JOURNAL OF DAIRY SCIENCE

Contents

Effect of Antioxidants in the Control of Oxidized Flavor Development in Stored Frozen Cream. I. Use of Ethyl Caffeate, Sustane, Tenox II and Tenox BHA. A. J. Gelpi, Jr., L. L. Rusoff and R. D. Skole 93

Field-cured and Field-baled Alfalfa Hay Versus Artificially Dried and Chopped and Pelleted Alfalfa Hays as a Source of Carotene and Roughage for Guernsey and Holstein Calves. H. D. Eaton, K. L. Dolge, R. D. Mochrie, J. E. Avampato and L. A. Moore 98

Factors That Affect the Formation of a Crystalline Deposit in Evaporated Milk. E. F. Deysher and B. H. Webb 106

The Ammonia Content and Formol Titration of Roller-dried Buttermilks as Indices of the Quality of the Source Creams. V. C. Van Devender, Jr., H. G. Lyon and E. W. Bird 116

Nutritive Values of Crops and Cows' Milk as Affected by Soil Fertility. II. The Essential Amino Acids in Colostrum and Milk Proteins. C. W. Duncan, Ge[illegible] Watson, K. M. Dunn and R. E. Ely 128

Cytochemical Reactions of Bovine S[illegible] and Seminal Plasma. R. M. Melampy, L. F. C[illegible] J. C. Porter 140

The Intracellular Proteinases of Certai[illegible] from Cheese and their Relationship to the Prot[illegible]heese. L. E. Baribo and E. M. Foster 149

Observations on the Tactual Flavor Q[illegible] Heated Milk. Stuart Patton and Donald V. Jo[illegible] 161

Fluorescence and Associated Changes Produced upon Storage of Evaporated Milk. H. D. Simonson and N. P. Tarassuk 166

The Relationship of Milk Energy and Total Protein to Per Cent Fat in Brown Swiss Herd Milk. S. D. Musgrave and G. W. Salisbury 174

Abstracts of Literature A9

UNITED STATES OF AMERICA

แผนกห้องสมุด กรม[illegible]

กระทรวงอุตสาหกรรม

ANNUAL MEETING
JUNE 24-26, '52
DAVIS, CALIFORNIA

Vol. XXXV, No. 2, February, 1952

Published by the

AMERICAN DAIRY SCIENCE ASSOCIATION

Duraglas Dairy Containers

DAIRY PRODUCT JARS
SQUAT HALF-PINT
TALL HALF-PINT
HANDI-ROUND QUART
HANDI-SQUARE QUART
PINT
OBLONG TWO-QUART
HANDI-SQUARE TWO-QUART
HANDI-ROUND TWO-QUART

...pass the test for true economy

To determine the cost of dairy containers, look beyond their first cost.

High-quality, returnable glass bottles are unequaled for keeping container costs low. That's why Duraglas bottles are so carefully controlled in manufacture to assure high quality and long life. You can depend on them to deliver dependably the high trippage that means *true economy.*

ACL—Each bottle can be a "talking salesman" for the dairy with Applied Color Lettering designs. Stock designs are always available, and our artists are ready to develop special designs.

CP TANKS MEAN DEPENDABLE SERVICE

For any bulk handling system investigate CP Progress Farm Cooling Tanks, because CP is a pioneer tank producer . . . and its refrigeration experience goes back more than 60 years!

Spick-and-span, compact, easy-to-clean CP Tanks—with built-in refrigeration sections at the sides and bottom—give you fast cooling and dependable storage after cooling.

CP Representatives in company-owned branches coast-to-coast will help you get the right tanks for any cooling or holding need. Ask for new Bulletin B-1020 today!

THE *Creamery Package* MFG. COMPANY

General and Export Offices: 1243 W. Washington Blvd., Chicago 7, Illinois

Branch Offices in 21 Principal Cities

CREAMERY PACKAGE MFG. CO. OF CANADA, LTD
267 King St., West, Toronto 2, Ontario

THE CREAMERY PACKAGE MFG. COMPANY, LTD.
Mill Green Road, Mitcham Junction, Surrey, England

JOURNAL OF DAIRY SCIENCE

OFFICIAL ORGAN OF
AMERICAN DAIRY SCIENCE ASSOCIATION

Published at
NORTH QUEEN ST. AND McGOVERN AVE., LANCASTER, PA.

F. E. NELSON, *Editor*
Ames, Iowa

Associate Editors

F. J. DOAN State College, Penn.	P. R. ELLIKER Corvallis, Ore.	H. A. HERMAN Columbia, Mo.	J. H. HETRICK Rockford, Ill.
L. A. MOORE Beltsville, Maryland	W. V. PRICE Madison, Wis.	V. R. SMITH Madison, Wis.	G. H. WISE Raleigh, N. C.

***Committee** on Journal Management*

J. K. LOOSLI, *Chail'man*

R. E. HODGSON	F. E. NELSON, *ex Officio*
\\'. V. PRICE	P. R. ELLSWOR'rH, *ex officio*

Subscriptions. Price, $10.00 per volume in North and South America; $10.50 in all other countries. Priccs are net, postpaid. New subscriptions and renewals are entered to begin with the first issue of thc current volume. Renewals should be made promptly to avoid a break in the series. Subscriptions should be sent to P. R. Ellsworth, 'rhe Ohio State University, Columbus 10, Ohio.

Subscriptions for the British Isles and British Empire, except for Canada and Australia, should be ordcred through our agents: Messrs. Bailliere, Tindall and Cox, 7 and 8 Henrietta Streets, Covent Garden, London, \V. C. 2, England. Subscriptions for Australia should be sent to our agent: John H. Bryant, Herbert St., St. Leonards, N. S. W., Australia.

Advertising copy should be mailed to P. R. Ellsworth, The Ohio State University, Columbus 10, Ohio. Advertising plates or cuts should be mailed direct to the Business Press, Inc., N. Queen St. and McGovern Ave., Lancaster, Pennsylvania.

Post Office Notices of undeliverable copies and changes of address should be sent to P. R. Ellsworth at the address above stated.

OFFICERS OF THE ASSOCIATION

H. A. BENDIXEN, *President* Pullman, WasII.	P. R. ELLSWORTH, *Sec-Treas.* Columbus, Ohio
H. B. HENDERSON, *Vice-President* Athens, Ga.	F. E. NELSON, *Journal Editor* Ames, Iowa

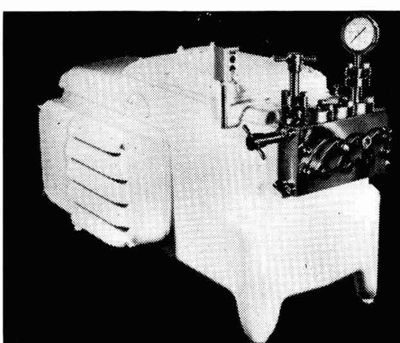
DIRECTORS

F. J. ARNOLD Ames, Iowa	R. B. BECKER Gaincs\'illc, Fla.	J. H. ERn Columbns, Ohio	I. A. GOULD Columbus, Ohio
L. A. MOORE Belts\'ille, Md.	E. J. PERRY New Bruns\\'ick, N. J.	C. W. TURNER Columbia, Mo.	

Entered as second-class matler April 13, 1934, at the postotlice at Lancaster, Pa. under the act of **March** 3. 1879.

WHY SETTLE FOR LESS THAN *Superhomo*

INDUSTRY'S MOST ADVANCED HOMOGENIZER



SUPERHOMO. Available in capacities from 50 to 2500 g.p.h. Design permits easy capacity increase at low cost.

If your present production is geared to an old-fashioned or inefficient homogenizer, the records may well show a waste of valuable man-hours in all departments. Every machine, every process in the plant will feel the effects of forced slowdowns, even work stoppages. It may also require overtime to process the daily quotas — and more overtime for cleanup.

If this is the case in your plant, you should start planning today to replace your present homogenizer with a Superhomo and eliminate these threats to profit.

HERE'S SUPERHOMO SUPERIORITY

Superior Homogenization
— simple, efficient homogenizing valve, single or two-stage operation. Replaceable stainless steel valve caps absorb 98% of the wear and provide improved performance at lower pressures.

Sanitary, Sturdy Cylinder Block
— solid stainless steel for greater strength and sanitation. All parts are easily removed from *front* of block for cleaning in 1/3 normal time.

"Extra Life" Plunger Vee Seals
— records prove that these neoprene seals can give as much as 1000 hrs. service at peak capacity. In addition, they mean greater sanitation, efficiency and economy.

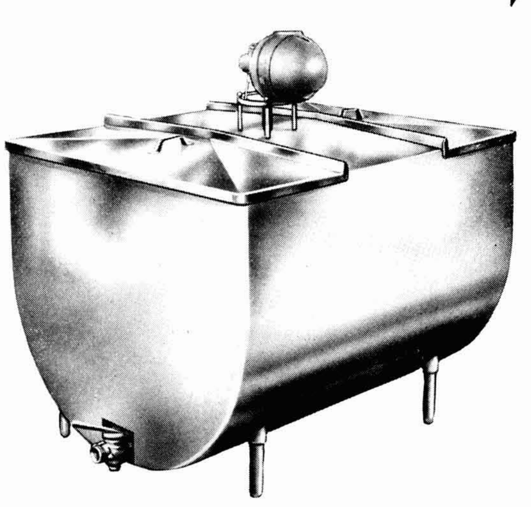
Rugged, Compact Frame and Drive
— mechanism is designed to give many years of trouble-free operation. Timken roller bearings reduce friction and wear. Lubrication is positive and automatic.

