable bearing on the magnitude of the refractive index obtained on the milk serum. Thus, such results are only comparable within studies employing one specified technique for securing the milk serum.

There have been few published studies on the refractive index of milk itself. However, Ramakrishnan and Banerjee (30), using an Abbé refractometer, measured refractive index on skimmilks, both individual and in bulk, from Indian cows. Individual results ranged from n_D^{20} = 1.3449 to 1.3480. Bulk milks ranged from n_D^{20} 1.3449 to 1.3477. Seasonal and geographical variations also were observed. Another Indian worker, Rangappa (31), determined the refractive index of milk and its rennet serum and ultrafiltrate. From these data he calculated that in a series of five samples having n_D^{20} = 1.3485 to 1.3506 and containing 5.5 to 6.6% fat and 14.3 to 15.2% solids-not-fat, the individual constituents made the following contributions to the refractive index n_D^{20}: casein, 0.0049–0.0060; serum proteins, 0.0021–0.0035; lactose, 0.0063–0.0067; and miscellaneous constituents, 0.0013–0.0022. Fat contributed nothing.

As shown in Fig. 14, the Abbé refractometer may be used to give reasonably satisfactory approximation of total solids content with a given sample of condensed skimmilk. This figure shows that total

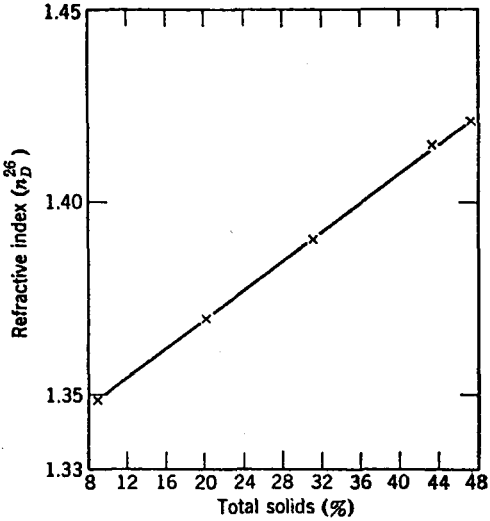


Fig. 14. The relation between total solids and refractive index in a sample of skimmilk.

solids is a straight line function of refractive index for the particular sample in question. A refractometer also may be used for determining sugar concentration in sweetened condensed milk (24).

FREEZING POINT

General

Certain properties of solutions depend on the number of solute particles (molecules or ions) present. These are known as colligative properties in contrast to constitutive properties which depend on the nature of the solute molecules. Important colligative properties of solutions are the depression of the vapor pressure and freezing point of the solvent, the elevation of the boiling point, and osmotic pressure. Measurement of any of these properties can be used for determining the concentration of solutes in a solution, but insofar as milk is concerned the depression of freezing point is by far of most interest and usefulness.

The freezing point of water is the temperature at which ice and water exist in equilibrium with each other; it is the temperature at which they have the same vapor pressure. The addition of a solute to water lowers the vapor pressure because it hinders the ability of the water molecules to escape from the surface. Thus it happens that ice and solution will be in equilibrium (i.e., have the same vapor pressure) at a lower temperature than pure water and ice. The amount of depression of the freezing point is proportional to the fraction of the total number of molecules which are solute molecules. Thus

$$\Delta T = T_o - T = k \frac{N_2}{N_1 + N_2}$$

where ΔT = freezing point depression,
T_o = freezing point of water,
T = freezing point of solution,
k = constant related to vapor pressure,
N_2 = number of solute molecules,
N_1 = number of solvent (water) molecules.

In dilute solutions N_2 is very small compared to N_1, and consequently

$$\Delta T = k' \frac{N_2}{N_1} = k' \frac{W_2/M_2}{W_1/M_1} = k' \frac{M_1 W_2}{M_2 W_1}$$

where W_1 and M_1 stand respectively for weight and molecular weight of solvent and W_2 and M_2 for weight and molecular weight of solute. In dilute aqueous solutions the freezing point of water is lowered by 1.86° C. for each mole of solute added to 1000 g. of water. Thus

$$\Delta T = \frac{1.86 \times 1000 \times W_2}{M_2 W_1} \qquad \text{(A)}$$

In chemistry, the freezing point depression ΔT is often determined to calculate the molecular weight of the solute M_2. In dairy chemistry, its principal use is to detect the additions of water and to compute its concentration.

Method of Determining Freezing Point

Since the difference between the freezing points of milk and water is only about 0.5° C., and since freezing point is used to calculate the quantity of added water, it is essential that the method of determining freezing point be as precise as possible. Experimental techniques for this purpose have been highly refined. Thermometers are used that can be read to 0.001° C. The technique is to supercool the solution and then seed with ice crystals. The temperature rapidly rises to the freezing point as ice separates out. In the case of pure water, the temperature remains constant until all of the water is frozen. However, when milk is supercooled and seeded the temperature rises to a point of momentary stability, after which it gradually falls. Milk behaves in this manner because the separation of ice crystals causes a concentration of the solutes with a resultant further depression of the freezing point. The momentary maximum observed after seeding is not exactly the "true freezing point" of the milk because a small amount of ice has formed before the maximum is reached. Some investigators have recommended the application of corrections to give the true freezing point, but general practice is to standardize the method, particularly the extent of supercooling, and to report without correction the results thus obtained. The apparatus and technique devised by Hortvet is of good precision and has gained wide acceptance. Details of this method are found in the methods of the AOAC (1).

Freezing Point of Milk

The freezing point of milk is its most constant physical property. The secretory processes of the mammary gland are such that the

osmotic pressure of milk is kept in equilibrium with that in blood. Any depression of the synthesis of lactose is compensated by increases in the concentration of sodium and chloride. It must not be thought, however, that the freezing point of milk is absolutely invariant. It varies somewhat but within very narrow limits. The vast majority of individual cow samples fall between −0.525° and −0.565° C. Actually, few fall outside the range of −0.530° and −0.550° C. The average is close to −0.540° C. Herd and bulk milk will exhibit an even narrower range of variation, unless water has been added either intentionally or accidentally.

The nutrition and environment of the cow and the stage of lactation have but little influence on the freezing point of milk. Colostrum has a slightly lower freezing point than normal milk—of the order of −0.570° to −0.580° C. There are a number of reports in the literature of the effects of feeding regime and water intake on the freezing point of milk. In one instance (4) where cows were housed without access to water at night and consumed large quantities of water during the day, the morning milk had a lower freezing point than the evening milk (−0.566° to −0.555° C. for morning versus −0.531° to −0.526° C. for evening). Differences as large as this are not usually observed, and it seems evident that much more needs to be learned about the effects of practices of feeding and watering cows on the freezing point of milk (26, 34).

Processing operations that do not involve either dilution or concentration of milk have a negligible influence on freezing point. Even rather severe heat treatment is without effect. In a survey of the composition of pasteurized bottled milks in eight cities (12), the freezing points of 135 samples ranged from −0.500° to −0.569° C., 119 of them falling between −0.525° and −0.555° C.

Souring, which involves a net increase in the number of molecules in solution as lactose (and sometimes citrate) is degraded, naturally results in a lowering of the freezing point. The available data on this point vary considerably in the change in freezing point which accompanies a given increase in titratable acidity, but it is of the order of 0.003° C. for every increase of 0.1 meq. of NaOH required to titrate 100 ml. of milk to the phenolphthalein end point. In detecting watering, the freezing point should be determined on unsoured samples for the greatest accuracy.

The freezing point is determined principally by the concentration of small molecules and ions in solution. Materials of high molecular weight contribute a negligible amount because they contain few mole-

cules per gram. Thus, a gram of lactose has about 1000 times the effect of a gram of β-lactoglobulin because its molecular weight is only about 1/1000 as great. Actually it is easy to calculate that lactose is a principal contributor to the freezing point depression of milk. In a milk containing 4.75% lactose and 12.5% total solids (i.e., 87.5% water) the freezing point depression due to lactose is:

$$\Delta T_{\text{lactose}} = \frac{1.86 \times 1000 \times 4.75}{342 \times 87.5} = 0.296^\circ \text{ C.}$$

$$\text{The remainder } 0.540 - 0.296 = 0.244^\circ \text{ C.}$$

is due to the various salts and miscellaneous small molecular weight compounds in solution.

As previously mentioned, the principal utility of freezing point is to assess for watering of milk. Equation A shows that if the solute remains constant in weight and composition, the depression of freezing point varies inversely with the amount of solvent present. Therefore it is simple enough to calculate the percentage of water added to a watered sample if the freezing point is known for the original authentic lot of milk. Unfortunately, in cases of suspected watering the original milk is not usually available and an average value is assumed. The official method of the AOAC assumes a freezing point for normal milk of -0.550° C. and specifies that added water be calculated as follows:

$$\text{Per cent added water} = \frac{0.550 - \Delta T}{0.550} \times 100$$

where ΔT is the freezing point depression of the suspected sample.

A tolerance of 3% is allowed, which is equivalent to specifying a minimum freezing point depression for authentic milk of 0.5335° C. The AOAC formula does not specify whether it refers to weight percentage or to volume percentage. Actually, for calculating the weight percentage of water added the term (100 — % total solids) should be substituted for 100. Thus

$$\text{Percentage added water} = \frac{0.550 - \Delta T}{0.550} \times (100 - \text{T.S.})$$

This is necessary because the freezing point depression varies inversely with the amount of water (100 — T.S.) per unit of solute. When tested on samples to which known percentages of water had been added, this latter equation gave nearly, but not quite, the correct result (13, 16).

The deviation seems to result from the fact that upon dilution small shifts in dissociation of some of the salts occur, with the result that the total number of particles in solution is altered.

For a more complete description of various aspects of the freezing point of milk, the reader is referred to *Richmond's Dairy Chemistry* (13).

ELECTRICAL CONDUCTIVITY

Since milk contains various kinds of ions, it can conduct an electric current. The ability of electrolytes to conduct a current is measured in terms of *specific resistance,* which is defined as the resistance in ohms of a column of solution 1 cm. long and 1 sq. cm. in cross-section. This measurement is made with an ordinary Wheatstone bridge—the electrolyte being contained in a cell between platinum electrodes. The conductivity or specific conductance is the reciprocal of specific resistance. It is expressed in terms of reciprocal ohms (i.e., ohm^{-1} or mhos). The specific conductance is computed from the measured resistance of the cell filled with solution.

$$\text{Specific conductance} = \frac{K}{R}$$

where K = "cell constant," i.e., a proportionality factor previously determined for the given cell with solutions of known specific conductance,

and R = measured resistance in ohms.

The specific conductance of milk is relatively low, being about 0.005 ohm^{-1} for normal whole milk. Rather wide ranges are reported in the literature, for example, from 0.003 to 0.010 ohm^{-1}. Undoubtedly many of the higher values represent milk from mastitic udders wherein the concentration of sodium and chloride is abnormally high. Most normal samples will fall in the range of 0.0040 to 0.0055 ohm^{-1} at 25° C. The principal ions responsible for the conductivity of milk are the sodium, potassium, and chloride ions since they are present in the free state in the greatest concentrations (see Chapter 5). As a matter of fact, a solution of sodium chloride having the same chloride content as milk—about 0.1%—has a conductivity of about 0.003 ohm^{-1}.

The specific conductance of whole milk is lower than that of skim-milk by about 10% (38). One reason for this difference is that the fat globules account for 4 to 5% of the volume of the whole milk,

in effect constituting an inert diluent. The volume occupied by the fat accounts for about half of the difference in conductivity between whole and skimmilk. The remainder may be due to actual obstruction of the flow of ions by the fat globules in whole milk.

The specific conductance increases by about 2%—that is, by about 0.0001 ohm^{-1}—per degree C. rise in temperature in the neighborhood of room temperature. This effect is probably due in part to increased dissociation of the weak electrolytes and in part to greater velocity of the ions as the viscosity of water decreases. In any event, for exacting work the temperature of measurement must be controlled and specified.

The effects of dilution and concentration on the specific conductance of milk are not simple. As milk is diluted conductance falls, but the change is proportionately less than the decrease in solids because the degree of ionization of the salts increases (9). On the other hand, concentration causes an increase in conductance up to a maximum at about 30% solids-not-fat (skimmilk), following which further concentration causes the conductance to decrease. This effect must stem from repression of ionization. The determination of conductivity is not of much value as a means of detecting and estimating added water because of the variability in conductivity of milk itself. It has, however, been employed successfully as an index of added neutralizers (10).

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CHAPTER

9

THE PHYSICAL CHEMISTRY OF MILK FAT GLOBULES

Chapter 2 has presented information on milk fat from the standpoint of fats and oils chemistry. An approach to milk fat that is of equivalent and perhaps greater importance concerns the physical chemistry of milk fat globules, with particular emphasis on the so-called membrane or immediate environment that surrounds these globules. There is hardly a dairy process or product that is not concerned with this phase of dairy chemistry. In this chapter an attempt has been made to present basic information on the subject. Detailed considerations in technology have been omitted since they are thoroughly covered in other textbooks (8, 21, 26, 29).

SIZE AND NUMBER OF FAT GLOBULES IN MILK

The bulk of the lipide material in milk exists in the form of an emulsion of tiny spherical droplets or globules dispersed in the milk plasma. These globules are readily observable with the microscope and can be enumerated and measured by appropriate microscopic techniques. Usually each globule is not measured precisely, but rather arbitrary size classes are established (e.g., 0.5–1.5 μ, 1.5–2.5 μ, 2.5–3.5 μ, etc.),

several hundred globules are observed, and the number or percentage falling into each class is determined. The literature contains a great deal of data on variations in fat globule size associated with breed of cow and produced by various processing operations.

Many texts quote Fleischmann's figures of 0.1 to 22 μ for the range of diameters of the milk fat globules. This wide range undoubtedly includes all or nearly all of the globules found in any milk. Usually, however, few globules over 10 μ in diameter occur in an unprocessed milk. The globules of 0.1 to 1.0 μ in diameter are difficult to measure and seldom are accurately enumerated and measured. Actually, these small globules, though undoubtedly numerous, account for only an insignificant portion of the total fat. Since the volume of a sphere varies as the cube of its diameter, the mere percentage distribution of the number of globules among different size classes gives an incomplete picture of the distribution of fat content. This is best presented as the percentage of the total fat in each size class.

Sometimes the average diameter or the average volume is computed as a means of comparing the size of fat globules in different milks. These are computed as follows:

$$\bar{d} = \frac{\Sigma nd}{N}$$

and

$$\bar{V} = \frac{\Sigma \pi n d^3}{6N}$$

where $\bar{d}$ = weighted average diameter,
$\bar{V}$ = average volume,
n = number of globules in each size class,
N = total number of globules measured,
d = diameter (midpoint of size class).

Obviously, results obtained in this way are approximations which approach the true value more closely as the range within each size class is made narrower and narrower. It must be stressed also that the true mean volume cannot be calculated by multiplying the cube of the mean diameter by $\pi/6$.

It is well known that the distribution of fat globule size is a breed characteristic and that cows of the Channel Islands breeds produce milk containing more of the larger globules than do those of the other breeds. This fact is illustrated in Fig. 1, constructed from data of Campbell (4). The range from the smallest globule to the largest is

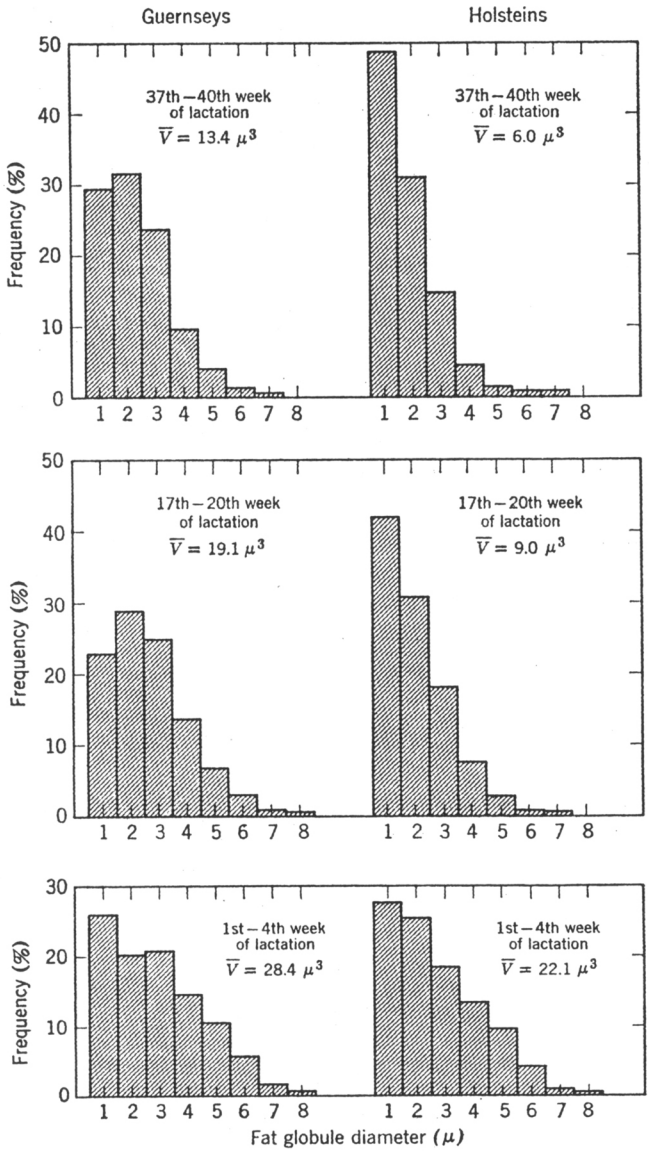

Fig. 1. Combined percentage frequency distributions of fat globule size for 10 Guernsey and 9 Holstein cows at three stages of lactation. Data of Campbell (4).

much the same in all milks, but Guernseys and Jerseys produce higher percentages of globules above 3 μ in diameter.

As lactation advances there is a shift toward smaller globules, as is also shown in Fig. 1. This effect of stage of lactation makes it difficult to determine if feed has any effect on fat globule size. Conflicting results have been reported on this point. There is some evidence that the first milk drawn at a milking contains a higher proportion of small globules and that the distribution shifts slightly toward the larger sizes as milking advances.

The number of fat globules in milk ranges from 1.5 to 3.0 billion globules per cm.^{3}. A given volume of Holstein milk of characteristic fat content and globule size contains about the same number of globules as the same volume of Guernsey milk. Milks do not differ so much in the number of globules as in their size distribution and consequently in the total amount of fat.

The surface area of the fat in milk is, of course, tremendous because of its extreme state of subdivision. Since the volume of a sphere is a function of the cube of the diameter ($\pi d^3/6$) and the surface a function of its square (πd^2), it is obvious that the specific surface (i.e., surface per unit volume) increases as the proportion of small globules increases, and vice versa. The surface-volume ratio is computed by dividing the total surface by the total volume of the globules measured (i.e., $S/V = 6\Sigma nd^2/\Sigma nd^3$). The numerical value of the surface-volume ratio depends on the units in which it is expressed. Thus, if d is expressed in microns (μ), surface in μ^2, and volume in μ^3, S/V has the dimensions of μ^2/μ^3 or reciprocal microns (μ^{-1}).

Campbell reported individual Guernsey and Holstein milks with surface-volume ratios of 1.26 μ^{-1} and 1.61 μ^{-1} respectively. If we assume that the Guernsey milk contained 5% fat and the Holstein milk 3%, we can calculate the total fat surface in these milks.

For Guernsey:

$$5\%\ \text{Fat is equivalent to } \frac{5 \times 1.032 \times 1000}{100 \times 0.90} = 57\ \text{cm.}^3\ \text{of fat per liter}$$

$$\text{Total fat surface} = 57\ \text{cm.}^3 \times 1.26 \times \frac{10^{-8}\ \text{cm.}^2}{10^{-12}\ \text{cm.}^3}$$

$$= 720{,}000\ \text{cm.}^2\ \text{per liter or about 700 sq. ft. per liter}$$

For Holstein:

$$3\%\ \text{Fat is equivalent to } \frac{3 \times 1.032 \times 1000}{100 \times 0.90} = 34\ \text{cm.}^3\ \text{of fat per liter}$$

$$\text{Total fat surface} = 34 \text{ cm.}^3 \times 1.61 \times \frac{10^{-8} \text{ cm.}^2}{10^{-12} \text{ cm.}^3}$$
$$= 550{,}000 \text{ cm.}^2 \text{ per liter}$$

STRUCTURE OF THE GLOBULES

The modern concept of emulsion stabilization holds that emulsions containing any appreciable amount of dispersed phase are stabilized by adsorption of a third phase at the interface. The emulsion of fat globules in milk plasma can be readily seen to be stable, that is to say, the fat does not separate out as such when milk is allowed to stand. To be sure, the fat globules rise to form a layer of cream, but it is readily demonstrated that even in highly concentrated creams the fat is still present in the form of globules. The question of the structure of the fat globules and the identity of the materials that stabilize the natural emulsion is most intriguing. This whole subject is thoroughly reviewed in the monograph by King (15).

A number of methods have been employed to determine the identity of these materials. In the first place, materials adsorbed on the surface of the fat globules will be concentrated in the cream when milk is separated. Comparative analyses of cream and skimmilk have demonstrated that phospholipides and the enzymes xanthine oxidase, aldolase, and alkaline phosphatase, as well as the metals copper and iron, are concentrated on the surface of the fat globules. Secondly, materials that are primarily fat-soluble would, if concentrated in the surface, be expected to be present in a larger concentration in small fat globules than in large ones because of the greater specific surface of the small fat globules. It has been found that the vitamin A and carotene contents of milk fat increase as the surface-volume ratio increases. This finding suggests that these materials are primarily oriented in the surface of the globules. A third method for studying the nature of the materials adsorbed at the surface of the fat globules is commonly known as the washing technique. This process involves repeated dilution of cream with water and reseparation. By this procedure the milk plasma constituents may be reduced to a negligible level. The fat globules of normal milk will withstand a large number of these washing steps without being destabilized and coalescing. It may be objected that any reversibly adsorbed materials will be removed by this process, and the objection is certainly valid. It is also possible that dilution of cream with water may actually favor adsorp-

tion of non-membrane material. Such adsorption might be predicted since water has greater surface tension than skimmilk. Nevertheless, a number of materials are so closely and tightly bound to the fat globules that they remain attached thereto through a series of washing treatments. These closely bound materials may be isolated by churning the washed cream, whereupon they may be recovered from the resulting buttermilk and butter serum. The material thus obtained consists of a mixture of proteins, a mixture of phospholipides, and a high melting triglyceride fraction composed almost entirely of long chain, saturated fatty acids. The organization of the various substances in the surface membrane of the fat globules is not completely understood. What little information is available indicates that it probably is arranged somewhat according to polarity, with the hydrophobic groups oriented primarily to the lipide core of the globule and the hydrophylic groups to the polar aqueous medium. Thus an interface is established with a gradient from one phase to the other. In Fig. 2 is presented a diagram, which is in keeping with this concept of the organization of the membrane. The term *membrane* is perhaps misleading in a sense because it may convey the connotation of a continuous elastic layer or a little sac enclosing the fat. This conception does not appear to be the case. Rather, the membrane seems to consist of a group of materials adsorbed and oriented in the interface between the fat globules and the plasma.

Available data on the concentration of each of the constituents in the membrane is somewhat conflicting. Such findings are not surprising since many of the analyses were made after washing, which undoubtedly removes some of the adsorbed materials. Table 1 gives a general idea of the concentrations of the membrane protein, phospholipides, and iron and copper per 100 g. of fat.

Certain high-melting triglyceride molecules of the milk fat appear to be closely linked to the phospholipides of the membrane. This triglyceride fraction may be isolated primarily from the butter serum after churning. Determination of the quantity of this material is rather arbitrary and empirical since it cannot be separated sharply from the butter fat of residual fat globules and butter granules. Unpublished data of the senior author indicate that 0.2 to 0.4 g. of high melting glycerides* can be isolated per 100 g. of fat.

The carotenoid and vitamin A contents of the fat remaining in skimmilk are both higher than that of the entire whole milk fat. Since

*Portion of lipides of washed cream butter plasma which was insoluble in ethanol at 24° to 25° C.

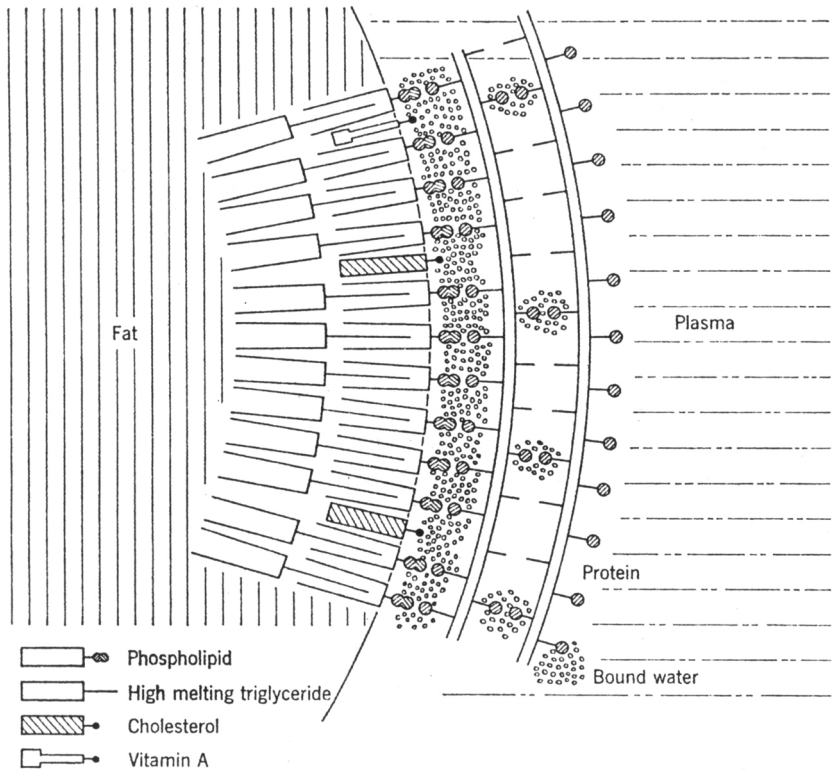

Fig. 2. Physical structure of the fat globule membrane as proposed by King (15).

the fat of skimmilk represents only the smallest of the fat globules, having a higher surface-volume ratio, the reported differences in carotenoid and vitamin A contents imply that these materials are concentrated in the fat globule surface rather than being distributed randomly throughout the globule. White and co-workers (31) further demonstrated a correlation between surface-volume ratio and carotene and vitamin A contents of a number of samples of whole milk. Both by washing experiments and by analyses of skimmilk and cream, the enzymes alkaline phosphatase, xanthine oxidase, and aldolase have been shown to be associated at least in part with the fat globules in milk.

The membrane constituents are spread very thinly over the surface of the fat globules. Thus, Jenness and Palmer (11) calculated that 34 to 49 μg. of protein and 15 to 22 μg. of phospholipide were present

Table 1. CONCENTRATION OF SOME CONSTITUENTS IN THE FAT GLOBULE MEMBRANE

Constituent	Concentration per 100 g. Fat	Method	No. of Samples	Reference
Protein	0.44–2.22 g.	Skim vs. cream	13	1
	0.29–0.82 g.	Skim vs. cream	10	2
	0.81–0.84 g	Protein insoluble in NH_4OH	2	3
	0.46–0.71 g.	Washed cream*	5	4
	0.46–0.86 g.	Washed cream†	19	5
Phospholipides	0.35–0.42 g.	Skim vs. cream	Numerous	6, 7, 8, 9, 10
	0.68 g.	Skim vs. cream	1	11
	0.19–0.35 g.	Washed cream*	5	4
	0.23–0.43 g.	Washed cream†	19	5
Iron	100–150 μg.	Skim vs. cream (centrifugal)	6	12
	200–300 μg.	Skim vs. cream (gravity)	2	13
Copper	40–50 μg.	Skim vs. cream (centrifugal)	2	12
	10–59 μg.	Skim vs. cream (gravity)	12	13

* Washed 4 to 8 times.

† Washed 6 times.

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per 100 sq. cm. of fat surface in cream that has been washed six times. (Such creams are perfectly stable and the fat globules do not coalesce.)

The nature of the individual components in the fat globule membrane and their interrelation with one another are poorly understood. The protein component consists of a number of individual proteins, several of which are enzymes. This protein mixture differs markedly in properties from any of the well-known proteins of milk plasma. In particular the nitrogen content is very low, amounting to only 12 or 13%. Furthermore, some peculiarities in amino acid composition have been noted. It seems futile to speculate to any extent about the composition of this protein mixture until its individual components can be isolated and analyzed individually. Likewise, the phospholipides of the membrane consist of a group of compounds rather than a single entity.

It appears that a complex exists between the protein and the phospholipide in the membrane, because the phospholipide cannot be removed by treatment with non-polar solvents such as ether or petroleum ether. The complex can be disrupted with alcohols, a feature which can be used to advantage in recovering the milk lipides and in preparing certain of the enzymes associated with the membrane. The lipoprotein complex is probably held together by physical or secondary valence-type forces.

Recently Morton (18) has presented evidence that the lipoprotein complex of the globule membrane exists as particulate matter in the form of *microsomes*. Microsomes may be defined as submicroscopic lipoprotein particles obtained from dispersions of animal tissue. The isolation of these microsomes was achieved by their concentration into the cream layer of milk by centrifuging, by washing the fat globules of the cream, and then releasing the lipoprotein particles from the globules into the washed cream buttermilk by churning. Final concentration of the microsomes in the washed cream buttermilk was accomplished by high-speed centrifuging. These particles were observed to range in size from less than 30 to 200 mμ. They are brown in color and contain about 22% of total lipide material, which is largely phospholipide. They also contain nucleic acid and a hemochromogen (probably cytochrome C) and display alkaline phosphatase, xanthine oxidase, diaphorase, and diphosphopyridine nucleotide-cytochrome C reductase activity. The microsomes of milk were observed to resemble closely those recovered from mammary gland tissue, and it is postulated that the tissue microsomes may pass into the milk in the secretion process.

It is not clear at this juncture what the relations are between these observations on lipoprotein particles and the findings of the many earlier investigations on fat globule membrane material. It is notable, however, that the milk microsomes of Morton have a distinct affinity for the fat globule surface and that they contain many of the components that have been demonstrated in the fat globule membrane, including phospholipides, proteins, xanthine oxidase, alkaline phosphatase, and a red pigment. This is to be expected since these particles were isolated by the classic method of Palmer which has been the basis for recovering membrane material in most investigations. The significance of Morton's findings appears to lie in the fact that membrane material exists in organized units that probably have metabolic significance. No doubt subsequent investigations will do much to clarify the possible significance of such lipoprotein particles in milk.

It is important to emphasize that the fat globule surface is undoubtedly in equilibrium with the plasma to some extent. Some of the membrane constituents are found in skimmilk. Morton even found that skimmilk contains some microsomes, although most of these particles are carried with the fat globules. It has already been pointed out that the membrane isolated from washed cream is comprised of those materials that are so tightly adsorbed that they are not released or are only sluggishly released during the washing process. All readily reversible material is removed. Furthermore, there is evidence that some of the membrane constituents are shifted from the interface to the plasma, and vice versa, with fluctuations in temperature and with agitation. This point indicates the delicate and complex physical balance in the milk system and is undoubtedly responsible for some of the variations in analytical results.

THE CREAMING PROCESS

After being drawn from the cow, normal milk will form a cream layer on standing. This layer may be evident in as little as 20 or 30 minutes. However, complete formation of the layer may require at least several hours. The presence of a cream layer on milk may be important from the consumer standpoint, but, beyond this, the phenomenon involves properties of fat globules, such as clustering, that are broadly significant in the behavior of milk and various of its products.

Fundamental factors in creaming. The rate of rise of a spherical particle in a liquid medium is represented by the following equation, which in essence is Stokes' law:

$$V = \frac{2gr^2 (d_1 - d_2)}{9\eta}$$

where V is the velocity of rise in centimeters per second,
r is the radius of the sphere in centimeters,
d_1 is the density of the dispersions medium,
d_2 is the density of the sphere,
g is the force of gravity (981 dynes), and
η is the viscosity of the liquid in poises.

A number of investigations have confirmed that the fat globules of milk rise in close accord with Stokes' law. However, average fat globules of approximately 4μ in diameter would take several hours to rise 1 inch according to the Stokes equation. It is clear that other factors must enter into the phenomenon for a cream layer to appear on milk in as little as 30 minutes. The principal factor that aids the creaming process is clustering of the individual globules. As the milk is drawn from the udder the globules exist largely as separate entities. In the quiescent state, globules in the milk begin to rise. As shown in the equation, the velocity of rise is directly proportional to the square of the radius of the globule. Therefore the larger globules overtake the smaller ones rather quickly. At this point the factor of clustering comes into play. When a large globule comes in contact with a smaller globule it is held there, and the two joined globules rise together at an even more rapid rate primarily because of their greater effective radius. Incidental to their rise they come in contact with other globules until clusters of substantial size are formed, which rise much more rapidly than any of the individual globules. It is true that these clusters do not rise strictly in accord with Stokes' law since they are irregular in shape and contain considerable occluded serum.

It then is logical to ask: What causes clustering? What makes the fat globules stick together? These questions have never been answered in a completely satisfactory manner. The work of Dunkley and Sommer (7) has established that a protein classified as a euglobulin (also known as fat agglutinin) in the milk is essential to the clustering of fat globules and the formation of a normal cream layer. It has not been definitely established that this protein is identical with the euglobulin fraction of the immune proteins discussed in Chapter 4.

The protein that facilitates creaming appears to act in much the same manner as bacterial agglutinins and alters the surface property of the fat globules in some way to make them adhere to one another. That euglobulin of milk is surface active on fat globules has been shown by Sharp and Krukovsky (24) and Dunkley and Sommer. When the milk fat is in a solid state, separation of the milk yields a skimmilk that is euglobulin-poor, whereas separation at 122° F. yields a skimmilk that is euglobulin-rich. The nature of the forces created by deposition of euglobulin on the surface of fat globules which enhances their powers to adhere has not been elaborated. On the assumption that low interfacial tension would be necessary to permit the globules to cluster and high electrokinetic charge would be the force tending to hold them apart, it seems probable that the effect of euglobulin is felt through an alteration of one of these forces. The evidence to date indicates that electrokinetic charge on the fat globules is not of sufficient magnitude to be a significant factor in clustering. Therefore changes in interfacial tension appear to afford the best explanation of the role of euglobulin in fat globule clustering. That surface tension activity is associated with the euglobulin is suggested also by the tendency toward excessive foaming in euglobulin-rich skimmilk.

A distinction between the terms *clusters* and *clumps* should be made at this point, although this distinction is not ordinarily made in technological usage. Sommer differentiated the two as follows: clusters are formed only at low temperatures and are dispersed by warming to 50° C. (122° F.); clumps are formed at elevated temperatures and, once formed, are not easily dispersed. The term *clumps* frequently is employed to describe the fat aggregates that form under certain conditions in homogenization of milk, cream, or ice cream mix (see section following on homogenization).

Factors in the creaming process. So far as variations in the milk itself are concerned, maximum cream layer volume is secured with milks of high fat content and relatively large fat globules. Such milk will contain more clusters of larger size with greater amounts of occluded serum. However, exclusive of these factors, there are two main variables that influence the creaming process. These are temperature treatment and agitation.

In the case of raw milk, the deepest cream layer volume is secured when the milk is cooled promptly and quickly to 50° F. and stored at 40° F. or lower. Cream layer volume in pasteurized milk, equivalent to that obtainable with the raw milk, can be secured if the milk is (*a*) processed without unduly rigorous agitation, (*b*) pasteurized at minimal conditions (143° F. for 30 minutes or 161° F. for 15 seconds),

and (*c*) promptly cooled and held at 35° to 40° F. Excessive heat treatment can irreversibly denature the euglobulin of milk. Thus, as heat treatments of milk are intensified beyond that for conventional pasteurization, progressive loss of cream layer volume occurs (see Fig. 3). Adsorption of euglobulin on the surface of the fat globules

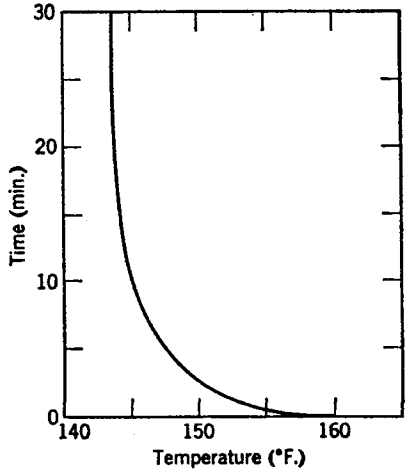

Fig. 3. Curve representing maximum time-temperature treatments to which milk may be subjected without reduction in creaming ability. Data of Whittaker et al. (32).

after pasteurization is favored by rapid cooling to low temperatures. Apparently the adsorption process is favored by having the fat in a solid or semisolid state. Where the cooling process is slow the adsorption of euglobulin does not go to completion, and as a consequence the clusters are small, rise slowly in the milk, and form a shallow, indistinct cream layer. The normal pasteurization process is somewhat like "wiping the slate clean" so far as the creaming process is concerned. Exclusive of agitation so rigorous during processing as to disrupt the normal emulsion of the fat, pasteurization can be considered as a treatment to regain the normal creaming potential of the milk. In the heating process all of the euglobulin is desorbed from the fat globule surface, and on cooling conditions can be made optimum for the readsorption process.

In general, the two principal factors involved in agitation and its effect on cream layer volume are intensity and temperature. As a rule excessive agitation should be avoided because of its disrupting effect on normal cluster formation and the possibility of its denaturing euglobulin. Excessive agitation is to be avoided particularly after the

milk has been pasteurized. Normal agitation during the pasteurization process is without effect on creaming potential for the globules are dispersed rather completely at that time. The creaming process in cold milk may actually be facilitated by mild agitation since such treatment would favor larger clusters which would be more loosely packed. Precise information on this point is lacking.

Cream layer analysis. For reasons of both research and quality control, various analyses of the cream layer of milk may be conducted. In addition to measurement of the cream volume and fat content of the cream layer, analyses to detect certain unethical practices for expanding the cream layer can be made. It is also of interest on occasion to examine the cream layer for bacteria, leucocytes, and foreign matter, since these types of suspended material are swept into the cream layer during the creaming process.

Graduated cylinders of from 100 to 1000 ml. capacity may be used satisfactorily for determining the cream volume of a sample of milk. Immediately after cooling, the milk is placed in the graduated cylinder and then held at the desired temperature for a specified period of time. Usually the holding temperature is 35° to 40° F., and the time period 24 hours. Measurements can also be carried out using quart milk bottles. One method employs a graduated gauge, the contours of which are adapted to the neck of the bottle and which will read volume or per cent volume from the top of the bottle down its side. Such gauges are reasonably accurate for most purposes; but there are substantial variations between bottles regarding the volume in the neck. Another possible source of error in this type of measurement concerns the level to which the bottle is filled. Lacking such a gauge, it is possible merely to mark the level of the cream layer and then determine the volume it occupies by decanting the contents to that level into a measuring device.

The cream volume of milk may vary from 12 to 20% or more depending upon the fat content, globule size distribution pattern, processing, and other factors, as discussed previously. The cream layer in milk can be isolated by allowing the creaming process to proceed in a separatory funnel. By slowly draining off the skimmilk phase, the cream layer can be analyzed for volume as well as fat content. Separation of the two phases can also be accomplished fairly satisfactorily by siphoning off slowly the lower phase in a conventional milk bottle. The fat content of the cream layer of normal milk usually averages 20 to 22%. The fat content of the skimmilk ranges from 0.3 to 0.4%.

Two unethical practices that are used to expand the volume of the cream layer are the addition of homogenized cream or superheated condensed milk. Detection of the addition of homogenized cream is accomplished by microscopic examination. The presence of clumps of small fat globules that are amorphous and abnormal in appearance is indicative of the practice. The effects of adding superheated condensed milk to expand cream layer volume have been studied extensively by Smith and Doan (25). Abnormal ratios of fat to casein and fat to solids-not-fat as well as fat content in the cream layer enable detection of this practice. They suggest that the fat to casein ratio should not be less than 10.0, the fat to solids-not-fat ratio should not be less than 3.00, and the fat content in the cream layer should not be less than 22%.

SEPARATION

As explained in the preceding section on creaming, the fat globules of milk have a normal disposition to separate by rising from the skimmilk phase. This is a relatively slow and inefficient means of recovering cream and the cream separator has proven extremely useful in both hastening the process and increasing its efficiency. Such machines develop force equal to several thousand times that of gravity.