See your Cherry-Burrell representative today about a Superhomo with the right capacity for your plant. Ask him for bulletin G-463 that gives you full details, or write us direct.

CHERRY-BURRELL CORPORATION
General Sales and Executive Office:
427 W. Randolph Street, Chicago 6, Ill.
Milk and Food Plant Equipment and Supplies
FACTORIES, WAREHOUSES, BRANCHES, OFFICES OR DISTRIBUTORS AT YOUR SERVICE IN 56 CITIES

trendmaker in a great industry

Save Labor with Mojonnier BULK MILK SYSTEM



Cooling milk in a stainless steel bulk tank saves labor in the milk house, and when combined with tanker pick-up, overall labor requirements are even more sharply reduced. In addition, with bulk milk, quality is up and spillage and drainage losses are eliminated.

If you need new milk cooling equipment you owe it to yourself to write for "The Bulk Cooling Story" — Mojonnier Bulletin 240. Address:

MOJONNIER BROS. CO.
4601 West Ohio Street, Chicago 44, Illinois

Mojonnier Bulk Milk System

FLAV-O-LAC FLAKES

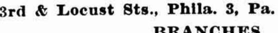
UNIFORMITY IS IMPORTANT!
Leading agricultural schools and colleges agree that the regular weekly use of FLAV-O-LAC FLAKES assures uniformity of smoothness, aroma and flavor in fermented milk products.

YOGHURT CULTURE

FLAV-O-LAC FLAKES
(shown) produce a quart of the finest starter on a single propagation. Single bottles $2.00.

SPECIAL FLAV-O-LAC FLAKES "40"
produce 40 quarts of starter on a single propagation. Single bottles $3.75. Plus postage. Free Cultural Manual of Fermented Milk Products on request.

THE DAIRY LABORATORIES



23rd & Locust Sts., Phila. 3, Pa.
BRANCHES
New York Baltimore Washington
See our catalog in Dairy Industries Catalog

AMERICA'S FAVORITE BRAND MARSCHALL RENNET MARSCHALL DAIRY LABORATORY, INC. MADISON, WIS.
MARSCHALL CHEESE COLOR

Marschall RENNET and COLOR

Strong, clean Rennet is important in the making and curing of cheese. Use Marschall Rennet generously for maximum results.

MARSCHALL DAIRY LABORATORY, INC.
MADISON 3, WISCONSIN

JOURNAL OF DAIRY SCIENCE

VOLUME XXXV | FEBRUARY, 1952 | NUMBER 2

EFFECT OF ANTIOXIDANTS IN THE CONTROL OF OXIDIZED FLAVOR DEVELOPMENT IN STORED FROZEN CREAM. I. USE OF ETHYL CAFFEATE, SUSTANE, TENOX II AND TENOX BHA

A. J. GELPI, JR., L. L. RUSOFF AND R. D. SKOLE

Louisiana Agricultural Experiment Station, Baton Rouge

With a declining butter market and an expanding ice cream industry, a considerable amount of fluid cream goes into frozen storage to be utilized at some later date. The utilization of stored frozen sweet cream for the ultimate manufacture of ice cream is by far the most profitable way of disposing of surplus milk, particularly that produced in fluid milk sheds.

The development of oxidized flavors in stored frozen cream presents a serious economic problem. This problem with its complexities has opened many avenues for research by numerous workers in this and related fields. The prevention of oxidized flavor in cream has been investigated by many workers using various substances and procedures including high temperatures and homogenization (5), ascorbic acid (3, 4) and nordihydroguaiaretic acid (NDGA) (6, 7). Stull *et al.* (6) found NDGA will retard oxidized flavor development in concentrations of 0.00125 to 0.005 per cent (butterfat weight basis) in sweetened frozen cream during storage for 12 mo. Stull *et al.* (7) also compared the effect of NGDA on unsweetened frozen cream with and without added Cu for 11 mo. The cream failed to hold up when Cu was added, regardless of the quality of cream used.

METHODS

Trial 1. In this study ethyl caffeate[1] and Sustane[2] (butyl hydroxyanisole) were used as antioxidants. Cream of average quality containing 40 per cent fat was pasteurized at 150° F. for 30 min., cooled to 40° F. and divided into two equal portions. One portion received 0.5 ppm. of Cu as $CuSO_4$. The antioxidants were dissolved in small amounts of warm glycerol, added to the cream and thoroughly distributed with a mechanical agitator. The two portions of cream were treated with the antioxidants at 0.04, 0.022 and 0.002 per cent levels on a fat basis, with and without 0.5 ppm. Cu added.

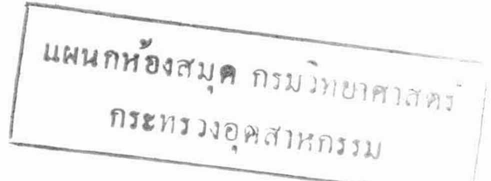
The treated cream was placed in 0.5-pt. standard glass milk bottles with parch-

Received for publication Aug. 1, 1951.

[1] Obtained from green coffee and prepared by Edwal Corp. N. Y. C.

[2] Courtesy Universal Oil Products Co. Chicago, Ill.

Copyright 1952, by the AMERICAN DAIRY SCIENCE ASSOCIATION



ment paper under plug caps to eliminate a possible source of cappy or other similar flavors. Immediately after bottling the samples were placed in storage at −10° F. Organoleptic examinations were made of samples every 30 days for a 6-mo. period and then again at the end of 12 mo.

Trial 2. Comparisons were made using ethyl caffeate and Sustane which were used in the first trial, Tenox II[3] (butylated hydroxyanisole + citric acid + propyl gallate in propylene glycol) and Tenox BHA[3] (butylated hydroxyanisole without synergists).

The procedure used was similar to that in the first trial with the exception that the antioxidants were dissolved in small amounts of 95 per cent ethyl alcohol

TABLE 1

The effect of adding ethyl caffeate and Sustane to summer cream stored at −10° F.

Treatment	Oxidized flavor[a] after storage time (mo.) of:						
	1	2	3	4	5	6	12
Control	−	−	−	−	+	+	+
0.04% ethyl caffeate	−	−	−	−	−	−	−
0.022% ethyl caffeate	−	−	−	−	−	−	−
0.004% ethyl caffeate	−	−	−	−	−	−	+
Control + 0.5 Cu ppm.	−	−	+	+	++	++	+++
0.04% ethyl caffeate + 0.5 ppm. Cu	−	−	−	−	?	−	−
0.022% ethyl caffeate + 0.5 ppm. Cu	−	−	?	−	+	+	+
0.004% ethyl caffeate + 0.5 ppm. Cu	−	−	+	+	+	++	++
Control	−	−	−	+	++	++	+++
0.04% Sustane	−	−	−	−	−	−	+
0.022% Sustane	−	−	−	−	−	−	+
0.004% Sustane	−	−	−	+	+	+	+
Control + 0.5 ppm. Cu	+	+	++	++	++	++	++
0.04% Sustane + 0.5 ppm. Cu	−	+	+	+	+	++	+++
0.022% Sustane + 0.5 ppm. Cu	?	+	+	+	+	+	+
0.004% Sustane + 0.5 ppm. Cu	−	?	?	?	+	+	+

[a] + = Slightly oxidized; ++ = Pronounced oxidized; +++ = Very pronounced; − = Negative; ? = Doubtful

in place of glycerol. Also, an additional series of samples were stored in 0.5-pt. friction-top tinned cans. The antioxidants imparted no detectable color, odor or flavor to the cream.

RESULTS AND DISCUSSION

Trial 1. Table 1 gives the effect of adding ethyl caffeate and Sustane on oxidized flavor development in frozen, stored summer cream. Samples of cream with added ethyl caffeate showed no evidence of oxidized flavor after 12 mo. of storage, with the exception of the one containing 0.004 per cent ethyl caffeate, which had a slight oxidized flavor after 12 mo. When Cu was added, no oxidized flavor appeared after 12 mo. of storage with the 0.04 per cent level of ethyl

[3] Courtesy Tennessee Eastman Corp. Kingsport, Tenn.

caffeate. The sample containing 0.022 per cent ethyl caffeate with Cu gave slight evidence of oxidized flavor beginning at 5 mo., while cream containing the 0.004 per cent concentration became slightly oxidized at 3 mo. This sample showed less oxidized flavor development when compared to the control plus copper.

Sustane at the 0.04 and 0.022 per cent levels without added Cu inhibited the oxidized flavor development for at least 6 mo. After 12 mo. of storage, only slight oxidized flavor was apparent. The cream containing 0.004 per cent Sustane became slightly oxidized at 4 mo. without increasing in intensity through 12 mo., while the control cream sample showed increasing intensity of oxidized flavor beginning at 4 mo.

TABLE 2

The effect of adding ethyl caffeate and Sustane to winter cream stored at −10° F.

Treatment	Oxidized flavor[a] after storage time (mo.) of:					
	1	2	3	4	5	6
Control	−	−	−	+	++	+++
0.04% ethyl caffeate	−	−	−	−	−	−
0.02% ethyl caffeate	−	−	−	−	−	−
0.004% ethyl caffeate	−	−	−	−	−	−
Control + 0.5 ppm. Cu	+	+	++	++	+++	+++
0.04% ethyl caffeate + 0.5 ppm. Cu	+	+	++	+++	+++	+++
0.02% ethyl caffeate + 0.5 ppm. Cu	+	+	++	+++	+++	+++
0.004% ethyl caffeate + 0.5 ppm. Cu	+	+	++	+++	+++	+++
0.04% ethyl caffeate in tinned cans	−	−	−	?	+	++
0.02% ethyl caffeate in tinned cans	−	−	−	?	+	+++
0.004% ethyl caffeate in tinned cans	−	−	−	?	+	+++
Control	−	−	−	+	+++	+++
0.04% Sustane	−	−	−	−	−	−
0.02% Sustane	−	−	−	−	−	−
0.004% Sustane	−	−	−	−	−	−
Control + 0.5 ppm. Cu	+	+	++	++	+++	+++
0.04% Sustane + 0.5 ppm. Cu	+	+	++	+++	+++	+++
0.02% Sustane + 0.5 ppm. Cu	+	+	++	+++	+++	+++
0.004% Sustane + 0.5 ppm. Cu	+	+	++	+++	+++	+++
0.04% Sustane in tinned can	−	−	−	−	+	++
0.02% Sustane in tinned cans	−	−	−	−	+	++
0.004% Sustane in tinned cans	−	−	−	−	+	++

[a] See table 1.

All treated samples containing added Cu showed some indication of oxidized flavor after the first month of storage. Some of the samples were classified as doubtful. Even with added Cu, the Sustane appeared to delay oxidized flavor when compared to the control sample containing added Cu.

Both ethyl caffeate and Sustane definitely were of value in preventing the development of oxidized flavor in the frozen stored summer cream. Ethyl caffeate was a little better than Sustane as an antioxidant.

Trial 2. In this phase ethyl caffeate and Sustane were used on winter cream. Also, two additional antioxidants, Tenox II and Tenox BHA, were used.

The data are presented in tables 2 and 3. All antioxidants used were effective in preventing oxidized flavor development during the 6-mo. storage period when no Cu was added. All samples with added Cu became oxidized within a month of storage. The copper-treated samples in trial 2 developed the oxidized flavor in a shorter period of time than those in trial 1. This difference might be accounted for by the fact that the cream used in trial 1 was produced in the spring of the year at a time when the cows were consuming an abundance of green feed and high quality roughages, which probably supplied sufficient carotene to possibly delay oxidized flavor development, as suggested by Henderson (2) and Brown *et al.* (1). On the other hand, the cream used in trial 2 was produced in the fall of the year and during a prolonged dry spell when little green feed and principally poor quality roughages were fed. This observation on the seasonal quality of the cream used may be an important factor in the storage of cream. The four antioxidants appeared to be equally effective in the prevention or delay of oxidized flavor development.

TABLE 3

The effect of adding Tenox II and Tenox BHA to winter cream stored at −10 F.

Treatment	Oxidized flavor[a] after storage time (mo.) of:					
	1	2	3	4	5	6
Control	−	−	−	+	++	+++
0.04% Tenox II	−	−	−	−	−	−
0.02% Tenox II	−	−	−	−	−	−
0.004% Tenox II	−	−	−	−	−	−
Control + 0.5 ppm. Cu	+	+	++	++	+++	+++
0.04% Tenox II + 0.5 ppm. Cu	−	+	++	+++	+++	+++
0.02% Tenox II + 0.5 ppm. Cu	−	+	++	+++	+++	+++
0.004% Tenox II + 0.5 ppm. Cu	−	+	++	+++	+++	+++
0.04% Tenox II in tinned cans	−	−	−	−	−	?
0.02% Tenox II in tinned cans	−	−	−	−	−	?
0.004% Tenox II in tinned cans	−	−	−	−	−	?
Control	−	−	−	+	++	+++
0.04% Tenox BHA	−	−	−	−	−	−
0.02% Tenox BHA	−	−	−	−	−	−
0.004% Tenox BHA	−	−	−	−	−	−
Control + 0.5 ppm. Cu	+	+	++	++	+++	+++
0.04% Tenox BHA + 0.5 ppm. Cu	+	+	++	+++	+++	+++
0.02% Tenox BHA + 0.5 ppm. Cu	+	+	++	+++	+++	+++
0.004% Tenox BHA + 0.5 ppm. Cu	+	++	++	+++	+++	+++
0.04% Tenox BHA in tinned cans	−	−	−	−	−	−
0.02% Tenox BHA in tinned cans	−	−	−	−	−	−
0.004% Tenox BHA in tinned cans	−	−	−	−	−	−

[a] See table 1.

Samples without added Cu stored in tinned cans developed the oxidized flavor in 4 to 5 mo., with the exception of those treated with Tenox BHA, which held up through the 6-mo. period. Thus, the type of storage container also is a factor in storing frozen cream.

The data indicate that (a) quality of the cream, (b) season of the year the cream is obtained and (c) type of container used for storage are some of the factors which may affect the keeping quality of frozen stored cream.

SUMMARY

Studies on the delay or prevention of the development of oxidized flavor in stored frozen cream with and without added Cu were conducted using ethyl caffeate, Sustane (butylated hydroxyanisole), Tenox II (butylated hydroxyanisole with synergists, and Tenox BHA (without synergists) as antioxidants. Samples containing 40 per cent pasteurized cream from milk produced in early summer and from milk produced in the fall were stored at – 10° F. in 0.5-pt. standard glass milk bottles and in 0.5-pt. friction-top tinned cans. Results of the two trials indicated that the antioxidants were effective in preventing the development of oxidized flavor for at least 6 mo. or longer in the absence of added Cu. In the presence of added Cu, however, ethyl caffeate at the 0.04 per cent level in summer cream was effective through 12 mo. but was ineffective in winter cream. None of the other antioxidants used was effective beyond 5 mo. of storage. Samples containing antioxidants and stored in metal cans without added Cu failed to hold up for a 6-mo. period.

ACKNOWLEDGMENTS

The authors wish to express their appreciation to I. I. Rusoff of General Foods Corp., Hoboken, N. J., for his suggestions in the design of this work and for supplying the ethyl caffeate; and to Eric West of the Dairy Department, Louisiana State University, for help in judging the samples.

REFERENCES

(1) BROWN, W. C., VAN LANDINGHAM, A. H., AND WEAKLEY, C. E., JR. Studies of the Effect of Carotene and Ascorbic Acid in the Feed of the Cow on the Susceptibility of the Milk to Copper-induced Oxidized Flavor. J. Dairy Sci., **22**: 345–351. 1939.

(2) HENDERSON, J. L. The Relation of Nutrition of the Cow to Development of Oxidized Flavor in Milk. Milk Plant Monthly, **28**: 26, 67. 1939.

(3) KRUKOVSKY, V. N., AND GUTHRIE, E. S. Ascorbic Acid Oxidation—a Key Factor in the Prevention of Tallowy Flavor in Milk. J. Dairy Sci., **28**: 565–569. 1945.

(4) KRUKOVSKY, V. N., AND GUTHRIE, E. S. Vitamin C, Hydrogen Peroxide, Copper and the Tallowy Flavor in Milk. J. Dairy Sci., **29**: 293–306. 1946.

(5) MCFARLAND, G. C., AND BURGWALD, L. H. Prevention of Oxidized Flavors in Frozen Cream by Homogenization and High-temperature Pasteurization. J. Dairy Sci., **23**: 494–496. 1940.

(6) STULL, J. W., HERREID, E. O., AND TRACY, P. H. A Study of the Use of the Antioxidant Nordihydroguaiaretic Acid in Dairy Products. II. Its Antioxygenic Properties in Unsweetened Frozen Cream. J. Dairy Sci., **31**: 1024–1026. 1948.

(7) STULL, J. W., HERREID, E. O., AND TRACY, P. H. A Study of the Use of the Antioxidant Nordihydroguaiaretic Acid. J. Dairy Sci., **32**: 301–305. 1949.

FIELD-CURED AND FIELD-BALED ALFALFA HAY VERSUS ARTIFICIALLY DRIED AND CHOPPED AND PELLETED ALFALFA HAYS AS A SOURCE OF CAROTENE AND ROUGHAGE FOR GUERNSEY AND HOLSTEIN CALVES[1]

H. D. EATON, K. L. DOLGE, R. D. MOCHRIE AND J. E. AVAMPATO

Animal Industries Dept., Storrs Agricultural Experiment Station, University of Connecticut, Storrs,

AND

L. A. MOORE

Bureau of Dairy Industry, U. S. D. A., Washington, D. C.