The rate of rise of fat globules in milk through the force of gravity is accurately expressed by the Stokes equation and the force constant (g) for gravity in this equation is 981 dynes. To determine this constant in terms of the acceleration produced in a centrifugal machine, such as a separator, the following equation is used:

$$g = \frac{(2\pi S)^2 R}{(60)^2}$$

in which S is bowl speed in r.p.m. and R is distance of a given globule from the axis of rotation. By using the expression of g in the Stokes equation and reducing the resulting equation to simplest terms, the rate of separation (V) is given as:

$$V = \frac{0.00244(d_1 - d_2)r^2 S^2 R}{\eta}$$

It becomes evident from this equation that the rate of separation of fat globules will increase with increase in r, the radius of the fat globules; S, the speed of the separator; R, the distance from the bowl axis; and with decrease in η, the viscosity of the milk. Temperature

is a factor of related importance since it influences r, η and $(d_1 - d_2)$, as discussed subsequently.

Efficiency of separation. There are a number of factors that influence efficiency of separation or, more particularly, the fat content of the skimmilk and cream. These factors fall into three general groups, namely, (*a*) the design and state of repair of the particular separator, (*b*) certain characteristics of the milk to be separated, and (*c*) the manner in which the separation is carried out. For any given separator in good working order, the relative efficiency in performance is more or less fixed, so that the separator itself does not serve as a variable. With respect to the milk, the principal factors are size of fat globules and fat content. Actually these two factors are related, since higher fat test is closely correlated with larger average globule sizes, mentioned earlier in this chapter. Fat globules of diameter 2 μ and lower are very incompletely recovered by the cream separator. Thus the distribution pattern of globules sizes may have some bearing on the efficiency of separation. The total amount of fat in a given milk will determine the fat content of the cream using a given setting of the cream or skimmilk screw in the separator. This results from the fact that adjustment of these screws (there being either one or the other on a separator) determines a ratio of discharge for the cream and skimmilk fractions. If the machine is so adjusted as to yield five volumes of skimmilk for one volume of cream, this ratio will be constant irrespective of the fat content of the milk going into the separator. Consequently, variations in the fat test of the milk will reflect themselves in the fat test of the cream.

The principal variable of importance in the separating process is temperature of the milk. Temperature bears on the following factors: (*a*) the difference in density between fat globules and the skimmilk phase, (*b*) the viscosity of the milk, and (*c*) the radii of the fat globules. Referring to the equation (p. 279) dealing with velocity of separation (V), increase in temperature tends to increase the difference between density of the globules and their suspending medium $(d_1 - d_2)$, decrease viscosity of the milk (η) and increase the radii of the globules (r). Sharp (22) has evaluated the effect of these temperature sensitive variables in the separating process through measurement of $(d_1 - d_2)$ and η. A value, K, which varies with temperature is thus derived. By inserting it in the equation for velocity of separation, we have:

$$V = Kr^2RS^2$$

It can be seen that the velocity of separation varies directly with the magnitude of K. If K is measured at a series of temperatures and the values are corrected for change in radii of the fat globules due to temperature, the per cent increase in K may be plotted as shown in Fig. 4. This figure reveals that K, and thus efficiency of separation,

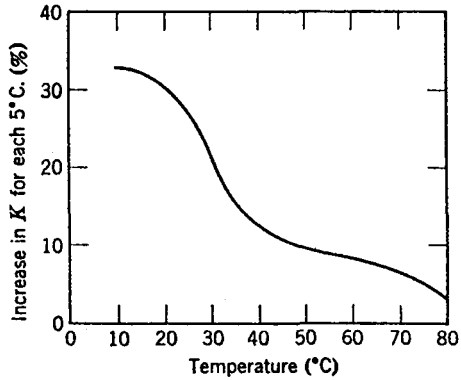

Fig. 4. Increase in the effective force of separation with temperature as represented by per cent increase in K, a factor that evaluates decrease in viscosity, increase in fat globule radii, and increase in density difference between plasma and fat of milk with increase in temperature. Data of Sharp (22).

increase substantially to approximately 35° to 40° C., above which range the percentage of increase becomes smaller and more constant. Sharp has estimated that by increasing the separating temperature of milk from 5° to 40° C. the effective force tending to separate the cream increases 380%, but in going from 40° to 80° C. the increase is only 95%.

In keeping with the findings of Sharp, temperatures of approximately 85° to 90° F. have been used widely in the past for separation of milk. Currently there is a trend towards lower separating temperatures, which, in general, have been made possible through improvements in the design and efficiency of separators. Cold milk separators, which operate at 50° to 60° F. and yield a skimmilk testing 0.01% fat by the Babcock method, are now employed in industry. A problem that is developing in this connection concerns the use of bulk tanks and refrigerated tank trucks in the production and transport of raw milk. As a consequence of these innovations, milk is coming into the plant at much lower average temperatures than was formerly the case.

Thus, if raw milk is separated as received it is well below the 50° to 60° F. temperature optimum of most cold milk separators.

Skimming efficiency is considered to be optimum when the skimmilk tests no more than 0.01% fat by the Babcock method. Such efficiency is possible with most of the available separators provided that they are in good working condition and the milk is separated within recommended temperatures. Fat test of the skimmilk is an obvious means of evaluating separator performance.

The rate at which milk is fed to a separator influences separating efficiency simply because the longer the milk is in the separator the longer it is exposed to the action of the centrifugal force. It can be seen also in this connection that the speed of the separator bowl is involved in a similar manner. The lower the bowl speed, the lower the centrifugal force is, the slower and less complete the separation, and the higher the loss of fat in the skimmilk.

Physical and chemical effects of separation. Although the primary effect of separation is to produce lipide-rich and lipide-poor fractions from milk, depending upon the temperature, several other effects have been noted. These concern foaming properties of the skimmilk, distribution of a globulin fraction of milk, and viscosity of cream. It seems probable that these factors are all related through release of greater amounts of fat globule membrane material into the skimmilk at higher separating temperatures. The work of Dunkley and Sommer (7) and of Sharp and Krukovsky (24) has established that when milk is separated at about 50° F., a substance that promotes clumping and creaming of the fat remains associated with the cream. However, when milk is separated at 120° F. this same substance goes into the skimmilk. By the same token, milks separated under such conditions will show respectively that foaming of the skimmilk and low viscosity of the cream are associated with higher separating temperatures, whereas little tendency to foam of the skimmilk and high viscosity of the cream are associated with the lower separating temperatures. This information may be summarized as follows:

	Separating Temperature	
Variable	50° F.	120° F.
Euglobulin content		
Cream	rich	poor
Skimmilk	poor	rich
Tendency of skim to foam	little	much
Viscosity of cream	high	low

CLARIFICATION

Clarification of milk is practiced primarily to remove material that is considered extraneous or undesirable. This includes dirt and debris which has gained entry to the milk incidental to its production, as well as leucocytes, epithelial cells from the udder, and to some extent bacteria. Actually, both separators and clarifiers accomplish this purpose; however, the principal difference between the two machines is that a clarifier has only one outlet and does not accomplish separation of the milk, and it usually has a bowl designed to accommodate a larger amount of sediment. This is necessary since much larger volumes of milk ordinarily are clarified than are separated. Machines that are designed to perform either clarification or separation have been made available in recent years.

The normal operating speeds of clarifiers, 4000 to 8000 r.p.m., are presumed to be without significant effect on the fat globules of milk. Depending on the temperature conditions, discussed in the section on separation, there may be some release of membrane material into the skimmilk phase as a result of the centrifugal force. However, this matter has received very little study. Skimmilk passed through a supercentrifuge at 25,000 r.p.m. shows as much as 30% of its phospholipides (measured as lipid phosphorus) deposited in the bowl sediment. Even the sediment of clarifiers and separators operated at normal speeds has been reported to contain substantial amounts of phospholipides, as well as certain enzymes (see Chapter 6), and the materials mentioned above.

HOMOGENIZATION

Homogenization is among the several basically important processes of the dairy industry. It was first considered in connection with milk around the turn of the century, but the process did not gain real impetus in the United States until the early 1930's. The fact that milk, cream, ice cream mix, evaporated milk, certain types of cream cheese, and various dry milk products are homogenized during processing gives some indication of the importance of this step.

In essence, homogenization breaks up the already small fat globules of milk into even smaller globules and stabilizes the emulsion to an extent that prevents any noticeable rising (cream layer formation) of

the fat. There are a variety of homogenizers employed by the dairy industry which in general operate on very similar principles. Milk to be homogenized is pumped under pressure of several thousand pounds through a constricted orifice in which process the native fat globules are disintegrated into smaller globules. The various detailed aspects of this processing as it is applied to milk and its products are discussed extensively in most text books of the dairy manufacturing field. In addition, the book by Trout (29) comprehensively treats the subject of homogenized milk and reviews the literature to 1950.

Effects on Milk

Appearance. Homogenization renders milk more opaque and eliminates its tendency to form a cream layer. The appearance is almost chalk-white, although a faint golden yellow character may be observed. When drained from the bottle, properly homogenized milk leaves a clean appearance on the sides. There is little or no indication of streaks or speckiness. A further consideration regarding appearance is the coloring power of homogenized milk and cream in coffee. Homogenization enhances the ability of these products to color coffee, presumably owing to the finer dispersion of the fat and the greater quantity of protein associated with the fat in a homogenized product.

Homogenized milk foams much more readily than unhomogenized milk. The reason for this is not known. A foam promoting factor may be associated with the native fat globule membrane. When milk is separated at high temperatures (100° to 120° F.), material goes into the skimmilk which makes for much greater foaming than is normally encountered in cold separated milk. It is possible that the same factor may be effectively dispersed into the milk plasma by the homogenization process.

Unless homogenized milk is clarified, it may show an unsightly sediment, composed of leucocytes and foreign matter. Normally this material is swept into the cream layer in unhomogenized milk, but in the homogenized product it settles to the bottom. Clarification following homogenization will prevent the defect. Clarification before homogenization does not give as satisfactory results.

Size and disposition of fat globules. Two primary effects of homogenization on milk are a reduction in size of globules and an increase in globule surface area. The data presented in Table 2 reveal the effect of homogenization at various pressures on reduction

Table 2. SIZE OF FAT GLOBULES IN MILK HOMOGENIZED AT VARIOUS PRESSURES *

Authority	Average of Diameters in μ at Homogenization Pressures in Pounds					
	0	500	1500	2500	3750	4500
Buttenberg (1903)					0.80†	
Wiegner (1914)	2.9				0.27	
Marquardt (1927)	3.88			1.56		0.97
Trout et al. (1935)	3.89	2.50	1.91	1.38		0.97
Tracy (1948)	3.71	2.39	1.40	0.99		0.97

* Data compiled by Trout (29).

† Not average diameter but most often observed.

of globule diameter. In general it can be seen from these data that as the homogenizing pressure increases globule size decreases. According to the formula previously presented, the surface per unit volume is related to diameter as follows:

$$S/V = 6\Sigma nd^2/\Sigma nd^3$$

In other words it is inversely proportional to the diameter. In homogenization the number of globules and the total surface increases but the total volume remains constant. Thus, if a given quantity of fat dispersed as uniform globules of 5 μ diameter is broken down to globules of 1 μ diameter the S/V is increased from 1.20 μ^{-1} to 6.00 μ^{-1}, a five-fold increase. Considering a more practical example, let us suppose that the lot of Guernsey milk, described by Campbell (p. 268) and having a value of $S/V = 1.26$, is homogenized to yield uniform globules of 1 μ diameter. The value of S/V is now 6.00 or an increase of about 4.8-fold in surface per unit volume of fat. The total fat surface, assuming 5% milk, has increased from 720,000 cm.^{2} per liter to 3,456,000 cm.^{2} per liter. These calculations are presented merely to indicate the order of magnitude of the increase in surface to be expected upon homogenization. Obviously it is erroneous to attempt to calculate the increase in surface from the mean diameters ($\bar{d} = \Sigma nd/N$) before and after homogenization. The calculation must be made by summing the volumes and areas of the fat globules in each size class. It becomes more precise as the limits of each size class are made narrower.

The changes that occur at the fat serum interface in milk as a result of homogenization are not well understood. It seems certain that the native state of the membrane is substantially altered by

homogenization, but precisely what happens to the native constituents of the fat globule surface is not known. In the case of the new surfaces that are created, some adsorption of material is predictable in order to satisfy the resulting free surface energy. It is stated in the literature that large amounts of casein are adsorbed on the surface of the fat in homogenized milk. Data on this point are so limited as to preclude any conclusions regarding either the quantity or precise identity of the material adsorbed. A study by Brunner et al. (2) suggests that the material is primarily a mixture of milk plasma proteins.

The mere reduction of the fat globules of milk to one-half or one-third their original size does not adequately account for the properties imparted by homogenization. Homogenized milk shows little tendency to form clusters of fat globules which are essential in the normal creaming process of milk. When only the skimmilk phase is homogenized and recombined with unhomogenized cream, creaming is inhibited. This suggests that the agglutinin-like material that promotes clustering of the globules in unhomogenized milk has been denatured in some manner. Further, homogenization has a destabilizing effect on the proteins of milk. This loss of stability is detectable by both alcohol coagulation and heat treatment. Although fat clumping is not normally a problem in homogenized milk it does occur in homogenized creams and "half-and-half" products. Clumping of the fat in these products is to be avoided insofar as possible, since it may result in formation of a cream plug and substantial differences in fat content between the top and bottom of the product. Factors that favor fat clumping in homogenized products are (*a*) single stage homogenization, (*b*) high homogenization pressures, (*c*) low homogenization temperatures, and (*d*) high fat content. Most homogenizers, in one manner or another, enable use of a second stage at relatively lower pressures (500 lb.) to aid in breaking up fat clumps that may have formed during the first stage of homogenization.

Flavor. The subject of flavor has been considered at length elsewhere (Chapter 12). However, there are several aspects that concern homogenized milk in particular and will bear brief mention here. In the minds of many, homogenized milk is a richer tasting beverage than the unhomogenized product. Whether such richness is due to somewhat greater viscosity resulting from homogenization, the more uniform distribution of fat, or some other intangible factor is not known. Homogenization also affects milk with regard to several important flavor defects; namely, those termed "oxidized," "rancid," and "sunlight." For reasons unknown, homogenization renders milk

much less susceptible to copper-induced oxidized flavor. The fact that the fat in homogenized milk seems to be occluded or enmeshed in a much greater quantity of milk protein may have some bearing on this matter. Since copper gains entry to milk through the aqueous phase and must be carried from there to the site of oxidizable lipide material, it is evident that large quantities of adsorbed protein may form an effective barrier toward the copper. Another explanation is that an ordered physicochemical relationship, involving a number of milk components, is necessary for oxidation of phospholipides at the fat serum interphase of unhomogenized milk and the formation of oxidized flavor. When this state is thoroughly disorganized by homogenization the tendency toward the defect is reduced. Further factors in this connection may involve generation of antioxidants or metal ion sequestering agents by homogenization.

The homogenization of raw milk or the contamination of pasteurized homogenized milk with even very limited amounts of raw milk will lead to hydrolytic rancidity and rancid flavor. It is therefore essential that milk lipase be completely inactivated in homogenized milk (see Chapter 6).

Homogenization creates greater susceptibility in milk to the development of sunlight flavor. There are actually two defects involved in this off-flavor; namely, lipide oxidation, and photolytic changes in the milk serum with the production of disagreeable-smelling and tasting sulfur compounds. The literature has not always made clear this distinction. Presumably, homogenization increases the tendency toward both components of the off-flavor. This difference between the homogenized and unhomogenized product, as well as variations related to copper-induced oxidized flavor and stability of the proteins, suggest that there are unknown but discrete chemical changes as well as the more obvious physical changes produced in milk by homogenization.

Curd tension. Homogenization lowers the curd tension of whole milk but does not affect that of skimmilk. The degree of reduction varies between whole milks and optimum results are secured in the range of 2000 to 2500 pounds pressure in the homogenizing process. Two explanations have been offered to account for this reduction in curd tension. One is that the finer dispersion of fat in the homogenized product creates points of weakness in the curd. The other theory contends that the adsorption of substantial amounts of casein to the surface of the fat globules in homogenized milk leaves a considerably diluted serum which cannot form a firm curd. These theories are not necessarily mutually exclusive and the effect may result from a combination of both. The effect of homogenization pressure on the

curd tension of a sample of milk is shown in Fig. 5. This subject has been reviewed comprehensively by Trout (29).

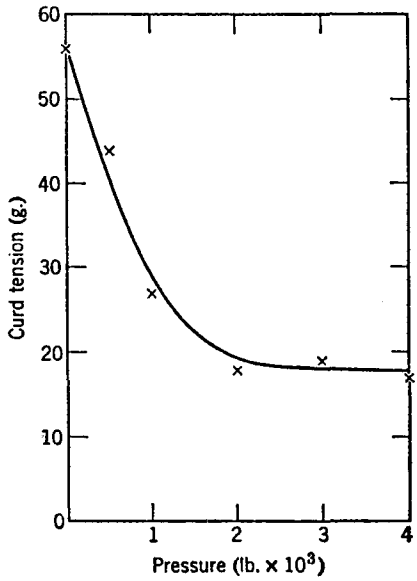

Fig. 5. The effect of homogenization pressure (single stage) on the curd tension of a 4.2% fat milk. Data of Doan (6).

Theories of homogenization. The phenomenon of homogenization has been explained on several theoretical bases: (*a*) shattering by impact, (*b*) explosion on release of pressure, (*c*) shearing between layers of liquid under flow, and (*d*) cavitation. The relative merits of the first three of these have been discussed by Sommer (27). More recently, Loo et al. (16) have presented evidence supporting cavitation as a primary cause of homogenization. Cavitation is perhaps adequately explained as the formation of vapor cavities within the medium due to a substantial drop in pressure as milk leaves the valve clearance followed by collapse of the cavities in the milk as it passes into a region of higher pressure. This collapsing or implosion is visualized as shattering the globules in a similar but reverse manner to that of explosion. Sommer objected to the shattering-by-impact theory on the basis that the milk on passage from the valve does not discharge into free space but into the liquid, which completely fills the valve assembly. In the same vein, he objected to the theory based on explosion through release of pressure since liquids are not very

compressible and there is no free escape space for the liquid. He favored the explanation of homogenization resulting from the shearing action of laminar flow of liquid. However, the probability of considerable turbulent flow is admitted.

There does not appear to be adequate evidence that the valve assembly during homogenization is completely filled with liquid. It seems quite possible that the jet of liquid leaving the valve under high pressure may act in the manner of a water aspirator regarding residual bubbles of air, or air adsorbed on the surface of the metal, causing the formation of vacuoles which would be filled almost instantaneously with water vapor. If these subsequently collapse, some shattering might be expected in the manner proposed by Loo et al. In any event it would appear worth while to attempt securing some evidence on whether a gaseous phase of any sort exists beyond the homogenizer valve, since three of the four proposed theories predict such a state.

Efficiency of homogenization. A widely accepted microscopic method for determining efficiency of homogenization is that known as the Farrall Index. This index may be defined as the number of fat globules 2 μ in diameter which can be obtained from all the fat in a homogenized sample appearing in globules larger than 2 μ, as measured under specified conditions. In this analysis a microscope with 1000× magnification, an ocular micrometer, and dark field illumination are employed to measure the number of globules in a specified amount of diluted milk sample, which fall into various size ranges. The number of globules in five fields falling into the various ranges shown in the following form are noted:

FORM FOR CALCULATING FARRALL INDEX

(Efficiency of homogenization)

Field	Size range of globules in					
	2–2.5	2.5–3	3–4	4–5	5–6	6–7
1	____	____	____	____	____	____
2	____	____	____	____	____	____
3	____	____	____	____	____	____
4	____	____	____	____	____	____
5	____	____	____	____	____	____
Total	____	____	____	____	____	____
K	1.4	2.6	5.4	11.4	21	34
$K \times$ Total	____	____	____	____	____	____

Farrall Index = sum of all $K \times$ totals.

The globules in each size range are totaled for the five fields and these totals are multiplied by a factor K which relates diameter for each size range in terms of 2-μ-sized globules. The sum of these values corrected for diameter yields the Farrall Index. It will be evident that under conditions of the determination, a properly homogenized sample contains few if any globules above 2 μ in size. Values between 5 and 7 are considered to be excellent from the standpoint of homogenization efficiency. The details of this analysis are given elsewhere (29). In addition to microscopic examination of the milk, efficiency of homogenization can be evaluated on the basis of the degree of fat separation after 48 hours standing. In such an analysis, samples from the top and lower portion of the bottle contents are analyzed for fat. The United States Public Health Service has defined homogenized milk to be satisfactory when the top 100 ml. of a quart sample does not vary in fat content more than 10% from the rest of the sample. Additional methods for evaluating efficiency of homogenization depend on (*a*) turbidity and (*b*) accelerated creaming through dilution and centrifugal separation. These methods are reviewed and discussed at length by Trout (29).

Analysis of homogenized milk for fat. In general, the Babcock and Mojonnier tests for the fat content of milk give satisfactory results with homogenized milk. In order to overcome the occurrence of charred material in the fat columns by the Babcock method, techniques of minimizing the action of the sulfuric acid are rather generally employed. These consist of using sulfuric acid of slightly lower specific gravity and adding the acid at longer intervals and in smaller portions with thorough mixing. The literature on this subject has been reviewed (29).

EQUILIBRIA BETWEEN SOLID AND LIQUID FAT

Commercial handling of dairy products frequently involves treatments that cause partial solidification of the fat. The extent and kind of crystallization markedly influences the churnability of cream and the physical structure of butter. Consequently, the problem of solidification and melting of milk fat is of great importance and interest in the dairy industry. The physical behavior and properties of milk fat throughout the temperature range from nearly complete

solidification to complete liquefaction and above need to be considered. For a review of these properties of fats in general, the student should consult the monograph by Bailey (1). A review by Mulder (19) summarizes the crystallization behavior of milk fat.

The numerous glyceride components of milk fat have a certain degree of mutual solubility in both the liquid and solid states (i.e., they form liquid solutions and probably also solid solutions or mixed crystals upon cooling). Cooling the fat below about 30° C. causes crystallization, and progressive lowering of the temperature causes successive glyceride fractions to crystallize. Slow and gradual cooling causes large crystals to be formed since only a few nuclei of the highest melting (and most insoluble) glycerides form at first, and subsequent cry tallization occurs on these nuclei. If the cooling were infinitely slow it might be expected that homogeneous mixed crystals would be present at any stage of the cooling and that the composition of the crystals would change progressively as the temperature is lowered. Actually, however, with cooling within a few hours or days it is probably more accurate to visualize the process as consisting of deposition of successive layers of differing composition on the original nuclei. Rapid cooling, such as that obtained by placing melted fat at 0° C., produces numerous small fine crystals.

There is extensive evidence that triglycerides can exist in more than one crystalline form, the particular modification obtained depending on the manner of crystallization. The forms differ in arrangement of molecules in the crystal lattice. This phenomenon of *polymorphism* manifests itself in multiple melting points, the sample when warmed melting at a given temperature and then solidifying with further warming as the original unstable form is converted to a more stable one. It may also be detected by inflections in (*a*) heating and cooling curves (temperature plotted versus time), (*b*) calorimetric curves (heat input plotted versus temperature), and (*c*) dilatometric curves (expansion plotted versus temperature). Furthermore, examination of the crystal structure by polarized light or by x-rays can be used to differentiate the forms. Polymorphism is readily demonstratable in simple glyceride systems such as tristearin or tripalmitin. It is much less readily observed in complex glyceride mixtures and in natural fats, either because it does not occur or because the phase transformations of certain components tend to counteract those of others in the mixture.

The possibility that milk fat may exist in polymorphic modifications

is suggested by the fact that it exhibits a double melting point if examined soon after quick solidification. Mulder (19), for example, observed that a sample, solidified quickly by cooling at 0° C. and then warmed within two hours, would melt at 19° C., solidify on further warming, and melt again at about 30° C.

It is evident from the foregoing paragraphs that the state of the fat in milk or cream depends not only on the temperature but also on previous temperature history, including rate of cooling and time at a given temperature. Two principal properties of fat are employed to follow changes in the crystalline status of milk fat in dairy products. These are the latent heat of fusion released and the expansion that occurs upon melting. Milk fat has a specific heat of approximately 0.5 cal./g. and a heat of fusion of about 20 cal./g. In order to calculate the percentage of fat that is solid in a given sample at a given temperature, the amount of heat required to shift it to another temperature is determined. This may be done either by measuring the wattage required to heat the sample electrically or by the "method of mixtures," in which the sample is mixed with a known amount of some other substance (e.g., a cream sample might be mixed with skimmilk) at a known temperature and the temperature of the mixture determined. In any event, the percentage of solid fat is calculated as follows:

$$\text{Per cent solid fat} = \frac{100[H - S(t_2 - t_1)]}{F}$$

where H = heat input per gram to raise temperature from t_1 to t_2,
S = specific heat (cal./g.),
F = heat of fusion (cal./g.).

Of course, if this method is applied to a system such as cream, allowance must be made for the heat required to raise the temperature of the cream plasma from temperature t_1 to t_2. The calculation is exact only if the heat of fusion is constant over the entire melting range. This condition is fulfilled in the case of pure compounds, but obviously is not in the case of highly complex natural fats such as milk fat, in which fractions of varying heats of fusion melt at different temperatures. Nevertheless, the method is of some value for comparative purposes.

The coefficient of cubical expansion of milk fat is about 0.00079 ml./ml./° C. in either the solid or the liquid state. The expansion on

melting or melting dilation amounts to about 0.045 ml./ml. Dilatometry (the measurement of expansion) may be used to determine the percentage of solid fat in a sample. The technique is similar to the calorimetric method in that the coefficient of expansion is analogous to specific heat, and the melting dilation to the heat of fusion.

$$\text{Per cent solid fat} = \frac{100[D - E(t_2 - t_1)]}{M}$$

where D = total expansion from temperature t_1 to t_2,
E = coefficient of expansion (ml./ml./° C.),
M = melting dilation (ml./ml.).

Here again the expansion of cream plasma must be taken into account. Table 3 shows data on the percentage of solid fat in cream quickly cooled to 0° C. and then warmed to various temperatures. These data were obtained by the dilatometric method. Milk fat is not completely

Table 3. Effect of Cooling Conditions on Percentage of Fat in Cream Present in the Solid State *

Temperature, ° C.†	Winter Cream, %		Summer Cream, %	
	Cooled at −18° C.	Cooled at 0° C.	Cooled at −18° C.	Cooled at 0° C.
2	98	88	92	76
10	79	81	68	69
18	47	49	34	37
26	16	16	14	13
34	3	2	2	1
42	0	0	0	0

* Data of Mulder and Klomp (20).

† Temperature to which the sample was warmed after being quickly cooled and held at −18° or 0° C. overnight.

solid even at 0° C. In order to solidify it completely and at the same time keep it in the dispersed state in globules, glycerol can be added to the cream to prevent freezing of the water in the plasma.

Calorimetric and dilatometric techniques can also be employed to indicate the effects of various treatments upon the solidification of

milk fat. For example, Fig. 6 shows that fat in bulk solidifies at a higher temperature than fat in the form of globules and that homogenization hinders solidification.

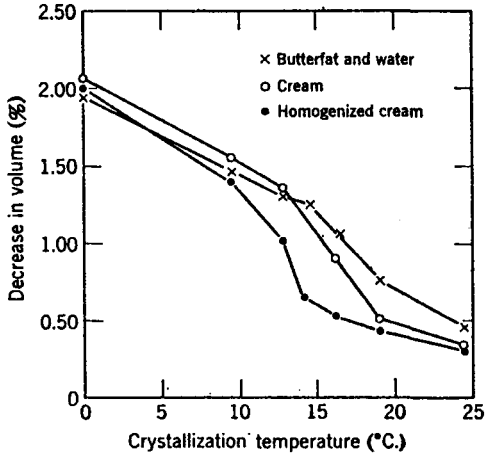

Fig. 6. Effect of degree of dispersion on solidification of milk fat. All samples originally at 50° C. were cooled to the temperatures indicated and held 18 hours before measuring the contraction. Data of Mulder (19).

CHURNING AND THE STRUCTURE OF BUTTER

The mechanism of the age-old process of churning has been the subject of much experimentation and speculation. Churning was long developed as an art but only in comparatively recent times has its mechanism been partly described in scientific terms. Churning occurs upon agitation of milk or cream when the fat is partially solid and partially liquid. The efficiency of churning or churnability is measured in terms of the time required to produce butter granules and by the loss of fat in the buttermilk. Figure 7 illustrates the marked effect of temperature on the churning time and the failure of butter to form in a reasonable time outside of the normal range of churning temperatures.

Conventional churning, by which a large proportion of the world's butter is still manufactured, involves agitation in a partially filled chamber. Under this condition, a large amount of air is incorporated into the cream in the form of bubbles. The resulting whipped product occupies a larger volume than the original cream. As agitation is

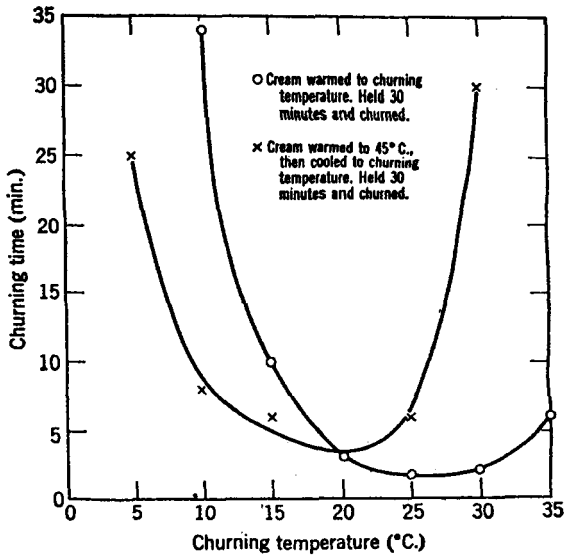

Fig. 7. Relationship between time and temperature of churning, churning time in minutes. All cream originally equilibrated at 2° or 3° C. Data of Holland and Herrington (9).

continued, the whipped cream is observed gradually to become coarser in texture and eventually the fat forms visible semisolid granules of butter which rapidly increase in size and separate sharply from the fluid buttermilk. The remainder of the buttermaking process consists of removing the buttermilk, kneading the butter granules into a coherent mass, and adjusting the contents of water and salt to the levels desired.

Any explanation of the mechanism of churning must take into account the following factors:

1. The function of the air.
2. The release of stabilizing materials from the fat globule surface into the buttermilk.
3. The differences between the structure of cream and butter.
4. The temperature dependence of the process.

Some workers have held the opinion that incorporation of air is an absolute requirement and that churning is entirely dependent on foaming. Actually, it has been demonstrated that churning can be produced in the absence of air, although air and foaming greatly accel-

erate the process. Release of protein and phospholipide (probably lipoprotein complex) from the surface of the fat globules has been amply demonstrated. In unwashed cream this release is best shown by analysis for phospholipides since it is difficult to analyze specifically for the membrane protein in the presence of the large amount of the plasma proteins. Ordinarily about one-half of the total phospholipides of the cream is liberated into the buttermilk during the churning process and one-half is retained by the butter. With washed cream, in which the release of both the membrane proteins and phospholipides can be followed, Jenness and Palmer (11) found that the buttermilk contained 0.17 to 0.46 g. of protein and 0.07 to 0.17 g. of phospholipide per 100 g. of original fat in the cream. The butter plasma contained 0.16 to 0.30 g. of protein and 0.11 to 0.22 g. of phospholipide per 100 g. of original fat. It is evident that, in churning, the complex becomes divided in such a way that a relatively protein-rich fraction is released to the buttermilk (protein-phospholipide ratio = 2.4–3.8), whereas a relatively phospholipide-rich fraction is retained in the butter (protein-phospholipide ratio = 1.0–2.0).

Cream is easily shown to be an emulsion of the oil-in-water type. It is readily diluted by water or skimmilk. Microscopically, the fat globules, although in contact with each other and deformed, may be clearly seen to be separate and distinct. Butter, on the other hand, is primarily of the water-in-oil type. It contains numerous water droplets dispersed in a continuous fat medium. Furthermore—and this complicates the issue somewhat—normal butter contains a certain amount of fat still in the globular form and dispersed in the free fat. King (14) has shown that these fat globules in butter can be enumerated and measured microscopically after carefully diluting the butter with a butterfat fraction liquid at the temperature of measurement. The final question demanding explanation is the fact that churning occurs only within a certain range of temperature.

The theory of churning propounded by Van Dam and Holwerda (30) and by King (14) seems to account for the known facts rather satisfactorily. This theory is based largely on microscopic observations of the fat globules during the churning process. Not every worker in the field subscribes to all of the details of the theory but, in general, it accounts for the observed phenomena. According to this theory, the fat globule membrane substance spreads out on the surface of air bubbles, partially denuding the globules of their protective layer. Furthermore, a liquid portion of the fat itself exudes from the globule and partially or entirely covers the globule, rendering it hydrophobic.

In this condition the globules tend to stick to the air bubble and thus are collected by a process that is essentially similar to the flotation process used for the concentration of minerals in ore technology. Free fat is a foam destabilizer, and when sufficient free fat has spread on the surface of the air bubbles the latter collapse, collecting the partially destabilized globules that were clinging to them into clusters cemented together by the free fat. Buttermilk contains a large proportion of its total fat in the form of particles of colloidal dimensions. Furthermore, buttermilk fat has a lower iodine number than the total fat of the cream from which it is churned. It is visualized that the colloidal fat of buttermilk arises from the scattering of the free fat and phospholipides coating the air bubbles when the latter collapse. The clusters of globules formed by collapse of the air bubbles may be again "flotated" on other bubbles and the process continued until the clusters attain the size of butter granules.

According to this theory, cream does not churn at low temperature because only a small amount of the fat is liquid and a layer of free liquid fat cannot form on the surface of the globules. At high temperature, where the fat is entirely liquid, the globules are destabilized by churning but do not stick together to form clusters and butter granules.

As previously stated, the speed of churning depends on the temperature and thus on the proportions of solid and liquid fat in the globule. Brunner and Jack (3) observed that even at the same final degree of solidification, cream that had been warmed to the churning temperature churned faster than cream that had been cooled to the churning temperature. They visualized that this difference is due to the fact that in cream warmed to the churning temperature the liquid fat is primarily near the surface, but in cream cooled to the churning temperature the liquid fat is entrapped in the interior of the fat globules surrounded by a layer of solid fat.

The true continuous phase of butter is then a liquid portion of the fat. In this are embedded the water droplets and partially destabilized fat globules. The fraction of the total fat in butter that is present in globular form depends on the degree of working. More vigorous or more prolonged working reduces the proportion of globular fat. Some data on this point are shown in Table 4.

As stated previously, foaming is not an absolute requirement for churning since butter can be formed in cream agitated in the absence of air. Apparently, in this case the globules are brought in contact with each other in the interior of the cream by violent agitation.

Table 4. EFFECT OF WORKING ON THE NUMBER AND SIZE OF FAT GLOBULES IN BUTTER *

Sample	Working Time, min.	Globules per 1 mm.^{3}, millions	Globular Fat, %	Free Fat, %
I	10	16.5	79	21
II	20	13.4	56	44
III	30	9.1	42	58
IV	40	6.6	28	72

* Data of King (12).

Again, however, free fat exuded from the globules and partially coating their surface is visualized as cementing them together.

In recent years, a number of continuous churning processes have been devised. It is not necessary to review the details of these processes at this time. They have been adequately described by Wiechers and De Goede (33) and by King (13). Suffice it to say that these processes consist either of violent agitation of normal cream (Fritz method) or 80% cream (Alfa and New Way processes), or of breaking the emulsion at a temperature at which the fat is liquid and re-emulsifying some of the aqueous phase back into the fat (Cherry-Burrell and Creamery Package methods). Obviously, butters prepared by these several processes will differ in structure, particularly in the proportions of globular and free fat and in texture. For example, Mohr and Baur (17) found ordinary churned butter to contain 17.8 to 28.2% of the fat in globular form, Alfa butter 21.3 to 32.7%, and Fritz butter 11.6 to 16%. Butters made by processes involving breaking of the emulsion and re-emulsification contain no globular fat.

The hardness and spreadability of butter is determined by the fatty acid composition of the fat and by the temperature history before, during, and following churning. Other things being equal, butter will be softer the higher the degree of unsaturation of the fat. Although summer butter is usually intrinsically softer than winter butter, the butter maker can control the hardness and spreadability by adjustment of the temperatures during the process. Fundamentally, temperature variations control consistency through their influence on the degree and type of crystallization of the fat. Control of temperature is involved at the following stages of the process:

1. Rate of cooling and temperature to which cream is cooled after pasteurization.

2. Holding temperature before churning.
3. Churning temperature.
4. Temperature of washing and working the butter.
5. Temperature at which the butter is stored.

Rapid cooling of cream to a low temperature tends to produce a harder butter than slow cooling. Rapid cooling causes a larger proportion of the total fat to solidify than slow or stepwise cooling. Furthermore, rapid cooling causes the formation of numerous small fine crystals which entrap some of the liquid phase. Both of these factors then contribute to increase the hardness.

Holding the cream at a low temperature before churning markedly increases the hardness of the butter, as may be seen in Table 5.

Table 5. EFFECT OF CREAM AND BUTTER HOLDING TEMPERATURES ON SPREADABILITY AND HARDNESS OF BUTTER *

Churning No.	Cream Storage Temperature,† ° F.	Hardness Butter Stored at‡ 36° F., g.	Hardness Butter Stored at‡ −6° F., g.	Resistance to Spreading Butter Stored at‡ 36° F., g.	Resistance to Spreading Butter Stored at‡ −6° F., g.
1	38	291	250	883	725
2	38	308	261	899	750
3	48	285	223	836	669
4	48	266	207	811	662
5	58	197	178	641	526
6	58	200	183	641	599

* Data of Huebner and Thomsen (10).

† Cream held 16 hours at temperature indicated and tempered quickly to 52° F. for churning.

‡ Butter stored at temperature indicated for 14 days and tempered at 55.5° F. for 48 hours before testing for spreadability and hardness.

Obviously, the lower the temperature of holding the greater is the degree of crystallization at the time of churning. Thus there is less free oil to escape into the continuous free fat phase of the butter and the butter consequently is harder.

The temperature of churning is not varied primarily to regulate the consistency of the butter. Rather, it is adjusted to produce butter in a reasonable period of time and to prevent the excessive losses of fat in the buttermilk that occur if the temperature is too high. The temperatures of the wash water and of working the butter are impor-

tant determinants of the consistency of butter. Warm washing and working yields a butter with a high proportion of liquid fat. Solidification of this fat in subsequent storage results in an interlocked crystalline structure and yields a hard and sometimes crumbly butter. On the other hand, cold washing and working yields a butter with a lower percentage of liquid fat and with the crystalline structure rather thoroughly broken. Such butter hardens to a smaller extent in storage. In general, the hardness in storage increases rapidly in the first few weeks and does not change much subsequently. Hardening is not so pronounced at low temperatures of storage (Table 5) as at high, probably because the low temperature affords less opportunity for the formation of a reticular interlaced crystalline structure. The hardness developed during storage can be destroyed, at least in part, by reworking the butter and much of it is lost in the printing process.

ANHYDROUS MILK FAT (BUTTEROIL)

Milk is a perishable commodity and its production fluctuates during the year. Flush periods, which occur in spring, early summer, and to some extent in the fall of the year, pose surplus problems. Some areas have an abundant milk supply and others may have little or none. All of these factors tend to encourage the development of stable forms of milk and its more useful components, the fat and non-fat phases. Although butter is by far the most important storage product so far as the fat phase is concerned, in recent years significant amounts of milk fat have been converted to the anhydrous form. The principal advantage of anhydrous milk fat, compared with butter, is that refrigerated storage is not strictly necessary for reasonably good keeping quality. The product also implies some saving from the standpoint of shipping weight and storage space since it contains no water and is essentially a pure fat product.

Anhydrous milk fat is more commonly known as butteroil in the United States. The term *butteroil* is somewhat a misnomer since large-scale manufacture of the product (directly from milk on a continuous basis involving no conversion to butter) is practiced. However, the product can be manufactured from butter as described below. Anhydrous milk fat is by no means a modern product. Its manufacture in various forms has been recorded for centuries and it is known by various names throughout the world. The distinctive physical properties and flavor of the product as well as its keeping quality have

been appreciated in nearly all the countries of Europe, the Middle East, and Asia. In the Middle East a product known as *maslee* is produced, a similar product of India is called *ghee,* and in Egypt the term *samna* is used. In some other areas similar commodities are made from ewe's and goat's butter. Because manufacture is on a small local scale, there is considerable variation in methods used and in flavor and keeping quality of the finished product.

Methods of Manufacture

"Boiling-off" process. This method is of world-wide significance and is the means by which the creams and butters from the milk of various animals is converted into *ghee, samna, maslee,* etc. Although there are many local variations in cooking time and temperature relationship and in the particular raw material used, they all have in common the use of temperatures somewhat in excess of 100° C. to drive the water from the cream or butter and to precipitate the milk solids. The resulting product is a light to dark brown oil with pleasing flavor and aroma and excellent storage qualities.

Gravity separation and filtration. Simple gravity separation of anhydrous milk fat from melted butter, with or without the aid of washing and filtration steps, have been employed for manufacture. Fat losses by methods based on this principle are relatively high and the methods are slow and cumbersome, particularly where filtration is used. In general, such methods yield products of relatively high moisture content and poor keeping quality.

Centrifugal methods. Most of the commercially feasible methods of manufacturing anhydrous milk fat are based on the use of the centrifugal separator. There are many variations ranging from the handling of small lots of cream or butter up to multiequipment, continuous processes that will produce anhydrous milk fat at the rate of 2000 lb per hour. Perhaps the simplest setup for manufacturing anhydrous milk fat involves the use of a cream separator and a vacuum pan. The milk is separated to cream and the cream churned to butter. The butter is melted and treated with several volumes of warm water and the mixture passed through the separator after adjusting it in the manner to recover plastic cream. The crude milk fat is washed and reseparated several times and dried to a minimum moisture level in a vacuum pan.

A continuous method of manufacture involves feeding a specially designed separator with cream at the rate of 4000 to 5000 pounds an

hour. Before entering the separator, the cream passes through an agitating unit that destabilizes the emulsion and then through a preheater that adjusts its temperature to about 140° F. The 90% fat product coming from the separator is diluted with warm water and neutralized if necessary. The product then passes through a second separator that concentrates the fat to a level in excess of 99.5%. Final drying is accomplished in a Vacreator. The resulting anhydrous milk fat may be stored in bulk or fed to a chiller that adjusts it to a semi-plastic condition. The product is finally packed in cans, with suitable precautions being taken to exclude oxygen. Where large-scale manufacture is required, the advantage of the continuous method is obvious. However, the capital outlay for equipment for this process is very substantial and, unless the anticipated demand is large and continuous, it may not be warranted. Continuous operations of the type described here are reported to be capable of producing 10,000 tons of anhydrous milk fat annually from a single installation. Methods, not unlike the continuous process described, have been developed in Australia and New Zealand.

Recent literature on this technology has been published (5).

De-emulsification with surface active agents. In methods for preparing milk lipides in a pure dry state from their native environment in fluid milk, a breaking of the natural emulsion is essential. In the methods described above, the emulsion is broken by physical means, either through special destabilizing-agitating type equipment or by churning cream into butter and melting the resulting butter. An additional principle that may be used in this connection is de-emulsification of heavy cream with surface active agents (28). A study of 97 commercially available surface active agents revealed that the majority of those useful for the purpose of de-emulsifying cream were of the anionic type. A pilot scale operation for the manufacture of anhydrous milk fat by this process is as follows: to cream testing approximately 40% fat is added 3% by weight of a suitable surface active agent, such as Tergitol 7. The mixture is continuously agitated and heated in a hot water bath to 180° F. for 15 minutes. The oil layer, which separates on standing, is removed and washed with water several times by passage through a cream separator, and the product is then dried under vacuum. Anhydrous milk fat properly manufactured by this process is closely comparable in properties to that obtained by melting, washing, and refining sweet cream butter.

It is of interest that simple organic liquids will also accomplish the de-emulsification of cream (28). When cream (40% fat) is heated

with such compounds, maximum demulsifying activity within a given homologous series occurs in the vicinity of the normal C_3 to C_6 members. As groups, the acids, alcohols, and amines are most effective; the aldehydes, ketones, and esters are least effective. The mutual solubility of the effective compounds in both the fat and non-fat phases of the cream, with a consequent lowering of interfacial tension and a release of globule-adsorbed materials, seems to offer a plausible explanation of the de-emulsification process. Although solvent de-emulsification and extraction techniques are used regularly in the analysis of milk and milk products for fat content, they have never been used for the commercial preparation of anhydrous milk fat. This is in direct contrast to the seed oil industry where such methods are widely employed.

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CHAPTER

10

PHYSICAL CHEMISTRY OF THE CASEINATE-PHOSPHATE PARTICLES IN MILK

GENERAL DESCRIPTION OF THE PARTICLES

Casein exists in milk as complex particles or micelles containing calcium, inorganic phosphate, magnesium, and citrate, in addition to the casein proteins. Various names have been applied to this system; in the discussion in this chapter it will be designated as the calcium caseinate-phosphate complex, which appears to be the most commonly used term at present. Many of the problems of dairy technology revolve around the behavior of the caseinate system and particularly the aggregation of the particles by heat, salts, acid, and rennin. Therefore a study of its composition and properties is a most important phase of dairy chemistry. The subject has been reviewed very concisely by Pyne (17).

The calcium caseinate-phosphate particles in milk vary considerably in size (i.e., they are polydisperse). A number of methods have been used in measuring the size distribution of these particles. Modern-day electron microscopes indicate that they are roughly spherical and range in diameter from less than 30 to more than 300 mμ. The actual size distribution observed by some Swiss workers for normal milk is given in Table 1. The particles with diameters from 40 to 160 mμ are most

Table 1. SIZE DISTRIBUTION OF CASEINATE-PHOSPHATE PARTICLES IN SKIMMILK

Diameter, mμ	Apparent Molecular Weight, millions	Frequency, % Sample 1	Sample 2
40–80	10–81	20.8	32.0
80–120	81–266	35.5	34.0
120–160	266–625	23.3	23.7
160–200	625–1220	13.9	8.0
200–240	1220–2025	4.5	2.0
240–280	2025–3280	2.0	0.4

Data of Nitschmann (10).

numerous. Calculations of the size of these particles have also been made from their velocity of sedimentation in a centrifugal field and from their ability to scatter light. Both methods indicate that the range is about the same as that found by the electron microscope, that is, 30 to 300 mμ. Needless to say, the caseinate-phosphate particles are exceedingly numerous. It has been calculated that there are present 5–15 $\times$ 10^{12} such particles per milliliter of milk.

The composition of the caseinate particles can be determined by separating them in some manner from the serum. The caseinate may then be analyzed directly, with due allowance for entrapped serum, or its composition may be calculated from differences in composition between the original skimmilk and the serum. Methods used for separation of caseinate from serum for analytical purposes include high-speed centrifugation, rennet coagulation, ultrafiltration, and equilibrium dialysis. These were mentioned in Chapter 5 in connection with the analysis for the dissolved salt constituents of milk. Such analyses indicate a composition approximately as given in Table 2. This composition varies considerably among individual milks, not only in total amount of caseinate but also in the amounts of calcium and inorganic phosphate present per unit of casein.

The phosphorus designated as organic phosphorus represents that esterified with the serine and threonine of the protein. The so-called inorganic phosphate is an acid soluble fraction that is associated with the casein and calcium. Since the caseinate particles vary in size, it has been of interest to determine if the composition varies with size. When the caseinate is fractionally centrifuged, it is found that the colloidal calcium is brought down in a constant ratio to the casein

Table 2. COMPOSITION OF CASEINATE-PHOSPHATE PARTICLES IN SKIMMILK

Constituent	Concentration	
	Milk, g./100 ml.	Caseinate, g./100 g.
Casein	2.6	94
α-casein	2.0	73
β-casein	0.5	18
γ-casein	0.1	3
Calcium	0.080	2.9
Magnesium	0.004	0.1
Organic phosphate (as phosphorus)	0.022	0.8
Inorganic phosphate (as phosphorus)	0.038	1.4
Citrate (as citric acid)	0.015	0.5

Average data compiled from various sources. See particularly reference 8.

nitrogen. Likewise, except for some very large particles that sediment early in the centrifugation and contain a higher proportion of phosphate, there is a relatively constant relation between casein nitrogen and acid soluble inorganic phosphorus. These facts indicate that the particles do not vary greatly in composition with size. It is known that α-, β-, and γ-caseins differ markedly in esterified phosphorus content. Nevertheless, the ratio of organic phosphorus to casein nitrogen remains constant during centrifuging, which shows that these three components of casein are either all present in the same particles or that (a less likely probability) they are present in separate particles with the same distribution of sizes.

It has never been decided unequivocally exactly how the calcium and inorganic phosphate are attached to the caseinate particle. One school of thought has favored the idea that the particles consist of a double calcium salt of phosphate and casein. Other workers have considered that the inorganic phosphate is present as a calcium phosphate protected physically by calcium caseinate. The experimental evidence for either of these points of view is meager and inconclusive.

Many attempts have been made to represent the caseinate particles in terms of specific salt structures, but these have little if any factual basis. Data are simply not available to support any specific double calcium salt structure of casein and phosphate or any specific mixture of calcium caseinate and calcium phosphate. We simply know that in a large proportion of the particles at least the atom ratio of

calcium : organic phosphorus : inorganic phosphorus is of the order of 5:2:2 (8).

There has been some question also as to whether the colloidal inorganic phosphate is present in the dicalcium or tricalcium form (i.e., whether all three or only two of its ionizable groups are combined with calcium). Most workers incline to the view that it is mainly tricalcic because the addition of neutral oxalate to milk causes a rise in pH. This result would be expected from the reaction of oxalate with tricalcic phosphate because removal of calcium as the insoluble oxalate would liberate trivalent $PO_4^{\equiv}$ ions which combine with H^+ ions of the milk to form $H_2PO_4^-$ and $HPO_4^=$.

The caseinate-phosphate particles bind a considerable quantity of water and various attempts have been made to measure the extent of this hydration. Since the binding of water involves a gradient in activity of water molecules extending from free water in the solution to maximally immobilized water at the surface of the particle, it is obvious that the precise amount of water classified as bound will depend on the method of measurement. By determining the viscosity of skimmilk and whey it may be calculated that the caseinate particles appear to bind about twice their weight of water. On the other hand, the ratios of water to lactose in whey and in caseinate particles centrifuged from skimmilk indicate that an amount of water equal to about 50% of the weight of the particles is so tightly bound that it cannot function as a solvent for lactose.

It is important to emphasize that the caseinate-phosphate particles are undoubtedly in equilibrium with the milk serum in which they are suspended. Thus, any change in the ionic concentrations in the serum will be reflected in changes in the particles. For example, removal of calcium by an ion exchanger will cause some of the calcium in the particles to come into solution. Furthermore, there have been suggestions that milk contains small quantities of one or more of the caseins in "monomeric" form in solution and that these are in equilibrium with the particles. If this is indeed the case, it would be expected that temperature fluctuations and other treatments would vary the ratio between monomeric and particulate casein.

DESTABILIZATION OF THE PARTICLES

The caseinate-phosphate particles in milk exist in a rather precarious equilibrium with the milk serum. They are extremely sensitive to

changes in ionic environment, because casein is primarily stabilized in solution by the charge carried by the particles and also because casein binds divalent ions such as calcium and magnesium very tenaciously. Much dairy technology is concerned with processes that destabilize the caseinate particles. In some of these processes, destabilization is sought and in other cases it is avoided. In the following paragraphs, some general principles involved in the destabilization of the caseinate system by various means are discussed. The important phenomenon of heat destabilization is discussed in Chapter 11.

pH and Acidity

The caseinate particles are very sensitive to changes in pH. It will be remembered that casein is defined as that protein which is precipitated from milk by adjustment of pH to its isoelectric point at 4.6. The dispersibility of the caseins depends primarily upon the pH. Addition of acid to milk by either bacteriological action or chemical means causes progressive removal of the calcium and phosphate from the caseinate-phosphate micelles. When the acidification has been carried to pH 5.2 to 5.3, the casein precipitates. Although the protein is not yet at its isoelectric point, the micelles have insufficient stability at this pH to remain in solution. The fact that the micelles precipitate at pH 5.2 to 5.3, where they still contain some calcium, complicates the problem of preparing mineral-free casein. Prolonged leaching with acid, washing with water, redispersion, and reprecipitation may be used to remove all of the mineral salts from casein.

The manufacture of cottage cheese involves the acid destabilization of the caseinate particles by the bacteriological production of acid in the milk. Under these conditions, with the milk in a quiescent state during the production of acid, the destabilization of the caseinate particles leads to the formation of a smooth gel occupying the entire volume originally occupied by the milk. It is visualized that in this gel the caseinate particles form a three-dimensional network, thus entrapping the aqueous phase and forming a semisolid system. When heat is applied at the cooking stage of the process, the caseinate particles become more closely knit together, water is expelled, and the clot shrinks. Control of the characteristics of the cottage cheese curd is attained by judicious adjustment of the pH and the cooking conditions.

Even at pH values well above the isoelectric point, casein may precipitate because of various factors, including the hydrogen ion

concentration, acting in concert. For example, the sensitivity of casein to coagulation by heat is enormously increased by decreasing the pH a few tenths of a unit below the normal value for milk. The converse is true at pH values above normal. Likewise, the stability of milk to rennet coagulation is greatly decreased by reducing the pH slightly.

Salts and Ions

The calcium caseinate phosphate micelles of milk are readily precipitated from solutions by saturating with various salts, such as saturating with sodium chloride or half-saturating with ammonium sulfate. Such precipitation obviously is due to neutralization of the charge and dehydration of the particles. Doan and Warren (7) have shown that the salting-out is reversible, that is, the protein can be put back into solution merely by diluting. However, if the salted-out protein is held in the presence of saturated sodium chloride at room temperature for a period of weeks, an irreversible reaction seems to occur, so that the protein can no longer be redispersed on dilution. Up to a certain time of holding (8 to 12 weeks) the redispersion can be effected by heating at 150° or 200° F., but thereafter even this treatment will not redisperse the protein. The nature of such irreversible changes, which cause the protein to be no longer redispersible on dilution, has not been satisfactorily elucidated.

The caseinate particles are particularly sensitive to changes in the divalent cation content of the medium. Such ions as calcium and magnesium are strongly bound by casein and serve to cause aggregation of the particles. It can readily be demonstrated by use of the electron microscope, or by turbidity or light-scattering procedures that increases in the concentration of calcium and magnesium cause aggregation of the particles, and decreases in the concentration of these ions cause dispersion of the particles to smaller sizes. The effects of divalent cations also come into play in connection with the destabilizing action of other agents such as rennin and heat. In both of these cases the concentrations of calcium and magnesium and the variations therein are of paramount importance in determining the stability of the particles. Phosphate and citrates ordinarily exert an action opposite to that of calcium and magnesium because they form undissociated complexes with calcium and magnesium, thus decreasing the effective concentration of the calcium and magnesium ions. Sodium and

potassium oppose the effects of calcium and magnesium, because these ions tend to cause a higher degree of dispersion and hydration of the caseinate particles.

Freezing

There has been considerable interest in the development of frozen concentrated milk as a means of transporting milk to areas not adequately served by a fresh milk supply. Unfortunately, attempts to manufacture such products have encountered the difficulty that casein becomes destabilized during storage of the frozen product. The flocculation progresses gradually; at first the flocs that are observed are readily dispersible, but as storage continues, they become very difficult to redisperse. The flocculation closely resembles that observed by Doan and Warren when casein was held in saturated sodium chloride brine.

Interestingly enough, the destabilization of the caseinate coincides with crystallization of lactose from the product (see reference 19). In frozen concentrated milk, lactose is present in a greatly supersaturated state and will eventually crystallize, at least at temperatures down to —5° F. or so. At extremely low temperatures, crystallization of the lactose may be inhibited indefinitely. Flocculation of the caseinate occurs as soon as the lactose has crystallized. Evidently lactose exerts some sort of protective action in the system when it is in the dissolved state, but loses this protective action when it crystallizes out. Possibly lactose in the dissolved state binds sufficient calcium to stabilize the system and releases this calcium upon crystallization. Other sugars that do not crystallize during the frozen storage also exert a protective effect.

At present, the maintenance of stability in frozen concentrated milk can only be achieved by sufficiently low temperature storage, hydrolysis of a large part of the lactose, or removal of a large part of the calcium by ion exchange treatments. It is interesting that casein recovered from destabilized frozen milk has the same sensitivity to precipitation by calcium as casein isolated from fresh milk. The protein itself seems to be unchanged even though the particles have been flocculated. A similar type of insolubilization occurs in dry milk containing over 7% of moisture when the lactose crystallizes. The parallelism between the insolubilization in frozen and dry milks is so great that it appears that a single phenomenon is involved.

CLOTTING BY RENNIN

Coagulation of the caseinate-phosphate micelles in milk can be produced by a great number of proteolytic enzymes of both plant and animal origin. However, in the dairy field, the clotting of these particles by rennin is of major interest because the process is so widely used in cheese making. Rennin clotting has been used for a great number of years, but the precise mechanism by which it operates has remained a fascinating and intriguing problem that has not as yet been entirely explained. The following paragraphs give a general account of the process and some recently discovered clues as to its mechanism. For further information, the reader is referred to the two excellent reviews of the subject by Berridge (3, 4).

Rennin is an enzyme found in the fourth stomach or abomasum of suckling calves. It is replaced by pepsin in older animals. The enzyme is prepared commercially for use in cheese making as a salt extract of dried calf stomachs. Such an extract containing the enzyme is called rennet or rennet extract. The enzyme rennin itself has been purified considerably by various workers and even brought to crystallization. There is some question as to whether any of the crystalline products as yet described actually is the pure enzyme completely uncontaminated by any other protein. Purified rennin is a typical globulin protein with a molecular weight of approximately 40,000. Its isoelectric point is approximately pH 4.5. Rennin is a proteolytic enzyme that hydrolyzes the peptide bonds of proteins. Its optimum pH for activity is at 3.8. In this respect it differs from pepsin, whose optimum pH is in the neighborhood of 2.0. It should be emphasized that commercial rennet extract usually contains pepsin in addition to rennin. Purified crystallized rennin appears to be free of pepsin.

Action of Rennin on Casein

In general, workers in the field subscribe to a theory proposed by Hammarsten in 1897, which states that the process occurs in two stages: first, a specific action of rennin on the caseinate, and second, coagulation or clotting of the product under the influence of calcium salts in the milk. It is easy to separate the two stages by manipulating the temperature or the calcium content. Thus, if milk is treated with the enzyme at a low temperature (below 15° C.), it will not normally

clot; but the enzyme evidently has acted because if milk is treated with rennin at a low temperature and is then warmed to 37° C., it clots very quickly. In like manner, if milk is dialyzed to remove the calcium or a portion thereof and then treated with the enzyme at 37° C., it will not clot, but on re-adding calcium, clotting will occur very quickly. Rennin acts on simple sodium or potassium caseinate systems devoid of calcium; such solutions will coagulate very quickly when calcium is added following rennin treatment. It appears that rennin induces a specific irreversible change in the casein. This change is only very slight since the altered product (often called "para-casein") does not differ detectably from the original casein in most properties.

The most spectacular difference in properties between the intact and rennet-treated casein is the sensitivity to precipitation by divalent cations, such as calcium, as illustrated in Fig. 1. The difference seems

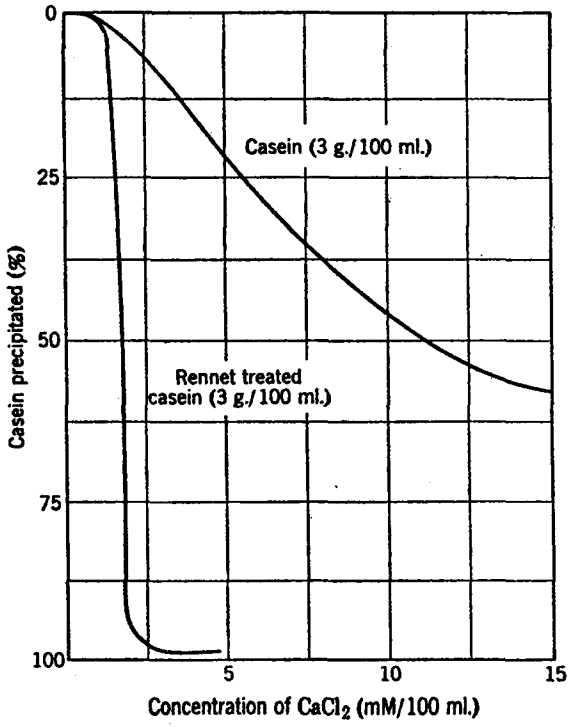

Fig. 1. Precipitation of casein and rennet treated casein by calcium at pH 6.5–7, 35° C. Data of Nitschmann and Lehmann (12).

to be a quantitative one. In view of the close similarity between the casein and paracasein, it is not surprising that the mechanism of the primary action of rennin has long remained obscure. Some recent workers (11) feel that the primary action consists of a mild proteolysis of the casein involving the liberation of a small amount of a peptide or peptides from the molecule. Rennin has been found to have this action on α-casein but not on β- or γ-caseins. About 2% of the total nitrogen of α-casein is thus liberated in the form of low molecular weight peptides before the product is rendered coagulable with calcium. This is indeed a pertinent observation, but the liberation of peptide has not been established unequivocally as the reaction that is responsible for the increase in sensitivity to calcium ions. Since α-casein appears to be a heterogeneous mixture of a number of proteins, it will need to be determined if rennin acts on only one of these. Finally, information is needed as to precisely which bonds in the protein are broken by the enzyme and what kind of structural alterations are thereby produced.

It is conceivable that a limited and specific proteolysis involving the rupture of a few strategic bonds by rennin acting at a pH far from its optimum makes enough groups available so that a three-dimensional network can be formed.

Clotting and Coagulation

In discussing the action of rennin on milk, the terms *coagulation* and *clotting* will be used. The former refers to precipitation in the form of loose separate flocs, and the latter denotes the formation of a smooth gel or clot occupying the entire volume originally occupied by the milk. The system clots when there are sufficient particles present and bonds of sufficient strength form a continuous three-dimensional network. Otherwise loose separate flocs occur. Various conditions as described below determine whether coagulation or clotting is obtained.

The two most widely used measures of the phenomena are the coagulation time and the strength of the curd. Coagulation time is determined merely by adding rennin to the milk at a definite temperature and determining the time required for flocs to form. This may be conveniently done in an apparatus as described by Berridge (2), in which the sample and rennet are placed in a tube, which is rotated at an angle in a thermostatically controlled bath. With such an appara-

tus, the formation of flocs in the flowing film of milk on the inner surface of the tube is easily observed. As ordinarily used, the clotting time determination does not distinguish between the two phases of the reaction, but rather measures the time for completion of both. Undoubtedly, in many cases the two phases overlap each other.

The other method of measuring rennin clotting is to determine the curd strength or curd tension of the clot or gel produced by the action of rennet in a definite time at a definite temperature. Obviously this is applicable only to conditions that produce clotting. The force required to push a knife through the curd is measured. Apparatus for this purpose has been devised in connection with studies of the strength of curd produced in milk with pepsin and acid (6). Such apparatus works equally well for the determination of the strength of the clot produced in milk by the action of rennin. Rennet clotting time and the rennet curd tension of individual milks usually exhibit an inverse relation (see Fig. 2).

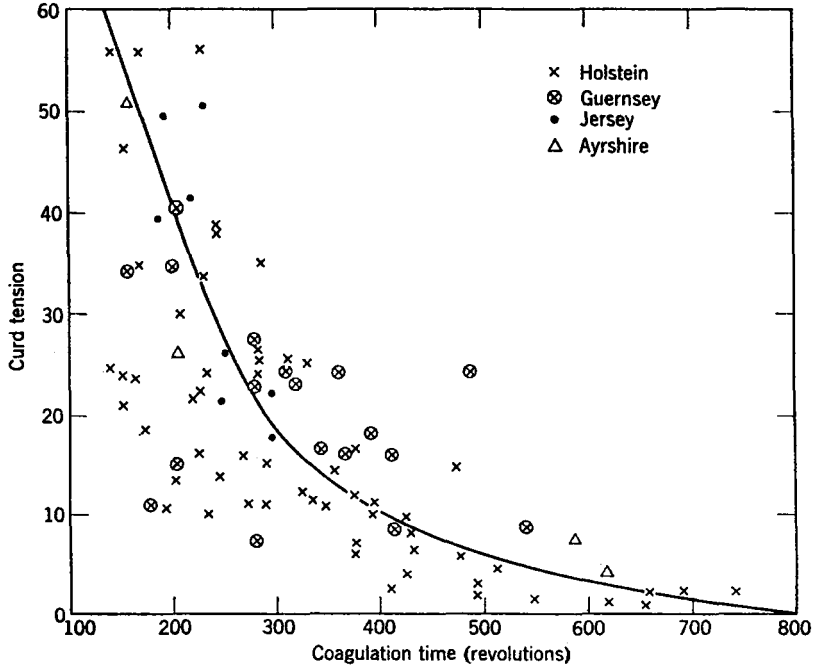

Fig. 2. Relation between rennet clotting time and rennet curd tension. Data of Kelley, Swanson, and Price (9).

Factors Affecting the Clotting Process

Many experiments in the literature have been interpreted as showing the effect of certain factors on the action of rennin. Since the coagulation phase of the phenomenon has usually been used as the test, it is often difficult to ascertain whether the factor exerts its effect on the action on the enzyme or on the coagulation. In the following discussion, an attempt will be made wherever possible to state which of the phases is being affected by the variable under consideration.

Hydrogen ion concentration. The time of rennet coagulation and the strength of clot produced depend very markedly on the hydrogen ion concentration. The reaction proceeds faster and the clot is stronger as the pH is lowered below that of milk. This effect of pH is undoubtedly in large part due to an effect on the activity of the rennin. The pH probably also affects the stability of caseinate particles directly as well as indirectly by the release of calcium ions from dissolved and colloidal complexes as the pH is lowered.

Concentration of calcium ions. Figure 1 shows that the rennin-altered casein is very sensitive to coagulation by calcium ions. Exceedingly small changes in the calcium ion concentration in the system can markedly affect the speed of curdling and the strength of the clot. The effect of calcium ions is not specific since other divalent and polyvalent cations also produce the effect. Of course, cations of higher valence are more effective. Pyne (16) has shown that the level of calcium affects not only the second or clotting stage, but also is important for the first or enzymatic stage of the reaction.

Concentration of caseinate and of colloidal calcium phosphate. The number of particles in a gel contributes to its strength, and consequently the concentration of caseinate micelles materially influences the curd tension. The higher rennet curd tension of Jersey milk compared with Holstein milk is due, among other things, to the higher concentration of caseinate in the former. Fat globules do not form an integral part of the clot. Their presence dilutes and weakens it. Homogenization, by increasing the dispersion of fat, reduces the curd tension of whole milk. It has been known for many years that the colloidal calcium phosphate in the caseinate micelles is involved in determining whether a clot forms or coagulation occurs when a caseinate system is treated with rennin. A gel will form upon rennet treatment of an artificial system of calcium caseinate containing 5% protein, but the presence of the colloidal phosphate is essential to the

production of a smooth gel or clot at the casein concentration observed in milk. Differences observed between milks in the strength of the clot produced by rennet are due in part to variations in the concentration of colloidal calcium phosphate as well as in the total casein concentration. The presence of the colloidal calcium phosphate probably does not greatly affect the vulnerability of calcium caseinate to the attack by rennin, but rather exerts its influence by sensitizing the particle to precipitation by calcium ions.

Temperature. When excess rennin is added to milk, the maximum speed of coagulation occurs at about 40° to 42° C. There is no coagulation below 10° or above 65° C. The maximum firmness of the clot occurs at about the same temperature as the maximum speed. Berridge (1) was able to segregate the effects of temperature on the two stages. He found that the temperature coefficient of the first stage was of the same magnitude as that of chemical reactions in general, that is, the reaction rate approximately doubled for an increase in temperature of 10° C. over the temperature range of 0° to 40° C. On the other hand, when the first stage was allowed to go to completion at 0° C. and then samples were warmed to various temperatures, the temperature coefficient of the second stage was extremely high, the rate increasing by a factor of 1.3–1.6 per 1° C. High temperature coefficients of this sort are characteristic of protein denaturation. Berridge postulated that the second stage of rennet coagulation might involve a denaturation-type reaction in which the molecules uncoil and unfold after some essential link has been split in the first stage of the reaction.

Variations in temperature produce effects on rennet clotting time and curd tension that are probably due to differences in concentrations of calcium ions and of colloidal calcium phosphate. Thus a sample of milk warmed quickly to 37° C. after being held for 24 hours at 5° C. clots more slowly and yields a weaker gel than a sample cooled quickly to 37° C. from 50° or 55° C. This difference appears to be related to the transfer of colloidal calcium and phosphate from the colloidal to the dissolved state upon cooling, and its sluggish reversal upon warming.

Previous heat treatment of milk. The speed of coagulation and the strength of clot are decreased if milk is heated at temperatures of 65° C. or over and subsequently cooled and treated with rennin. Heat treatment itself exerts a detrimental effect on clotting, but further slow increases in clotting time occur if milk is held after heat treatment. This phenomenon is illustrated in Fig. 3 and is called

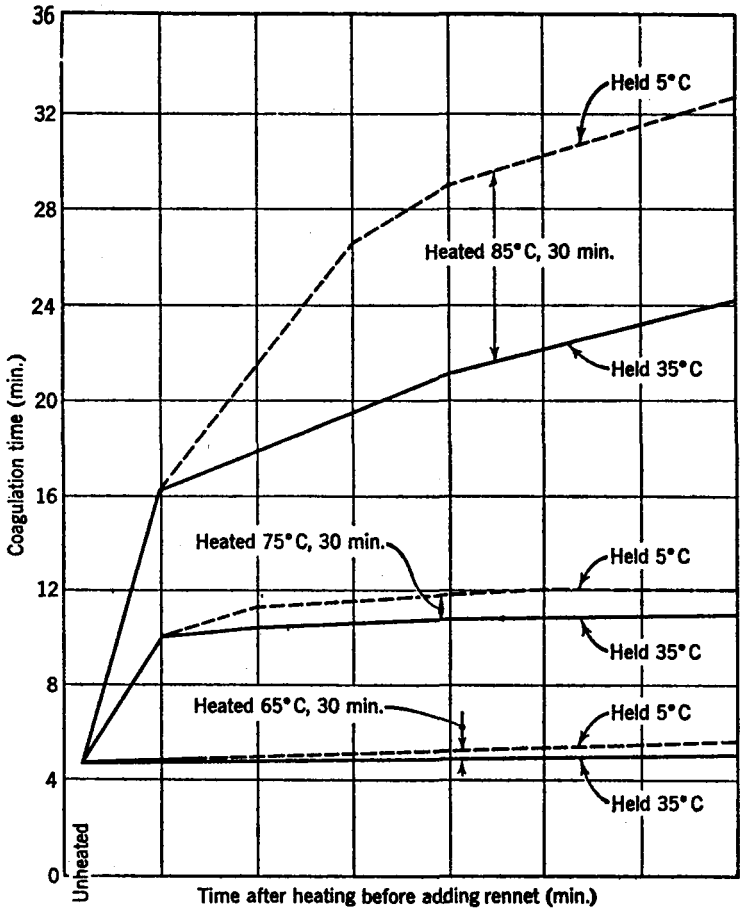

Fig. 3. Effect of heat treatment and subsequent holding on rennet coagulability. Data of Powell (14).

"hysteresis" or delayed action. These effects of heat treatment seem to be entirely on the second stage of the reaction. The ability of rennin to attack the casein is not altered by heat treatment. Pyne (15) has shown that the hysteresis is accompanied by the release of small amounts of calcium and phosphate from the caseinate particles. It may thus be suggested that heat treatment exerts a detrimental effect that is partially counteracted by the transfer of dissolved calcium and phosphate from the solution to the colloidal phase. As this additional colloidal calcium and phosphate leave the colloidal particles upon holding the treated milk, the ability to coagulate with rennin becomes progressively poorer. This whole phenomenon seems to be rather

poorly understood as yet. Experiments in the senior author's laboratory have indicated that if artificial systems are prepared, containing caseinate but devoid of β-lactoglobulin, neither the effect of heat treatment nor the hysteresis occurs. These findings imply that the β-lactoglobulin is involved in some way in the effect of heat on the rennet coagulability of the caseinate.

Rennin Clotting of Buttermilk

Sweet cream buttermilk forms a very weak clot with rennin, compared with the clot produced by the corresponding skimmilk (13, 18). Two principal reasons have been advanced to explain this phenomenon. In the first place, the fat globule membrane material released into the buttermilk by the churning process has been found to lower curd tension. The mechanism by which the membrane material exerts its action has not been satisfactorily elucidated. Secondly, certain free fatty acids are produced by lipolysis of the fat and some of these, particularly capric, lauric, oleic, palmitic, and stearic acids, have been found to inhibit curd tension. Buttermilk would not always contain significant quantities of these free fatty acids, but it is an interesting observation in its own right that they do exhibit this effect. If such acids are incorporated into skimmilk at or above their melting points and the sample then aged at low temperature and warmed to the clotting temperature of 35° to 37° C., clotting is greatly inhibited. In many cases no clot will form at all.

It has been suggested that the fatty acids inhibit clotting by being adsorbed on the caseinate particles. It is much more likely, however, that they produce the effect by tying up some of the calcium ions as insoluble salts. The senior author has observed, for example, that oxalic acid, which would not be expected to be adsorbed on the caseinate particles, exhibits an identical effect on clotting. Oxalic acid, of course, ties up calcium ions by forming an insoluble precipitate of calcium oxalate.

Curd Tension

The strength of the curd formed in cows' milk by the action of rennin or pepsin has been employed considerably as an index of the suitability of the milk for feeding of human infants (see Chapter 13). This subject was of great interest in the period 1925–1945 when "soft curd milk" was a watchword of the fluid milk industry. Several

different coagulants have been suggested for curd tension tests. Some of these are calcium chloride, rennet extract, and pepsin. An official method adopted by the American Dairy Science Association (6) in 1941 employs 10 ml. of 0.45% pepsin in 0.08*N* hydrochloric acid per 100 ml. of milk.

Milks from individual cows differ considerably in curd tension; in general, soft curd milks are those with low contents of solids-not-fat. Natural soft curd milk from selected cows was sold in certain markets in the past, but they gave way to milks rendered "soft curd" by certain treatments. Mastitis infections lower curd tension by depressing the casein content and raising the pH; naturally it was important to avoid infected animals when selecting a herd for the production of soft curd milk.

Among the treatments that have been employed to decrease curd tension are dilution, heating (see Chapter 11), acidification below pH 5.9, homogenization (see Chapter 9), partial decalcification with ion exchangers, incubation with proteolytic enzymes, and sonic vibrations. Evaporated milk, having been highly heat-treated, forms only a weak curd, if any. It is widely used for the feeding of infants. The whole subject of soft curd milk has been reviewed by Doan (5).

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CHAPTER 11

THE EFFECTS OF HEAT ON MILK

INTRODUCTION

Purposes of heat processing. Of all the steps employed in the processing of milk, heating is by far the most important. Without heat processing the dairy industry would come to an abrupt halt. It is required by law that certain dairy products receive specified heat treatments. Other dairy products, such as concentrated and dry milks, gain their identity only through heat treatment. The purposes of heat processing may be summarized as follows: to meet public health requirements, that is, pasteurization and sterilization, to remove water, to destroy enzymes, to facilitate mixing and blending processes, that is, ice cream mix and processed cheese, to achieve incubation temperatures, that is, cheese and cultured dairy products, and to impart desirable properties.

In a general sense the main objective in applying heat to dairy products is preservation. Keeping quality from a microbiological standpoint is the overriding consideration, although in the case of concentrated and dried products the effort may be toward preservation from chemical deterioration as well. This latter preservation is accomplished through removal of water (solvent) and is based on the

principle that many chemical reactions are either slowed or completely inhibited in the absence of a solvent. Some other properties that may be imparted to dairy products as a result of heat treatment are: increased viscosity, reduced curd tension, resistance to oxidation, changes in flavor and color, and alterations in protein stability.

Processes employing heat treatment. There are a substantial number of milk processing operations that employ heat either incidentally or purposely to achieve the objectives and properties just mentioned. Although it will not be necessary to dwell at length on the various processes, since they are primarily technological and are discussed extensively in the many texts on milk and its products, a presentation of their essential qualities seems pertinent for a proper understanding of subsequent discussion on the physical and chemical effects of heat. In considering the processes, the reader should bear in mind that frequently they are superimposed on one another. For example, virtually all milk is pasteurized as a preliminary step to any other processing, primarily to arrest any microbiological change and to destroy certain enzymes. Following pasteurization, milk may be concentrated with the aid of heat in a vacuum pan and subsequently dried with the aid of heat. Thus the effects of heating may be cumulative in any given dairy product.

It must be remembered that there is a definite trend toward higher temperatures and shorter heating times in processing milk and its products. The aim of this trend is to achieve continuous processing with its beneficial effects and to meet requirements regarding destruction of microorganisms and enzymes, while minimizing detrimental effects on flavor and other properties. As opposed to the older processes involving 15 minute to half-hour holding periods and relatively lower temperatures, there is an increasing amount of experimentation and application of short-time heat processing employing temperatures up to 300° F. (150° C.) and exposure times down to a few seconds. Citing the maximum temperature and its holding time can be misleading. The time required to reach this temperature and to cool from it is also important. It should be emphasized that present day H.T.S.T. processing minimizes coming-up and cooling-down time as well as holding time.

Comparisons of the effectiveness of various heat treatments in such processes as destroying bacteria, inactivating enzymes, denaturing proteins, and producing browning are often presented as plots of the logarithm of time versus temperature required to produce a given extent of reaction. Such plots, referred to by bacteriologists as

"thermal death time curves," are generally linear, at least over reasonably short ranges of temperature. They provide convenient methods of (*a*) summarizing a large amount of information on the effect of temperature on the rate of a reaction, (*b*) determining equivalent conditions at various times and temperatures, (*c*) determining the sum of the effects of a series of heat treatments, and (*d*) comparing the time-temperature requirements of different reactions. These plots of log time versus temperature represent an approximation of a well-known equation in physical chemistry known as the Arrhenius equation. This equation states that a linear relation exists between the logarithm of the rate of reaction and the reciprocal of the absolute temperature. Since for any given extent of reaction the log of reaction rate is proportional to log of time and since for short temperature ranges (40° C. or so) an approximately linear relation holds between T the absolute temperature and its reciprocal $1/T$, the plot of log time versus temperature is linear. Plots of this type are presented in this chapter (Fig. 5). A word of caution must be interjected at this point. It is not justifiable to extrapolate a plot of log time versus temperature much beyond the points from which it was constructed because over a wide temperature span it may be a curve rather than a straight line.

Pasteurization. The purpose of pasteurization is to destroy all pathogenic bacteria and the bulk of the non-pathogenic organisms. Basically, there are two methods in use in the industry, the holder process and the high temperature short time (H.T.S.T.) process. Because of variations in the susceptibility of pathogenic bacteria to heat destruction in the various fluid dairy products, the extent of heat treatment necessary to accomplish pasteurization varies somewhat from one product to another. Table 1 presents pasteurizing treatments generally employed for the three principal fluid dairy products. Requirements for the pasteurization of skimmilk are the

Table 1. HOLDER AND HIGH-TEMPERATURE SHORT-TIME (H.T.S.T.) PASTEURIZING TREATMENTS FOR FLUID DAIRY PRODUCTS

	Milk	Cream	Ice Cream Mix
Holder	143° F., 30 min.	145°–150° F., 30min.	160°F., 20 min.
H.T.S.T.	161° F., 15 sec. 200° F., 3 sec.*	Not generally used	175°F., 25 sec. or 194° flash†

* For Roswell-type heaters.

† For the Vacreator.

same as those for milk. These are the treatments usually sanctioned by public health authorities. However, there are many variations, mainly of a minor degree, among requirements of local health ordinances.

Sterilization. The primary purpose of heat sterilization of milk is to destroy *all* microorganisms and their spores. Although there is some limited production of unconcentrated sterilized milk in this country, the main application of heat sterilization is in the manufacture of evaporated milk. This product is a 2 : 1 concentrate (approximately 8% fat and 26% total solids) of whole milk, packed in cans, sealed, and heat sterilized. The sterilization of evaporated milk is usually accomplished under continuous agitation and temperatures of approximately 241° F. (116° C.) for 15 minutes. There is considerable variability in this processing. At the temperatures mentioned the coming-up and cooling-down times are not necessarily always uniform. Minor variations between plants with respect to both temperature and time are essential in contending with variations in behavior of milk supplies toward the sterilizing treatment. Further, the pattern regarding use of forewarming temperatures (see below), which has some bearing on the amount of heat required for sterilization, is not consistent. In the case of retort sterilization of the product, it can be assumed that the minimum heat treatment is approximately 241° F. for 15 minutes plus some variable degree of heating incident to forewarming and condensing. Sterilization of evaporated milk is not limited strictly to the holder process. At least one commercial product is manufactured by sterilizing treatment in the vicinity of 300° F. for several seconds, followed by aseptic packing. Equivalent times and temperatures for sterilization as measured by the destruction of bacterial spores are presented in Fig. 5.

Forewarming. The term *forewarming,* or *preheating,* is employed largely in connection with the manufacture of condensed and evaporated milks. Forewarming enables introduction of the milk into the vacuum pan at a temperature well above the boiling point inherent within the pan, thus facilitating removal of water. In addition, it imparts to milk certain qualities that are manifest in the finished evaporated milk as satisfactory protein stability and viscosity properties. In general, temperatures from 190° to 212° F. (88° to 100° C.) are employed in forewarming. There currently is a trend toward preheating above 212° F. The time employed for holding milk at the forewarming temperature will depend upon the temperature used, the stability of the milk, the type of forewarming equipment used, and the

degree of concern that the manufacturer may have for color and flavor of the finished evaporated milk. A 10-minute holding period seems ample to insure uniform temperature in a hot-well of milk. When forewarming temperatures are substantially elevated, some compensation by reduction of the holding time is usually made.