The need for excellent quality roughage in limited whole milk calf feeding systems has long been recognized (10, 14) and the inadequacy of poor or average quality hay to meet the calf's carotene requirement demonstrated (3, 5). The actual production of excellent quality hay in the northeastern states has not been realized (1), particularly in Connecticut, because of unfavorable weather conditions (11). Artificially dried and pelleted alfalfa hay when compared to field-cured and field-baled alfalfa was found to be a superior source of carotene and roughage for Holstein calves up to 105 days of age (3). Whether these results were applicable to smaller breeds was not determined. Therefore, a further comparison between field-cured and field-baled alfalfa hay and artificially dried and chopped or pelleted alfalfa hays as a source of carotene and roughage for Guernsey as well as Holstein calves was undertaken.

EXPERIMENTAL

Animals. Eighteen male calves, nine Guernseys from Lyman Farm, Middlefield, Conn., and nine Holsteins from the Mansfield State Training School and Hospital, Mansfield, Conn., were obtained during the months of October, November, and December, 1950. The Guernsey calves were allowed to nurse their dams for approximately 24 hr. and the Holstein calves were separated from their dams shortly after birth. The calves then were transported to the University research barn and placed in individual tie stalls in a separate portion of the barn in which the temperature was maintained at a minimum of 10° C. A randomized block design was used with the three different types of hay and two breeds of calves. The first three calves of each breed were assigned at random to the three hay groupings and successive trios were treated in a similar manner. Since preliminary data (13) indicated that Jersey calves did not readily consume pellets of the size used in the previous experiment with Holstein calves (3), a smaller pellet was made for the Guernsey calves. However, in the presentation of the data, no differentiation is made in size of pellet.

Received for publication Aug. 6, 1951.

[1] This study was made in part with funds provided by the Research and Marketing Act of 1946, through a contract between the Storrs Agricultural Experiment Station and the Bureau of Dairy Industry.

The three alfalfa hays estimated to be in initial bloom when cut were produced and processed in the same area and manner as previously described (3). The hay, which was field-cured and field-baled, required 3 days from cutting to storage. This hay was not rained upon. The average chemical composition of the hays as fed is given in table 1. The field-cured, field-baled hay as fed graded U. S. no. 2 alfalfa hay and contained on the average 0.7 per cent timothy, 0.5 per cent foreign material and 38 per cent leafiness of legumes. The per cent color of the hays were 39, 61, 70 and 71 for the field-baled, the chopped, the small pellets and the large pellets, respectively. A sample of the chopped hay averaged 2.8 ± 1.5 cm. in length and the passing of a 100-g. sample through a series

TABLE 1
The mean chemical composition of the feeds fed[a]

Feed	Composition						
	Dry matter	Per cent of dry matter					Carotene
		Protein	Fat	Fiber	N.F.E.	Ash	
	(%)						(*mg./lb.*)
Field-cured field-baled alfalfa hay	89.43	18.63	1.75	31.28	37.77	10.56	3.88
Field-cured field-baled alfalfa hay weighback	89.65	15.90	1.34	35.77	37.53	9.46	1.73
Artificially-dried and chopped alfalfa hay	90.99	21.28	3.01	26.70	37.62	11.39	22.24
Artificially-dried and pelleted-small alfalfa hay	91.55	19.59	3.66	27.08	37.50	12.18	22.74
Artificially-dried and pelleted-large alfalfa hay	92.04	20.54	3.45	26.07	38.60	11.33	23.02
Calf starter	89.91	20.36	3.44	6.93	63.49	5.78	0.25
Depletion ration	87.86	16.48	3.01	13.32	61.64	5.54	0.08

[a] The herd milk contained 191 γ of carotene/lb. and 178 γ of vitamin A/lb.

of U. S. standard sieves resulted in 54.6 per cent of the sample being retained by a no. 12 sieve, 38.6 per cent by no. 40, 4.8 per cent by no. 60, 1.5 per cent by no. 80 and 0.5 per cent by no. 100. The small pellets were cylindrical in shape, 0.7 cm. in diameter and 0.6 ± 0.2 cm. in length, and the large pellets were 1.7 cm. in diameter and 0.9 ± 0.2 cm. in length.

The Guernsey calves, which were permitted to nurse for 24 hr. after birth, received 7 lb. of Holstein herd milk daily the second through the seventh days and 6, 5, 4 and 2 lb. daily for successive 7-day periods thereafter. Each Holstein calf received 8 lb. of its dam's colostrum the first day and 8 lb. of Holstein herd milk daily for the second through the seventh days. Thereafter each received 7, 5, 4, and 2 lb. per day for the second, third, fourth and fifth weeks, respectively. After the seventh day all calves were allowed free access to hay and water and a

maximum of 2.5 lb. of starter[2] per Guernsey calf per day and 3.0 lb. of starter for Holsteins. All hays were fed so as to allow a minimum weighback of 10 per cent.

When each calf reached the 106th day of age, it was placed on a low-carotene ration so that the vitamin A storage could be determined. This ration, the "depletion" ration[3] was fed according to the following formula $Y = 0.0561W^{0.87}$ (where Y = the lb. of feed required and W = the weight of the calf in lb.) It was derived using Morrison's mid-interval, T.D.N. requirements for 300-lb. growing dairy cattle (9) and Brody's calculation (2) of Morrison's suggested weight relationship for the maintenance requirement of dairy cattle. The amounts of the depletion ration fed were adjusted to the weight of individual calves at successive 7-day intervals. When the blood plasma vitamin A value remained less

TABLE 2

The effect of type of alfalfa hay on the total feed consumption and total carotene intake of Guernsey and Holstein calves

Feed	Breed	Field-cured and field-baled alfalfa hay	Artificially-dried and chopped alfalfa hay	Artificially-dried and pelleted alfalfa hay
Milk (*lb.*)	Guernseys	119.0	119.0	119.0
	Holsteins	126.0	126.0	126.0
Starter (*lb.*)	Guernseys	194.3 ± 6.6	188.8 ± 3.7	193.4 ± 1.6
	Holsteins	237.6 ± 8.0	234.9 ± 6.5	169.8 ± 57.2
Hay (*lb.*)	Guernseys	125.9 ± 18.9	133.4 ± 21.5	140.3 ± 12.4
	Holsteins	167.8 ± 29.6	220.2 ± 9.9	235.2 ± 30.3
Carotene (*mg.*)	Guernseys	792 ± 125	3263 ± 617	3006 ± 195
	Holsteins	882 ± 100	4365 ± 505	4775 ± 735

than 4.0 γ per cent for two consecutive 7-day intervals, each calf was considered depleted of its vitamin A stores as defined by Jacobson *et al.* (5).

Scours was treated as previously described (3), except that sulfamethazine was not administered unless scours was accompanied by rectal temperatures of 103° F. or above.

Samples and analyses. Procedures used were identical to those previously reported (3) but in addition, height at withers, heart girth and girth of paunch were measured at 14-day intervals to the nearest 0.5 in., as described by Brody (2). The data presented in tables 2, 3 and 4 are arithmetic means and their standard errors.

[2] The starter contained per ton 519.5 lb. of cracked corn, 400 lb. crimped oats, 300 lb. wheat bran, 140 lb. linseed oil meal (expeller process), 280 lb. soybean oil meal (expeller process), 140 lb. dried skimmilk, 40 lb. B-Y 500-potency dried fermentation solubles, 0.5 lb. irradiated yeast (Standard Brands type 9-F), 20 lb. bone meal, 10 lb. iodized salt and 150 lb. cane molasses.

[3] A mixture by weight of one-third of dried beet pulp and two-thirds of a grain mixture composed of the following: 419.5 lb. ground barley, 500 lb. crimped oats, 500 lb. wheat bran, 150 lb. linseed oil meal (expeller process), 150 lb. soybean oil meal (expeller process), 200 lb. cane molasses, 40 lb. B-Y 500-potency dried fermentation solubles, 20 lb. steamed bone meal, 20 lb. iodized salt, and 0.5 lb. irradiated yeast (Standard Brands type 9-F) per ton of mixture.

RESULTS AND DISCUSSION

Feed consumed. There were no real differences in either total milk or starter consumed (fig. 1 and table 2). The rather low average starter consumption of those calves fed the pelleted alfalfa was due primarily to one Holstein calf which had a particular aversion to starter. This calf consumed a total of 55 lb. of starter, while the other two Holstein calves in the group averaged 222 lb.