From a practical standpoint there seems to be no question that the extent of forewarming treatment can be a factor in the color, and presumably the flavor, of evaporated milk. The tendency is for successive heat treatments of milk to have cumulative effects. Milks that have been rigorously forewarmed (in excess of about 100° C. for 10 minutes) will show a greater degree of browning with a given concentration and sterilization process than milks receiving more moderate forewarming (3). For unconcentrated skimmilk sterilized at 120° C. for 20 minutes, the first additive effects of forewarming (100° C.) on color were noted between 15 and 30 minutes holding time (22). Pilot-scale experimentation with a milk supply will ordinarily reveal a forewarming treatment that is sufficient to impart desired properties of body and protein stability to an evaporated milk and yet will have no additive effect on color.

Condensing. The purpose of the condensing operation is the removal of water. For all practical purposes, heating under vacuum is the sole means employed in the dairy industry. Concentration by mechanical removal of ice crystals or sublimation of ice, although feasible in other segments of the food industry, have never proved practical in application to milk. With the widespread use of multiple effect evaporators for condensing milk, it is not possible to specify precisely the degree of heat treatment to which milk may be subjected in the condensing operation. In some of the low temperature evaporators the heating effect is essentially nil. In a single effect vacuum pan the evaporating temperature usually ranges between 110° and 140° F. (43° to 55° C.). Though milk may be exposed to only slightly elevated temperatures during condensing, the significant factor to remember is that application of heat is essential to the operation, either in the process of forewarming or within the pan to facilitate evaporation.

Drying. The purpose of drying milk and milk products is to remove virtually all the water in them with a minimum amount of irreversible physical and chemical change. Of the various processes that are used to dry milk, spray drying is by far the most important. Lesser quantities of milk and other dairy products are roller dried. Spray drying consists of atomizing concentrated milk into a stream of

hot dry air. The moisture-laden air is continuously replaced in the drier and the dehydrated particles of milk are usually removed through the combined effects of gravity and dust collection systems. In the roller process for drying milk, the concentrated milk is deposited in a thin film on a metal drum heated with steam. During the course of one revolution of the drum the milk film is deposited, dried, and removed from the drum surface by an appropriately placed knife edge. Double drums placed with extremely small clearance and rotating counter to one another are used more frequently than a single drum.

It is very difficult to assess the effects of heating on milk during the drying process. The fact that the milk is in a progressing state of concentration suggests that some of its constituents may be rendered more labile to the effects of heat, whereas others may be stabilized. Moreover, the cooling effect of evaporation must be taken into account. In spray drying the length of time that dried milk remains in the drier is a factor, but in the roller process the speed of the drum will determine how long the milk film is in contact with the heated metal surface. In spray drying, air inlet temperatures ranging from 160° to 350° F. (71° to 177° C.) may be employed, and in atmospheric drum-drying processes temperatures of 194° to 270° F. (90° to 132° C.) are used. The precise temperature conditions vary with the particular drying equipment.

Current trends toward use of minimum heat treatment in the processing of milk have focused interest on the actual temperature that a particle of dry milk achieves during the spray drying process. There is no simple method of measuring the temperature of such a minute particle. It has been postulated that the temperature of the particle never exceeds the exhaust air temperature of the drier. Measurement of temperatures at the surfaces to which the dried particles fall appears to confirm this postulation. However, it can readily be seen that with driers operating at an air inlet temperature of 300° F. and an exhaust temperature of 160° F., the maximum temperature achieved by the particle can be very significant from the standpoint of heat damage.

In general, atmospheric roller drying of milk is more problematical regarding heat damage than is the spray drying process. Roller-dried milks frequently contain burnt particles. Not only is the overall potential for heat damage in milk by the process greater than with spray drying, but extreme care must be taken to avoid uneven deposition and removal of the milk film. Although roller drying under

vacuum overcomes these difficulties and, indeed, yields a much better dried product, the process has not been widely adopted by the industry.

Superheating. The main purpose of superheating is to increase the viscosity of the fluid dairy product so treated. The process is applied primarily to condensed whole or skimmilk to enhance its properties as an ingredient in ice cream mix. When superheated condensed milks are used in ice cream manufacture the need for a stabilizer is largely or wholly overcome, and the resulting ice cream will exhibit very satisfactory body and texture characteristics when rapid turnover of the product in distribution channels is possible. The actual superheating process is accomplished by direct injection of superheated steam into the condensed milk. Heating is normally to a temperature of 185° F. (85° C.) for a period of time sufficient to give the desired increase in viscosity. Some experience with this end point is essential since it will vary from one milk to another and since overheating will result in complete destabilization of the milk protein. The process is in effect a method of partially coagulating the milk protein with a resulting increase in viscosity.

Homogenization. The primary purpose of homogenizing milk is to break down the fat globules into a finer and more stable state of dispersion. Heating the milk is somewhat incidental to the process. Ac ıally, homogenization can be carried out, although not satisfactorily from the standpoint of breaking down fat globules, without heating. The two main reasons for heating milk prior to homogenization are to liquefy the fat and to inactivate milk lipase. If milk lipase is not inactivated by suitable temperature treatment, homogenization will create conditions which promote rapid lipolysis of milk fat, resulting in a disagreeable rancid flavor caused by liberation of lower fatty acids from the glycerides. It is feasible to homogenize milk either before or after pasteurization. For the purpose of inactivating lipase the milk should be heated to at least 140° F. (60° C.). Homogenization has many effects on the physical and chemical properties of milk that are not directly attributable to heat treatment. This matter is discussed further in Chapter 9 and in the text by Trout (31).

Miscellaneous. There are many other situations where the application of heat is essential in the processing of dairy products. These include adjusting milk to satisfactory temperatures for incubation with starter organisms in the manufacture of cheese and cultured dairy products. The satisfactory blending of the ingredients for processed cheese manufacture requires heat treatment to approximately 160° F.

(71° C.) in a steam-jacketed kettle. The manufacture of ice cream mix utilizes heat, incidental to pasteurization, for a number of purposes. Such treatment accomplishes the melting of butter, the solubilizing of dry sugar, and the dispersion of stabilizer, among other things.

Finally it must be borne in mind that storage conditions for certain dairy products are comparable to heat treatment. What may be accomplished during a few minutes at temperatures above 212° F. may also be accomplished by a lengthy storage period at room temperature. One of the best examples of this type of parallel change is afforded by sweetened condensed milk. When a can of this product is submerged in boiling water for a half hour it is converted into a brown semisolid pudding with an attractive caramel flavor. The same type of change in this product results from two years storage at room temperature.

It will be seen from the foregoing that the heat treatment of milk and its products is indeed of broad significance. An understanding of milk processing and its problems can be no more than superficial without some appreciation of the physical and chemical effects that heat may have on the medium. The following information regarding the effects of heat on milk has been included to meet this need. Although other chapters of this book have treated the subject as it relates to individual constituents, in the present treatment intact milk and milk products serve as the basis of discussion.

THE EFFECTS ON THE MILK SALTS

The composition and properties of the milk salt system have been discussed in Chapter 5. In this section the effects of heat on the milk salts and their ionic balance is considered. The subject is closely allied to effects on the milk protein, particularly protein stability, matters that are treated in the following section.

The heat-induced changes in the milk salt system may be grouped into three categories: (*a*) Readily reversible shifts in the ionic balance produced by changes in temperature. It should be possible to collect information on this point although none seems to be currently available. Of particular interest would be determination of the ionic conditions in hot milk at various temperatures. (*b*) Sluggishly reversible shifts in ionic balance. The reversal is slow enough so that it can be followed in cooled milk after heating. (*c*) Irreversible shifts in ionic balance.

In describing the effects of heat on milk salts, it is necessary to consider the effects of all possible variations in temperature to which the milk may be subjected. The equilibria undoubtedly change continuously after the milk is drawn from the cow. The particular equilibrium that holds in a sample at any instant is determined by the temperature at that instant and by the temperature history of the sample. The temperature range that needs to be considered extends from below the freezing point to 200° C. or so.

Concentration of milk markedly shifts the salt equilibria and consequently needs to be taken into consideration in discussing the effects of heat. A concentrated product may not behave like the original unconcentrated milk from which it was prepared. As pointed out in Chapter 5, concentration of milk results in a shift of phosphate and citrate to the colloidal phase and a reduction in pH.

The observed effects of heat treatment on the ionic equilibria of milk are as follows.

Loss of CO_2. Milk as secreted by the cow contains about 20 mg. of CO_2 per 100 ml. (or about 10% by volume). This gas is rapidly lost from milk, owing to the low content in the air. The loss is essentially irreversible. Ordinarily, commercial milk may contain about half of the original CO_2 (i.e., about 10 mg. per 100 ml.). The loss of CO_2 from milk is accelerated by heating and agitation.

It would be anticipated from Fig. 7, Chapter 5, that removal of CO_2 would affect the balance in the rest of the system. Such effects on the pH and titratable acidity of milk can be determined, uncomplicated by other effects of heat, through vacuum removal of CO_2. Such treatment has been found to raise the pH and reduce the titratable acidity (see Chapter 8). It would be expected that the removal of CO_2 and the consequent rise in pH would be reflected in a decrease in dissolved calcium and phosphate and probably also in calcium ion activity. As far as is known, such an effect has not been demonstrated.

Transfer of calcium and phosphate to colloidal state. Since calcium phosphate is less soluble at high temperature than at low, it would be expected that the concentrations of dissolved calcium and phosphate in milk would be decreased by heat treatment. This effect actually was observed by Söldner in 1888 and many reports on it have appeared in the literature since that time. Almost all workers observed a decrease in dissolved calcium and phosphate upon heating, but there is a good deal of quantitative variation among the data of various investigators, as may be seen in Table 2.

Table 2. Loss of Dissolved Calcium and Phosphate Produced by Heating Milk as Reported by Various Investigators

Worker	Method of Separating Phases	Heat Treatment Temp.	Heat Treatment Time	Loss in Dissolved Ca, mg./100 ml.	Loss in Dissolved P, mg./100 ml.
Söldner	Clay filter	Boil	...	9.0–10.0	4.4–5.6
De Vries and Boekhout	Chamberland filter	Boil	2 hr.	1.7–3.9	...
Grosser	Bechold filter	Boil	5 min.	4.9	+2.6
			15 min.	6.7	−0.4
			30 min.	0.2	+1.3
Rupp	Clay filter	68.3° C.	30 min.	0.7	0.1
Bell	Pasteur	60° C.	30 min.	3.0	0.9
	Chamberland	65.6° C.	30 min.	1.0	0.9
	filter	71.2° C.	30 min.	2.0	0.9
		76.7° C.	30 min.	5.0	2.6
		82.3° C.	30 min.	4.0	1.8
Mattick and Hallet	Dialysis for	46–49° C.	30 min.	0	4
	18 hr. vs.	57–61° C.	30 min.	0	1
	3% NaCl	63–66° C.	30 min.	3	0
	parchment	74–77° C.	30 min.	3	1
	membrane	96–98° C.	30 min.	4	1
Lampitt and Bushill	Dialysis vs. H_2O colloidion membrane	100° C.	1 hr.	5.2	3.6

At least three possible causes can be suggested for these discrepancies.

1. The transfer of calcium and phosphate to the colloidal state is slowly reversed upon holding the milk cold after heating (see Fig. 1). Thus the results obtained will depend on the time elapsed after heating before a sample of ultrafiltrate or dialysate is prepared for analysis. For many of the data in the literature this time is not specified. In order to follow the reversal, a method for separation of phases must be employed by which a sample of dissolved phase can be prepared shortly after heating.

2. Some workers may have used ultrafilters to separate the phases that allowed passage of some of the colloidal particles of raw milk.

3. Variations in behavior occur between milk samples. Studies in the senior author's laboratory have revealed some rather large variations among samples in the extent of transfer to the colloidal state produced by a given heat treatment as well as in the reversal to

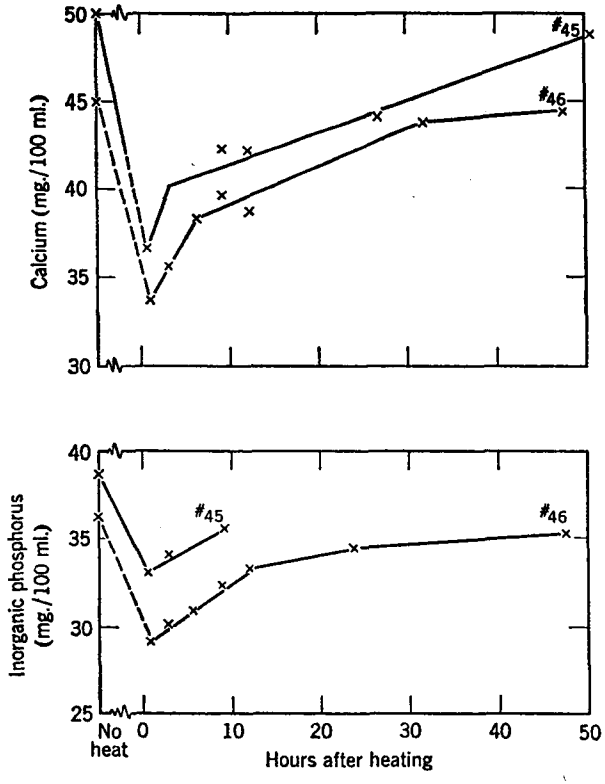


Fig. 1. Ultrafilterable calcium and phosphorus in milk heated at 78° C. for 30 minutes.

solution upon holding after heating. The level of CO_2 in the milk does not appear to be an influencing factor. The effect of time and temperature of heating on the amounts of dissolved calcium and phosphate in one sample of milk is given in Fig. 2. There seems to be no constant ratio between the Ca and P insolubilized under such conditions.

It may be presumed that the dissolved calcium and phosphate transferred to the colloidal state by heat treatment becomes connected in some way (perhaps adsorbed) to the colloidal micelles of caseinate-phosphate. The exact nature of such a connection is not known and it must be reiterated that the original structure of the micelles is not known either. It is important to recognize, however, that extensive changes in the structure of the micelles may be produced by heat

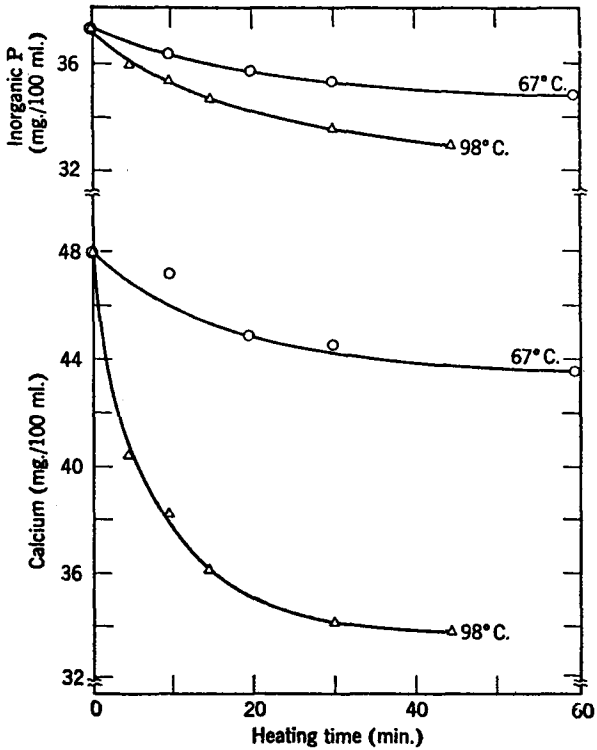


Fig. 2. Effect of time and temperature of heating on dissolved calcium and phosphate measured at 5° C. within one hour after heating.

treatment. The mere fact that the level of dissolved calcium and phosphate tends to revert to the original value after heat treatment does not mean that the original conditions are regained throughout the system. Furthermore, aggregation of the caseinate-phosphate micelles may occur (reversibly or irreversibly).

Effect on acidity. The removal of CO_2 causes an increase in pH and a decrease in titratable acidity as already pointed out. This effect is counteracted upon heat treatment by hydrogen ions liberated by insolubilization of the calcium and phosphate. The effect is as though the following reaction occurred:

$$3Ca^{++} + 2HPO_4^{=} \rightarrow Ca_3(PO_4)_2 \downarrow + 2H^+$$

In some cases these two opposing factors so nearly counterbalance each other that there is no net change in pH.

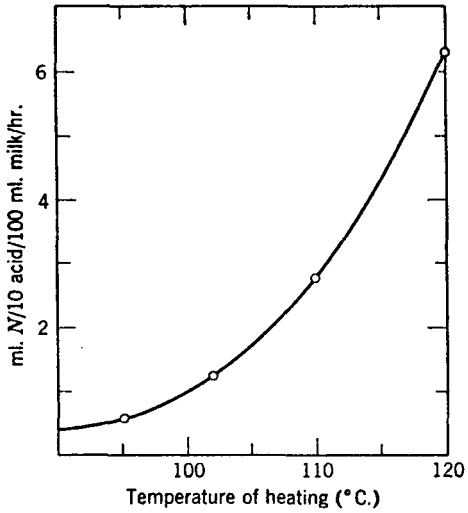


Fig. 3. Effect of temperature of heating on rate of production of acid in milk. Data of Whittier and Benton (35).

High temperature heat treatment results in the production of acids (lactic and formic among others) by degradation of lactose (Fig. 3). This increase in acidity greatly alters the ionic equilibria in the milk.

Lack of effect on citrate. Possible effects of heat on citrate in milk have been investigated considerably. Several workers have demonstrated that citrate is not *destroyed* by heating milk at temperatures up to boiling or by autoclaving. However, since calcium citrate is less soluble at high temperature than at low, the possibility has to be considered that heat treatment causes a precipitation of calcium citrate or its transfer to the colloidal state. On the basis of available data, heat appears to have little, if any, effect on the amount of dissolved citrate in milk.

EFFECTS ON THE MILK PROTEINS

Serum Proteins

Denaturation and related changes. Matters of very practical interest attach to the heat-induced changes involving the milk serum proteins. Such phenomena as cooked flavor, development of anti-oxygenic properties, impairment of clotting properties or imparting of

soft curd characteristics to milk, prevention of age-thickening in evaporated milk, and improvement in the baking qualities of non-fat dry milk are related either directly or indirectly to heat effects on the serum proteins. This group of proteins amounts to approximately 0.6 to 0.7% of the milk, and since they reside in the whey following removal of the caseins from skimmilk by acid or rennet coagulation, they are also designated the "whey proteins." The most important of these in the present consideration is β-lactoglobulin, which represents about 50% of the total serum proteins. It figures most prominently in heat-induced changes of milk.

Since the turn of the century, certain isolated observations have been made regarding the effects of heat on milk, effects which now seem possible to explain fairly completely on a single basis. The changes concerned are: evolution of H_2S, formation of cooked flavor, lowering of oxidation-reduction potential, development of antioxygenic properties, and lowering of curd tension. The common cause of these phenomena is heat denaturation of the milk serum proteins. It appears that in the native state the milk serum proteins have a definite coiled configuration which is maintained by hydrogen bonding, hydration, and secondary valence forces. When these proteins are exposed to heat above a certain critical level the native configuration is disrupted and the characteristic properties of the proteins are altered. One important manifestation of heat denaturation of these proteins is a change in solubility. They become very much less soluble in acid at pH 4.6 and in saturated sodium chloride. Thus they precipitate from heated milk with the casein upon acidification or saturation with salt.

Figure 4 presents data concerning the effects of various heat treatments on the heat denaturation of these proteins in skimmilk as measured by precipitability with saturated sodium chloride according to the method of Harland and Ashworth (10). The rate increases regularly as the temperature is raised from 68° to 80° C. In Fig. 5 the data have been replotted to show the equivalent time-temperature treatments required for denaturations for 5 and 40%. Also in Fig. 5 is plotted a line representing normal pasteurization requirements of 63° C. for 30 minutes or 71° C. for 15 seconds. The denaturation of the serum proteins requires somewhat more drastic heat treatment than normal pasteurization. Results of this kind are, in fact, a composite for the denaturation of the proteins in the mixture. The individual proteins exhibit differing degrees of susceptibility to heat denaturation, and the situation is further complicated by interactions among them. When milk is heated at various temperatures and the casein and the

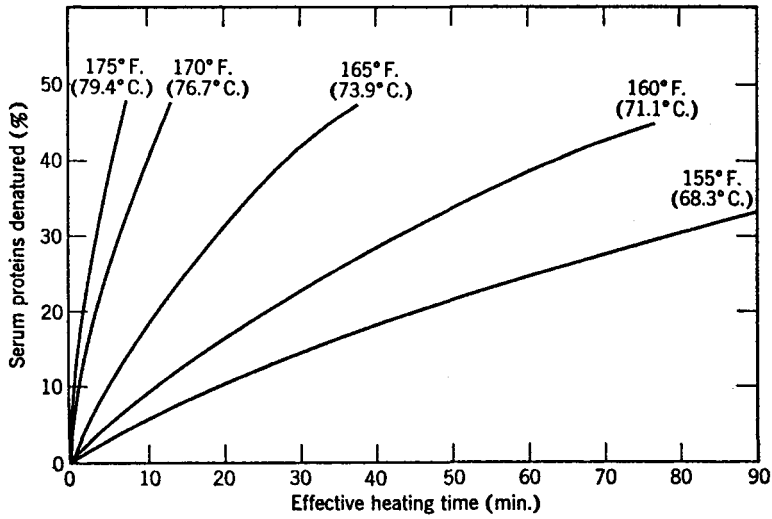


Fig. 4. Heat denaturation of milk serum proteins in skimmilk as measured by precipitability with saturated NaCl. Data of Harland et al. (12).

denatured serum proteins thrown out by acidification, electrophoretic analyses of the resulting wheys show that the immune globulins are rendered acid precipitable by the least heat treatment, followed in order by the blood serum albumin, the β-lactoglobulin, and finally the α-lactalbumin, which is very resistant to heat denaturation (20).

Another significant property of milk serum proteins affected by heat concerns the reactivity of their sulfhydryl (—SH) groups. These groups are apparently occluded or bonded in such a way in the native protein as to be relatively unreactive. However, when the protein particle is subjected to sufficient heat it uncoils and the groups become more accessible and reactive.

The activation of —SH groups in milk by heat treatment first becomes evident at about 167° F. (75° C.) as measured by the nitroprusside test (17). A reagent that shows similar sensitivity to such change is thiamine disulfide. Data pertaining to the detection of sulfhydryl activity with this latter reagent under various conditions of heating are presented in Fig. 6. In addition to illustrating the activating effect of heat on sulfhydryl groups in skimmilk, Fig. 6 also indicates the susceptibility of these groups to oxidation, as shown by the curve for the deaerated sample. The lability to oxygen of these groups is the basis of explanation for the antioxygenic properties of milk so heated. By the same token, —SH groups are powerful

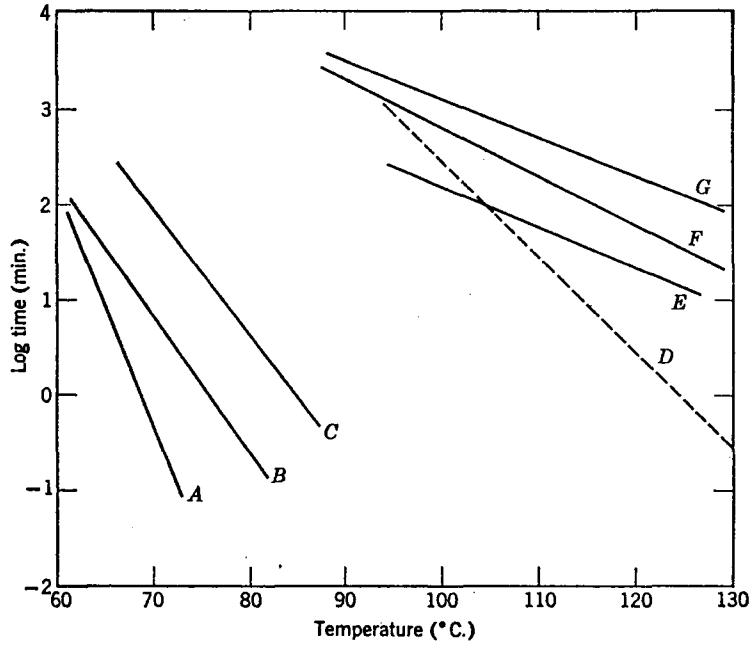


Fig. 5. Time-temperature relations for: *A*—pasteurizaton; *B* and *C*—5 and 40% denaturation, respectively, of serum proteins (12); *D*—sterilization (7); *E*—browning (4); *F* and *G*—heat coagulation of 18 and 9% solids skimmilk, respectively (34).

reducing agents and probably account for the lowering of the oxidation-reduction potential that simultaneously accompanies their activation in milk. Data revealing this drop in oxidation-reduction potential are presented in Chapter 12. As explained in that chapter, the evolution of volatile sulfides and the appearance of cooked flavor in heated milk closely parallels activation of the —SH groups. H_2S seems partly responsible for the cooked flavor and it is notable that the amino acid cysteine, the principal site of —SH groups in the milk serum proteins, liberates H_2S when heated in aqueous solution. The exact chemistry of this decomposition is not known, although it may be effected in the following alternative ways:

$$HOOC—CHNH_2—CH_2—SH + H_2O \rightarrow HOOC—CHNH_2—CH_2—OH + H_2S$$

or

$$2HOOC—CHNH_2—CH_2—SH \rightarrow (HOOC—CHNH_2—CH_2)_2S + H_2S$$

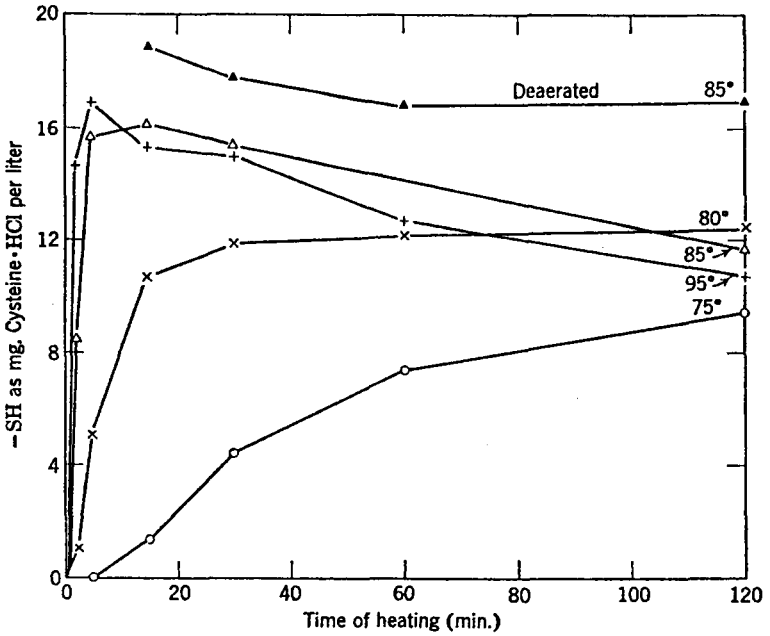


Fig. 6. Activation of sulfhydryl groups by heating milk as measured by thiamine disulfide. Data of Harland et al. (11).

Of the milk serum proteins, β-lactoglobulin is the main origin of —SH groups. The proteins associated with the fat globule membrane, which may or may not be in part similar or identical to the milk serum proteins, also serve as an origin. The available information indicates that the latter source is somewhat more readily activated by heat treatment. Ordinarily the nitroprusside reaction is positive for cream after a momentary temperature treatment of 160° F. (71° C.), or approximately 7° F. lower than that for skimmilk.

Changes in the curd forming properties of milk as a result of heating have at least two practical aspects, the one being development of soft curd characteristics, which is significant from the standpoint of infant nutrition; and the other concerning impairment of milk protein clotting in the manufacture of cottage cheese. In all probability these phenomena are directly traceable to the flocculation of the serum proteins, a result of heat denaturation. It has not been established whether the soft curd characteristics of highly heated milks are due to chemical or purely physical changes. One theory holds that the flocculated particles of serum protein represent points of weakness in the

curd. The soft curd characteristics of homogenized milk are explained in much the same manner, except that it is minute particles of milk lipide rather than denatured protein that inhibit the cross-linking mechanism of curd formation. Another explanation is that the serum proteins react chemically with the casein, thus destroying the capacity of the casein to build a firm curd. In support of the latter explanation, electrophoretic data indicating interaction of β-lactoglobulin with casein components can be cited. On the other hand, studies of this matter based on sulfur distribution in the proteins removed from heated milk by supercentrifugation have yielded conflicting results. A recent investigation by Sullivan et al. (27), employing serum proteins labeled with S^{35}, indicates that substantial quantities of denatured whey proteins are removed with the casein when heated milk is supercentrifuged. The precise mechanism notwithstanding, the soft curd characteristics of heated milk appear plausibly to be a general result of heat denaturation of the milk serum protein.

It has long been known that raw milk contains a deleterious factor, or factors, that depresses loaf volume and slackens dough when it is incorporated into bread. This effect can be overcome by heat treatment. The improvement in baking quality produced by heat treatment has been found to parallel approximately but not exactly the denaturation of the serum proteins. Thus the extent of denaturation of the serum proteins has come to be widely used as an index of baking quality of lots of non-fat dry milk solids of unknown history. Actually, at least three factors seem to be involved. These are (*a*) a deleterious, unknown protein fraction that follows the immune globulins when milk serum proteins are fractionated (this fraction is inactivated by heat treatment), (*b*) a deleterious effect of casein, and (*c*) a deleterious effect of freshly heated β-lactoglobulin due to the active sulfhydryls that it contains. The fundamental mechanism by which the milk proteins exert their effects on dough and bread is not known, but certainly it must involve some sort of interaction with the gluten proteins that weakens the structure of the latter.

There are two additional phenomena of milk that may result from heat treatment of the serum proteins. These are loss of capacity to form a normal cream layer and an increase in the reflectance (whitening) of milk. As discussed elsewhere (Chapter 9), certain of the globulins of milk are responsible for normal creaming since they promote the formation of clusters among the rising fat globules. Overpasteurization of milk initiates denaturation of the globulins, which causes the cream layer formed in such milk to be shallow and

indistinct. The subject of color changes produced in milk by heat has been investigated recently by Burton (4). He has noted that before browning, milk shows increased whiteness due in all probability to heat flocculation of serum proteins, changes in the state of casein aggregation, and conversion of soluble calcium to insoluble salts.

The Caseinate System

The caseinate particles of milk have been discussed in detail in Chapter 10. Casein is not considered to be a denaturable protein in the sense that the milk serum and many other proteins are. Casein, when heated alone in solution, is not altered in solubility, viscosity, and optical rotation in the range of heat treatments that denature the serum proteins. Consequently the changes that are wrought in casein as a result of heat treatment depend upon different mechanisms from those that operate with the serum proteins. Since they are stabilized by charge, the caseinate particles are extremely sensitive to changes in ionic environment. They readily aggregate with increase in concentration of calcium or magnesium ions and break up with decrease in concentration of these ions. Since their equilibrium dispersion in milk is rather precarious, minor changes in salt balance and pH easily upset this equilibrium and tend to destabilize and precipitate the casein particles.

The problem of protein stability is of tremendous practical importance in the dairy field. The successful processing of most dairy products is predicated on operations that will avoid destabilization of the proteins and, even further, will insure their stability during storage when necessary.

Destabilization of the caseinate particles by heat treatment causes them to aggregate to form a three-dimensional network entrapping some of the milk serum. The process is first manifested by an increase in viscosity, and with more drastic heat treatment by actual coagulation of the caseinate with a separation of the system.

Protein stability is of particular interest and importance in the manufacture of evaporated milk. The conventional evaporated product must be able to withstand sterilization in the can without coagulating. A slight gelation is permissible if it is readily dispersible by mechanical agitation. Actually, some thickening during sterilization is desirable to inhibit separation of the fat and because too thin a product gives the impression of an insufficient degree of concentration. Another aspect of the protein stability problem has come into promi-

nence in recent years with the use of high-temperature short-time heat treatments to produce sterile concentrated milks of light color and bland flavor. Such products are often of low viscosity initially, but frequently gel during storage. A certain degree of destabilization of the protein is sought in the manufacture of non-fat dry milk for use in bread because this enhances the water-holding capacity of the milk in dough.

In general there is a straight-line relation between the temperature and the logarithm of the time necessary to produce coagulation. This is shown in Fig. 5 for temperatures from 80° to 130° C. This linear relation may not always hold true; some workers have found significant variations from it, and sometimes the order of stability of a series of samples at one temperature is reversed at another. It is at once evident from Fig. 5 that coagulation of the caseinate particles requires very much more drastic heat treatment than the denaturation of the serum proteins. This graph also indicates the effect of concentration; skimmilk of 9% solids concentration is more stable than after evaporation to 18% solids. The heat stability decreases progressively with increasing concentration of solids. This decrease in stability may be seen in Fig. 7, which also illustrates the effect of forewarming. Such a reduction in stability with increasing concentration of solids is to be expected because of the increased concentration of destabilizing ions and the reduced pH (see Chapter 5).

It was pointed out in Chapter 10 that the stability of the caseinate particles is influenced by a large number of factors. Heat treatment is one of the more significant of these, but it cannot be considered as an isolated factor. To a large extent it acts through and in conjunction with such factors as pH, salts, and serum proteins in producing the observed effects.

The only known direct effect of heat on casein is degradation with the release of esterified phosphate and hydrolysis of peptide linkages. For example, Howat and Wright (14) observed extensive dephosphorization and hydrolysis of peptide bonds during heating of sodium and calcium caseinate sols at 120° C. for periods up to five hours. The extent to which such hydrolytic changes occur in the sterilization of milk has not been definitely established. Furthermore, it is not known whether or not these changes are the critical factors causing destabilization. They would seem to merit further study.

As pointed out in Chapter 10, the caseinate particles are very sensitive to slight changes in pH, being destabilized by lowering and stabilized by raising it. High temperature heat treatment produces

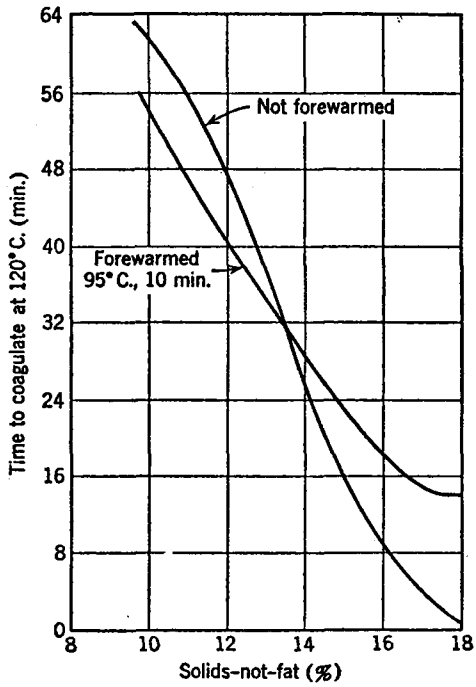


Fig. 7. Effect of solids concentration on heat stability. Data of Webb and Holm (34).

increased acidity through cleavage of ester phosphate from the casein and through production of acids, such as lactic and formic, by degradation of lactose. The latter acids are produced very slowly up to 90° C., but the rate increases rapidly as the temperature is raised (Fig. 3). At 100° C. and higher the acid produced in this manner far overshadows any effects from elimination of carbon dioxide. Increases in these acids can be expected to bring calcium and phosphate back into solution, thus increasing the calcium ion concentration and decreasing the stability of the caseinate particles. It seems probable that generation of such acids is only a practical consideration in milks which receive substantial heat treatment above 100° C.

Small quantities of polyvalent cations (such as calcium and magnesium), especially when acting in conjunction with heat and lowered pH, are very effective in destabilizing the caseinate particles. Unfortunately the stability of the caseinate system toward heat may not be simply a function of these cations. Some few milks apparently are

stabilized by added calcium and destabilized by ions such as phosphate and citrate that sequester calcium.

Observations such as these are the basis for the well-known "salt balance" theory first suggested by Sommer and Hart (26). This theory holds that optimum stability depends on a certain ratio of calcium and magnesium ions to those of phosphate and citrate. The concept has proved very useful in developing practical procedures for controlling the stability of evaporated milk during sterilization. In practice the evaporated milk to be sterilized is treated, as a series of samples on a pilot scale, with graded levels of phosphate or calcium salts, the latter being rarely if ever necessary. The samples are then sterilized and after cooling the minimum level of added salt that imparted satisfactory stability is noted and used to stabilize the lot of milk to be sterilized.

The Sommer and Hart theory is indeed useful in this connection; however, the phosphate and citrate salts that are used are almost invariably those of sodium and it seems important to consider the possible significance of this ion regarding stability. Calcium ions are divalent and thus offer the possibility of cross-linking and polymer formation in the casein system. Sodium ions, on the other hand, are monovalent and would tend to oppose polymer formation. Moreover, the addition of disodium phosphate, which is commonly used as the stabilizing salt, would have some tendency to raise the pH of milk slightly, which also would favor stability. Such factors as these suggest that the sodium ions of these stabilizing salts as well as the phosphate and citrate ions may be of significance. A procedure for stabilizing evaporated milk by ion exchange treatment based on replacement of calcium and magnesium with sodium ion has been developed (18).

It has often been suggested that one or more of the serum proteins interact in some manner, either chemically or physically, with the casein when milk is heated. Aqueous dispersions of casein are extremely stable toward heat treatment, but the addition of even small amounts of milk serum protein renders the casein much more susceptible to heat coagulation. It has been demonstrated that small natural increases in the so-called lactalbumin content of milk cause a considerable decrease in the sterilization time required to coagulate evaporated milk. One possible clue in this matter concerns the very potent destabilizing action that added H_2S has on evaporated milk. H_2S is released during the heat denaturation of serum protein.

It is well known that a forewarming treatment of the milk before

condensing greatly increases the stability of the evaporated product during sterilization. Such preheating treatments are somewhat less severe than those required to coagulate the milk. For example, a treatment of 95° C. for 10 minutes is commonly employed. Figure 7 illustrates the effect of forewarming on the stability of skimmilk of varying solids concentration. It is important to note that heat treatments of this magnitude make unconcentrated milk less stable to subsequent sterilization, but they greatly stabilize the 2:1 concentrate. High-temperature short-time forewarming has been found to produce greater stability in the evaporated product than the treatment of 95° C. for 10 minutes. The data of Webb and Bell (32), shown in Table 3, exhibit the great superiority of forewarming at 150° C. for

Table 3. COMPARISON OF CONVENTIONAL AND HIGH-TEMPERATURE SHORT-TIME FOREWARMING ON THE HEAT STABILITY OF EVAPORATED WHOLE MILK
(26% total solids)

Forewarming Treatment		Coagulation Time at 115° C.	
Temp., ° C.	Time	No Salts Added, min.	Optimum Salt Addition, min.
95	10 min.	19	32
150	25 sec.	74	93

Data of Webb and Bell (32).

25 seconds. High-temperature short-time forewarming even permits the production of milk concentrated to 32 to 37% total solids that is stable to sterilization.

The mechanism of the forewarming effect is not known. Specifications for optimum forewarming, like those for added stabilizing, have been developed by trial and error. Suggestions have been made that forewarming exerts its influence through shifts in salt equilibria, possibly through precipitation of calcium phosphates that decrease in solubility with increase in temperature, or through denaturation of the serum proteins. To date a conclusive statement cannot be made as to the mechanism involved.

There is a trend toward high-temperature short-time treatments for sterilization of evaporated milk. A glance at Fig. 5 indicates the underlying reason for the superiority of such methods. The sterilization curve is steeper than those for coagulation and browning. Thus,

in the high-temperature short-time zone there is more leeway for the production of a sterile product with a minimum of brown discoloration and cooked or caramelized flavor. Exploitation of these potentialities has been made possible by the development of heating equipment in which the temperature and exposure time can be controlled and of machinery for canning the product aseptically after sterilization. Products made in this way have some very serious drawbacks. They are of such low viscosity initially that separation of the fat is a problem. Worse still is the fact that after a few weeks or months in storage at room temperature they undergo a rapid increase in viscosity, culminating in an actual gelling.

The phenomenon of gelation seems to be a quite different form of destabilization of the caseinate particles from the usual coagulation by heat. A smooth gel is formed, occupying the entire volume of the milk. It does not shrink or synerese. When first formed it can be restored to the sol state merely by shaking. Gradually it becomes irreversible. The fact that gelation occurs in the high-temperature short-time sterilized product but not usually in conventional evaporated milk suggests that the milk proteins pass through a "critical structural" state during the course of heating. In this state they are able to form a cross linked gel network. The H.T.S.T. treatment leaves them in this state, but conventional sterilization carries them through and beyond it. In further support of this hypothesis, it has been found (30) that the gelation can be delayed greatly by heating the concentrate at 82° to 93° C. for 30 minutes prior to sterilization. This process evidently carries structural changes in the proteins beyond the critical zone. It can be applied without increasing the brown color and caramelized flavor.

It can be seen from the foregoing that the relation of heat treatment to the various factors affecting protein stability is indeed complex. Many of the considerations here have special practical value in connection with concentrated and dried milk products. Those interested in the technological aspects of the problem are urged to consult Hunziker's text (15), the monograph by Whittier and Webb (36), and the comprehensive review on dry milk products by Coulter et al. (6).

Nitrogen Distribution

It is generally recognized that normal pasteurization has no measurable effect on the nitrogen distribution in milk. However, heat

treatments in excess of those employed for pasteurization show progressive changes in this pattern for milk. The principal changes which become evident with increasing heat treatment are an increase in the apparent casein nitrogen with a corresponding decrease in albumin-globulin nitrogen and an increase in the proteose-peptone and non-protein nitrogen fractions. These changes are attributed to the coagulation of the albumin and globulin fractions of milk and their recovery with the casein in the acid precipitation fractionating procedure; the increases in proteose-peptone and non-protein nitrogen result from fragmentation of the milk proteins. Data representative of those that are normally encountered in this connection are presented in Table 4. Part of the increase in non-protein nitrogen is due to the formation of ammonia.

Table 4. THE EFFECTS OF STERILIZING HEAT TREATMENT (120° C. AT 30 MIN.) ON THE NITROGEN DISTRIBUTION OF SKIMMILK

	Pasteurized, % N	Sterilized, % N
Total N	0.5333	0.5270
Non-casein N	0.1103	0.0719
Non-protein N	0.0300	0.0401
Globulin N	0.0197	0.0188
Casein N	0.4230	0.4551
Albumin N	0.0353	0.0000
Proteose N	0.0253	0.0319
(Data above calculated as percentage of total N)		
Non-casein N	20.68	13.64
Non-protein N	5.63	7.61
Globulin N	3.69	3.57
Casein N	79.32	86.36
Albumin N	6.62	00.00
Proteose N	4.74	6.05

Data of Menefee et al. (21).

BROWNING

One readily demonstrable effect resulting from the prolonged heat treatment of milk is browning. Browning is of broad significance in the food field. At times it is sought such as in the toasting, baking, and frying of foods, whereas in other instances it is clearly considered to be

a defect. Generally speaking, with the exception of caramel and butterscotch confections, the development of browning in milk and milk products is objectionable. In the normal course of handling, processing, distribution, and consumption, pasteurized milk exhibits no tendency to brown. However, certain concentrated and dried milk products, which are processed at high temperatures and are stored at room temperature for appreciable time, are subject to browning. Evaporated milk is particularly susceptible to the defect. The processing and storage conditions employed for this product invariably impart some degree of browning. When conditions are optimum, sweetened condensed milk, dried milks, dried whey, and dried ice cream mix also will exhibit browning. Somewhat less problematical because of their low lactose and protein contents are sterilized, unconcentrated milks and creams and dried cream products.

Fundamentally, there appear to be three main types of browning: (*a*) caramelization or non-amino browning of sugars, (*b*) amino-sugar or Maillard-type browning, and (*c*) oxidative browning. Caramelization browning may be defined as the heat decomposition of sugars as a function of pH and buffers in the absence of amino compounds. It requires a relatively high order of activational energy. On the other hand, Maillard-type browning requires a relatively low order of energy for its initiation and exhibits autocatalytic qualities once it has started. It may be defined as that browning which results from the interaction of amino compounds (proteins, amino acids, etc.) with sugars. It is by far the most important type of browning in the food field and is the type that prevails in milk and its products. Oxidative browning, with which we are not directly concerned here, is typified by the color change that occurs when the surface of fruits or vegetables is bruised.

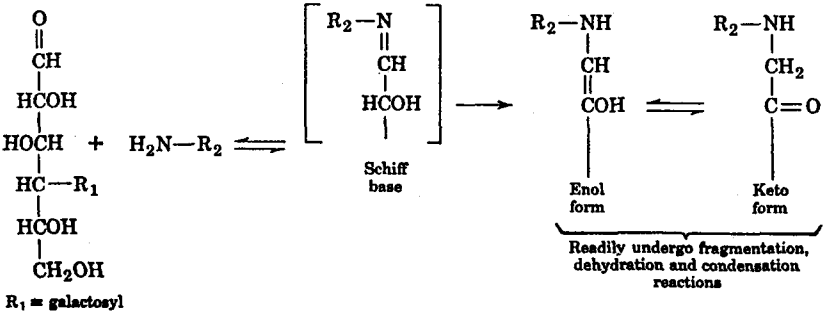
Reactants and chemical mechanisms. The two principal reactants in the browning of milk and milk systems are lactose and casein. In aqueous solution or in the dry state under suitable conditions of humidity, these two substances will undergo browning readily, whereas neither will discolor when so treated alone. There is evidence that suggests that the milk serum proteins and the phosphate salts may make minor contributions to browning of milk systems; however, they are distinctly of secondary importance.

The chemistry of browning in foods is highly complex and in an effort to unravel the problem, model systems have been used by many investigators. In place of the complex media presented by foods, simple aqueous solutions of a sugar, usually glucose, and various amino

compounds, particularly amino acids, have been employed. The information that has been compiled as a result of this work on simplified systems is very comprehensive in contrast to the limited findings for milk. Those interested in the basic chemistry of browning should consult the reviews by Hodge (13) and Danehy and Pigman (8). Browning of milk and its products also has been reviewed (23). Many of the observations that have been made on simplified systems find close parallel in those made regarding the browning of milk and its products.

Between the established fact that casein and lactose are the primary reactants and that a brown product is a resultant of the reaction, there lies an area of chemistry that is largely unknown. The initial reaction, at least in the dry state, between glucose and casein involves a 1 : 1 reaction of the glucose with the ϵ-amino group of lysine. Such a 1 : 1 reaction between these components has not been demonstrated in an aqueous system, presumably due to instability of the addition product under such conditions. In addition to the amino groups of lysine, the other basic amino acids, arginine and histidine, may be involved secondarily.

The precise nature of the interaction between the lactose and amino groups of lysine has not been established, but it seems probable that the first formed condensate, among other things, undergoes the Amadori rearrangement, the products of which can lead to sugar fragmentation, formation of furans, reductone-like reducing substances, fluorescent substances, and brown pigments. The critical intermediate that can promote such changes is the N-substituted 1-amino-1-deoxy-2-ketose. Compounds of this type have been isolated recently from evaporated milk and dried skimmilk by techniques of tryptic hydrolysis and paper chromatography (1). They are presumed to result according to the following scheme:



Little additional information can be offered at this time regarding the intermediate chemistry of browning in milk. In essence, the phenomenon appears to be a catalytic decomposition of lactose, through dehydration, fragmentation, and condensation, by the amino groups of casein. These reactions lead to the formation of brown pigment and many associated changes which are discussed below.

The brown pigment (melanoidin). The brown substance or substances formed in milk systems as a result of high heat treatment or storage under suitable conditions have received very limited study. The material appears to be highly complex and is probably a mixture of polymers. For this reason it is difficult to attribute physical and chemical properties to it with certainty since there are no criteria of purity for such materials. The pigment appears to be unsaturated and probably owes its light-absorbing capacity to this property. It seems to contain reductone (R—COH=COH—R) and carbonyl (R—CHO and R—CO—R) groups, which may account for the strong reducing properties associated with it. It can be decolorized by bromine, which fact also is supporting evidence of unsaturated and reducing groups. For reasons mentioned earlier, the pigment from milk has not been satisfactorily analyzed for elemental composition. Brown pigments from simplified systems reveal the presence of significant amounts of nitrogen. One property of browning in milk which has been subject to debate concerns attachment of the pigment to the milk protein. This association has been noted by many investigators. One school of thought maintains that the process is a physical adsorption. However, nothing short of protein decomposition is completely effective in freeing the pigment. Thus, in part at least, the binding seems to be chemical.

Factors Affecting Browning

Fluid milks. In this section, unconcentrated, evaporated, and sweetened condensed milks are considered. Factors bearing on browning in dry milk products are treated in the next section. The principal factors that affect browning in fluid milk systems are: (*a*) heat treatment, (*b*) total solids concentration, (*c*) pH, (*d*) properties of the milk, (*e*) storage time and temperature, (*f*) oxygen, and (*g*) various added compounds.

Unquestionably, heat treatment is the most important single factor in the browning of fluid milk. In the case of evaporated milk it presents a twofold consideration in the processing steps of forewarming

and sterilization. Where forewarming is for a significant period of time between 185° and 212° F. (85° and 100° C.), the heat effects may be additive to those of sterilization so far as browning is concerned. Table 5 shows the effects of forewarming treatment at 100° C. on the color

Table 5. THE EFFECT OF PREHEATING AT 100° C. FOR VARIOUS TIME INTERVALS ON THE AMOUNTS OF COLOR DEVELOPED IN THE SAME SAMPLES OF SKIMMILK WHEN AUTOCLAVED AT 120° C. FOR 20 MINUTES

Preheating Time, min.	Optical Density	
	After Preheating	After Autoclaving
0	0.016	0.105
7.5	0.016	0.107
15	0.016	0.105
30	0.017	0.125
45	0.016	0.138
60	0.020	0.144

Data of Patton (22) secured at a wavelength of approximately 520 mμ using the method of Choi et al. (5).

of sterilized unconcentrated skimmilk. Although no increase in optical density was evident up to 30 minutes holding time of forewarming, in a concentrated product such as evaporated milk holding times of 10 to 15 minutes would have a significant effect on the color of the sterilized product. It has been shown that high-temperature short-time processing of evaporated milk will produce a product relatively free of browning (2, 25, 29). Figure 5 illustrates that the rate of browning increases less rapidly than the rate of destruction of spores (sterilization) as the temperature is raised. For this reason there is a trend in the industry toward application of such methods to both forewarming and sterilization.

As the concentration of the milk solids increases, the extent of browning with any given level of heat treatment increases. The role of lactose concentration in this phenomenon is demonstrated in Fig. 8, which shows color development in an autoclaved casein dispersion at various levels of lactose. Another factor of substantial importance in the heat-induced browning of fluid milks is pH. Figure 9 portrays the effect of pH between 5.7 and 7.5 on the amount of color development in various milk fractions as a result of autoclaving. Related to this factor are the alkaline stabilizing salts used in the manufacture

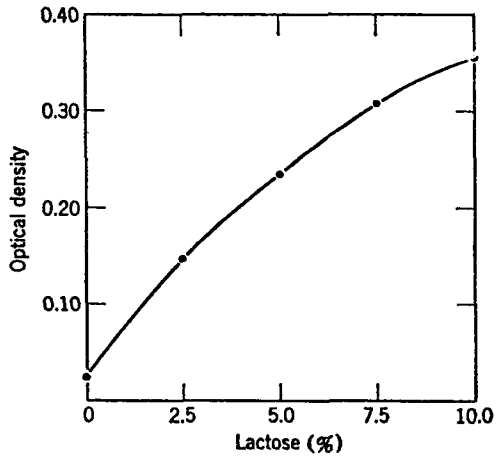


Fig. 8. Color development (browning) in an aqueous casein suspension as a result of autoclaving (124° C., 20 minutes) in the presence of various quantities of lactose (see footnote, Table 5).

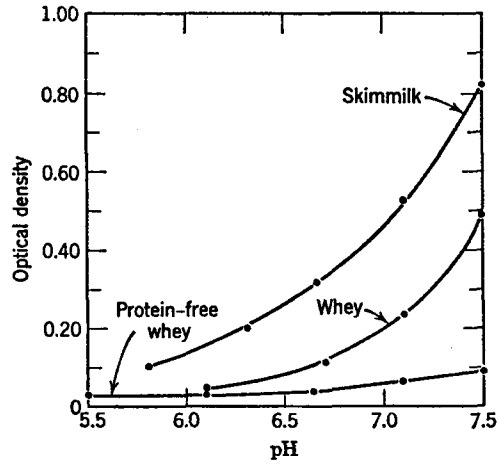


Fig. 9. The amounts of color (browning) developed by various milk fractions at different pH values as a result of autoclaving (120° C., 20 minutes) (see footnote, Table 5).

of evaporated milk. In pilot sterilization trials conducted to determine the optimum amount of stabilizing salt required by a batch of evaporated milk, color intensity in the samples invariably increases with increased level of stabilizer, and it seems probable that the effect is primarily due to pH.

From early years it has been observed by evaporated milk manufacturers that with a given set of processing conditions the amount of browning in the freshly manufactured product will vary from one lot of milk to another. It appears, therefore, that there are certain variables in milk as a raw material which are related to its potential for browning. These differences have been attributed mainly to normal variations between milks regarding pH and protein concentration. The compound urea has been reported to favor browning in milk, and variations among milks concerning tendency to brown may result from differences in urea content.

An additional factor comes into play with concentrated products stored at room temperature. Both time and temperature of storage are involved, and color intensity increases with storage time and is greatest at elevated storage temperatures, as shown in Fig. 10. It is interesting

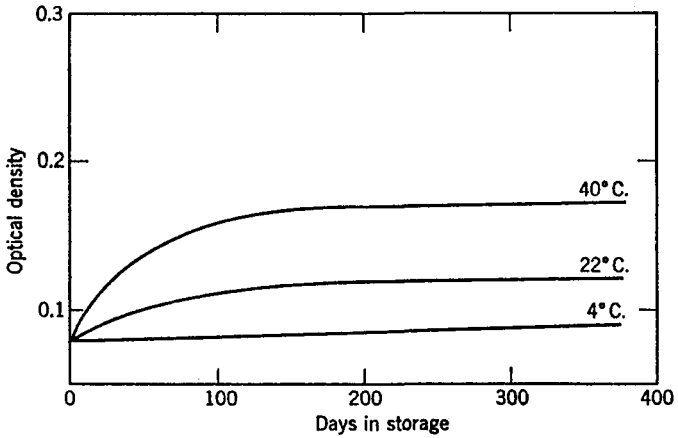


Fig. 10. The effect of storage temperature on color development (browning) in a commercial sample of evaporated milk (see footnote, Table 5).

to note from this figure the strong inhibitory effects that refrigerated storage has on color development.

Oxygen has been reported to be a factor favoring browning of milk. At most its role is secondary since exclusion of oxygen is not completely preventive. It seems probable that oxygen may play its role in conjunction with —SH groups. These groups are generated in the heat denaturation of the milk serum proteins and have a definite inhibitory effect on browning. Since —SH groups are readily destroyed by

oxygen their preservation through elimination of oxygen may be beneficial in preventing browning.

In investigational work on the browning of milk, many compounds have been studied regarding their promotive and inhibitive effects. The reducing sugars, such as lactose and glucose, favor browning to a much greater extent than sucrose. This is of practical significance in the manufacture of sweetened condensed milk since sucrose proves to be a much better additive than corn sugar from the standpoint of discoloration. A number of sugar fragments also have been studied. Glyceraldehyde and dihydroxyacetone are strongly promotive of heat-induced browning in milk. The addition of hydroxymethylfurfural to milk slightly increases the degree of browning as a result of heat treatment. Acetaldehyde and methylglyoxal have no effect; formaldehyde at very low concentrations favors browning, whereas at concentrations of 50 p.p.m. or better it is inhibitory. Sodium bisulfite, a compound used to prevent browning in a number of foods, is effective in inhibiting the phenomenon in milk. Its specific mode of action in this connection is not known. Hydrogen peroxide has also been reported to prevent browning in milk.

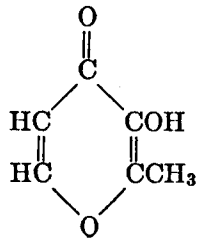
Dried products. Browning as such is not a primary problem in dry milks. The main reason for this lies in the absence of water. In any food system browning is very definitely favored by the presence of water, and in the instances where browning has been encountered as a problem with dried milk products, it nearly always is associated with excessive moisture content. Milks dried to moisture levels below 5% show essentially no change in color even during two years of storage at 37° C. Initially satisfactory moisture levels are no guarantee against browning. Since dry milk products are very hygroscopic they tend to imbibe water readily from humid atmospheres. Thus, packaging is also an important consideration. When moisture levels are excessive, storage time and temperature can be a factor in the browning of dry milks. As with concentrated fluid products, degree of browning varies directly with storage time and temperature. The subject of browning and related changes in dry milk systems has been reviewed by Coulter et al. (6).

The most important preventives for browning in milk and milk products are to keep heat treatments, storage times, and temperatures at the acceptable minimum. In the case of dry milk products, minimum moisture content also should be sought, both in processing and packaging.

Changes Related to Browning

The progressive decomposition of a reducing sugar in the presence of a protein is accompanied by many changes, only one of which is browning. In addition, fluorescent and reducing substances, various sugar fragments, and flavor compounds are formed. Many of these are detectable before the onset of browning and it should be emphasized that from a practical standpoint some of these changes, particularly those relating to flavor, may be far more important than any discoloration. When browning has become evident in a dairy product, the point of palatability has long since passed. From a research standpoint it seems very important to undertake work that will shed additional light on the early (colorless) stages of the browning reaction. Such changes appear to be the cause of the many stale flavors encountered in stored milk products. Although the flavors resulting from the browning reaction in milk undoubtedly result from a complex mixture of compounds, perhaps of greatest significance is caramelized flavor, which is discussed in Chapter 12.

Compound formation. A substantial number of compounds, which are largely lactose fragments, have been revealed to result from the prolonged high temperature treatment of milk. These include furfuryl alcohol, furfural, hydroxymethylfurfural, maltol, acetol, methylglyoxal, and acetaldehyde; butyric, propionic, acetic, formic, lactic, and pyruvic acids; NH_3, H_2S and CO_2. As discussed in Chapter 3, the bulk of these products come directly from lactose decomposition. Butyric, propionic, and pyruvic acids, NH_3 and H_2S, are exceptions. NH_3 and H_2S no doubt result from protein, and possibly urea, decomposition. Pyruvic acid and CO_2 conceivably could come from either milk protein or lactose. The origin of the other acids mentioned has not been determined. The compound maltol,



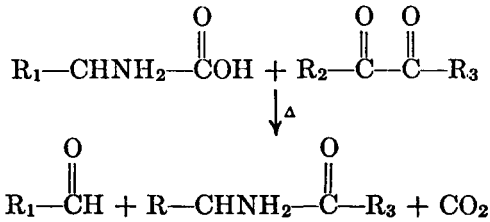
is a lactose fragment that appears to require catalysis by amino groups for its formation. When lactose is destroyed in heated milk, galactose, but not glucose, accumulates. It seems probable therefore that the bulk of the above-mentioned compounds arise from the glucose portion of the lactose molecule.

Studies at the junior author's laboratory, involving addition of lactose labelled with 1-C^{14} to milk, have revealed substantial recoveries of radio-activity in the maltol and formic acid produced by heat treatment. Thus it seems clear that at least these two compounds are formed from the glucose portion of lactose and more particularly from that part of the glucose containing the hemiacetal (reducing) carbon.

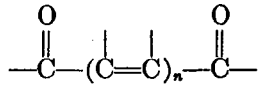
Reducing substances. Heated and dried milks contain a complex reducing system involving —SH compounds, ascorbic acids, and substances associated with the browning reaction. The latter have been detected or measured in a number of ways including methods based on use of the following reagents: Benedict's solution, methylene blue, indophenol, and acid ferricyanide. The reducing substances associated with browning exhibit the properties characteristic of reductones. No specific reductones have been identified as resultants of the browning reaction in heated milk.

Fluorescent substances. Incident to browning, fluorescent substances are produced in milk. In the ether extract of highly heated skimmilk, compounds showing both blue and yellow fluorescence under ultraviolet light are present. The chemical character of these compounds has not been revealed although some general observations regarding their presence in evaporated and dried milks have been made (16, 25, 29).

Strecker degradation. A reaction which is of demonstrated significance in simplified browning systems, but for which there is little direct evidence as yet in the case of milk, is the Strecker degradation (13, 24). In essence, it accomplishes the conversion of an amino acid to an aldehyde containing one less carbon; it may be represented as follows:



As shown, the reaction requires a dicarbonyl compound conforming to the following type structure in which n may be zero or an integer:



Sugar fragments such as methylglyoxal and pyruvic acid fulfill these structural requirements. From a practical standpoint, this reaction may be important in converting non-odorous amino acids into flavorful aldehydes. Moreover, recent evidence indicates that light or certain varieties of bacteria, as well as heat, may accomplish the essence of the reaction.

Loss of nutritive value. Although the information is meager and not necessarily incontrovertible, there is sufficient evidence in the literature to warrant mentioning the adverse effects that heat treatment and browning may have on the nutritive value of milk and its products. There are at least four considerations in this regard, as follows: (*a*) lowered consumption of browned products because of poor palatability, appearance, and physical properties; (*b*) loss of nutritional value due to vitamin and essential amino acid destruction; (*c*) loss of biological value and digestibility of proteins; and (*d*) production of toxic substances or metabolic inhibitors. As might be expected, lysine and the sulfur-containing amino acids cystine, cysteine, and methionine are principal among those that suffer some slight destruction in the high temperature heat treatment of milk.

Some of the vitamins in milk are significantly labile to heat treatment. This subject, as well as others related to the nutritive value of heated milks, is considered in Chapter 13 and in published reviews (19, 23).

Measurement of Browning

The study of browning, whether it be in milk and milk products or simplified systems thereof, requires a method of measuring color. Basically there are three methods which are used for this purpose: visual comparison, spectral transmission measurements, and reflectance measurements. Visual comparison can be simply a matter of side-by-side comparison of the treated sample with a control in a uniform environment. This procedure makes manifest fine differences in color and, by using sufficient numbers of observers, data concerning color differences can be treated statistically. Quantitative evaluation by visual comparison necessitates the use of standards, and a highly

refined method of this type has been developed by Webb and Holm (33). Reflectance methods have been applied to measurement of browning in milk with very satisfactory results. Since milk is essentially opaque, reflectance appears to be the most valid method of measuring its color. In this method the reflectance spectrum of the sample is ordinarily obtained with a suitably designed spectrophotometer and a magnesium carbonate reference standard. An extensive study of variations in the reflectance spectrum of milk as a function of heat treatment has been made by Burton (4). Browning is manifest in the reflectance spectrum of milk primarily through loss of absorption towards the blue end of the visible spectrum. Methods of measuring browning in milk systems that depend on transmitted light require that the system be transparent. A technique that has proven very satisfactory for this purpose is tryptic digestion of the milk proteins followed by acidification and filtration (5, 22). This method is versatile and comparatively simple. It has been applied to dry milk products, evaporated milk, fluid milk and simplified milk systems. The method can employ any conventional photoelectric colorimeter provided with a suitable filter and it shows satisfactory agreement with the reflectance method.

OTHER HEAT-INDUCED CHANGES

There are additional changes induced in milk by heat treatment, some of which have been partially clarified. It is well known that heating milk will inactivate the numerous enzymes that the medium contains. The susceptibility of any given enzyme native to milk to inactivation by heat treatment appears to be a unique property of the particular enzyme. Thus, some are readily destroyed by pasteurization, for example, phosphatase, whereas others are essentially unaffected by such treatment, for example, lactoperoxidase and xanthine oxidase. This matter is considered in detail in Chapter 6.

One of the most vacuous areas concerning knowledge of the effects of heat on milk relates to the lipides. A few points in this connection, such as the formation of δ-decalactone, have been discussed in the chapter on milk lipides. However, information on many points of interest are unavailable. Some questions that may be raised in this area are: Does high temperature heat treatment of milk cause any fat hydrolysis? Does such treatment cause isomerization of double bonds within the unsaturated fatty acids? Does heat treatment promote

interchange of the fatty acids within the triglycerides? What bearing does heat treatment have on the formation and destruction of peroxides in milk fat? Does polymerization of unsaturated milk fat acids occur as a result of heat treatment? Since milk lipides are finely dispersed in an aqueous medium, does any hydration of double bonds in the lipides occur on heating? Not only have these questions remained largely unanswered but investigational work to date seems to have ignored the possible interaction of lipides with non-lipide constituents in milk under the influence of heat treatment.

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CHAPTER

12 FLAVORS AND OFF-FLAVORS IN MILK AND ITS PRODUCTS

GENERAL CONSIDERATIONS

Flavor is of primary importance in foods. A food may be wholesome, nutritious, attractively packaged, and reasonably priced, but if its flavor is poor it will be rejected by the consumer. In no instance is this truer than with milk and its products. Thus, the effort on the farm and in the milk processing plant is to produce products with good flavor. This objective is a dominant factor in milk production, from when and what the cows shall be fed to what equipment and temperature treatment shall be used in milk processing. Even the metal composition of milk handling equipment and the color of milk bottles are directly concerned with the flavor of milk. As a consequence, some knowledge of flavor is indispensable in the production of dairy products that are consistently acceptable to the consuming public.

The subject of flavor in milk and its products brings together fields of science that are rather distantly related. These are dairy technology, chemistry, and psychology. Dairy technology is directly concerned with the control of flavor. Chemistry bears on the subject through the profound effects that minute chemical changes can have on flavor and

through the relation of structure to the flavor of compounds. That phase of psychology that deals with sensory perception, in this case taste and odor, is naturally a basic consideration in flavor. It brings to the subject the unique and difficult aspects of human behavior and individual differences. It introduces the statistical concept into the matter and places flavor outside the realm of an exact science.

The nature of flavor. Flavor is a sensory perception, something that one is equipped to perceive through suitable receptors; namely, the nose, mouth and related structures. Flavor has three basic components, olfactory, gustatory, and tactual, which are concerned respectively with the odor, taste, and feel of a flavor stimulus. Olfaction deals with odor, and the odor of food taken into the mouth becomes apparent as vapors pass up the back of the nose into the olfactory area. Whereas variation in odor is almost limitless, taste is concerned with a relatively few sensations that are detected in the mouth, and more particularly on the tongue. These are: sweetness, sourness, saltiness, and bitterness. In order to produce such sensations, which are detected by the taste buds, compounds must be in solution. The third, and perhaps most frequently overlooked, aspect of flavor is the tactual, that is, the way the substance feels in the mouth. The sensations of smoothness, graininess, tenderness, chalkiness, etc., are examples of tactual flavor responses. In the field of dairy products the defects of sandiness in ice cream resulting from lactose crystallization, and chalkiness, a sensation reminiscent of milk of magnesia, in reliquefied dry milks are examples of tactual flavor responses.

In the evaluation of flavor it is important to be able to differentiate the three basic components from the overall response. The net flavor response to a food may be bad, but from the standpoint of product improvement, it is important to know in exactly what respect it is bad. Does it feel bad in the mouth, is it too sweet, sour, salty, or bitter, or is the aroma objectionable? With respect to olfactory responses, two simple considerations are helpful to bear in mind: detection of odor depends on air circulation through the olfactory area; in order for a compound to be odorous it must volatilize. Holding the nose or clogging of the nasal passages, as sometimes occurs when a person is afflicted with a cold, prevents air circulation and the detection of odors. The volatility of odorous materials would seem to be a foregone conclusion. However, we frequently hear reference to the odors of proteins and other high molecular weight substances. It is not logical that such materials have odor except to the extent that they are

decomposed and contaminated with compounds of lower molecular weights and appreciable vapor pressures.

Individual differences. Individuals differ in their sensitivity to various flavor stimuli, and they have flavor memory and patterns of likes and dislikes. One concept of first importance in this connection is the threshold. As with any of the other senses, there are taste and odor stimuli of a barely detectable intensity. These are said to be in the threshold area. Stimuli of lower intensity are not perceived and

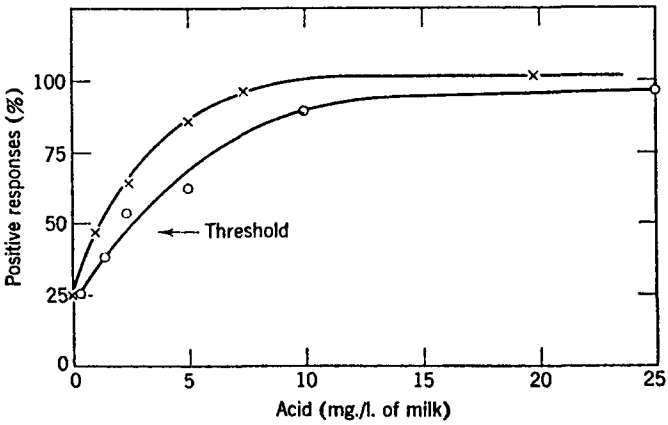


Fig. 1. Averaged data for nine untrained observers concerning perception of butyric (o—o) and caproic (x—x) acids in milk.

those of greater intensity are readily and consistently detected. Thus the threshold value for any flavor or odor will lie somewhere between 0 and 100% detection. Generally, that concentration of stimulus that will produce a positive response 50% of the times it is applied is defined as the threshold value. This point is illustrated in Fig. 1, in which average data for nine untrained observers concerning the detection of butyric and caproic acids added at various levels to homogenized milk are presented. These data vary somewhat from the sine-shaped curve that should be obtained under ideal conditions. The relatively large numbers of positive judgments at low and zero concentrations indicate considerable guessing as is usually the case with untrained observers. The flavor threshold values for butyric and caproic acids revealed by the study are approximately 25 and 14 mg./l. respectively.

The data in Fig. 1 are an average from nine observers. As with all types of behavior, there are variations among individuals concerning

a flavor threshold for a given compound in a particular medium. In the case of lactose in water, it has been shown that thresholds may vary between such extremes as 2 and 40 g./l. within a small group of taste observers. A difference in thresholds for butyric acid in milk is illustrated in Fig. 2. These results clearly show that one observer could detect the acid at approximately one-fifth the threshold concentration required for the other observer. The point here is that

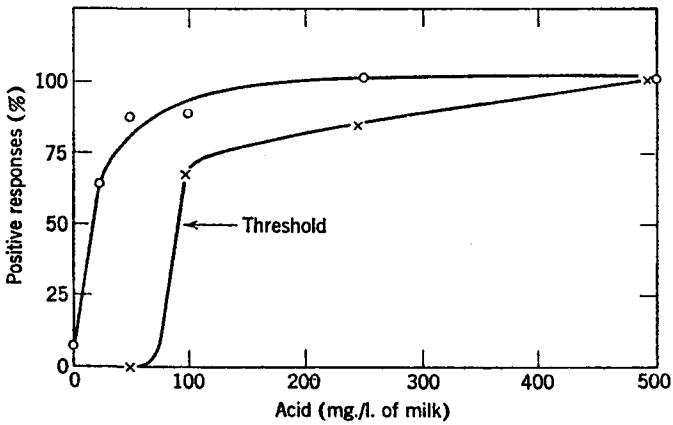


Fig. 2. Difference in perception of butyric acid in milk between two untrained observers.

individuals differ in their sensitivity to various flavor stimuli and that no two people gain exactly the same impression, although it may be similar in most respects, when evaluating flavor in a food. Some judges of flavor imply that if they detect an off-flavor in a product, "it is there!" and if others cannot detect it, "they are flavor blind." Such a dogmatic approach has little to recommend it, for no individual can reflect average behavior toward all flavor stimuli.

Just as there is a difference among individuals in quantitative flavor sensitivity so is there a qualitative variation in their responses. No gathering around a dinner table would be complete without comments regarding food likes and dislikes. This factor is not entirely unrelated to the concept of threshold. A flavor stimulus in its intensity factor alone can range through pleasantness to the distasteful, and what may be just pleasingly perceptible to one may be overpoweringly objectionable to another. However, there are many other factors, beyond our consideration here, that bear on the matter of food likes and

dislikes. A complex of individual experiences, habits, and hereditary factors determine one's attitude toward a particular food.

Measurement of flavor. The two principal means of measuring flavor are psychometric evaluation and chemical analysis. Of the two the former appears to be more important. Chemical measurement of flavor is based on the premise that a flavorful compound is present in sufficient quantity to be measured by a suitable chemical method of analysis. This type of measurement has the precision characteristic of the physical sciences. There are two reasons why such a method for measuring flavor has limited utility. First, flavor compounds fre-

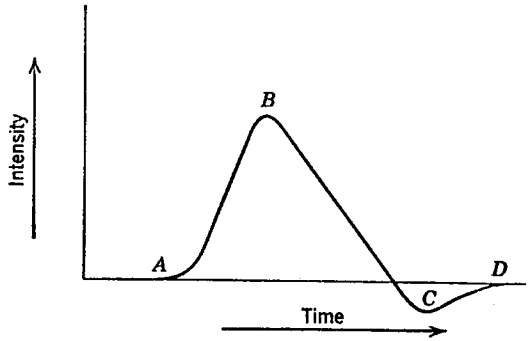


Fig. 3. A hypothetical stimulus-response curve for perception of flavor. *A*—application of stimulus; *B*—registration of maximum response; *C*—negative response due to fatigue; *D*—full recovery of perception mechanism.

quently are encountered at levels that defy detection by chemical means, but which are readily evident psychometrically. Second, chemical methods cannot evaluate the like-dislike aspect of flavor, nor can they readily reveal small qualitative differences. Obviously, where possible, an effort is made to apply chemical methods of measuring flavor since they can eliminate the vexing human biases that are an ever-present danger in psychometric methods. In considering the factors that influence measurement of flavor by psychometric means, it is well to have in mind the basic characteristics of the stimulus-response curve, an example of which is reproduced in Fig. 3. In this hypothetical curve, the stimulus is applied at point *A*, or in the case of flavor evaluation, the material is placed in the mouth. Depending upon the nature of the stimulus, there is a time lag following point *A* during which period the substance is being detected by the receptor organs and the message is being carried to the central nervous system. The length of this time lag may be expected to vary with the intensity

and nature of the stimulus and the sensitivity of the individual. Similarly, these same factors determine the position of point *B* (maximum response). It appears probable that magnitude of response to the stimulus falls off because maximum response for the degree of stimulus has been registered, fatigue or blocking of receptors occurs, and the stimulus becomes dissipated through swallowing, exhalation, dilution, and possibly decomposition. Point *C* represents negative response which may be anticipated theoretically as a result of fatigue. By administering the same stimulus with rapid repetition, the responses become progressively weaker. This is particularly true of strong stimuli which impart considerable after-taste. In time, *D* is reached when full recovery of the mechanism has occurred. A casual consideration of the curve in Fig. 3 makes evident that its shape and the position of the significant points will vary widely depending upon the stimulus material and the condition of receptor organs. Some substances which are slow to go into solution will show a considerable lag period following point *A* and a slow buildup to point *B*. Materials with strong after-taste will show a gradual slope between points *B* and *C*. Some stimuli will be quickly perceived and dissipated, soon leaving the mouth in a clean, refreshed condition. Such factors as these are of definite importance in determining the number of samples that can be evaluated by one individual in a given period of time.

In addition to the nature of the substance being evaluated, the quantity of material, its temperature, whether it is pleasant or unpleasant to the individual observer, and whether rest periods and rinses of the mouth are afforded will be significant variables in the psychometric measurement process. A good example of the importance of temperature is evident with ice cream. The coldness of this product has a numbing effect on the tongue and mouth and the levels of flavoring agents used in it are suitable for the frozen product, but seem a little drastic in ice cream mix at room temperature.

There can be a number of objectives in the measurement of flavor. These are: (*a*) quantitative, or measurement of flavor intensity, (*b*) qualitative, or description of flavor components, (*c*) comparative or determining the presence and extent of difference in flavor between samples, (*d*) acceptance or like-dislike testing, (*e*) ranking or scoring products for flavors and off-flavors, and (*f*) threshold determinations. Means of achieving these objectives are important in basic food research, product development and improvement, and consumer acceptance studies. For this purpose a variety of psychometric methods are

available and, in fact, development of improved methodology is currently an active area of research. The existing methods have various advantages and disadvantages and care must be used both in their application and in the interpretation of results derived therefrom. Since, in such an area of experimentation, we are dealing with behavior, statistics becomes an indispensable tool. For the benefit of readers interested in this field of work, several references (7, 8, 23, 34, 35, 43) on methods for evaluating flavor have been included in the list of selected references at the end of the chapter.

In the dairy industry a few basic and important products have been manufactured for a long number of years and it has become the custom to evaluate these products on the basis of a scoring system, a part of which is devoted to flavor, body, and texture. These scoring systems, described extensively in a text by Nelson and Trout (29), are so well established that some dairy products are bought on the basis of their score. This type of scoring system involves the use of expert judges and to the extent that these judges can reflect an average response to the product, their scores may be indicative of product quality on a consumer acceptance basis. The fact that product scoring methods are useful, well established, and largely the ones currently employed in the dairy field, does not mean that other methods are not needed. On the contrary, a need exists for aggressive application of the best available methods for evaluating flavor in dairy products, especially for those which frequently pose flavor problems.

Flavor identification research. With respect to milk and its products, flavor research has three general objectives: prevention of off-flavors, preservation of normal or desirable flavors, and contraction or elimination of ripening and aging processes required for flavor development in certain products. The research means of achieving such worthwhile objectives are deserving of consideration. Emphasis in the present discussion will be placed on the aromatic component of flavor. This is not to say that the gustatory aspect, sweet, salt, sour, etc., or the tactual aspect, body and texture, are not of prime importance. However, of these considerations, aroma in foods is most variable, most difficult to control, and least understood.

For obvious reasons chemical characterization of the compound or compounds responsible for a flavor is a logical first objective. This is true whether the flavor is desirable or objectionable. Information concerning specific chemical identity greatly facilitates development of conditions that favor formation and preservation of pleasing flavors, as well as means of inhibiting off-flavors. It will be clear from the

following material in this chapter that such information is lacking with respect to a large number of flavors of interest in the dairy field.

The chemical characterization of flavor substances in foods has four basic steps; namely, isolation, concentration, purification, and identification. Conventional techniques of organic chemistry to accomplish these steps have been available for many years. The critical consideration with naturally occurring flavor substances is to find methods that are operative on a microscale. Flavor compounds effectively present at levels of parts per million, or even per billion, are commonplace. Some recently developed research tools have done much to alleviate the need in this area. These are chromatographic methods and vastly improved and widely available spectrophotometers.

The versatility of chromatography makes it as close to indispensable as any tool can be in flavor identification work. It supplies at once data useful in both isolation and identification. Although it is not possible to dwell on the many applications of chromatography, as an example, special mention is made here of 2-4-dinitrophenylhydrazones (DNP's) of aldehydes and ketones. Although use of the reagent is primarily confined to carbonyl compounds, these are rather frequently involved in flavor. As crystalline derivatives, DNP's are particularly valuable for identification purposes because of their ease of preparation, purification, and wide range of melting points. Their chromatographic behavior with both column and paper techniques has rendered them even more useful. As a case in point, Fig. 4, as well as data by others (10, 18, 20), reveals the ease with which various of the lower molecular weight carbonyl compounds can be separated as DNP's. Cutting the spots from such chromatograms and heating them with levulinic or other suitable carbonyl acids frequently will regenerate the parent compounds sufficiently to enable their presumptive identification by odor. Spectral properties of these derivatives are an additional aid toward their identification (1). Two investigations that nicely demonstrate the utility of chromatograhy of DNP's in flavor research are that by Forss et al. (10, 11) on oxidized flavor in skimmilk and that by Jackson and Morgan (20) on malty flavor in milk.

Infrared spectral data can be of great aid in identification work. The complete coincidence or superimposability of infrared spectra for a known and an unknown compound in the 2- to 15-μ region is generally considered excellent evidence that the two compounds are identical. The beauty of this application to flavor research lies in the fact that as little as 1 mg. of pure compound will ordinarily yield a

satisfactory spectrum, that the spectrum furnishes evidence regarding functional groups, and that the compound is available for further tests and observations following spectral analysis. Figure 5 demonstrates such data, which were used effectively in the identification of δ-decalactone as the compound responsible for a coconut-like flavor in stored milk fat (28).

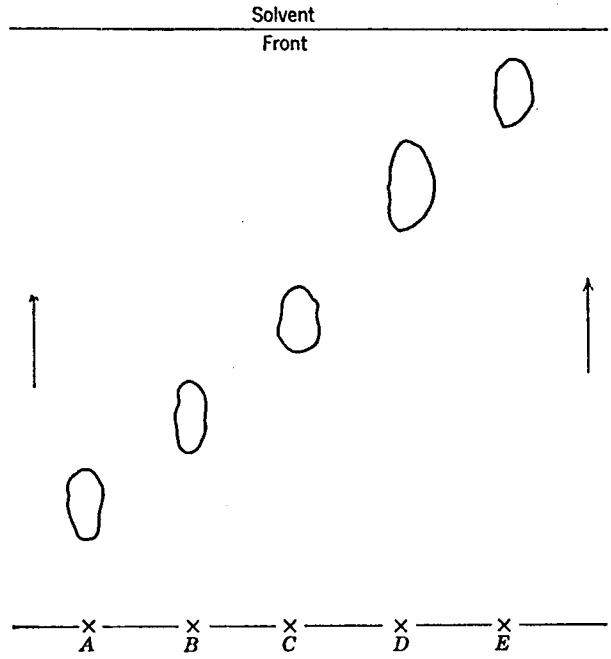


Fig. 4. Paper chromatographic separation of 2,4-dinitrophenylhydrazones. Solvent, heptane-rich layer from 2:1 heptane-methanol. Equilibration, 17 hours in an atmosphere from the methanol layer. Running time, one hour and 40 minutes. *A*–formaldehyde; *B*–acetaldehyde; *C*–acetone; *D*–isovaleraldehyde; *E*–*n*-heptaldehyde.