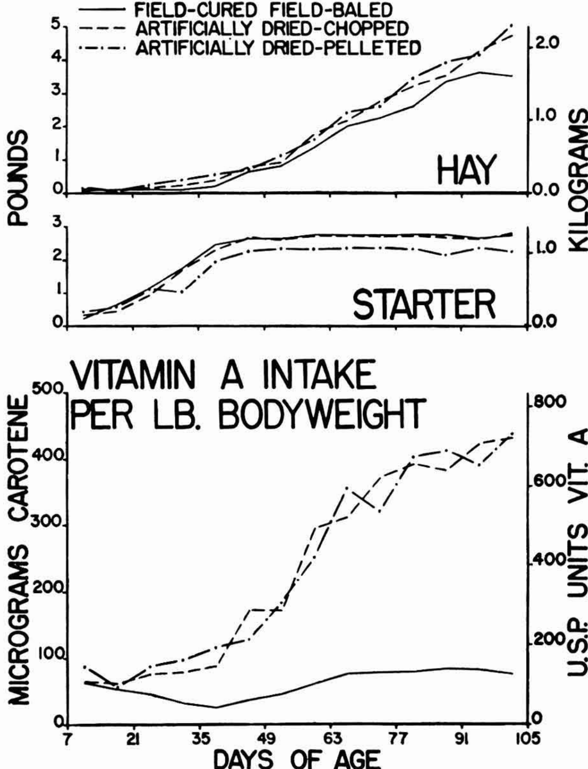


FIG. 1. The effect of type of alfalfa hay on the mean daily intake by 7-d. peroids of vitamin A per lb. bodyweight and on the mean daily consumption by 7-d. periods of hay and starter by young dairy calves.

Both types of dehydrated alfalfa hay were consumed (fig. 1 and table 2) in greater quantities ($P < 0.05$) than the field-baled hay and Holsteins ate greater amounts of hay ($P < 0.01$) than did the Guernseys. When the amounts of hay eaten were adjusted to the initial weight of the calves at 7 days of age, the differences in consumption between types of hay were reduced ($P < 0.10$) and the breed difference was accounted for.

Calculation of the total carotene intake[4] (table 2) and the mean daily carotene

[4] All feeds fed were included in the calculations and 0.25 γ of vitamin A was considered equivalent to 0.60 γ of carotene.

intake per pound of bodyweight by 7-day periods[4] (fig. 1) indicated the superiority of the artificially dried alfalfa hays in supplying the carotene requirements of the young dairy calf. Inspection of figure 1 shows that at no time did the average carotene intake of those calves receiving the artificially dried alfalfa hays fall below the level recommended by the National Research Council's Committee on Animal Nutrition (7) or the Moore *et al.* physiological requirement for carotene (8). In contrast, those calves fed the field-baled alfalfa hay did not receive sufficient carotene according to the National Research Council's standards until after 8 wk. of age and were borderline in carotene intake according to Moore's standards from 21 to 49 days of age.

Growth data. Both liveweight and heart girth measurements (table 3) adjusted to the initial value at 7 days of age were greater ($P < 0.10$) in those calves fed the artificially dried alfalfa hays than in calves fed the field-baled hay. An additional adjustment for total dry matter intake accounted for a part of these ration differences by reducing the degree of significance to $P < 0.20$. No ration effects were observed for either height at withers or girth of paunch. When adjustment was made for the initial value at 7 days of age, Holstein calves made greater increases in height at withers ($P < 0.05$), heart girth ($P < 0.05$) and girth of paunch ($P < 0.01$) than did Guernsey calves.

TABLE 3

The effect of type of alfalfa hay on various growth criteria in Guernsey and Holstein calves

Breeds	Field-cured and field-baled alfalfa hay		Artificially-dried and chopped alfalfa hay		Artificially-dried and pelleted alfalfa hay	
	Value on 7d.	Value on 105d.	Value on 7d.	Value on 105d.	Value on 7d.	Value on 105d.
			Liveweight (lb.)			
Guernseys	74 ± 6	178 ± 10	73 ± 4	188 ± 10	71 ± 4	181 ± 3
Holsteins	95 ± 4	223 ± 9	93 ± 7	250 ± 11	108 ± 5	260 ± 24
			Height at withers (in.)			
Guernseys	28.7 ± 0.9	34.2 ± 0.7	28.5 ± 0.3	34.2 ± 0.3	28.7 ± 0.7	34.0 ± 0.6
Holsteins	30.8 ± 0.4	36.3 ± 0.2	30.3 ± 1.4	35.7 ± 0.7	30.8 ± 0.4	36.2 ± 0.3
			Heart girth (in.)			
Guernseys	29.3 ± 0.4	37.5 ± 0.5	28.8 ± 0.2	37.5 ± 0.5	29.5 ± 0.5	38.0 ± 0.6
Holsteins	30.8 ± 0.6	39.3 ± 0.4	31.0 ± 1.2	40.8 ± 0.6	31.7 ± 0.3	42.0 ± 1.2
			Girth of paunch (in.)			
Guernseys	29.2 ± 0.3	46.5 ± 0.3	29.0 ± 0.8	47.7 ± 1.2	29.5 ± 0.8	45.0 ± 1.2
Holsteins	29.5 ± 0.9	49.5 ± 1.6	29.7 ± 0.9	53.8 ± 1.3	30.7 ± 1.4	54.5 ± 3.1

Vitamin A metabolism. Plasma carotene and vitamin A levels and time to deplete the calves of their vitamin A stores (table 4) were greater in those animals which received the artificially dried hays than in the animals fed field-baled hay. When adjustments were made for the initial value at 7 days of age, the differences in plasma carotene were of the order of $P < 0.01$ and plasma vitamin A of $P < 0.05$. An additional adjustment for the mean carotene intake per mean pound of bodyweight accounted for the ration differences in plasma caro-

tene. It should be mentioned that there was a suggestion of greater utilization of the carotene in the chopped alfalfa than in the pelleted ($P < 0.10$). This can be seen by inspection of plasma carotene levels in table 4. A similar additional adjustment for plasma vitamin A levels, although feasible statistically, does not appear to be valid because of the lack of close correlation between blood plasma levels of vitamin A and carotene or vitamin A intake (6) and a possible antagonism between carotene and vitamin A metabolism as recently reviewed by Wise *et al.* (15).

The mean depletion time (table 4) was 5.8 wk. for the calves fed field-baled hay, 9.3 wk. for those fed chopped hay and 7.7 wk. for those fed pellets. These differences were not statistically significant. Adjustment of the depletion time for mean daily carotene intake per pound of mean bodyweight failed to reveal any significant differences between rations in utilization of the carotene. Assum-

TABLE 4

The effect of type of alfalfa hay on the mean blood plasma carotene and vitamin A levels and the mean time to deplete the vitamin A stores of Guernsey and Holstein calves

Breed	Mean carotene intake per lb. av. bodyweight	Plasma carotene		Plasma vitamin A		Depletion time
		Value at 7d.	Mean 14–105d.	Value at 7d.	Mean 14–105d.	
	(γ)	(γ%)		(γ%)		(*wk.*)
		Field-baled and field-cured alfalfa hay				
Guernseys	68 ± 7	73 ± 9	69 ± 9	21.1 ± 2.6	17.7 ± 2.3	4.7 ± 1.5
Holsteins	59 ± 3	13 ± 3	55 ± 4	12.0 ± 2.3	13.7 ± 0.4	7.0 ± 0.0
		Artificially-dried and chopped				
Guernseys	283 ± 35	33 ± 5	215 ± 48	17.2 ± 3.0	20.3 ± 1.6	10.0 ± 1.0
Holsteins	274 ± 21	23 ± 2	147 ± 46	15.1 ± 1.5	17.2 ± 1.1	8.7 ± 2.2
		Artificially-dried and pelleted				
Guernseys	261 ± 16	60 ± 8	168 ± 26	17.2 ± 1.8	19.3 ± 2.8	6.3 ± 0.9
Holsteins	285 ± 26	19 ± 6	108 ± 17	11.9 ± 2.2	17.7 ± 1.6	9.0 ± 0.6

ing the carotene to be of equal value from the three types of hay, linear regression equations of depletion time (Y) on mean daily carotene intake per pound bodyweight (x) for Guernseys was $Y = 3.27 + 0.0183x$ and for the Holsteins, $Y = 5.88 + 0.0113x$. Although derived from limited data exhibiting a high degree of variability, these equations are in agreement with an observation of a more rapid depletion rate in Guernsey male calves than in Holstein male calves (4).

Scours. Four calves fed the field-baled hay had scours for a total of 17 days, four calves fed the chopped hay for a total of 25 days and four calves fed the pelleted hay for 27 days. Conversion of each calf's per cent days free of scours to the appropriate angle (12) and subsequent analysis of variance of the angles did not show differences between rations. However, there was a suggestion of a breed difference in that Guernsey calves appeared to have a greater incidence of scours ($P < 0.10$) than Holstein calves.

These data are in general agreement with those previously reported for Hol-

stein calves (3). Although the magnitude of the differences for the Holsteins does not entirely agree with last year's data, the trend is the same. Guernsey calves differ in some respects from Holstein calves, but in general follow the same trends as the Holsteins.

SUMMARY AND CONCLUSIONS

Eighteen 7-day old male calves, 9 Guernseys and 9 Holsteins were used in a comparison of the relative value of field-cured and field-baled alfalfa hay with artificially dried and chopped and pelleted alfalfa hays as a source of carotene and roughage. Both types of artificially dried hay were eaten in greater quantities accompanied by greater increases in liveweight and heart girth, higher levels of blood plasma carotene and vitamin A and greater vitamin A stores than field-baled alfalfa hay. Holstein calves made greater increases in height at withers, heart girth and girth of paunch than did Guernsey calves.