A most profound development that holds considerable promise in application to flavor research is gas-liquid chromatography. Several of its attributes appear to be ideal: it is extremely efficient in obtaining complete separation of components, it is applicable to volatile materials, and a few milligrams of material constitute an adequate sample charge to the apparatus. Discussion of the method, apparatus, and applications has been published (21, 22).

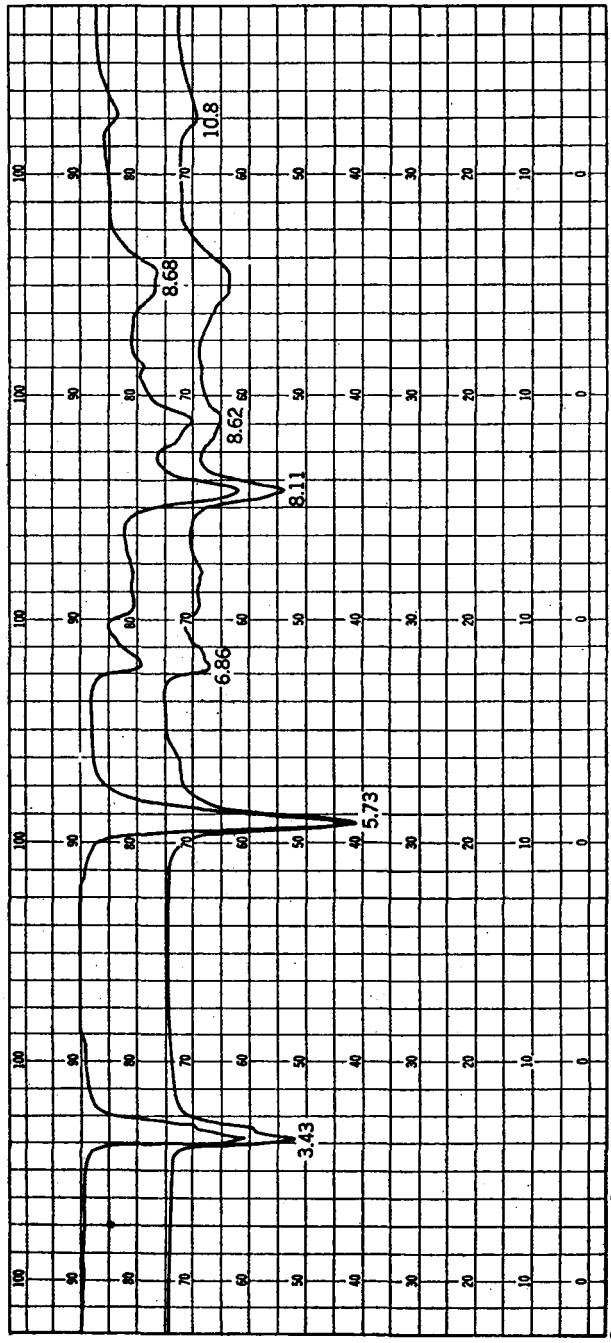


Fig. 5. Infrared spectra of authentic δ-decalactone (upper); and compound isolated from deteriorated milk fat (lower).

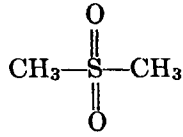
NORMAL FLAVORS

Flavors of food products may logically be divided into two groups, that is, the normal or natural flavors that are desirable, and off-flavors or those that are not typical of the products and that usually but not always are undesirable flavors. For the purposes of this discussion normal flavors will include a consideration of milk and various ripened and cultured dairy products. No effort will be made to treat the rather substantial field of added flavors which mainly concerns frozen desserts and, to a lesser extent, milk and certain processed cheeses.

Flavor of milk. From a gustatory standpoint, milk is both sweet and salty. Normally it is neither bitter nor sour. Apparently the degree of sweetness or saltiness varies considerably between individuals. With some, sweetness is the dominant note, whereas others detect only a slight saltiness, and yet others derive only a flat sensation from tasting milk. Exclusive of the observer, milk appears to vary in its sweet-salt taste character. Since lactose varies inversely with the chlorides content of milk, milks high in chlorides taste somewhat more flat or salty than normal. Milks high in chlorides are secreted by cows at the end of lactation and during times of udder infection (astitis). Milk is described as having a faint characteristic flavor. Presumably this refers to the olfactory component and the description reveals two significant points; namely, milk has very little flavor, and its flavor is not easily characterized. At the present time it cannot be said with certainty what compounds contribute to the characteristic flavor of normal milk; however, it seems probable that certain of the low molecular weight compounds present in trace quantities, such as acetone, acetaldehyde, butyric, and certain other free fatty acids contribute to the flavor. A pronounced flavor of any kind is considered abnormal to milk, although incidence of abnormal flavors in some local milk supplies becomes so regular on occasions as to be considered normal and the milk to be quite acceptable. Although roughness or chalkiness is sometimes evident in certain processed dairy products, normal milk is smooth feeling in the mouth.

Milk contains traces of the volatile product, methyl sulfide, $(CH_3)_2S$, boiling point 38° C. The flavor threshold for this compound is approximately 12 p.p.b. in distilled water. At slightly above threshold levels it has a malty or cowy flavor character. The odor of

this compound is detectable on cow's breath, and the sulfone derivative



has been demonstrated in cow's blood. Thus, it seems quite probable that methyl sulfide is a significant contributor to the flavor of milk.

Flavor of cultured dairy products. The number of so-called cultured and fermented dairy products is large. However, only a few are of broad significance, the main ones being cultured buttermilk, butter, and cottage cheese. In general, the remarks will be confined to the development and nature of flavor and aroma in these products.

The odor and flavor of cultured dairy products may be ascribed most logically to certain carbonyl compounds and volatile acids, which are by-products of the lactic acid fermentation. Principal among these are diacetyl, acetoin (acetylmethyl-carbinol), acetone, acetaldehyde, and butyric, propionic, acetic, and formic acids. Of the compounds mentioned, diacetyl is by far the most important. Optimum production of the flavor and aroma compounds is best achieved through use of a mixed culture of selected strains rather than by use of any one pure culture. The most commonly used organisms are *Streptococcus lactis, S. citrovorous,* and *S. paracitrovorous.* The latter two organisms are considered to be particularly beneficial in the production of diacetyl, and their action is facilitated by acid conditions created by fermentation of lactose to lactic acid by *S. lactis.* A mechanism that logically accounts for the production of diacetyl from pyruvic acid and acetaldehyde (or for that matter, indirectly from citric and lactic acids), is presented in Fig. 6. With reference to the importance of various enzymes in this mechanism, the findings of Juni (26) and Singer and Pensky (39) should be consulted. It must be emphasized that this is not necessarily the only mechanism of diacetyl formation that may be operative in dairy products. The literature is not completely clear regarding the initial substrate from which diacetyl is formed. The bulk of evidence indicates that the citrates of milk are the primary source. However, diacetyl formation from lactose in the absence of citrates has been demonstrated. On the other hand, it is well known that citrates, when added to milk, will facilitate production of diacetyl.

As revealed in Fig. 6, the compounds 2,3-butylene glycol, acetoin,

and diacetyl are related through oxidation-reduction mechanisms. This is of practical importance with respect to the flavor of cultured products. The amount of available oxygen in the medium will be a determining factor concerning the relative proportions of these compounds which will exist. In the absence of oxygen and in a fermentation exhibiting a strong reducing potential, primarily the non-odorous 2,3-butylene glycol will predominate. When oxygen is in reasonable supply, diacetyl and acetoin formation are favored. As a consequence,

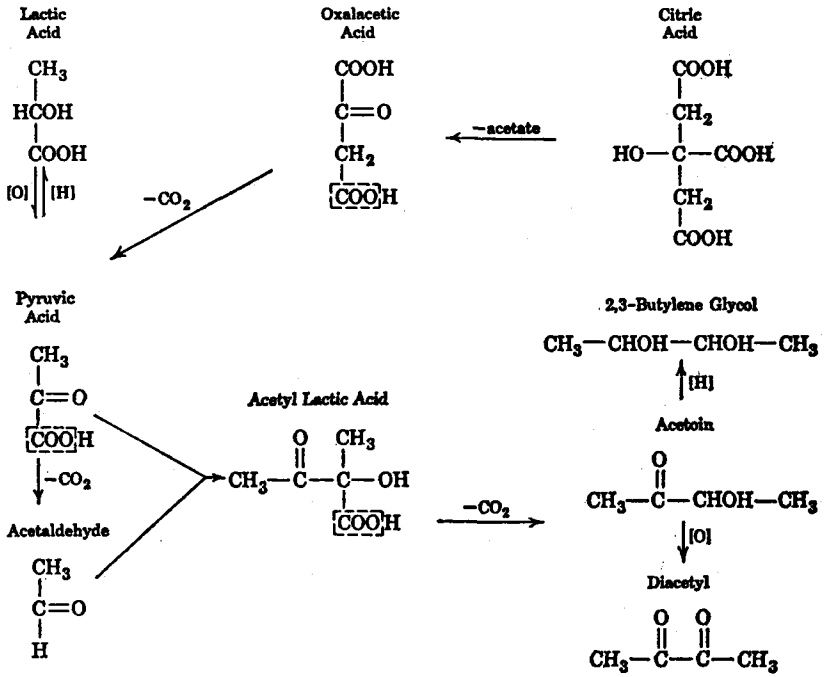
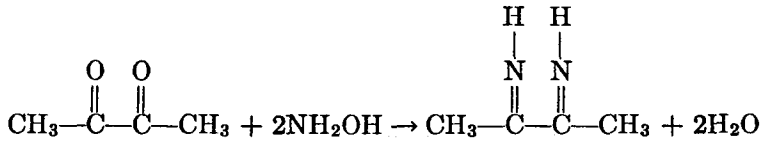


Fig. 6. A scheme for the formation of diacetyl and related compounds from pyruvic and other acids.

the aeration and aging of cultured dairy products frequently enhance their flavor and aroma. It is worth noting that diacetyl is readily detectable at a level of 1 p.p.m. in milk. As with many flavor compounds, diacetyl can be distinctly obnoxious when present in excessive concentration. Of course, the tolerance of individuals for such compounds at elevated levels will vary. Factors favoring production of diacetyl in cultured products are: (*a*) the use of mixed cultures composed of *S. lactis*, *S. citrovorous*, and *S. paracitrovorous;* (*b*) the

addition of up to 0.15% citric acid; (*c*) vigorous agitation with the incorporation of air; and (*d*) aging in the cold. Ordinarily acetoin content will substantially exceed the level of diacetyl in a starter. Ranges for diacetyl and acetoin contents respectively in starter are 1 to 3 mg./l. and 30 to 350 mg./l. Prill and Hammer have shown that a combined value of 580 mg./l. for diacetyl and acetoin can be achieved through addition of citric acid to starter. The transfer of flavor and aroma compounds from starter and cream to butter is relatively inefficient (5 to 10%). Therefore, the addition of starter distillate to butter during the working process is practiced especially for butter markets, which favor a strong diacetyl flavor in the product.

The manner of analyzing for diacetyl and related compounds is of interest in connection with starter, butter, and various varieties of cheeses. A satisfactory method in general use involves the reaction of diacetyl with hydroxylamine to form dimethyl glyoxime (36). This reaction is as follows:



The glyoxime so formed produces a red color when treated with an ammoniacal ferrous sulfate reagent. This color can be measured photometrically in comparison with that produced by a standard dimethyl glyoxime solution. Acetoin may be determined simultaneously by oxidizing a second sample with ferric sulfate which converts the compound to diacetyl. The difference between values for the oxidized and unoxidized samples yields the acetoin content. This method for dairy products (Prill and Hammer's) has been used perhaps most widely to date. There are many other suitable methods for measuring diacetyl and its related compounds, but few of these have been specifically adapted to dairy products.

Acetaldehyde is considered to be a flavor compound of importance in yoghurt in particular and fermented milks in general. A sensitive method for measuring this compound based on its color reaction (blue) with sodium nitroprusside reagent in the presence of certain amines has been developed (38).

Cheese flavor. This subject clearly is difficult to treat. There are so many varieties of cheese which obviously differ with respect to flavor that it is not possible to generalize on the subject. Moreover, the principal flavor compounds in most varieties of cheese have not

been elaborated. To date no single compound has been proposed as clearly having cheese-like odor and flavor properties. On the contrary, it would appear that any given cheese derives its characteristic flavor and aroma from a mixture of compounds. Some observations regarding cheese flavor in some of the more common varieties of cheese follow.

In the case of cheddar cheese, the presence of milk fat is essential for development of characteristic flavor. No compound or group of compounds is known that will give a reasonable reproduction of cheddar flavor and aroma. According to Dacre (6), the principal flavor compound(s) is neutral, volatile with steam, and susceptible to oxidation or reduction. The neutral volatile distillate from cheddar cheese has been shown to contain acetaldehyde, ethyl alcohol, butyraldehyde, ethyl acetate, and ethyl butyrate, but none of these singly or in combination is a source of cheddar aroma. Limited amounts of the lower free fatty acids are consistently present in well-ripened cheddar cheese. The flavor of mold-ripened cheeses, such as Roquefort, Stilton, blue, and Gorgonzola, is derived in part from free fatty acids and methyl ketones (5, 14, 30). Straight chain methyl ketones containing 3, 5, 7, 9 and 11 carbons have been demonstrated in blue cheese and of these 2-heptanone is most characteristic of the aroma. Various combinations of such ketones and the lower fatty acids achieve a fair semblance of mold-ripened cheese flavor. Indole, a compound with a definite fecal odor, has been demonstrated as a normal constituent of Camembert and Limburger cheeses (3). A characteristic flavor component of Swiss cheese is propionic acid. This cheese is usually observed to be somewhat sweeter than other varieties and such sweetness is usually attributed to certain of the amino acids and salts of hydroxy acids which are sweet tasting. It seems probable that diacetyl contributes, at least to a limited extent, to the flavor of most varieties of cheese.

In recent years extensive investigations into the amino acid composition of various cheeses have been conducted. These studies have revealed no clear-cut correlation between appearance and disappearance of amino acids from cheese, either singly or in combinations, and the development of the typical cheese flavor. It has been shown that the addition of the amino acid leucine to cracker doughs imparts a cheese-like flavor to the resulting crackers during baking. More simply, heating solutions of leucine with dicarbonyl compounds, such as methyl glyoxal, will produce this flavor. The presence of methionine (traces) in this reaction benefits the aroma. The resulting aroma is

on the order of a toasted cheese character. It is clear that the chemical nature of cheese flavor is unresolved and constitutes a challenging area for research. A knowledge of the particular flavor compounds involved would be a distinct help in determining optimum conditions for manufacture, ripening, and blending of the various cheeses. For further discussions of cheese flavor see references 3, 16, 42.

OFF-FLAVORS

Among foods, milk is particularly susceptible to off-flavors. In this regard, the cow constitutes the initial problem by acting as a condenser for odor substances in feeds, weeds, and barn air. In a matter of minutes following inhalation by the cow, strong odors may be reflected in the flavor of the milk. Other factors that contribute to the off-flavor problems are the essential lack of flavor in normal milk, the sensitivity of milk to chemical change, and the excellent qualities of milk as a fermentation medium. Off-flavors in milk and its products are divided here into three aspects: chemical flavor deterioration, microbiological flavor deterioration, and absorbed flavors. Under chemical aspects, those off-flavors produced by heat, light, air, etc., are considered. The role of bacteria in producing off-flavors is discussed under microbiological deterioration. The topic of absorbed flavors is primarily concerned with the relation between the cow's metabolic activity and the flavor of milk, particularly as affected by ingestion of feeds and weeds.

Chemical Flavor Deterioration

Oxidized flavor. The most important single flavor defect of milk and a number of its products is oxidized flavor (13). Such terms as *cardboard, metallic, oily,* and *tallowy* also are used to describe this off-flavor. It seems probable that oxidized flavor is a general term that can be applied to a number of closely related off-flavors. These off-flavors have oxidation of lipide materials in common. However, the nature of the specific flavor compounds, their concentrations, their origins, or modes of formation must differ. For example, the oxidized flavor of fluid milk is not identical to the oxidized flavor of dry whole milk or butteroil.

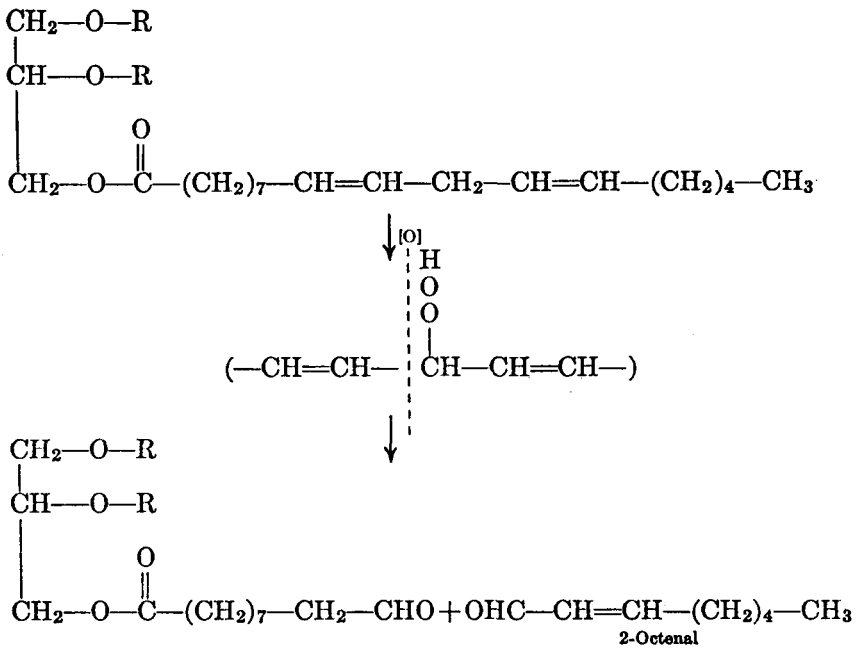
The bulk of evidence indicates that the phospholipides of milk serve as the origin of oxidized flavor in fluid milk. When milk is

separated, approximately one-third of the phospholipides are found in the skimmilk; the other two-thirds are associated with the material of the fat globule membrane. The exposure of the phospholipides to the milk serum makes them more accessible than the fat to attack by oxygen. Evidence has been obtained showing the fat in oxidized milk to have undergone little change in iodine value (unsaturation), whereas the iodine value of the phospholipides will have been lowered considerably under these conditions. In addition, sweet cream buttermilk, which is particularly high in phospholipides, is very susceptible to oxidized flavor development. Moreover, butteroil free of phospholipides can be prepared and stored for months under suitable conditions without development of oxidized flavor, whereas it is very difficult to prepare phospholipides without the immediate appearance of this defect. Phospholipide oxidation is accompanied by a flavor termed *cardboard* or *cappy*. Milk fat, undergoing oxidative deterioration, exhibits an oily flavor and aroma resembling that of linseed oil. In addition to this flavor, milk fat also gives rise to a coconut-like off-flavor during its deterioration (discussed later, this chapter). Thus, the bulk of evidence suggests that the phospholipides are the principal source of oxidized flavor in fluid milk products.

A number of factors are essential to the development of oxidized flavor in fluid milks. Of first importance is atmospheric oxygen. Unless definite precautions have been taken to prevent it, milk is normally saturated with air. This amount of air (oxygen) is more than sufficient to enable the development of oxidized flavor, providing other necessary conditions prevail. That oxygen is essential to flavor development is revealed by the fact that deaerated milk does not undergo oxidation, even when catalyzed by such potent factors as copper and sunlight. Consequently, various antioxygenic procedures serve as a basis for prevention of the off-flavor. It is probably true that if any normal milk were held long enough the only essential requirements for oxidized flavor development would be the phospholipides as substrate and air as the oxidizing agent. However, it is known that milks vary considerably in their susceptibility to development of the off-flavor. The reasons for such variations are not known with certainty, but it seems safe to assume that differences in levels of antioxygenic materials between milks must be a factor. A convenient classification of milks regarding their susceptibility to the off-flavor is (*a*) spontaneous, for those milks that spontaneously develop the off-flavor within 48 hours after milking, (*b*) susceptible, for those milks that, after contamination with divalent copper, will

develop the off-flavor within 48 hours, and (*c*) resistant, for those milks that will exhibit no oxidized flavor, even after contamination with copper and storage for 48 hours.

The chemical mechanism of oxidized flavor development in milk is complex and only partially established to date. The off-flavor process discussed here forms one aspect of lipide oxidation, which subject is dealt with in Chapter 2. The primary substrate from which the flavor compounds are formed appears to be the highly unsaturated fatty acids, such as linoleic, arachidonic and others contained in the phospholipides and glycerides. Oxygen is known to attack the methylene groups adjacent to the double bonds in these acids, an action that results in the formation of hydroperoxides. Such hydroperoxide compounds are unstable and presumably lead to secondary oxidation products, some of which are derived by cleavage of the carbon chain. It is these cleavage products of low molecular weight that are responsible for off-flavors in oxidized lipides. In several lipide systems, α-β unsaturated aldehydes (R—CH=CH—CHO) have been shown to be significant off-flavor compounds. A study of the oxidized flavor compounds in skimmilk treated with copper has revealed the presence of a series of such aldehydes (10, 11). These ranged in carbon chain lengths from C-5 to C-11, and some doubly unsaturated (2,4-dienal) members also were demonstrated. Of these compounds, 2-octenal containing 8 carbons and 2-nonenal containing 9 carbons appear to have the most characteristic oxidized flavor properties. Both of these compounds are capable of imparting flavor to milk at levels well below one p.p.m. Evidence that carbonyl compounds containing isolated double bonds contribute to oxidized flavor also has been secured (27). Despite the considerable number of compounds isolated and identified, the scarcity of information concerning both the origin and mechanism of oxidized flavor development does not permit a precise scheme of the phenomenon at this time. The essence of the reaction as it may transpire with a glyceride containing linoleic acid follows:



There are several factors that tend to accelerate oxidized flavor development in milk and its products. These are certain trace metals, ascorbic acid, and light. Of the trace metals, cupric ions are most important. Copper, even when present in milk at levels of a fraction of a part per million, is a very potent catalyst of oxidized flavor development. To a lesser yet significant extent, ferric ions are also effective catalysts of the process. Thus copper-containing processing equipment, and cans and equipment exhibiting iron rust should be kept from contact with milk. It appears that copper and iron are more effective as catalysts of lipide oxidation when proteins are present.

The behavior of ascorbic acid in the phenomenon of oxidized flavor development is anomalous. When other conditions are promotive, the amount of ascorbic acid in milk, that is, 10 to 20 mg./l. tends to favor production of oxidized flavor. However, when milk is fortified with 50 to 100 mg./l. of ascorbic acid, the compound acts as an effective antioxidant. Ascorbic acid is actually a strong reducing agent and it would be expected that at any concentration it would exhibit some antioxidant activity. The fact that the reduced form of the compound may establish an equilibrium with the dehydro (oxidized) form indicates a possible role as hydrogen donor and acceptor of

importance in the lipide deterioration mechanism. Recent evidence indicates that ascorbic acid can catalyze both oxidative attack at the carboxyl end of long chain fatty acids and the free radical mechanism of hydroperoxide type oxidation. In the case of milk, a one to one ratio of reduced to dehydro ascorbic acid appears optimum for promoting oxidized flavor development.

The chemical deterioration of lipides in the presence of oxygen and catalyzed by light does not appear to differ materially from other autoxidative mechanisms of lipide deterioration. When milk is exposed to light, as it frequently is on the customer's doorstep, oxidized flavor forms part of the overall off-flavor produced. The relative importance of the oxidized component of the flavor depends among other things on the relative amount of lipide material in the product. Under such conditions, milk (particularly unhomogenized) and cream show a substantial degree of oxidized flavor, whereas skimmilk exhibits primarily sunlight flavor. For a consideration of the latter, see subsequent discussion.

The preventive measures for oxidized flavor generally are considered to be adequate; but practical procedures for eliminating the defect, once it has developed in a dairy product, are not available. Although oxidized flavor is still a significant problem with certain products, the dairy industry has substantially overcome widespread incidence of the off-flavor through an intensive campaign to eliminate copper-bearing alloys from milk processing equipment. The industry for the most part is on a stainless steel basis except in special cases. In most situations where dairies have a chronic oxidized flavor problem, the trouble can be traced to a few remaining copper-containing units in the processing line. Unquestionably the elimination of equipment that may contribute copper contamination to milk is one of the best preventive measures against oxidized flavor.

Despite the fact that copper has been largely ruled out as a possible promoting agent in the problem, bulk milk supplies seem to have become somewhat more susceptible to oxidized flavor in recent years. A correlation has been noted between improvement in milk quality and incidence of the defect. It is considered that a definite relation exists because of the reducing action of bacteria in inadequately cooled milks and in milks that have been heavily contaminated incident to their production. The antioxygenic action of bacteria may result in two ways, first through using up available oxygen in the medium, and second, through elaboration of antioxygenic compounds. In some areas the addition of a small quantity of buttermilk starter to milk

during the coming-up time for pasteurization has been employed satisfactorily as an antioxygenic procedure.

A common practice with a number of dairy products is to use processing temperatures in the range that activate sulfhydryl (—SH) substances. These substances, which originate in the β-lactoglobulin and proteins associated with the fat globulin membrane, are strongly antioxygenic and are effective in preventing oxidized flavor. Activation of —SH groups for practical purposes commences in milk at temperature treatments above 73° C. (170° F.). Although such treatment produces cooked flavor, this flavor is generally held to be less objectionable than oxidized flavor. Moreover, in stored dairy products the cooked flavor is slowly dissipated and after several months cannot be detected at all.

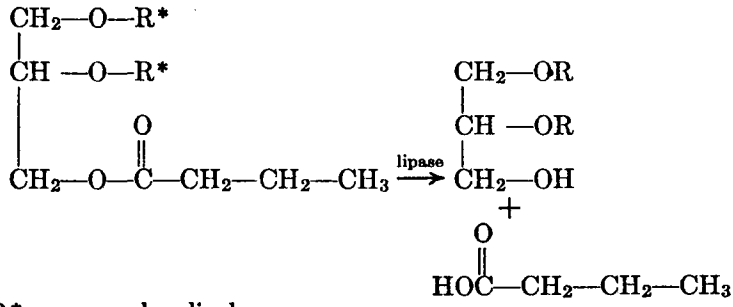
In addition to high heat treatment there are other processing measures that are helpful in combatting oxidized flavor. These are homogenization and deaeration. It is well established that homogenization renders milk much less susceptible to oxidized flavor. The precise reasons for this are not known. If anything, we might expect that the large increase in fat surface obtained by homogenization would aggravate the problem. However, this is not the case and we are led to the conclusion that either the disorientation (physical) of the fat globules destroys the conditions conducive to flavor development, or that the combined effects of high pressure and heat during homogenization cause discrete but significant chemical changes of an antioxygenic nature. With respect to deaeration, its effectiveness obviously rests on the essential role of oxygen in the off-flavor mechanism. Deaeration is not a widespread practice with fluid milks since, for the most part, it is possible to produce milk of good quality without this step. Nonetheless, deaeration is helpful in removing the more volatile off-flavors from milk as well as rendering the milk more resistant to both oxidized and sunlight flavors.

Another form of the deaeration measure for preventing oxidized flavor is the various degassing processes used for stored dry milk products. The objective here is to remove as much oxygen as practicable from the packaged product and to store it either in the vacuumized condition or with the air replaced by an inert gas such as nitrogen.

One of the most straightforward means of preventing oxidized flavor in dairy products is through treatment with antioxidants. The use of such agents is prevented by law in most areas. Antioxidants employed in milk and its products fall in several categories: (*a*)

phenolic-type compounds which interrupt the chain reaction mechanism of lipide oxidation resulting from free radical and hydroperoxide formation, (*b*) strong reducing compounds, which lower the oxidation-reduction potential to a point unfavorable for lipide oxidation, and (*c*) tryptic-type antioxidants, which appear to alter the milk proteins in a manner rendering them antioxygenic. The exact means by which antioxidants act, especially in a complex system such as milk, is a matter of speculation. Most of the texts on lipides afford entree to the subject for the interested reader. Some other measures that are known to discourage development of oxidized flavor in milk are adequate rather than submaintenance levels of feeding for the cow, feeding of green succulent feeds, feeding small quantities of menadione (a phenolic compound), complete removal of any residual chlorine used in sanitizing equipment, and use of colored glass or heavy paper milk bottles to prevent the oxidative effects of light.

Rancid flavor (hydrolytic rancidity). As discussed elsewhere in this text all raw milk contains active lipases. These lipases have the ability to hydrolyze glycerides either partially or completely, with the result that free fatty acids are liberated. Since butyric acid is the principal acid responsible for rancid flavor in dairy products and since only a very limited degree of lipolysis is required for formation of flavor, the essence of the reaction causing rancidity may be represented as follows:



R* = an acyl radical.

It is evident from the above reaction that the quantitative conversion would involve the formation of three moles of fatty acid and one mole of glycerol from one mole of triglyceride; hydrolysis only to the diglyceride and one mole of fatty (butyric) acid is shown.

The lipases of normal raw milk at the time of secretion of the milk are inactive. Generally this inactivity is ascribed to the fact that the lipases are present in the aqueous phase of the milk and are pre-

vented from contacting the fat because of materials adsorbed on the surface of the globules. Thus it is clear that anything that will alter the membrane and permit adsorption of lipase on the fat (such as homogenization, vigorous agitation, and warming and cooling) will promote rancidity. Rancidity is one of the most characteristic flavors that develop in dairy products. Milk fat is the only common fat that contains substantial amounts of the lower fatty acids, and since these acids are particularly odorous, disagreeably so in high concentrations, it is natural that rancid flavor would be distinctive of milk products. Although high levels of free fatty acids in milk render it unacceptable for human consumption, it is worth noting that rancidity is not in every sense an off-flavor. The pleasant flavor of many types of cheese is due, at least in part, to free fatty acids. The characteristic flavor of certain types of milk chocolate results from the use of rancid milk solids in the formula. In addition, it seems probable that the normal, faint, characteristic flavor of milk and cream results in part from trace levels of free fatty acids (44). Of the several lower fatty acids (butyric, caproic, and caprylic), the most characteristic rancid flavor is imparted by butyric acid. The flavors of caproic or caprylic, although equivalent to and possibly exceeding butyric acid in potency, are atypical.

For practical purposes, pasteurization destroys the lipases of milk. Thus the problem of preventing rancidity is largely a matter in proper handling of raw milk. Factors that deform the milk fat globules and contribute to rancidity of unpasteurized milk or cream are as follows: (*a*) prolonged and excessive agitation, especially when accompanied by foaming; (*b*) homogenization; (*c*) separation or clarification; (*d*) warming cold milk to 80° or 90° F. and cooling again to low temperatures; (*e*) freezing and thawing of milk; (*f*) secretion of milk during advanced lactation; and (*g*) air drying of unheated milk. The first three factors are particularly important when the milk is warm. One of the most common causes of rancid flavor in milk results from the mixing of raw milk or cream with homogenized pasteurized milk. Even very limited contamination of homogenized milk with raw product can be problematical in this regard. Another significant cause of rancidity which has become of much importance in recent years originates in the bulk handling of milk. The layout of bulk handling systems on the farm has frequently led to conditions that favor rigorous agitation and foaming of the warm raw milk. Such conditions are highly conducive to the development of rancid flavor. The equipment that appears to offend most often in this connection is

pipeline milkers with risers in the line and combinations of pumps and milk level actuating devices that promote foaming. It seems probable that each farm poses special problems with regard to the installation of bulk handling equipment and that as more experience is gained and more caution is employed in design and installation of such systems the rancidity problem will be alleviated.

For obvious reasons it is of interest at times to measure degree of rancidity in milk and milk products. There are three general methods for this purpose: organoleptic, surface tension measurement, and titration with base. The organoleptic or flavor evaluation method is perhaps the most important since the way a food product tastes is always a primary consideration. Unfortunately, people vary in their response to flavor and odor, and for scientific purposes the value and interpretation of such data may be problematical. Milk undergoing fat hydrolysis usually shows a decrease in surface tension. This effect presumably may result both from the acid liberated and the mono- and diglycerides which are formed. Surface tension data are not easily reproduced. In addition, the surface tension of normal milks exhibits an appreciable range, and for data to be meaningful the surface tension of milk both before and after lipolysis is required for proper interpretation. However, it is true that rancid samples of milk will exhibit lower values for surface tension on an average than non-rancid samples. The third general method for measuring rancidity involves titration of the free fatty acids present in the extracted fat with a standard alkali solution. Most of these methods, although satisfactory for comparative purposes, are rather arbitrary. They either do not recover all of the free fatty acids or they may recover acids other than those associated with the fat. Alkali titration methods are quite sensitive and seem to show good correlation with organoleptic data.

For additional discussion and review of the rancidity problem, see reference 17.

Sunlight flavor. Incident to processing, distribution, and storage, milk and its products are exposed to light. As might be expected, light has detrimental effects on milk (41). It would be desirable to avoid exposing dairy products to light if for no other reason than to protect vitamins. However, the deleterious effects of light on the flavor of milk is also an important consideration. The knowledge gained to date indicates that there are two principal off-flavors induced in milk by light; these are oxidized flavor and sunlight flavor. The latter is also known as activated flavor and is described by such

terms as *burnt* and *cabbagey*. Although short periods of exposure to indirect daylight or indoor exposure to artificial light are contributive to the problem, bright direct sunlight is of first importance. Milk is most apt to encounter such conditions when packaged in uncolored glass bottles and when placed on the customer's doorstep during home delivery.

The amino acid methionine appears to serve as the specific origin of sunlight flavor. Since methionine does not exist as such in milk but as one of the components of the milk proteins, fragmentation of the proteins must occur incident to development of the off-flavor. It is known further that riboflavin (vitamin B_2) is directly involved in the mechanism of off-flavor formation. The exposure of various milk constituents to sunlight in the absence of riboflavin produces no sunlight flavor. The milk proteins readily produce the off-flavor when exposed in the presence of this vitamin. The removal of riboflavin from milk by selective adsorption renders the milk resistant to development of the off-flavor, and increasing the level of riboflavin in milk increases the degree of off-flavor produced on exposure to light. The principal off-flavor compound has been postulated as 3-mercaptomethylpropionaldehyde, more commonly known as methional (31). This compound can be detected readily in milk at a level of 1 part in 20 million. The mechanism for sunlight flavor formation is represented by the following reaction scheme:

$$\underset{\text{Methionine}}{CH_3—S—CH_2—CH_2—CHNH_2—COOH} \xrightarrow[\text{riboflavin}]{\text{light}} \underset{\text{Methional}}{CH_3—S—CH_2—CH_2—CHO} + CO_2 + NH_3$$

Factors related to sunlight flavor development are as follows: (*a*) the intensity of light, (*b*) the duration of exposure, (*c*) certain properties of the milk, and (*d*) the nature of the container.

The importance of intensity and duration of exposure to light can be seen in the comparative effects of a sunny and an overcast day. Flavor develops much more rapidly and intensely on a bright sunlight day. However, significant though lesser degrees of the off-flavor are imparted to milk even on heavily overcast days.

In connection with sunlight flavor, it is appropriate to mention briefly a hay-like off-flavor (47). This flavor has become a problem of importance because of a recent trend toward fortification of skim-milk with vitamin A. The limited study that has been given the problem to date indicates that the vitamin A itself is the origin of the off-flavor compound and that the tendency to produce the off-

flavor varies between sources and lots of the vitamin. Exposure to light and the presence of pro-oxidant conditions are reported to favor and antioxygenic conditions to inhibit development of the defect.

Heated flavors. Of all the processes to which milk and its products are subject, heating is perhaps most fundamental. Pasteurization, forewarming, preheating, superheating, and heat sterilization are indispensable processing procedures. Such treatment of milk is not without effect on its flavor and, in general, the more extensive the heat treatment is the more substantial the flavor change will be. It is held by discriminating observers that pasteurization of milk, either holder or short-time process, imparts a very slight change in flavor. In earlier years, when pasteurization was not so widely practiced, people accustomed to drinking raw milk could readily detect the slight flavor change caused by pasteurization. However, the use of pasteurization is quite universal now and, as a consequence, the flavor of good quality pasteurized milk generally is the flavor standard for milk so far as the consuming public is concerned.

When heat treatments in excess of those employed for pasteurization are used in the processing of milk, varying degrees of flavor change may be noted. At or about 74° C., momentary heating, a distinct cooked flavor commences to develop (12, 24). This flavor arises from the —SH groups activated by heat denaturation of β-lactoglobulin and proteins of the fat globule membrane (19). The flavor is specifically due to volatile sulfides, and hydrogen sulfide (H_2S) in particular. Although the precise mechanism of H_2S formation from the —SH groups in these proteins is unknown, factors affecting the quantity of sulfides produced in heated milk have been studied extensively by Townley and Gould (45). In general their finding shows that oxidizing conditions inhibit and reducing conditions favor sulfide formation. It is known that in the region in which cooked flavor is formed in milk there is a lowering of the oxidation-reduction potential. The exact reasons for this phenomenon have not been adequately demonstrated. Data by Josephson and Doan showing the relation between lowering of oxidation-reduction potential, degree of cooked flavor and —SH activity as a function of various heat treatments are presented in the following table. In this table —SH activity is presented in terms of the nitroprusside test, one of a number of methods that may be used for this purpose.

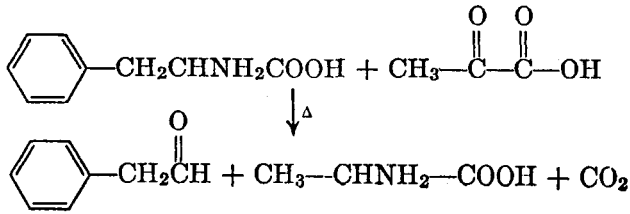
Table 1. THE EFFECT OF HEATING ON THE OXIDATION-REDUCTION POTENTIAL, FLAVOR AND NITROPRUSSIDE REACTION OF MILK

Heat Treatment	O-R (volts)	Flavor	Nitroprusside Reaction
None	0.2441	Normal	−
145° F., 30 min.	.2423	Normal	−
160° F., momentary	.2426	Normal	−
170° F., momentary	.2349	Cooked ±	±
180° F., momentary	.2331	Cooked ++	+
190° F., momentary	.2025	Cooked ++++	+++

It will be clear from the foregoing that the information available on cooked flavor leaves much to be desired. The reasons why heat treatment activates the —SH groups of certain of the milk proteins, causes a drop in oxidation-reduction potential, and favors the formation of volatile sulfides are obscured in the complexities of protein denaturation (see Chapter 4).

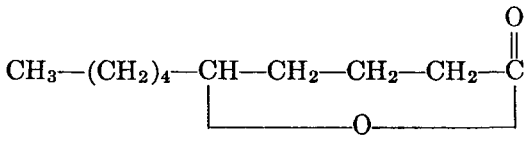
When heat treatment is prolonged or extended to temperatures well above 75° C., cooked flavor slowly gives way to caramelized flavor. There seems to be no good reason for presuming any cause and effect relation between these two flavors at this time. The chemical nature of caramelized flavor is unknown. At what point in the heat treatment of milk this flavor becomes evident and whether browning is always co-present with the flavor is not known. Degree of caramelized flavor is correlated positively with the magnitude of browning and associated changes. The fact that caramelized flavor does not develop in heated fluid whey suggests that casein is directly involved in the flavor development. The subject of browning, which seems indispensable in the fundamental consideration of caramelized flavor, is treated in Chapter 11.

Heat treatment may bring about the decomposition of α-amino acids to aldehydes of one less carbon. This reaction, in which certain dicarbonyl compounds are required, is known as the Strecker degradation (37) and its essence is expressed in the following equation, which employs phenylalanine and pyruvic acid as an example:



As mentioned elsewhere (Chapter 7), milk contains trace quantities of free amino acid, and these levels are enhanced by the hydrolytic effects of high temperature heat treatment on the milk proteins. Heat also produces in milk dicarbonyl compounds such as pyruvic acid and methylglyoxal. In addition, other lactose decomposition products and dehydro ascorbic acid can serve as suitable dicarbonyls for the reaction. Considering the variety of amino acids that are present in milk proteins, it seems clear that a great number of flavorful aldehydes could be produced by the Strecker degradation in milk. The plausibility of this reaction under the conditions suggests that it is a contributing chemical mechanism in the formation of caramelized flavor. In an effort to give at least a tentative definition to caramelized flavor, the following components are mentioned: (*a*) caramel or malty, resulting from sugar decomposition and Strecker degradation of amino acids; (*b*) stewed meat, arising from methionine decomposition and the presence of H_2S; (*c*) hydrolytic rancidity, caused by fat hydrolysis; and (*d*) coconut-like, resulting from the formation of a lactone in the milk fat.

The latter flavor, that is, that termed coconut-like, is of particular interest in view of its broad significance for products containing milk fat. Recent research (28, 33) has established that the coconut-like flavor compound is δ-decalactone, which has the following formula:



This lactone originates from milk fat and more particularly the low melting portion of the fat. Its precise origin and mode of formation are not known although cold storage prevents and heat treatment and elevated storage temperatures favor its formation. It has been established that δ-decalactone contributes to the storage flavor defect of the following products; butter oil (anhydrous milk fat), dry whole milk, dried cream, and evaporated milk. Its presence has also been noted in cheddar cheese.

A further effect of heat treatment on the flavor of milk concerns its tactual properties, that is, the way it feels in the mouth. Normally milk is smooth tasting and imparts no undue sensation of graininess or chalkiness to the mouth. However, heating milk considerably in excess of ordinary pasteurization treatment will impart a tactual flavor defect (32). It appears to resemble, in a mild degree, the sensation that milk-of-magnesia gives in the mouth. The available

information indicates that this defect is caused by insoluble aggregates of calcium and magnesium with the milk proteins. The defect is particularly marked at times in dry milk products, which suggests that when milk is dried and then reliquefied the milk components are never quite restored to their native state. Homogenization also has been reported to impart a tactual defect occasionally to milk.

The foregoing section on heated flavors in milk reveals the significance of heat treatment as an agent for producing off-flavors. As a result of this fact, the dairy industry is continuously searching for means of minimizing heat treatment in the processing of its products or other safe methods of preserving milk products. This problem is indeed challenging, for milk appears to be fully as sensitive to the agents that have been employed to date for long-range preservation as are the enzymes and bacteria, whose destruction is sought. Even if some innocuous means of preservation could be found, the question remains as to how long milk, a dynamic biological system, could be stored without an objectionable degree of autolytic decomposition.

Micro-biological Flavor Deterioration

The excellence of milk as a growth medium for nearly all types of bacteria, yeasts, and molds establishes microbiological activity as an important source of off-flavors in milk. The versatility of microorganisms in producing chemical changes makes the complexity and variety of off-flavors produced by such agents almost limitless. A current problem in this connection concerns bottled milk, which is being held longer and longer before use. Bulk storage of milk, every-other-day and three times weekly delivery, and the sale of milk through stores where the consumer buys a substantial supply of milk, all contribute to greater age of milk at the time it is consumed. For the most part, proper refrigeration and improved sanitation practices in the dairy industry have extended the keeping quality of bottled milk. However, psychrophilic (cold-tolerating) bacteria will sometimes grow in milk at 40° to 45° F. sufficiently to affect the flavor after four to six days (46). The action of these bacteria does not manifest itself in sourness, but rather in bitter, fruity, rancid, stale, and putrid types of off-flavors. Coagulation of the milk frequently is not evident. Psychrophiles are destroyed by pasteurization. However, some post-pasteurization contamination of milk is practically unavoidable. Frequently water supplies used in cleaning bottles and plant equipment are sources of these organisms. Thus it seems clear that the flavor and

keeping quality of bottled milk would be protected considerably through efforts to minimize contamination of the milk following pasteurization.

A good example of what can be done to clarify the chemistry of flavors microbiological in origin concerns the work that has been conducted on malty flavor. This off-flavor is primarily a problem with raw milk that has not been adequately cooled. On occasions it also has been reported as a defect in cream and butter. Malty flavor is produced by *S. lactis var. maltigenes,* which frequently contaminates equipment on the farm. The findings of Jackson and Morgan (20) have shown that the organism acts on the amino acid leucine to produce isovaleraldehyde in accordance with the following equation:

$$(CH_3)_2—CH—CH_2—CHNH_2—COOH \rightarrow (CH_3)_2—CH—CH_2—\overset{O}{\overset{\|}{C}}H + NH_3 + CO_2$$

Organoleptic tests revealed that as little as 0.5 p.p.m. of isovaleraldehyde added to milk will closely simulate the characteristic malty flavor and aroma.

Absorbed Flavors

In addition to chemical and microbiological deterioration of milk constituents, there is yet another important origin of off-flavors. In a general sense, this is covered by the term *absorption,* since entry of the flavor and odor compounds into the milk is more or less incidental to milk secretion. Flavorful substances may enter milk either before or after milking. Actually, the latter route as a flavor problem seems somewhat overdone and the former means of entry, that is, through the cow, is by far the most important. There are two pathways by which flavor and odor substances may gain entry into the milk via the cow. One is by the nose or mouth, to the lungs, to the blood stream, to the udder cells, and into the milk. The other is from the digestive tract to the blood, to the udder cells, and into the milk. In the first case the cow is actually acting as an odor trap, and where highly odorous feeds are fed and where barn air is heavily contaminated with odorous substances, this is the primary route of the off-flavor. This means of entry is characterized by the speed with which the off-flavor appears in the milk. For example, when a cow either eats or smells onions or garlic the odor is noted in the blood within a very few minutes and the flavor is detected in the milk within 20 to 30

minutes. In this process it is well to remember that the flavor substance is being given off in the breath, sweat, urine, and feces of the animal as well as in the milk and that the process is dynamic. As long as the odorous substance is being fed or inhaled, the build-up in the body will continue and when feeding or inhalation ceases the concentration of the odorous material will decrease first in the blood and subsequently in the milk, until no further deposition in the milk occurs. Flavor substances may also be absorbed into the blood from the digestive tract of the cow and then from the blood into the milk. As compared with the inhalation route, this process is considerably slower.

Feed and weed flavors. Off-flavors that are absorbed into the milk through the cow fall into fairly well-defined categories as judged by taste of the milk. These are: "feed," "weed," "cowy," "barny," and "unclean." The most problematical among these are the feed and weed off-flavors. The contributions of various feeds and weeds to off-flavors in milk have been studied rather comprehensively (40) and it is possible to list some of the consistent offenders as well as some that ordinarily have little effect.

Sources of Feed Flavor	Sources of Weed Flavor	Feeds with Little Effect
Onions	Garlic and chives	Sugar beets
Fermented silage	French weed	Dried beet pulp
Alfalfa	Mustard	Soybeans
Cabbage	Boneset	Carrots
Turnips	Buckhorn	Pumpkins
Rape	Pepper grass	Soybean hay
Kale	Skunk cabbage	Potatoes
Beet tops	Ragweed	Mangoes
Green barley	Wild tansy	Oats
Green alfilaria	Dog fennel	Rye
Clover hay	Tar weed	Peas
Distillers grains	Alanthus shoots	Corn
Brewers grains		Clover and grass
Musty hay or silage		Timothy hay
Citrus pulp		Most concentrates
		Tankage

There are a number of measures that generally are adequate to control feed and weed flavors. It is obvious that highly odorous and objectionable feeds should be given in limited quantities if at all. Irrespective of the nature of the feed, it is desirable to prevent cows

from eating for a period of three to five hours before milking. Stall feeding and pasturing after, rather than before, milking are preferred practices. Regarding weed flavors, it is desirable so far as possible to eliminate weeds from pastures. This can be accomplished by a number of good pasture management practices, including high level clipping to prevent weeds from going to seed, use of weed killers, and fertilization, renovation, and reseeding in instances where pastures have gone completely to weeds.

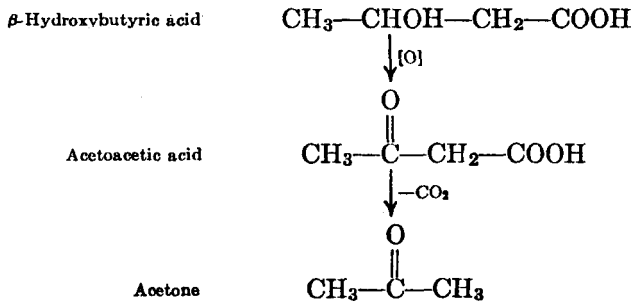
The question arises whether anything can be done to overcome weed and feed flavors, and for that matter other absorbed flavors, once they have gained entry into the milk. Although there is no foolproof method of removing any and all flavors of this type, aeration or heat coupled with vacuum treatment are sometimes very effective. The effectiveness varies with the particular compounds in question and more specifically with their volatility, flavor potency and concentration. The more volatile and less concentrated a flavor substance is in milk the more readily it can be removed by heat and vacuum treatment.

As mentioned previously, the capacity of milk to absorb odors directly from the atmosphere has been overemphasized. Milk can be stored in open milk cans in an atmosphere rank with silage, manure, onions, and the like for several hours with no detectable effect on its flavor. Moreover, milk will never absorb odor or flavor from barn or stable air during the course of customary milking. The off-flavors derived from cows breathing unclean stable air can be avoided by proper ventilation. In this connection it is of interest that morning milk frequently carries a barny taint when cows are stabled under conditions of poor air circulation. A final category regarding absorbed off-flavors concerns those picked up from improperly cleaned utensils and equipment. Residual disinfectant and any odorous substance clinging to utensils and equipment has the potential of imparting off-flavors in milk.

Cowy flavor. Cowy or barny flavor is particularly prevalent in raw milk supplies during the winter months. There may be three significant sources of this particular flavor: (*a*) tainted stable air, (*b*) abnormal silage fermentations, either due to temperature or age of silage, and (*c*) ketosis, a disease in cattle involving the endogenous energy metabolism.

Ketosis in dairy cows is fairly common, especially in the late winter or early spring and particularly right after calving. Analyses of the blood and urine of cows suffering from the disease show relatively high

concentrations of acetone bodies, and the milk from such animals contains somewhat lower concentrations of these substances. The compounds known as acetone bodies are: β-hydroxybutyric acid, acetoacetic acid, and acetone. Chemically these substances are related in the following manner:



Van Slyke's method (15) enables measurement of total acetone bodies as well as the individual components.

In the light of findings by Josephson and Keeney (25), a direct relation is indicated between the concentration of acetone in milk and the incidence and degree of cowy flavor. Their results show that the addition of acetone to milk imparts cowy flavor, that the threshold level for a barely detectable off-flavor is approximately 25 p.p.m., and with increasing concentrations of acetone up to 100 p.p.m. increasing degrees of cowy flavor are evident. Above this level a chemical or medicinal flavor character is manifest in the milk. It seems probable that some of the feed flavors observed in mixed milks may actually be due to low concentrations of acetone.

Chemical identity of absorbed flavor compounds. The amount of information available on the identity of compounds responsible for weed, feed, and related flavors in milk is almost negligible. The practical measures of preventing such flavors mentioned above have largely obviated this difficult area of work. However, it is of interest on occasions to learn the nature of these compounds, and in such instances the procedures and methods employed by Forss (9) in revealing benzyl mercaptan as an agent in the weed tainting of butter are worthy of note. Using similar techniques, Conochie (4) has identified indole and skatole as tainting compounds in the milk from cows fed *Lepidium hyssopifolium.*

The information presented in this chapter testifies to the far-reaching importance of flavor in milk and its products. The phrase "is not known" and variations thereof have been used throughout

almost to an embarrassing degree. In future dissertations on the subject it is hoped this will not be necessary. Excellent tools, both psychometric and chemical, are available to attack the fundamental and practical problems of flavor. The areas appropriate for study are many and the need is obvious.

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CHAPTER 13 NUTRITIVE VALUE OF MILK

So far as the purposes of this text are concerned there are two principal reasons for presenting information on the subject of nutrition. These are to attempt some orientation of the dairy science student regarding the field of nutrition, and to supply information on the nutritive value of milk as far as it is understood at present. The need for information on nutritional aspects of milk is so general and frequent that the subject seems within the province of this book. In addition, chemistry is a primary consideration in the stability of various nutrients in milk and milk products. However, the field of nutrition is vast and most ramifications of it are not considered here. To meet more extensive needs in this connection the list of selected references at the end of the chapter includes a number of very useful and informative texts on the subject (1, 2, 7, 10, 11, 15).

THE SCIENCE OF NUTRITION

Characteristics. Information in the field of nutrition should be approached with caution. This holds true for the advertising claims on a food package as well as the published results of scientific investi-

gations from reputable laboratories. It is not that the statements in either situation are necessarily incorrect, but careful interpretation to derive the true meaning and limitations of statements and claims regarding nutrition is required. Health and nutrition always have certain aspects as a popular fad, perhaps more so in present times than previously. As a consequence, the lay public often serves as a misguided interpreter and disseminator of information on nutrition. Even qualified scientists are frequently in error or disagreement regarding the findings of nutritional investigations. It will be clear therefore that a cautious approach to the field seems warranted.

The question is appropriately raised: Why can information on nutrition not be straightforward? and, Why is such information so often misleading? The answer stems first from the complexity of nutrition as a field of study, and secondly from the tendency of man to reduce complex information to simple and general terms that can be easily understood. The situation may be clarified by the following hypothetical example. The Food and Nutrition Board of the National Research Council recommends certain daily dietary allowances for the various nutrients, vitamins, and minerals. This information reaches the public in various ways, but let us assume that the person in question by one means or another comes by the understanding that the recommended daily dietary allowance for vitamin B_1 is 1.2 mg. Now the normal person with a healthy concern for his wellbeing will wish to make sure that he is getting the recommended allowance of this vitamin. Thoughts may come to mind that consumption short of the amount specified will lead to sickness. Before coming to such a conclusion, we might consider the following:

1. Ultimate knowledge of nutrition is held by none. The Food and Nutrition Board makes recommendations on the basis of the information available and there is much additional information it would like to have.

2. There is a difference between recommended daily dietary allowances and minimum daily requirements. Generally, the former is substantially higher than the latter and even the latter may make some allowance for a margin of safety.

3. There are extensive individual variations in requirements for a given nutrient. Actually the 1.2 mg. of thiamin is recommended for women of approximately 25 years of age, 121 pounds weight, and 62 inches height. The average requirement for an adult for vitamin A is 5000 international units per day, but the range among individuals

may be of the extremes, 2000 to 50,000 I.U. per day. The subject of variation is discussed further below.

4. It is commonly observed that the requirement for a certain nutrient varies with the nature of the diet. Some nutrients have sparing action on other nutrients and, in general, needs for any given vitamin or nutrient will be at a minimum in a well-balanced and varied diet as compared with a narrow, poorly balanced diet.

A further feature of the field of nutrition is that much of the investigational work is conducted with experimental animals. The rat, because of the structure and function of its digestive system, has been used to supply much guiding and valuable information for the field of human nutrition. However, direct extrapolation of findings on rats to performance and requirements in man obviously can lead to difficulties. There are many other species of animals employed in nutritional investigations, including the dog, chick, guinea pig, hamster, rabbit, and calf. Obviously these animals will show species differences and one very worthwhile question that can be posed concerning any research in the field of nutrition is, for what species of experimental animals do the findings apply?

Functions of food. Foods serve the following broad purposes: (*a*) growth, (*b*) reproduction, (*c*) supply of energy, (*d*) maintenance and repair, and (*e*) psychic needs such as pleasure and appetite satisfaction. It will be obvious that requirements in these particular categories not only will vary with the individual, but in some instances some of the stated functions of foods will not need to be served. For example, infants and children require food for both growth and maintenance whereas adults no longer require food for growth purposes. In the case of pregnant and lactating women, foods must perform the additional functions of supplying nutrients for development of the fetus and for synthesis of milk. Although the satisfaction of appetite and the pleasure derived from eating are somewhat incidental to meeting the body's basic needs for food, the importance of these considerations should not be underestimated. It is beyond question that whether a food is eaten and what amount of it is consumed will depend very substantially on how appetizing it is to a given individual. This fact clearly establishes the relation between such matters as food quality, especially its flavor, appetite, the pleasure of eating, and sound nutrition. It seems probable that, given an adequate food supply, people will eat primarily what they like and secondly what is good for them.

The functions of food are served specifically through the various nutritionally important food components, which are proteins, carbohydrates, lipides (fats), minerals, vitamins, and water. The various proteinaceous materials and structures of the body are synthesized and maintained from dietary sources of protein. When food protein is taken into the stomach it is broken down to its component amino acids, and these serve as the structural building blocks for the various proteins of the body. Certain amino acids can be synthesized in the human body from other exogenous material, but a number of them cannot. The latter are known as the essential amino acids. They are tryptophan, phenylalanine, lysine, threonine, valine, methionine, leucine, and isoleucine. Table 1 presents information on the quantita-

Table 1. ESSENTIAL AMINO ACIDS

Amino Acid*	For Maintenance of Nitrogen Balance in Adult Man	
	Minimum Daily Requirement	Recommended Daily Intake
L-Tryptophan	0.25	0.5
L-Phenylalanine	1.10	2.2
L-Lysine	0.80	1.6
L-Threonine	0.50	1.0
L-Valine	0.80	1.6
L-Methionine	1.10	2.2
L-Leucine	1.10	2.2
L-Isoleucine	0.70	1.4
L-Arginine	...	...
L-Histidine	...	...

Data by Rose (9).

* All members of this list are essential for rat growth.

tive requirements for these amino acids in the adult man. In a sense it is misleading to give data on requirements for amino acids since it has been observed that the amount of any one amino acid needed can be substantially influenced by the amounts of others made available, as well as by the general quality of the diet. For further information on the utilization of dietary proteins see references 1 and 16.

The principal function of carbohydrates and lipides is to supply energy. On an average, these dietary components provide 89% of the calcries in the American diet. The dietary intake of carbohydrates

and fats should not be limited to the extent that protein, which is essential for growth and maintenance, is utilized instead for energy needs. However, so far as the American diet is concerned, the problem is more one involving overconsumption of carbohydrates and fats, resulting in obesity.

Vitamins. A vitamin may be defined as an organic compound that is required in small amounts for satisfactory growth and maintenance and that is not synthesized within the body. The principal functions of vitamins are to facilitate transfer of energy and to regulate metabolism in the body. They do not serve as sources of energy or as structural units for the body to any significant extent.

The interrelated and essential chemical processes involved in the metabolism of the animal body are largely accomplished through the functioning of systems of enzymes. In such enzyme systems, vitamins, metal ions, and proteins all play an indispensable role. As pointed out in the chapter on enzymes, all enzymes are first and foremost proteins, but in order to be capable of functioning the protein of some enzymes must be supplemented with a prosthetic group. Although these prosthetic groups include a variety of compounds, many of the vitamins and metal ions act as such groups. The specific functions and mechanisms of action of the various vitamins, to the extent that they are known, are beyond the scope of this text. Appendix I-B contains a summary of information on the various major vitamins.

Minerals. The term *minerals* is widely employed in nutrition with reference to the various elements required by the body either in the form of compounds or their component ions. For example, the element phosphorus is required by the body but is not utilized as such. Rather it serves its function in the various forms of phosphate. Minerals have four principal functions in nutrition; namely, (*a*) as components of structural units, (*b*) as prosthetic groups in enzymes, (*c*) as activators of enzymes, and (*d*) as water-solubilizing agents for products of metabolism which otherwise would be water insoluble. It is considered here that their regulation of membrane diffusion processes is closely related to their water-solubilizing activities.

The elements that are required in relatively high concentrations in order to maintain normal growth and reproduction of the organism are sulfur, phosphorus, magnesium, calcium, nitrogen, sodium, potassium, and chlorine (as chloride). Certain so-called trace elements are also essential for animals, including iron, copper, zinc, manganese, and iodine. Cobalt also is probably required, at least in some species. A clear-cut requirement for the adult human has not been established.

The chapter on enzymes has presented information showing the significance of certain metals in the composition and functioning of various enzymes in milk. Table 2 presents further information on this

Table 2. SPECIFIC METALLO ENZYMES

Enzyme	Reaction	Metal
Carbonic anhydrase	$CO_2 + H_2O \rightleftharpoons H_2CO_3$	Zn
Dehydropeptidase	Glycyldehydrophenylalanine $\rightarrow NH_3$ + phenylpyruvic A	Zn
Glycylglycine dipeptidase	Glycylglycine $\rightarrow$ glycine	Zn
Carboxypeptidase	Carbobenzoxyglycyl-L-phenylalanine $\rightarrow$ phenylalanine	Zn
Alcohol dehydrogenase	Ethanol + DPN $\rightleftharpoons$ acetaldehyde + DPNH	Zn
Glutamic dehydrogenase	Glutamate + DPN $\rightleftharpoons$ ketoglutarate + DPNH + NH_3	Zn
Lactic dehydrogenase	Lactate + DPN $\rightleftharpoons$ pyruvate + DPNH	Zn
Inorganic pyrophosphatase	Pyrophosphate + $H_2O \leftarrow PO_4$	Mg
Fumaric hydrogenase	Fumaric acid + 2H $\rightarrow$ succinic acid	Fe
Catalase	$2H_2O_2 \rightarrow 2H_2O + O_2$	Fe
Peroxidase	H_2O_2 oxidation of aromatic amines and other compounds	Fe
Cytochromes	Electron transport	Fe
DPNH-cytochrome c reductase	DPNH + cytochrome c (Fe^{+++}) $\rightarrow$ DPN + cytochrome c (Fe^{++})	Fe
Uricase	Uric acid + $O_2 \rightleftharpoons$ allantoin + $H_2O_2 + CO_2$	Cu
Tyrosinase	Tyrosine + $1/2O_2 \rightarrow$ hallochrome	Cu
Laccase	Phenols $\rightarrow$ ortho and para quinones	Cu
Ascorbic acid oxidase	Ascorbic acid $\rightarrow$ dehydroascorbic A	Cu
Butyryl CoA dehydrogenase	Butyryl CoA-2e $\rightarrow$ crotonyl-CoA	Cu
Prolidase	Glycylproline $\rightarrow$ proline	Mn
Nitrate reductase	NO_3 + TPNH + $H^+ \rightarrow NO_2$ + $TPN^+ + H_2O$	Mo
Xanthine oxidase	Xanthine + $O_2 \rightarrow H_2O$ + uric acid	Mo
Aldehyde oxidase	Acetaldehyde + $O_2 \rightarrow$ acetate + H_2O	Mo

Data by McElroy and Nason (8).

point for a variety of enzymes in general. The role of trace elements in nutrition may be studied to advantage in a recent review on the subject (8).

Sometimes by oversight the important role that water plays in

nutritional well-being is taken for granted. It is to be emphasized that the indispensable chemical processes in the body that accomplish growth, maintenance, repair, reproduction, and lactation all take place in a solvent. That solvent is water.

Human nutritional requirements. A given individual's nutritional requirements are influenced primarily by the following considerations: the state of development, height and weight, occupation, and inherited characteristics. It is to be anticipated that, in addition to maintenance, infants and children will have growth requirements to satisfy. Since such a state involves less body weight, the additional requirement for growth may be somewhat offset by a lessened requirement for maintenance. It is also evident that need for overall nutrition is heightened in occupations requiring considerable physical activity. Perhaps the most important variable in human nutrition concerns the differences that an individual acquires through heredity. It is frequently cited that there are group variations as well as individual variations. For example, in certain parts of the world there are groups of people who theoretically should not be able to survive on the narrow and limited diets which they receive. It is well to bear in mind that in these groups natural selection over many generations may have been playing a role in determining those capable of surviving on such limited diets. It seems theoretically possible under such conditions that in the course of generations a group could be selectively bred which might have very limited requirements for certain of the essential nutrients. Of course, such diets are not necessarily without effect on the general well-being of the people involved. Using protein nitrogen intake as a criterion of the level of nutrition, Table 3 reveals the effects that nutrition may have on stature and

Table 3. EFFECT OF NATIONAL DIETS ON STATURE AND LONGEVITY

	Australia	U. S.	China	India
Protein N intake, g./day	18.1	15.7	11.1	9.8
Animal protein, %	69	57	8.5	16
Average height, cm.	172	170	158	161
Average weight, kg.	77.2	70.0	54.3	50
Life expectancy, years	65	64	30	27

Data by Albanese (1).

longevity of peoples from several areas of the world. Other factors, such as disease and sanitation, also can be involved in stature and life expectancy.

As information in the field of nutrition has become more comprehensive and reliable, recommendations have been made concerning daily dietary allowances for the maintenance of good nutrition in the normal healthy person in the United States. This information is reappraised regularly and is put out in one form by the Food and Nutrition Board of the National Research Council. The latest revision of this information was made in 1958 and this information, as published by the National Dairy Council, is reproduced as Appendix I-A to this book.

NUTRITIVE VALUE OF MILK AND MILK PRODUCTS

Milk. From a nutritive standpoint, milk frequently is defined as "the most nearly perfect food." Although its generally excellent food value is beyond question, milk is not a perfect food and has certain limitations. It supplies more of the essential nutrients in significant amounts than any other single food. However, its value as a food will show some variation among individuals, both from the standpoints of inherent capacity to utilize the medium and the particular state of development of the individual. Sufficient, though very limited, numbers of people exhibit allergy symptoms from ingesting milk to indicate that it is not beneficial in all respects for everyone. In the interest of stimulating consumption, milk has been recommended in the dietary of all age groups. This recommendation has merit but it seems evident that milk is most beneficial for the young.

A summary of some nutritive properties of milk are presented in Table 4. Milk is outstanding as a source of calcium, phosphorus, and riboflavin (vitamin B_2). It also is a good source of high quality protein. It contributes significantly to vitamins A and B_1 requirements. It is rather mediocre as a source of iron and copper and is distinctly inadequate in the amounts of vitamin C and nicotinic acid that it can supply. Milk as secreted by the cow is a poor source of vitamin D. However, nearly all milk is fortified with this vitamin to the extent of 400 I.U. per quart. The latter amount is the recommended daily dietary allowance for infants and children. Further discussion of fortified milks is presented later in this chapter. The information in Table 4 regarding concentration of vitamins, minerals, and vitamin-like substances is supplemented by tabular data on milk composition in Chapter 1, Table 1.

Table 4. Some Nutritive Properties and Components of Milk

Nutrient	Amt. in 1 Qt. Avg. Milk	Amt. per 100 kcal. Portion	Requirements per Day		Daily Consumption of Milk (qt.) Necessary to Satisfy Requirement			
			Adults	Children*	Adults	Classification†	Children*	Classification†
Energy	650 kcal.		3000 kcal.	2500 kcal.	4.6		3.8	
Protein	33 g.	5.0 g.	70 g.	70 g.	2.1	Good	2.1	Good
Calcium	1.12 g.	0.17 g.	0.8 g.	1.2 g.	0.71	Excellent	1.1	Excellent
Phosphorus	0.94 g.	0.14 g.	0.9 g.	1.2 g.	1.0	Excellent	1.3	Excellent
Iron	2.26 mg.	0.35 mg.	12 mg.	12 mg.	5.3	Fair	5.3	Fair
Copper	0.26 mg.	0.04 mg.	1.0 mg.	1.0 mg.	4.0	Fair	4.0	Fair
Iodine	0.04–0.07 mg.		0.05 mg.	0.15 mg.				
Vitamin A	500–1000 I.U. winter 2000–3000 I.U. pasture	75–460 I.U.	5000 I.U.	5000 I.U.	1.7–10.0	Fair to good	1.7–10.0	Fair to good
Vitamin D	5–15 I.U.	0.75–2.25 I.U.		400–450 I.U.			30–90	Poor
Thiamine	0.35–0.40 mg.	0.06 mg.	2.0 mg.	1.2 mg.	5.0	Fair to good	3.0	Fair to good
Riboflavin	1.5 mg.	0.23 mg.	2.5 mg.	1.8 mg.	1.7	Excellent	1.2	Excellent
Niacin	0.2–1.2 mg.	0.03–1.8 mg.	20 mg.	12 mg.	15–100	Poor	10–60	Poor to fair
Pantothenic acid	2.9 mg.							
Ascorbic acid	20 mg. (fresh milk) 5 mg. (past. milk)	0.75 mg.	75 mg.	75 mg.	3.7–15	Poor	3.7–15	Poor

* Ten- to 12-year olds.

† Excellent—10% requirement furnished by amount furnishing not over 100 kcal.
Good—10% requirement furnished by amount furnishings not over 200 kcal.
Fair—10% requirement furnished by 1 qt.
Poor—less than 10% requirement furnished by 1 qt.

One of the more recently established members of the vitamin B complex is B_{12}, for which milk is a good source. This vitamin, known as the so-called antipernicious anemia factor, exists in milk in amounts of from 3 to 10 μg. per quart. Although this is an infinitesimal quantity of material, vitamin B_{12} is effective at extremely low levels. For example, human beings suffering from certain forms of pernicious anemia will respond to the administration of as little as 1 μg. of vitamin B_{12} (5). Vitamin B_{12} is also known by many other names, perhaps most common of which is cobalamine. It is a highly complex molecule of empirical formula, $C_{63}H_{90}O_{14}N_{14}PCo$. The presence of cobalt in this vitamin is one of its notable properties. A minimum dietary requirement for vitamin B_{12} has not been established. In fact, it is not clear whether the normal human being requires an exogenous source since substantial quantities of the vitamin are produced by bacteria in the alimentary tract.

Milk products. In general, the nutritive value of milk products is based on the nutritive value of milk as influenced by processing measures. Processing can have a modifying effect in two ways. These are through separation, dilution, or concentration of the milk components; and physical and chemical effects of the various processing steps on the nutrients.

As an example of the first type of effect, let us consider the manufacture of butter. When whole milk is separated, the fat soluble vitamins A, D, E, and K will go almost exclusively into the cream. The content of these vitamins in the cream will be a function of the fat content of the cream. The various water soluble vitamins remain associated with the skimmilk. These are principally the various members of the B complex and vitamin C. It should be noted that the skimmilk portion of the cream will contain the same concentration of the water soluble vitamins as the skimmilk removed by separation. On a weight basis this group of vitamins will exist at lower levels in the cream because of the diluting effect of the fat. When the cream is churned to butter a further fractionation occurs, the fat soluble vitamins again disposing themselves largely with the fat and the water soluble vitamins following the buttermilk. Buttermilk does contain some fat, particularly lipide material from the surface of the fat globules, scuffed off during the churning process. The granules of churned butter occlude a small amount of buttermilk and this quantity is reduced further during the washing and working processes. Thus butter contains, as a result of manufacturing, only trace amounts of the nutrients associated with the non-fat phase of milk and represents

a concentrate of the nutrients associated with the fat phase. The actual content of fat soluble vitamins in butter varies rather widely with such factors as breed, feed, and season of the year. Generally speaking, butter is considered to be a rich source of vitamin A, a good source of vitamin D, and a fair source of vitamin E.

Another appropriate example regarding distribution of nutritive value as a function of processing concerns cottage cheese. Initially the product is made from skimmilk and is largely devoid of the fat soluble vitamins. When the skimmilk is set with rennet and starter and the whey expelled from the curd by heating, the curd becomes essentially a concentrate of protein (casein). On the other hand, the water soluble vitamins, soluble salts, and lactose are found in the whey. In order to obtain optimum quality in cottage cheese, the whey is drained either partially or more or less completely, and water is then added to the curd. This tends to remove more of the whey constituents, enhancing keeping quality, and helping to give the curd proper texture. It will be seen that the levels of the water soluble vitamins and minerals may be influenced by the way this step in the manufacturing is done. A further variable enters the picture depending on whether the cottage cheese is creamed or not. In summary, it can be said that the composition of cottage cheese from a nutritive standpoint will be influenced by moisture content, whey content, and the amount and fat content of added cream. An additional factor of probable importance concerns pH relations. The extent that colloidally dispersed and protein-associated calcium will be taken into solution varies with pH. Thus, with products made under more acid conditions lower calcium content is to be expected. A comparison of the composition and nutritive value of cottage cheese (without added cream) and of milk is made in Table 5. This table also presents information on nutritive components in a number of other dairy products. In connection with these data it is a point of interest that the iron and riboflavin contents of cottage cheese are somewhat higher than would be anticipated for water soluble constituents. Since both of these components are known to exist partially in combination with the proteins of milk, the data suggest some concentration effect of such proteins in cottage cheese curd.

The example concerning cottage cheese brings out another important point. Data on the nutritive value of any given dairy product are not meaningful as a generalization. One must know something about both the processing and the composition in order to interpret one sample of a product in terms of data on another sample. In the case of ice cream, it is obvious that fat content, serum solids content, amount

Table 5. QUANTITIES OF CERTAIN NUTRITIVE COMPONENTS IN MILK AND MILK PRODUCTS (100 g. portions)

Product	Water, %	Food Energy, cal.	Protein, g.	Fat, g.	Carbohydrate, g.	Ash, g.	Calcium, mg.	Phosphorus, mg.	Iron, mg.	Vitamins A, I.U.	Vitamins B_1, mg.	Vitamins B_2, mg.	Vitamins Niacin, mg.	Vitamins C, mg.
Milk (whole)	87.0	68	3.5	3.9	4.9	0.7	118	93	0.1	(160)	0.04	0.17	0.1	1
Milk (skim)	90.5	36	3.5	0.1	5.1	0.8	123	97	0.1	Trace	0.04	0.18	0.1	1
Butter	15.5	716	0.6	81	0.4	2.5	20	16	0.0	3300*	Trace	0.01	0.1	0
Cheddar cheese	37	398	25.0	32.2	2.1	3.7	725	495	1.0	1400	0.02	0.42	Trace	(0)
Cottage cheese	76.5	95	19.5	0.5	2.0	1.5	96	189	0.3	(120)	0.02	0.31	(0.01)	(0)
Dry milk (whole)	3.5	492	25.8	26.7	38.0	6.0	949	728	0.6	1400	0.30	1.46	0.7	6
Dry milk (skim)	3.5	362	35.6	1.0	52.0	7.9	1300	1030	0.6	(40)	0.35	1.96	1.1	7
Condensed milk†	27.0	320	8.1	8.4	54.8	1.7	273	228	0.2	(430)	0.05	0.39	0.2	1
Evaporated milk	73.7	138	7.0	7.9	9.9	1.5	243	195	0.2	400	0.05	0.36	0.2	1
Ice cream	62.1	207	4.0	12.5	20.6	0.8	123	99	0.1	520	0.04	0.19	0.1	1

Data from Watts and Merrill (13). Parentheses indicate imputed values.

* Year-round average.

† Sweetened whole.

of added sugar, and quantities of various additives such as fruits and nuts are extremely variable and that nutritive value of such products will also vary accordingly. The same thing holds true for cheese in that moisture, fat and protein concentration vary rather substantially both within and between varieties. A further factor of importance regarding cheeses is the actual synthesis of certain vitamins by the microorganisms they contain. With respect to nutritive components in concentrated and dried milk products, these are best interpreted on the basis of fluid milk equivalent.

Besides modifying the content of various nutritional constituents through concentration, fractionation, and dilution, processing may in a few instances destroy nutrients or enhance nutritive aspects of a product. Heat treatment and oxidation (storage in contact with air) are the main factors in destruction. Variable amounts of vitamin B_1 are destroyed by heat treatment. From 0 to 10% may be lost as a result of pasteurization and amounts from 30 to 50% may be destroyed by sterilization (240° F. for 15 minutes). Vitamin C is also destroyed by heat treatment but this effect appears to be primarily oxidative in nature. On an average, pasteurized milk contains about one-half of the original vitamin C content of fresh raw milk. The actual amount of the vitamin in pasteurized milk varies considerably since there is a progressive decline from day to day. From 60 to 100% of the vitamin C in milk is destroyed by sterilization. The loss of vitamin C during heat processing and storage of milk is determined primarily by a complex of factors in each sample. These factors are responsible for the level and poise of the oxidation-reduction potential (see Chapter 8). Both vitamins A and E are subject to oxidative deterioration, and although they appear to be quite stable in fluid dairy products, their variable losses are encountered in dry whole milk, depending upon processing and storage conditions. Sterilization and drying are reported to have minor and probably negligible effects on the biological value and digestibility of milk proteins (7).

Processing may be beneficial to the nutritive properties of milk through such phenomena as improvement in flavor, appearance, fat dispersion and curd tension. Lowering of curd tension is discussed subsequently.

FORTIFIED AND SPECIAL MILKS

Vitamin D milk. As information on the nature of vitamin D and its role in the prevention of rickets became known in the early 1920's,

attention was focused on milk as a plausible point for fortification with this vitamin. Vitamin D regulates the absorption and metabolism of the bone forming elements calcium and phosphorus. Milk is an excellent source of these elements. Three separate methods of fortifying milk with vitamin D were developed: (*a*) irradiation of milk with ultraviolet light to convert inactive provitamin D (7-dehydrocholesterol) to vitamin D, (*b*) feeding irradiated yeast to cows, and (*c*) addition of vitamin D concentrate to fluid milk. Development of all three of these methods dates to the early 1930's and gradually fortification with vitamin D concentrate has gained wide acceptance over the other methods. It was estimated in 1955 that 90% of the bottled milk in large metropolitan areas was fortified with vitamin D. It seems beyond question that the significantly lower incidence of rickets today stems in no small measure from the vitamin D milk fortification program. The standards established for vitamin D in fortified milk by the American Medical Association are that the milk must conform to the U. S. Public Health Service's Standards for grade A milk, it must contain at least 400 U.S.P. units of vitamin D per quart, and must contain no emulsifiers not approved by the Food and Drug Administration.

Multivitamin-mineral fortified milks. In an effort to make a good product still better, various milks fortified with vitamins in addition to D, as well as with minerals, have been developed and marketed. To afford the consumer a choice with regard to calories both whole milk and non-fat products have been offered. Perhaps most notable of these is the whole milk product fortified in such a manner as to supply the minimum daily requirement of the major vitamins and minerals in one quart of milk. The sales volume of this milk appears to be expanding steadily and its wholesomeness has recently been confirmed (12). Such milk is usually sold at a one- to two-cent premium per quart. On a much more limited scale, the skimmilk counterpart to this product is also merchandized. Another skimmilk, offered more widely, is fortified only with vitamins A and D. In order to overcome the rather flat flavor character of skimmilk, most of the fortified products contain added solids (total solids approximately 11%) and they are frequently heated somewhat in excess of pasteurizing treatment in order to bring out a mild degree of cooked flavor.

Multivitamin-mineral fortified milks are not without their problems. No completely satisfactory procedure for fortifying with vitamin C has been developed. Unfortunately, milk is notably inadequate with respect to vitamin C content and both added vitamin C and that which

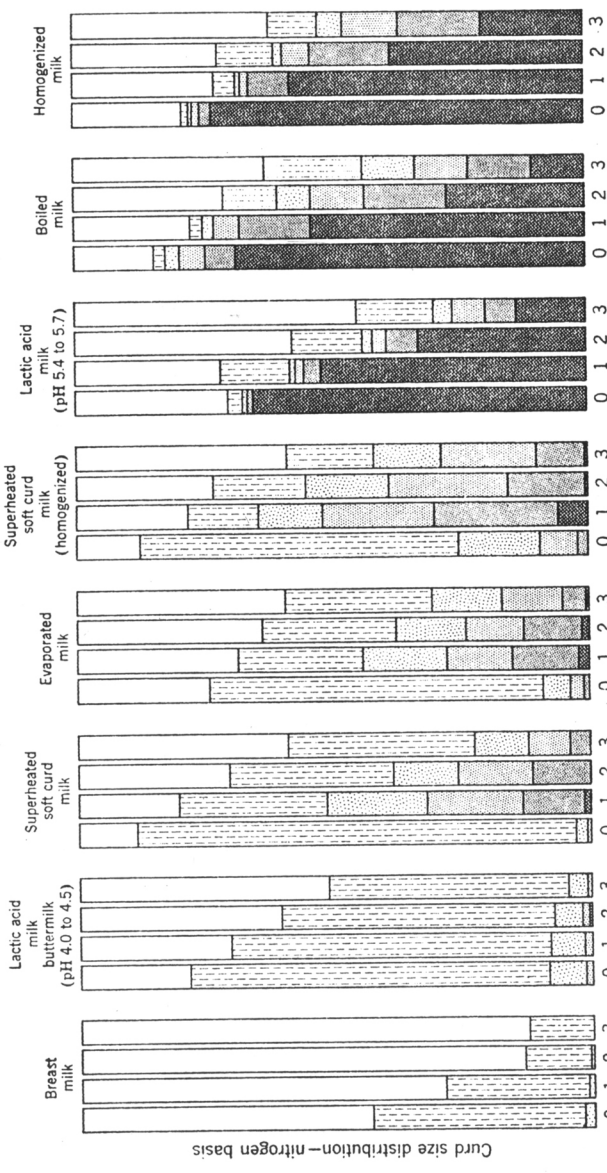
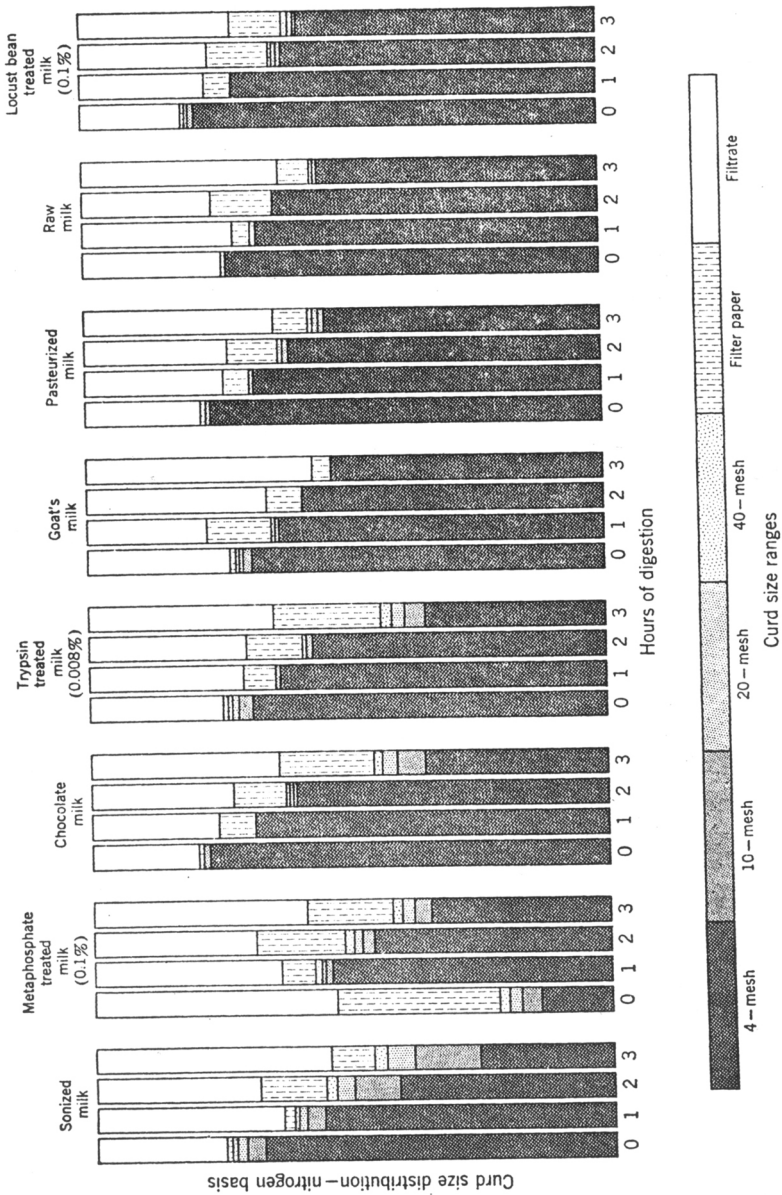


Fig. 1. The comparative curd characteristics and relative digestibility of various types of milk. Data of Doan and Dizikes (4).