In light of the results presented here together with those of the previous paper (3), the following conclusions appear to be justified:

(a) Holstein and Guernsey calves consume larger quantities of alfalfa hay as dehydrated pellets, or as dehydrated chopped, than as long, field-cured hay.

(b) Largely because of this higher consumption, calves grow faster on the dehydrated hays than on the field-cured hay.

(c) The dehydrated hays, used in these studies on unlimited feeding, provided adequate carotene to meet the calf's requirements from 7 to 105 days of age. The field-cured hays did not.

(d) The dairyman who must purchase hay for his calves might well consider using dehydrated-chopped or dehydrated-pelleted alfalfa. The relative costs of the hays would have to be considered, of course. Economic considerations were not a part of this study.

ACKNOWLEDGMENTS

The authors are most grateful to B. A. Donahue, Mrs. Priscilla Howker and C. W. Van Cor for technical assistance during the course of the experiment; to W. H. Hosterman, Inspection Div., Grain Branch, Production and Marketing Adm., U. S. D. A. for grading the hay samples and to H. J. Fisher, Conn. Agr. Expt. Sta., New Haven, for the proximate analysis of the feedstuffs. We also are indebted to Russel Garrigus of Mansfield State Training School and Hospital, Mansfield, Conn. and to Donald Gates of Lyman Farm, Middlefield, Conn. for procurement of the Guernsey calves, and to F. I. Elliott for aid in preparation of the manuscript.

REFERENCES

(1) BURKE, J. D. Results of a Two-year Survey of Hay Quality on New York Farms. Proc. of the 1950 Cornell Nutrition Conference for Feed Manufacturers. pp. 1–10. 1950.

(2) BRODY, S. *Bioenergetics and Growth.* pp. 475–483, 560–561 and 598. Reinhold Publishing Co., New York. 1945.

(3) EATON, H. D., CARPENTER, C. A., CAVERNO, R. J., JOHNSON, R. E., ELLIOTT, F. I., AND MOORE, L. A. A Comparison of U. S. No. 2 Field-cured and Field-baled Alfalfa Hay

with Artificially-dried and Ground and Pelleted Alfalfa Hays as a Source of Carotene and Roughage for Holstein Calves. J. Dairy Sci., **34**: 124–135. 1951.

(4) EATON, H. D., HELMBOLDT, C. F., AVAMPATO, J. E., JUNGHERR, E. L., AND DOLGE, K. L. Unpublished data. Storrs (Conn.) Agr. Expt. Sta., Storrs, Conn. 1951.

(5) JACOBSON, W. C., CONVERSE, H. T., AND MOORE, L. A. Effects of Vitamin A and Carotene Intake on Depletion Time of Young Dairy Calves. J. Dairy Sci., **32**: 418–428. 1949.

(6) LEWIS, J. M., AND WILSON, L. T. Vitamin A Requirements in Calves. J. Nutrition, **30**: 467–475. 1945.

(7) LOOSLI, J. K., HUFFMAN, C. F., PETERSEN, W. E., AND PHILLIPS, P. H. Recommended Nutrient Allowances for Dairy Cattle. National Research Council Bulletin. 1945.

(8) MOORE, L. A., SYKES, J. F., JACOBSON, W. C., AND WISEMAN, H. G. Carotene Requirements for Guernsey and Jersey Calves as Measured by Spinal Fluid Pressures, J. Dairy Sci., **31**: 533–538. 1948.

(9) MORRISON, F. B. *Feeds and Feeding.* 21st ed., p. 1148. The Morrison Publishing Co., Ithaca, N. Y. 1948.

(10) NORTON, C. L., AND EATON, H. D. Dry Calf Starters for Dairy Calves. Cornell Agr. Expt. Sta. Bull. 835. 1946.

(11) ROSS, V. E., AND FELLOWS, I. F. An Economic Evaluation of the Barn Finishing Method of Harvesting Hay. Storrs (Conn.) Agr. Expt. Sta. Bull. 277. 1951.

(12) SNEDECOR, G. W. *Statistical Methods.* 4th ed. p. 449. Iowa State College Press, Ames. 1946.

(13) THOMAS, J. W., AND MOORE, L. A. Unpublished data. Bureau Dairy Industry, U. S. D. A., Beltsville, Md. 1950.

(14) TURK, K. L., AND BURKE, J. D. Raising Dairy Calves and Heifers. Cornell Extension Bull. 761. 1949.

(15) WISE, G. H., ATKESON, F. W., CALDWELL, M. J., PARRISH, D. B., AND HUGHES, J. S. Effects of High Vitamin A Intake on Milk and Fat Yields and on Vitamin A Constituents in Milk, Blood, and Livers of Dairy Cows. J. Dairy Sci., **30**: 279–292. 1947.

FACTORS THAT AFFECT THE FORMATION OF A CRYSTALLINE DEPOSIT IN EVAPORATED MILK

E. F. DEYSHER[1] AND B. H. WEBB[2]

Bureau of Dairy Industry, Agricultural Research Administration, U. S. D. A., Washington, D. C.

The white crystalline deposit that occasionally forms in evaporated milk during storage has been shown to consist largely of calcium citrate (2). Small but varying quantities of tricalcium and trimagnesium phosphate are present, but in most cases 98 per cent of the crystalline deposit is calcium citrate (1).

The factors that affect the formation of calcium citrate crystals in evaporated milk have not been investigated extensively. The possibilities that season of the year, stage of lactation of the cow producing the milk and solids content of the finished product may affect the formation of sediment have been discussed by Hunziker (5).

Mojonnier and Troy (6) found that crystal formation was retarded during storage of evaporated milk at low temperatures. Additional studies of the chemical and physical factors that affect calcium citrate crystallization in evaporated milk are needed to aid in the prevention of crystal formation.

EXPERIMENTAL

Commercial evaporated milks and experimental samples, prepared in the Bureau laboratories with pilot plant equipment, were examined for the presence of calcium citrate crystals before and after storage at controlled temperatures. The milk from each can was carefully poured off, the sediment was thoroughly agitated with 25 per cent alcohol and the liquid decanted. This treatment was repeated several times until the wash alcohol showed no turbidity. The crystals were dried and weighed. Results were expressed as the weight of crystals present in a 14.5-oz. can of evaporated milk.

Ca, Mg, P and citric acid were determined by the methods used in earlier work (1).

The origins of the commercial milks differed. Some lots were obtained directly from the manufacturer who furnished descriptive data with the samples. Lots of eight or ten cans of evaporated milk of the same brand and code number were purchased from retail stores. Nine well-known brands of milk were represented in this latter group.

To study the effect of the stage of lactation upon the growth of crystals in evaporated milk, three groups of cows from the Bureau herd were selected as a source of milk samples. Each group consisted of five cows. The first group had freshened recently, the second was in the middle and the third in a late stage of

Received for publication Aug. 10, 1951.

[1]Retired June 30, 1950.

[2] Present address: National Dairy Research Labs., Oakdale, N. Y.

the lactation period. Composite samples from each lactating group of cows were manufactured into evaporated milk in the laboratory pilot-plant equipment. All milk used in the experiments on the influence of the lactation period on calcium citrate crystallization was produced in the months of October, November and December.

All experimental samples were prepared by forewarming raw milk at 203° F. for 10 min., concentrating it in a batch vacuum pan, homogenizing, cooling, standardizing and sterilizing it at 240°F. for 15 min. Processes used in the preparation of commercial evaporated milk are substantially the same as this laboratory procedure.

In some of the work, salt solutions were added to the concentrated milks before they were sterilized. Disodium phosphate was used as a stabilizer. Ten per cent solutions of sodium citrate, $CaCl_2$ or a normal solution of citric acid adjusted to pH 6.66 with NaOH and mixtures of these, were added to experimental samples to induce the rapid formation of calcium citrate crystals. When additions of Ca and citrate ions were made in the experiments reported in this paper, 9 ml. of 10 per cent $CaCl_2$ were added with stirring to 2,250 g. of concentrated milk. This was followed at once by the addition of 15 ml. of *N* citric acid (pH 6.66). Control samples were prepared by the addition of 24 ml. of water to 2,250 g. of milk. In each case the milk solids content of the samples was adjusted after concentration, so that the product would be of the desired concentration (usually 26 per cent total solids) after addition of the solutions.

RESULTS

A preliminary survey of 45 14.5-oz. cans of commercial evaporated milk, bearing 11 different brand labels, was made. The cans had been held at room temperature for about 2 yr. These samples contained from zero to 0.66 g. of calcium citrate crystals per can, with an average of 0.26 g. per can. Later, a shipment of three cases of 14.5-oz. cans of milk was examined, this milk being part of a large batch rejected because of excessive crystallization. These samples had been held in storage about 3 yr. The reaction of 30 cans opened ranged from pH 6.03 to 5.98 and the weight of crystals per can from 0.25 to 0.61 g. with an average weight per can of 0.37 g. A dozen cans from a third batch of commercial milk that was 2 yr. old contained an average of 0.52 g. of crystals per can and after storage for another year, cans from the same batch averaged 0.50 g. per can. Seven cans from a fourth batch of commercial milk received after they had been held in storage 2 yr. varied from 0.39 to 0.64 g. per can with an average of 0.52 g. Of approximately 300 cans of crystal-bearing laboratory and commercial milk from which the crystals were recovered quantitatively, washed and dried, the largest harvest of crystals was 0.74 g. The over-all average was 0.426 g. It should be emphasized that the 300 cans of milk in which crystals were found were actually screened from a much larger number which were crystal-free. Only cans containing crystals were received from outside sources; these must have been only a few from thousands of cans of crystal-free milk that were not seen.

Calcium citrate crystals did not usually develop in evaporated milk during the first few months of storage. Maximum development was during the storage interval from 4 to 20 mo. Crystallization often did not continue beyond 2 yr. and there was a tendency for the total crystal weight per can to be less at reactions below pH 5.95.

Table 1 shows the extent of crystal formation in six commercial brands of milk, together with the viscosity and reaction of the samples. There was significant crystal growth in only two of the six brands of milk. Those milks that were held beyond the 283-day storage period at 86° F. were not acceptable for most evaporated milk uses because of deterioration in color, flavor and physical stability.

TABLE 1

The formation of calcium citrate crystals (in grams per 14.5 oz. can) and the accompanying changes in the viscosity and acidity in evaporated milk during undisturbed storage at 86° F.

Storage time	Lot 1			Lot 2			Lot 3		
	Viscosity	pH	Crystals	Viscosity	pH	Crystals	Viscosity	pH	Crystals
(d.)	*(c.p.)*		*(g.)*	*(c.p.)*		*(g.)*	*(c.p.)*		*(g.)*
28	19.23	6.06	none	25.65	6.07	none	20.52	6.06	none
112	17.95	6.00	0.0016	29.07	6.03	0.019	17.10	6.02	none
172	18.98	....	0.014	42.75	....	0.058	20.52	....	none
283	....	....	none	64.12	5.95	0.195	23.09	5.92	0.0015
390	27.36	5.83	0.078	88.06	5.86	0.272	38.47	5.83	....
548	....	....	....	222.3	....	0.426	....	....	0.006
	Lot 4			Lot 5			Lot 6		
28	17.10	6.08	none	16.25	6.07	none	17.10	6.09	none
112	17.10	6.04	0.03	15.39	6.03	none	15.39	6.00	0.0027
172	27.36	....	0.01	17.10	....	none	18.17	....	0.027
283	59.85	5.93	0.01	47.02	5.95	none	55.58	5.91	0.155
390	55.57	5.88	0.005	38.47	5.84	none	116.15	5.83	0.244
548	47.02	....	0.031	....	....	0.004	29.90	....	0.386

Attempts were made to accelerate crystal formation in evaporated milk. Addition of $CaCl_2$ followed by citric acid to milk before sterilization always resulted in the development of crystals during storage. These usually appeared after the milk had been held 60 days at 86° F. The crystals continued to grow for about 250 days, after which the rate of crystal formation declined until it finally stopped when the milk was 1.5 to 2 yr. old. Some of these relationships are shown in figure 1.

The effect of making evaporated milk from milk produced at different times during the lactation period of the cow was studied. Results of one typical experiment are given in the lower part of figure 1. There was no significant difference between the quantity of crystals that formed in the milk produced early or late in lactation. Crystallization was stimulated about equally in the various samples by the addition of Ca and citrate ions.

Evaporated milk was seeded with minute quantities (6 mg. per can) of powdered calcium citrate before the milk was sterilized. This slightly accelerated the formation of crystals but did not materially change the quantity finally pro-

duced. In some cases only the seed was recovered at the end of the storage period. The recovered deposit was in the form of fine granules which did not resemble the aggregates that were normally seen.

Cans of commercial and laboratory-made evaporated milks were subjected to various kinds of agitation in an attempt to speed up the crystallization of calcium citrate. Two cases of commercial milk were stored at 86° F. for 4.5 yr. One case was turned over every month for 1 yr. and not turned thereafter. The

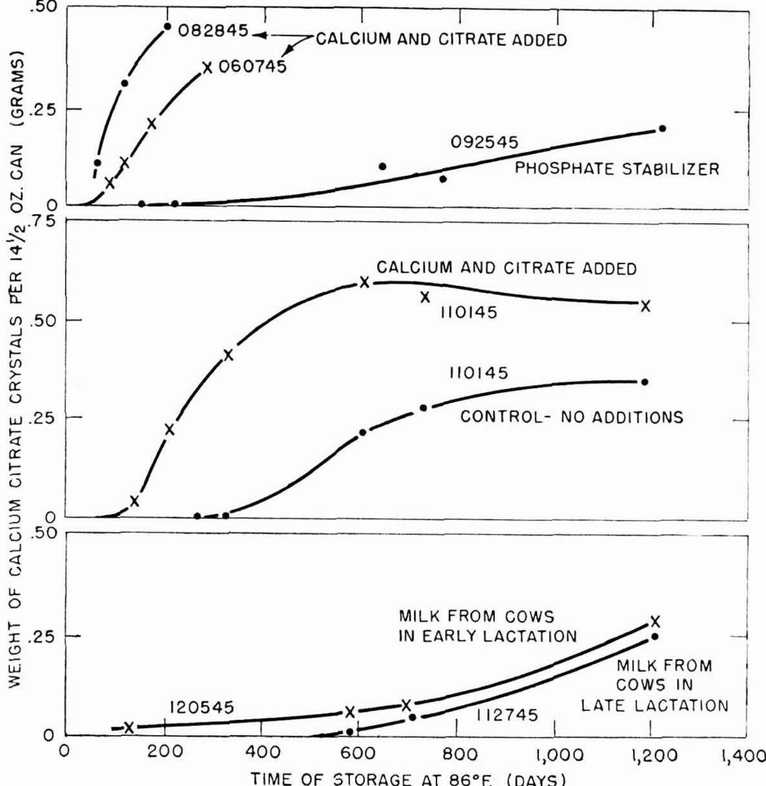


FIG. 1. The effect of time of storage on the development of calcium citrate crystals in pilot plant evaporated milk samples treated differently. The number on each curve refers to the date of manufacture.

other case was held without turning. No crystals developed in either case during the first 2 yr. of storage. Crystal development thereafter was approximately the same in the cans of both cases. After 4.5 yr. the average weight of crystals from all cans opened during the entire storage period was 0.45 g. per can in the unturned milk and 0.44 g. per can in the turned milk.

Cans of evaporated milk were placed in a continuously rotating device so that the cans received end-over-end rotation at 86° F. Several different batches of

TABLE 2

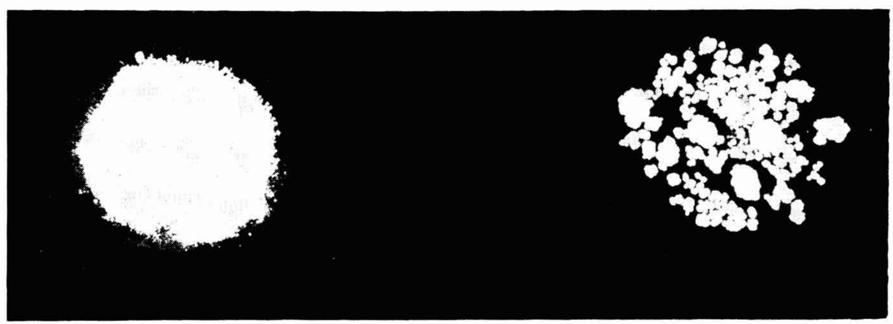
Effect of temperature of storage on the development of calcium citrate crystals in evaporated milk

Code	Type of production	Month of manufacture	Composition		Stabilizer/ 1,000 lb. evaporated milk	Storage period	Crystals after storage (Weight/14.5-oz. can)					
			Fat	T. S.			50°	60°	70°	77°	86°	104° F.
			(%)	(%)	(oz.)	(d.)	(g.)	(g.)	(g.)	(g.)	(g.)	(g.)
214[a]	Commercial	July	8.85	28.68	4	912	0.13	....	0.33	....	0.25	....
Lab.[b]	Pilot plant	December, January, February	8.0	26.0	none	135	....	0.12	....	0.24	....	0.23
p[c]	Commercial	December	4.08	24.35	none	113	0.00	....	....	....	0.02	....
						167	0.01	....	....	....	0.05	....
						227	0.02	....	....	....	0.16	....
						339	0.08	....	....	....	0.23	....
						446	0.28	....	....	....	0.37	....
						614	0.29	....	....	....	0.48	....
429	Commercial	May	8.06	26.15	5	757	0.01	0.00	0.00	....	0.01	....