Curd size distribution—nitrogen basis
Sonized milk
Metaphosphate treated milk (0.1%)
Chocolate milk
Trypsin treated milk (0.008%)
Goat's milk
Pasteurized milk
Raw milk
Locust bean treated milk (0.1%)
0 1 2 3
Hours of digestion
4—mesh
10—mesh
20—mesh
40—mesh
Filter paper
Filtrate
Curd size ranges

is present naturally are progressively destroyed during processing and storage. Some milk processors are offering milk fortified with a specific amount of vitamin C. Because this vitamin has been added does not mean that it is biologically active at the time of consumption. With the improvements that have been made in the keeping quality of milk, it currently is not uncommon for milk to be as much as a week or more old at the time of its consumption.

Although milk is only a fair source of iron, there has been some reluctance toward fortifying it with iron. Two possible reasons for a conservative approach regarding this element are that trace metals, particularly copper and to a lesser extent iron, are notably effective in promoting oxidative deterioration in milk products, and that iron appears to protect milk lipase against heat inactivation. The action of this enzyme leads to rancid flavors.

Vitamin A presents an additional problem with respect to flavor. A hay-like off-flavor has been traced to the addition of this vitamin to milk and various measures for its prevention have been presented (14). It appears that the principal difficulty involves the flavor quality of the vitamin A concentrate that is used. Concentrates that are relatively free of flavor and odor in the first place and that are not detectable in the milk immediately after addition do not ordinarily present a flavor problem. However, those concentrates that are marginal with respect to flavor at the time of addition to the milk usually create flavor problems that become progressively worse during processing, distribution, and storage of the milk.

Soft curd milk. For infant feeding purposes in particular, it may be desirable that milk on ingestion form small, soft curd particles which are quickly broken down in the stomach. As compared with breast milk, raw or conventionally pasteurized cow's milk produces a relatively hard curd which is slow to break down in the stomach. Various processing measures have been developed to soften the curd characteristics of milk. These include heat treatment, homogenization, culturing with suitable organisms such as in the manufacture of buttermilk, and treatment with minerals or sonic vibrations. The subject of curd characteristics and digestibility of milk has been reviewed (3) and data revealing these properties of milk treated in various manners are shown in Fig. 1.

Low sodium milk. Some instances of heart and kidney trouble make it necessary to reduce sodium intake in the diet very substantially. Since, on an average, milk contains approximately 480 mg. of sodium per quart, it is not satisfactory without alteration as a

component of a low sodium diet. On the other hand, because of its many other benefits, the exclusion of milk from the diet should be avoided if possible. In order to meet this dilemma, a low sodium milk has been developed through use of ion exchange resins (see Chapter 5 for information on the milk salts). A resin charged with potassium is used for sodium removal. In the process, the sodium content is reduced to approximately 20 to 50 mg. per quart, and the potassium concentration of the milk may be raised from approximately 1.2 g. to 2.3 g. per quart. As it is generally recommended that low sodium diets limit intake to 200 mg. of sodium per day, such treatment of milk allows a normal incorporation of milk in the diet. Low sodium milk has been well received by the medical profession.

Acidophilus milk. Some medical authorities propose that certain types of intestinal ailments are benefited by establishment of *Lactobacillus acidophilus* in the intestinal tract. This condition is accomplished by feeding milks containing large numbers of viable acidophilus organisms. For further comments regarding the possible value of such milks in the dietary and their methods of manufacture, see reference 6.

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APPENDIX I-A

RECOMMENDED DIETARY ALLOWANCES, REVISED 1958[1]

Formulated and published by the Food and Nutrition Board of the National Academy of Sciences-National Research Council, the Recommended Dietary Allowances, Revised 1958, has just become available. As with other editions, the Allowances are designed to indicate levels of intake of dietary essentials which can be expected to maintain in good nutrition, healthy persons living in the United States under present conditions. These allowances represent the combined judgment of many nutrition authorities who have evaluated, for this purpose, the available published evidence of human dietary requirements. In this issue of Dairy Council Digests, these Recommended Dietary Allowances are summarized as completely as space permits.[1]

NUTRIENTS NOT TABULATED

Carbohydrate and fat. These nutrients are essential for energy, providing about 90% of the calories in the national diet in the United

[1] Appendix I-A is a reproduction of *Dairy Council Digest* No. 5, Vol. 29 of September 1958, prepared and published by the National Dairy Council, 111 N. Canal Street, Chicago 6, Ill.

Table I. Food and Nutrition Board, National Research Council Recommended Daily Dietary Allowances,* Revised 1958

Designed for the Maintenance of Good Nutrition of Healthy Persons in the U.S.A.
(Allowances are intended for persons normally active in a temperate climate)

	Age, years	Weight kg.	Weight (lb.)	Height cm.	Height (in.)	Calories	Protein, g.	Calcium, g.	Iron, mg.	Vitamin A, I.U.	Thiamine, mg.	Riboflavin, mg.	Niacin,† mg. equiv.	Ascorbic Acid, mg.	Vitamin D, I.U.
Men	25	70	(154)	175	(69)	3200‡	70	0.8	10	5000	1.6	1.8	21	75	
	45	70	(154)	175	(69)	3000	70	0.8	10	5000	1.5	1.8	20	75	
	65	70	(154)	175	(69)	2550	70	0.8	10	5000	1.3	1.8	18	75	
Women	25	58	(128)	163	(64)	2300	58	0.8	12	5000	1.2	1.5	17	70	
	45	58	(128)	163	(64)	2200	58	0.8	12	5000	1.1	1.5	17	70	
	65	58	(128)	163	(64)	1800	58	0.8	12	5000	1.0	1.5	17	70	
	Pregnant (second half)					+300	+20	1.5	15	6000	1.3	2.0	+3	100	400
	Lactating (850 ml. daily)					+1000	+40	2.0	15	8000	1.7	2.5	+2	150	400
Infants	0–1/12§														
	2/12–6/12	6	(13)	60	(24)	kg. ×120	(See	0.6	5	1500	0.4	0.5	6	30	400
	7/12–12/12	9	(20)	70	(28)	kg. ×100	note §)	0.8	7	1500	0.5	0.8	7	30	400
Children	1–3	12	(27)	87	(34)	1300	40	1.0	7	2000	0.7	1.0	8	35	400
	4–6	18	(40)	109	(43)	1700	50	1.0	8	2500	0.9	1.3	11	50	400
	7–9	27	(60)	129	(51)	2100	60	1.0	10	3500	1.1	1.5	14	60	400
	10–12	36	(79)	144	(57)	2500	70	1.2	12	4500	1.3	1.8	17	75	400
Boys	13–15	49	(108)	163	(64)	3100	85	1.4	15	5000	1.6	2.1	21	90	400
	16–19	63	(139)	175	(69)	3600	100	1.4	15	5000	1.8	2.5	25	100	400
Girls	13–15	49	(108)	160	(63)	2600	80	1.3	15	5000	1.3	2.0	17	80	400
	16–19	54	(120)	162	(64)	2400	75	1.3	15	5000	1.2	1.9	16	80	400

* The allowance levels are intended to cover individual variations among most normal persons as they live in the United States under usual environmental stresses. The recommended allowances can be attained with a variety of common foods, providing other nutrients for which human requirements have been less well defined. See text for more detailed discussion of allowances and of nutrients not tabulated.

† Niacin equivalents include dietary sources of the preformed vitamin and the precursor, tryptophan. 60 mg. tryptophan equals 1 mg. niacin.

‡ Calorie allowances apply to individuals usually engaged in moderate physical activity. For office workers or others in sedentary occupations they are excessive. Adjustments must be made for variations in body size, age, physical activity, and environmental temperature.

§ See text for discussion of infant allowances. The Board recognizes that human milk is the natural food for infants and feels that breast feeding is the best and desired procedure for meeting nutrient requirements in the first months of life. No allowances are stated for the first month of life. Breast feeding is particularly indicated during the first month when infants show handicaps in homeostasis due to different rates of maturation of digestive, excretory, and endocrine functions. Recommendations as listed pertain to nutrient intake as afforded by cow's milk formulas and supplementary foods given the infant when breast feeding is terminated. Allowances are not given for protein during infancy.

States. In the body, there is ready interconversion of protein, carbohydrate, and fat. No specific recommended allowance for either carbohydrate or fat has yet been formulated. Combined intake of both should be sufficient to avoid utilization of protein for energy needs and provide storage fat for insulation and protection of the body. Complex carbohydrates provide bulk for intestinal contents. Alcohol consumed in beverages contributes calories. Food fat carries fat-soluble essential nutrients, that is, vitamins A, D, E and K; and polyunsaturated fatty acids such as linoleic and arachidonic acids, essential in the diet of many animals and possibly man. It is not yet possible to indicate the fatty acid mixture most favorable for support of human health. A diet of a wide variety of foods of vegetable and animal origin is most likely to maintain good health.

Water. Requirement for water varies with body heat production, renal solute load, concentrating capacity of kidneys, and losses from skin. Desire for water should govern intake except for infants, sick persons, or under conditions of extreme heat or excessive perspiration. Under ordinary conditions, water needs of young infants are met by milk mixtures supplying 150 ml. water per 100 calories; and adults by 72 to 100 ml. per 100 calories.

Sodium and potassium. Allowances for sodium and potassium have not been formulated. Intakes in the United States have been estimated at 3 to 7 g. sodium per person per day, depending upon added salts; and 1.5 to 4.5 g. potassium, depending on calorie intakes and choice of foods. Restriction of sodium intake is used in treatment of diseases characterized by edema, but requirement is not established. Potassium requirement is related to protein intake, and has been estimated as between 0.8 to 1.3 g. per day with intake of 100 to 110 g. protein and 1.6 g. sodium.

Phosphorus. Allowances for phosphorus should: equal those for calcium in children and women during last half of pregnancy and lactation, and be 1½ times that of calcium for other adults. When calcium and protein needs are met, it is assumed phosphorus needs are also met since these nutrients are closely associated in available foods.

Magnesium. Allowances have not been formulated for magnesium, but requirements are estimated at 13 mg. per kg. body weight during growth and 250 to 300 mg. per day for adults. Magnesium retention tends to parallel protein, calcium, and other mineral intakes.

Copper. Requirement for copper is about 2 mg. daily for adults, and 0.05 mg. per kg. body weight for infants and children. A varied American diet usually supplies needs.

Iodine. Requirement for iodine is small, but increases in adolescence and pregnancy. The need is met by regular use of iodized salt. Less than one-half the table salt sold in the United States is iodized.

Fluorine. Small amounts of fluoride are generally present in plant and animal tissues, especially bones and teeth. Extensive evidence indicates that during tooth development controlled intake of fluoride results in substantial protection against dental caries. The practice of adding fluoride at rate of one p.p.m. to water naturally low in this mineral is recognized as an important public health measure.

Trace elements. Evidence indicates that cobalt, zinc, manganese, molybdenum, and perhaps bromine are dietary essentials, as parts of complex compounds or functionally associated with enzyme systems. Amounts needed are exceedingly small and would appear to be provided by mixed diets. Antagonisms may occur between trace minerals with relatively slight increases of intake. Caution should be used in supplementation of diet with trace elements in absence of specific evidence of deficiency.

Vitamin B_6 group. Evidence indicates that daily intake of vitamin B_6 should be from 1 to 2 mg., an amount readily provided by ordinary mixed diets.

Vitamin B_{12}. About 1 μg. vitamin B_{12} daily, injected parenterally, will allow replacement for lack of absorption of this nutrient in pernicious anemia, postgastrectomy, bacterial, or parasitic interference, or basic defect of intestines as found in non-tropical sprue. In each case, oral administration of the vitamin is ineffective. Allowance for vitamin B_{12} has not been formulated for the normal person but appears to be amply provided by small amounts of animal food products, milk, meat, and eggs.

Folacin. It seems probable that dietary intakes of about ½ mg. of folic acid per day can be expected to cover any nutritional needs for folic acid activity. The actual requirement or allowance cannot be set at this time. While a useful compilation of folic acid activity in foods has appeared, dependable values on dietary intake of folacin under a variety of conditions are not available.

Pantothenic acid. While it is a practical certainty that pantothenic acid is required in human nutrition, the amounts needed are not established and allowances are not formulated. A 2500 calorie diet of foods of animal and plant origin supplies about 10 mg. pantothenic acid daily.

Biotin. The human need for biotin has been demonstrated. The

average American diet provides 150 to 300 μg. biotin per day, enough to maintain human health.

Vitamin E. This nutrient probably plays a role in human nutrition, but information is not available to use as a basis for recommended allowances. A daily intake of 14 mg. *d*-alpha tocopherol has been estimated for one adult population group in the United States.

Vitamin K. In absence of liver disease, there seems no reason to supplement maternal diets in this country with vitamin K. As synthetic water-soluble K, it may be administered to mothers in labor in dosages of 2 to 5 mg., or in dosages of 1 to 2 mg. to newborn babies in situations conducive to neonatal hemorrhage (e.g., prematurity, anoxia, erythroblastosis).

DISCUSSION OF TABULATED NUTRIENTS

Calories. The recommended adult calorie allowances are for a "reference" man and woman: both aged 25; living in a temperate climate, mean environmental (external) temperature 20° C.; weighing 70 and 58 kg. respectively; and moderately active physically, being neither sedentary nor engaged in hard physical labor as a major occupation. The calorie allowances should be adjusted for age, body size, climate, activity, pregnancy, lactation, and rate of growth.

ADJUSTMENT OF CALORIES FOR AGE should be made by a reduction of 3% for each decade between ages of 30 to 50 years, 7.5% for each decade from age 50 to 70 years, and a further decrease of 10% for age 70 to 80 years. Accordingly, calorie allowances at 45 years are 6% less and at 65 years are 21% less than at age 25. See Table II.

ADJUSTMENT OF CALORIES FOR BODY SIZE should be made by application of the following formulas:

$$\text{Cal. for men} = 0.95(815 + 36.6W)$$

$$\text{Cal. for women} = 0.95(580 + 31.1W)$$

where W = desirable body weight in kilograms. See Tables II and III.

ADJUSTMENT OF CALORIES FOR CLIMATE should be made by a 5% increase for first 10° C. decrease in mean environmental temperature of 20° C., and a 3% increase for each additional 10° C. decrease. For each 10° C. increase above 20° C., allowances should be reduced 5%. Suggested adjustments are for average exposure to external environmental temperature.

Table II. Calorie Allowances at Various Body Weights and Ages

(20° C. mean external temperature, moderate activity)

Desirable Weight		Calorie Allowances		
Kilograms	Pounds	25 Years	45 Years	65 Years
		Men		
50	110	2500	2350	1950
55	121	2700	2550	2150
60	132	2850	2700	2250
65	143	3000	2800	2350
*70	154	3200	3000	2550
75	165	3400	3200	2700
80	176	3550	3350	2800
85	187	3700	3500	2900
		Women		
40	88	1750	1650	1400
45	99	1900	1800	1500
50	110	2050	1950	1600
55	121	2200	2050	1750
*58	128	2300	2200	1800
60	132	2350	2200	1850
65	143	2500	2350	2000
70	154	2600	2450	2050
75	165	2750	2600	2150

* "Reference" man and woman.

Table III. Desirable Weights for Height

(Modified from Metropolitan Life Insurance Company Statistical Bulletin 23, 1942; 24, 1943. Age disregarded because weight gains beyond ages of 25 to 30 years considered undesirable.)

Height, in.	Weight in Pounds	
	Men	Women
58	...	112 ± 11
60	125 ± 13	116 ± 12
62	130 ± 13	121 ± 12
64	135 ± 14	128 ± 13
66	142 ± 14	135 ± 14
68	150 ± 15	142 ± 14
70	158 ± 16	150 ± 15
72	167 ± 17	158 ± 16
74	178 ± 18	...

ADJUSTMENT OF CALORIES FOR ACTIVITY cannot be achieved by any single scheme. Physical activity is the outstanding factor causing variability in calorie needs. The calorie allowances in Table I are for the average American. Persons doing heavy labor will seldom have a need greater than 25% higher than standard allowance. Weight change and general health will indicate adequacy or excess of calorie intakes. Adjustment of food intake to calorie needs is more efficient in the active than in the inactive child or adult.

ADJUSTMENT OF CALORIES FOR PREGNANCY AND LACTATION are needed beyond any adjustments for age, body size, and climate. An extra 300 calories per day for the last half of pregnancy and an extra 130 calories for each 100 ml. milk produced during lactation (about 1000 calories daily) are recommended. Increasing calorie intake is especially important for the active and not fully matured woman. Those women who become relatively inactive may not need extra calories during pregnancy. A weight gain of about 22 pounds for the gestation period is a healthful objective of the woman who enters pregnancy at desirable weight. A lesser gain is desirable for the obese woman and a greater gain may be desirable for the underweight woman.

CALORIE ALLOWANCES FOR INFANTS, CHILDREN, AND ADOLESCENTS are proposed as average and approximate allowances for feeding groups and are less applicable to individuals. Rate of growth, body size, and activity vary widely among children, especially adolescents, and greatly affect calorie needs. No allowances are given for infants for first month of life when breast feeding is indicated and calorie allowances of the mother are based on her providing the infant with 850 ml. of milk. From 2 to 6 months, 1000 ml. of mother's milk can provide the recommended 120 calories per kg. body weight. From 7 to 12 months, 100 calories per kg. body weight is recommended. Allowances for boys and girls have been grouped together again through age 12. About 100 calories increase in daily allowances has been made for children 1 through 12, except for the boys 10 to 12. Girls 13 to 15 also have 100 calorie increase in allowance, while boys 13 to 15 have a 100 calorie decrease and boys 16 to 20 a 200 calorie decrease in allowance.

Protein. Protein allowances recommended for normal, healthy, non-pregnant, non-lactating adults are based on one gram of protein per day per kg. desirable body weight, assuming a diet adequate in calories and other essential nutrients. Additional 20 g. daily is recommended for last 5 lunar months of pregnancy, and additional 40 g. for lactation to meet needs for milk production. Breast milk, the

desired source of nutrients for human infants, contains about 1.2 g. protein per 100 ml. Nursing infants receive 1.5 to 2.5 g. protein per kg. body weight during first six months. Protein allowances for such infants are included in allowances for the mother. Infants receiving cow's milk formulas receive 3.2 to 4.3 g. protein per kg. body weight with good growth and nitrogen retention. There is no evidence that protein intakes above recommended allowances are harmful. Essential amino acid requirements must be satisfied as well as the need for total protein. (This is readily accomplished by combining protein of animal and plant origin.)

Calcium. The adult calcium allowance of 0.8 g. per day, based on balance studies and on metabolic and dietary data pertaining to intakes and utilization of calcium, has been reaffirmed. Consumption of excessive calcium in foods has not been shown or seriously considered to be harmful. The ability of people without access to a relatively free food supply to adapt to low calcium intakes does not warrant recommending such intakes as desirable. Allowances for pregnancy and lactation provide for growth of fetus in third trimester and for milk production. Where there has been previous undernutrition, increased calcium intake should be started early in pregnancy. Previous recommendations for calcium intake of children and adolescents have been further substantiated by new evidence.

Iron. Allowances for iron have been lowered to 10 mg. per day as adequate for adult men. Dietary iron apparently is not utilized for the first 4 to 5 months of life. Equilibrium can be maintained on intakes of 0.15 mg. per kg. body weight per day, approximately the intake on an all-milk diet. Allowances during growth are based on increased hemoglobin and myoglobin content of the body, plus metabolic losses. Additional needs for pregnancy are estimated at 3 mg. per day above normal allowances. An equal amount is suggested for lactation. One mg. of iron may be secreted in human milk in one day.

Thiamine. Thiamine allowances are based on 0.5 mg. per 1000 calories consumed, since the need for this vitamin is related to the total calories of the diet. Adult intakes should not be less than 1 mg. per day. Needs may be increased during pregnancy. Higher intake is needed during lactation. Human milk contains an average of 0.15 mg. thiamine per liter, or 0.21 mg. per 1000 calories.

Vitamin A. Need for vitamin A is proportional to body weight. Allowances, as established in 1948, are based on minimum requirements with ample margin of safety, and estimate that the average

American diet provides two-thirds of the vitamin A value as carotene and one-third as preformed vitamin A.

Riboflavin. Riboflavin allowances were computed from protein allowances, using a factor of 0.025. This computation is based on conditions which simultaneously increase need for both nutrients (i.e., growth, body weight increases, pregnancy, lactation, etc.). Allowances for larger individuals should be increased by an additional 0.025 mg. riboflavin for each kg. above reference body weight. Although recommended levels exceed the riboflavin likely consumed by infants on human milk only, adequacy of human milk is not questioned. Cow's milk contains more than three times as much riboflavin as human milk.

Niacin. Allowances for niacin are expressed as niacin equivalents, assuming 60 mg. of the amino acid, tryptophan, may be converted to 1 mg. of niacin in the body. Adult allowances for niacin equivalents are based on requirements calculated according to body weight and calorie intake and increasing the value by 50%. Infant allowances are based on niacin equivalents of human milk. Allowances for pregnancy and lactation are increased by 6.6 niacin equivalents for each 1000 calories added to the diet.

Ascorbic acid. Allowances for ascorbic acid (vitamin C) are the same as the 1948 and 1953 revisions. The allowance for infants is based on the daily intake from human milk.

Vitamin D. The allowances for vitamin D recommended in 1953 are again proposed. Three hundred to 400 I.U. of vitamin D daily permits maximum retention of calcium in infancy when calcium intake is satisfactory. Maximum calcium retention is associated with excellent skeletal growth and early dentition. Larger amounts of vitamin D do not increase calcium retention or rates of growth or dentition. When milk intake is appropriate, good calcium retention occurs in children with 400 I.U. of vitamin D daily, and in adolescents with this amount or more. During pregnancy and lactation, when needs for calcium and phosphorus are greatly increased, moderate dosage of vitamin D increases utilization of these minerals.

Discussion. Nutrient requirements vary widely between individuals. The allowances provide quantities above highest estimated requirements. The allowances are conceived as being wholly adequate for maintaining good nutrition for healthy, moderately active persons throughout life. However, nutritional deficiencies do not necessarily exist if allowances are not all fully met. Values given allow for incomplete absorption or availability of certain nutrients, but do not

allow for losses due to storage, cooking, etc. These losses must be considered in diet planning. The quantities of nutrients recommended may be readily obtained from usual portions of commonly available foods in the United States. Some foods are important because of unique contributions to the diet. For instance, milk is an important source of protein, calcium, and riboflavin; citrus fruits and tomatoes provide relatively large amounts of ascorbic acid. Some individuals, such as pregnant and lactating women, especially need foods of high nutrient content without excess calories.

"The final objective of the recommended allowances must be to permit and to encourage the development of food practices by the population of the United States which will allow for greatest dividends in health and in disease prevention. This revision is dedicated to that end, and others will follow as new knowledge accrues permitting further progress towards this common goal."

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Editor's Note. While conversion of beta-alanine to pantothenic acid by microorganisms has been demonstrated, such conversion by human beings has not been demonstrated. Experimental work with animals does not suggest that the human body can make such conversion. This information is brought to your attention to correct any misunderstanding of the following statement from the final paragraph of p. 2 of *Dairy Council Digest,* **28:7** (Sept. 1957): "Low intake of some vitamins necessitates their synthesis in the body from amino acids (i.e., niacin from tryptophan and pantothenic acid from beta-alanine)."

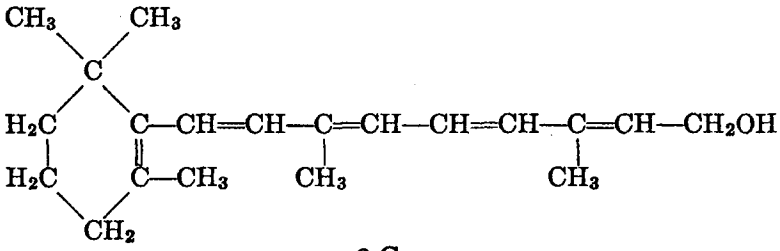
APPENDIX

SUMMARY OF INFORMATION ON THE VITAMINS[1]

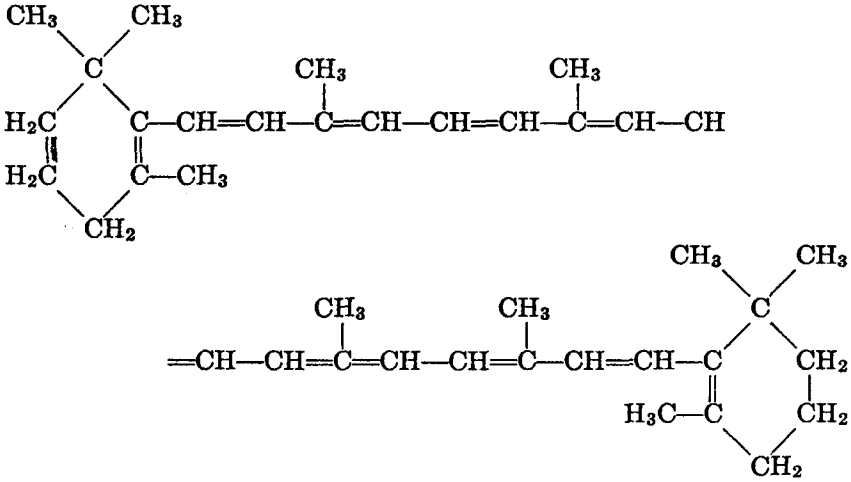
I-B

Vitamin Name, Active Forms Formula: Vitamin A (carotenes)†

VITAMIN A



β-CAROTENE



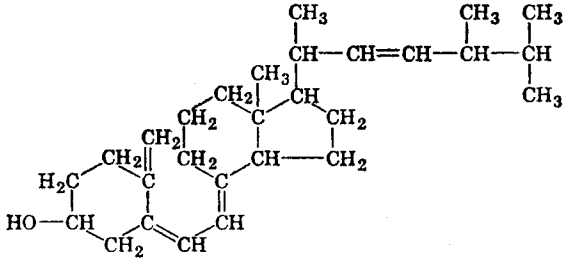
[1] Appendix I-B is information secured from *Dairy Council Digest* No. 1, Vol. 26 of September 1954, prepared and published by the National Dairy Council, 111 N. Canal Street, Chicago 6, Ill.

Note: For footnotes to this appendix see p. 431.

Minimal Dietary Requirement:* 10 I.U.‡ per day per kilogram body weight.
Food Sources: fish liver oils, liver, milkfat, egg yolk, yellow and green leafy vegetables.
Biological Role: Acts to initiate vision from light energy. Essential for growth and maintenance of epithelial tissue. Essential for normal reproduction and growth.

Vitamin Name, Active Forms Formula: Vitamin D (D_2,D_3)†

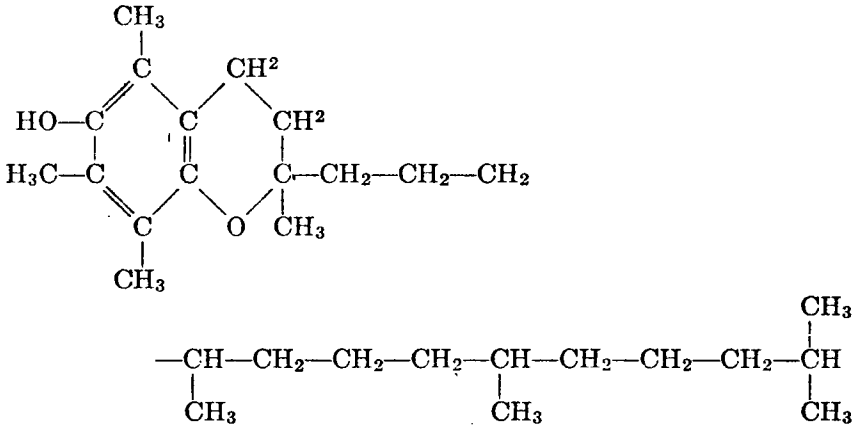
CALCIFEROL (vitamin D_2)



Minimal Dietary Requirement:* 100 I.U.§ per day for average growth.
Food Sources: fish liver oils, concentrates, vitamin D milk.
Biological Role: Influences metabolism of phosphorus and calcium, perhaps by functioning as a coenzyme for alkaline phosphatases, thus affecting intestinal and renal absorption of these minerals and their deposit in bone.

Vitamin Name, Active Forms Formula: Vitamin E (tocopherols)

α-TOCOPHEROL



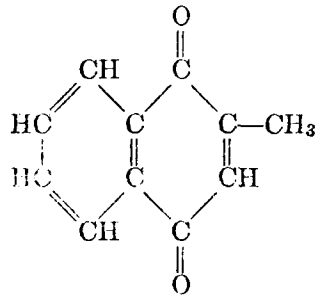
Minimal Dietary Requirement:* not yet demonstrated.

Food Sources: vegetable oils, green leafy vegetables, milkfat, and most common foodstuffs.

Biological Role: Is a strong antioxidant. As such may help prevent oxidation of unsaturated fatty acids and vitamin A in intestinal tract and body tissues. May act in oxidation-reduction reactions.

Vitamin Name, Active Forms Formula: Vitamin K (K_1,K_2,K_3)

VITAMIN K_3—"MENAPHTHANE" ("Menadione")
(2 methyl-1,4-naphthoquinone)



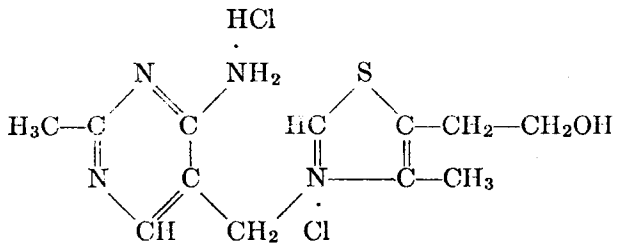
Minimal Dietary Requirement:* 1 mg. per day for infant.

Food Sources: green leafy vegetables, synthesized by microorganisms in intestinal tract.

Biological Role: Aids in production of prothrombin, a compound required for normal clotting of blood.

Vitamin Name, Active Forms Formula: Thiamine, Vitamin B_1

VITAMIN B_1 (hydrochloride)



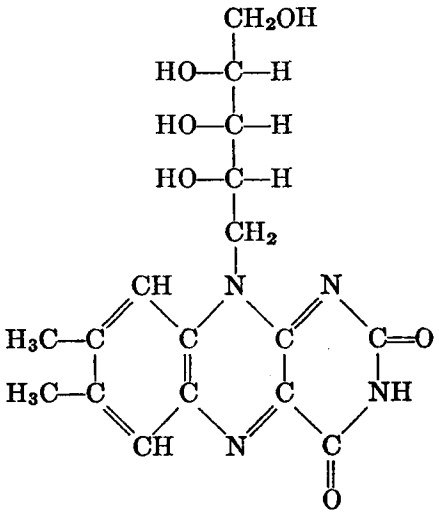
Minimal Dietary Requirement:* 0.23 to 0.3 mg. per 1000 food calories.

Food Sources: meats, legumes, whole grain, and enriched cereals and breads, milk, and dairy foods.

Biological Role: As part of cocarboxylase, aids in removal of CO_2 from alpha-keto acids during the oxidation of carbohydrates. May function similarly as part of lipothiamide.

Vitamin Name, Active Forms Formula: Riboflavin, Vitamin B_2, Vitamin G

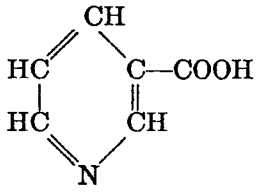
RIBOFLAVIN (6,7,dimethyl-9-D-ribityl, isoalloxazine)



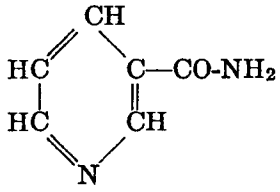
Minimal Dietary Requirement:* 0.5 to 1.0 mg. per day.
Food Sources: milk and dairy foods, organ meats, some vegetables, enriched cereals, and breads.
Biological Role: As part of flavoproteins, aids in removal of hydrogen from breakdown products of proteins, fats, and carbohydrates.

Vitamin Name, Active Forms Formula: Niacin

NICOTINIC ACID



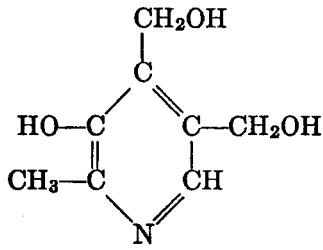
NICOTINAMIDE



Minimal Dietary Requirement:* 10 to 15 mg. per day.
Food Sources: many grains, meats, eggs, and milk have high niacin activity.
Biological Role: As part of coenzymes I, II, and III, aids in transfer of hydrogen in the oxidation of breakdown products of protein, fat, carbohydrate, steroid hormones, cholesterol, and vitamin A.

Vitamin Name, Active Forms Formula: Vitamin B_6, Pyridoxamine, Pyridoxal

PYRIDOXINE (vitamin B_6)
[2-methyl-3-hydroxy-4,5-di(hydroxymethyl)pyridine]

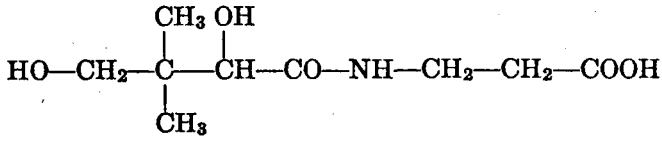


Minimal Dietary Requirement:* 0.15 to 3 mg. per day.
Food Sources: cereal bran and germ, liver, milk, egg yolk, most common foodstuffs.
Biological Role: As a coenzyme, aids in the synthesis and breakdown of amino acids and in the synthesis of unsaturated fatty acids from essential fatty acids. Acts in removal of CO_2, transfer of NH_2, removal of H_2S, and other amino acid reactions.

Vitamin Name, Active Forms Formula: Pantothenic Acid

PANTOTHENIC ACID

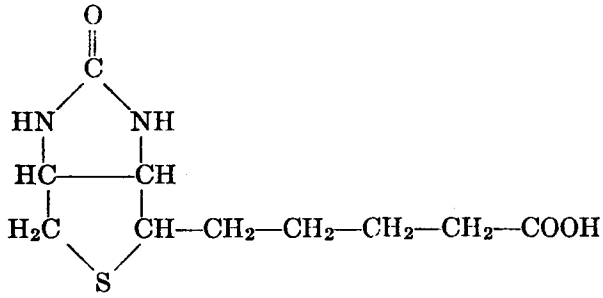
(α,γ-dihydroxy-β,β-dimethyl-butyryl,-β-alanide)



Minimal Dietary Requirement:* level not yet determined.
Food Sources: present in all plant and animal foods.
Biological Role: As part of coenzyme A, functions in the synthesis and breakdown of many vital body compounds, through activating two-carbon fragments provided by protein, fat, and carbohydrate.

Vitamin Name, Active Forms Formula: Biotin (vitamin H)

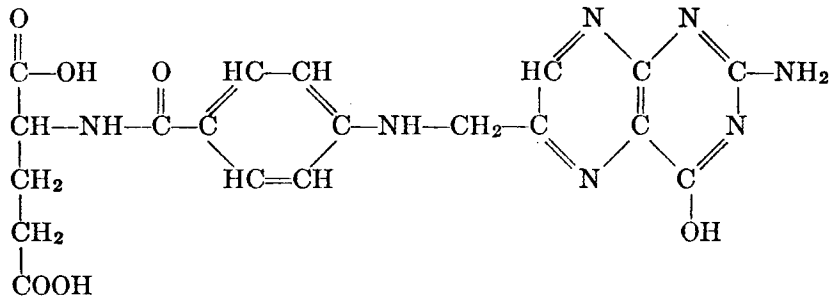
BIOTIN



Minimal Dietary Requirement:* level not yet determined, but very small.
Food Sources: organ meats, milk, eggs, most foods of plant and animal origin.
Biological Role: Appears to be involved in synthesis and breakdown of fatty acids and amino acids, through aiding the addition and removal of CO_2 to or from active compounds, and the removal of NH_3 from amino acids.

Vitamin Name, Active Forms Formula: Folic Acid, Folinic Acid

FOLIC ACID (pteroyl-glutamic acid)



Minimal Dietary Requirement:* not yet determined.
Food Sources: green leafy vegetables, organ meats, lean beef, wheat.
Biological Role: Essential for biosynthesis of nucleic acids and probably for normal fat metabolism. Involved in metabolism of single-carbon fragments. "Active single-carbon fragments" come from limited sources, primarily choline, methionine, glycine, and serine.

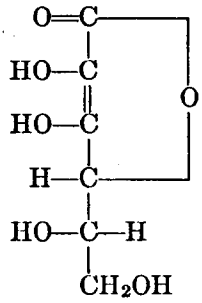
Vitamin Name, Active Forms Formula: Vitamin B_{12}
Cyanocobalamine, Cobalamine

Minimal Dietary Requirement:* not yet determined.
Food Sources: milk and dairy foods, meat, eggs, fermentation from some microorganisms.

Biological Role: Involved in the metabolism of single-carbon fragments. Essential for biosynthesis of nucleic acids and probably for normal fat metabolism.

Vitamin Name, Active Forms Formula: Ascorbic Acid, Vitamin C

VITAMIN C (l-ascorbic acid)



Minimal Dietary Requirement:* 10 to 25 mg. per day.

Food Sources: citrus fruits, tomatoes, melons, peppers, greens, most fresh plant foods, fresh milk.

Biological Role: Possibly functions as coenzymes in the oxidation of tyrosine. Is functionally related to folic acid and vitamin B_{12}. Essential for production of collagen, the basic substance of connective tissue. Related in some way to biosynthesis of steroid hormones.

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* Recommended dietary allowances are given in Appendix I-A.

† Toxic at high levels.

‡ I.U. vitamin A = 0.30 mcg. vitamin A alcohol, previously = 0.6 mcg. beta-carotene.

§ I.U. of vitamin D = 0.025 mcg. calciferol (D_2).

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DAIRY SCIENCE

Principles of DAIRY CHEMISTRY

Dairy technology is a broad domain that helps us to understand the significance of food for human health. Since recent decades, there has been observed a substantial scientific revolution in agriculture and food industry; technological advances and marked improvement has made this scientific expansion possible. The present recent edition has been updated to develop a body of knowledge that provides the biochemists, experts, researchers a book with solution allied with all the other aspects of the dairy technology. The organization of the book has emphasized on the rich array of dairy chemistry and represented in a simplest possible way.

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ISBN 978-93-86800-83-1