[a] Experimental composition. Av. of 5 cans held at each temperature.
[b] Average of 9 batches and 27 cans.
[c] Experimental.

milk were used for this experiment. The cans were rotated at 16 r.p.m. for periods up to 100 days. The rotation caused a churning of fat in the milk. At the end of the rotation period the cans were held in still storage until opened. Representative results for one batch of milk follow. The control samples held still for 1 yr. did not develop crystals. A sample rotated the first 34 days of a year storage time had 0.001 g. of crystals per can. Samples rotated the first 68 days of storage periods of 158 days and of 1 yr. contained 0.008 and 0.065 g. of crystals per can, respectively. Samples rotated the first 103 days of storage periods of 150 days and of 1 yr. contained 0.017 and 0.025 g. of crystals per can, respectively. It was concluded that the amount of agitation evaporated milk receives during ordinary handling is a factor of minor importance in the development of calcium citrate crystals in the milk.

High temperatures of storage promoted crystal development in evaporated milk. This was to be expected since the solubility of calcium citrate decreases with increasing temperature. However, uncontrolled factors must have affected



Storage at 50° F.	Storage at 86° F.
Crystal Weight 0.29 gr.	Crystal Weight 0.48 gr.
pH 6.18	pH 5.81

FIG. 2. Effect of temperature of storage for 614 days on the formation of calcium citrate crystals in 14.5-oz. cans of evaporated milk.

the rate and amount of crystallization in the experimental samples. There commonly was more rapid growth of crystals at the higher storage temperatures. The quantity of crystals that formed in four types of evaporated milk are given in table 2. The difference in the kind and quantity of crystals found in cans of milk *p* (table 3) after storage for 614 days at 50 and 86° F. is illustrated in figure 2.

Analyses of four samples of evaporated milk were made to determine whether the salt composition of the milk bore a relationship to salt crystallization during storage. The results are given in table 3. No correlation appears to exist between the Ca : P or citric acid ratios and crystal formation. Batches 214 and 218 were made in the same plant from milk obtained from the same supply, the one within 2 days of the other. Batch 214 was lower in total solids than 218 but it developed crystals while 218 did not. These results indicate that the salt con-

TABLE 3

The effect of the salt composition of evaporated milk on the crystallization of calcium citrate during storage at 86° F. for 3 yr.[a]

Code	Date of mfr.	Composition					Salt balance		Crystal formation	
		Total solids	CaO	MgO	P_2O_5	Citric acid	CaO : P_2O_5	CaO : Citric	Crystals first seen	Weight of crystals/ 14.5 oz. can after 1,000 d.
		(%)	(%)	(%)	(%)	(%)	(*ratio*)	(*ratio*)	(*d.*)	(*g.*)
214	7/1/43	28.68	0.358	0.052	0.477	0.376	1: 1.33	1: 1.05	600	0.25
218	7/3/43	30.15	0.392	0.055	0.529	0.397	1: 1.34	1: 1.01	none	....
M	11/1/46	26.0	0.350	0.047	0.438	0.322	1: 1.25	1: 0.92	146	0.38
563	6/9/44	26.0	0.341	0.046	0.462	0.352	1: 1.35	1: 1.03	none	....

[a] Stabilizers used in manufacture:
214— 4 oz. Na_2HPO_4/1,000 lb. evaporated milk.
218—12 oz. Na_2HPO_4/1,000 lb. evaporated milk.
563 and M—unknown.

centration as determined analytically was not the principal factor concerned with calcium citrate crystallization. Sample 218 contained much more phosphate stabilizer than sample 214. While this does not appear in the Ca : P : citric acid ratios, it may explain why crystals failed to develop in 218. Experimental milk 092545 (figure 1) also was phosphate-stabilized and it developed only a trace of crystals during the first 2 yr. of storage. The possible importance of phosphate as an inhibitor of calcium citrate crystallization was not apparent until the project was nearing completion. There then was no opportunity to study this point at greater length. Unfortunately stabilizer figures were not furnished with samples M and 563.

A large part of the soluble calcium in evaporated milk seems not to be available for the formation of calcium citrate. An average value for the crystal weights in all the crystal-bearing milks that were examined was 0.426 g. per 14.5-oz. can. An average figure for soluble CaO in fresh milk is 0.060 per cent. In a 14.5-oz. can of evaporated milk (411 g. of milk concentrated 2.11 to 1) there would be 0.126 per cent or 0.517 g. of soluble CaO if the processing treatment did not decrease soluble Ca. This could produce 1.7 g. of calcium citrate.

Soluble Ca was determined in the clear serum from two samples of evaporated milk. No. 1 was only 2 mo. old, while no. 2 was about 3 yr. old and without calcium citrate crystals. The milks were filtered through a pyroxylin filter and the sera analyzed to obtain the results shown in table 4. The 0.104 per cent soluble Ca in evaporated milk serum could form 1.44 g. of calcium citrate per 14.5-oz. can of milk. The highest amount of sediment found was 0.7 g. per can, indicating that more than half of the soluble Ca did not enter into the reaction to form the citrate salt. The anhydrous citric acid needed to form 0.7 g. of calcium citrate was 0.47 g. or 0.11 per cent, calculated on the basis of a can of evaporated milk. If the citric acid content of evaporated milk is assumed to be 0.35 per cent, then only a third of the available citric acid combined with Ca to form crystalline calcium citrate.

TABLE 4

Composition of the clear ultrafiltrate from 2 samples of evaporated milk

Milk no.	pH		Composition of ultrafiltrate		
	Milk	Ultrafiltrate	Solids	Ash	Soluble CaO
			(%)	(%)	(%)
1 (fresh)	6.10	6.08	14.93	1.27	0.104
2 (aged)	5.72	5.72	14.15	1.23	0.102

The data indicate that much of the Ca and citrate that appears to be present in the serum of evaporated milk does not precipitate as the insoluble crystalline salt during storage. Several reasons for this could be suggested. Nordbo (7) reported that only 20 per cent of the diffusable Ca of fresh milk was present as Ca ions. He also believed that a small amount of Ca was bound as a nonionized Ca-lactose compound, but that the concentration of this compound represented less than 1 per cent of the concentration of lactose. Hastings *et al.* (3) consider

that calcium citrate ionizes in two stages and that the secondary dissociation is quite incomplete, resulting in the binding of part of the Ca as a complex calcium citrate ion with one negative charge.

The solubilities of calcium citrate and of dicalcium phosphate are 0.25 and 0.024 g., respectively, per 100 ml. of water at 86° F. (4). If no other factors than solubility were involved it would be expected that the use of disodium phosphate stabilizer and the provision of an excess of phosphate would lower the concentration of ionized calcium to such an extent that it would preclude the formation of calcium citrate. The nature of the equilibrium between Ca ions and the calcium caseinate in the fresh and aged milk also may cause shifts in Ca ion concentration which are not now understood.

Whittier (8) has observed that if NaOH is gradually added to an acid solution of calcium citrate, a voluminous, highly hydrated precipitate occurs. The precipitate gradually dehydrates on standing and crystalline calcium citrate is formed. If this hydrated precipitate were present in fresh milk or formed during concentration of milk, then slowly dehydrated as the milk was held in storage, calcium citrate crystals might form.

SUMMARY

The average weight of the calcium citrate crystals found in 300 14.5-oz. cans of crystal-bearing evaporated milk was 0.426 g. per can. Many more cans were examined which contained no crystals.

The occurrence of crystals could not be correlated with the time during the lactation period of the cow when the milk was produced, the movement or agitation the milk received during storage, the salt composition of the milk as determined analytically, or the pH or viscosity of the milk.

Conditions usually favorable to the growth of calcium citrate crystals in evaporated milk were storage at temperatures above 60° F. and direct additions of $CaCl_2$ and citric acid solutions to the milk before sterilization. The period of maximum crystal growth was during the storage period from 4 to 20 mo.

The growth of calcium citrate crystals in several batches of evaporated milk apparently was retarded by addition of disodium phosphate as a stabilizer before sterilization and by storage at low temperatures.

REFERENCES

(1) DEYSHER, E. F., AND WEBB, B. H. The Chemical Composition of the Crystalline Deposit in Evaporated Milk. J. Dairy Sci., **31**: 123–126. 1948.

(2) GOULD, I. A., AND LEININGER, E. The Composition of the Solids which Deposit from Evaporated Milk during Storage. Mich. Agr. Expt. Sta. Quart. Bull., **30**: 54–56. 1947.

(3) HASTINGS, A. B., MCLEAN, F. C., EICHELBERGER, L., HALL, J. L., AND DACOSTA, E. The Ionization of Calcium, Magnesium and Strontium Citrates. J. Biol. Chem., **107**: 351–370. 1934.

(4) HODGMAN, C. D. *Handbook of Chemistry and Physics,* 32nd ed. Chemical Rubber Pub. Co., Cleveland, O. 1950–1951.

(5) HUNZIKER, O. F. *Condensed Milk and Milk Powder,* 7th ed., p. 294. Published by the author, La Grange, Ill. 1949.

(6) MOJONNIER, T., AND TROY, H. C. *Technical Control of Dairy Products.* 1st ed. Mojonnier Bros. Co., Chicago, Ill. 1922.
(7) NORDBO, R. The Concentration of Ionized Magnesium and Calcium in Milk. J. Biol. Chem., **128**: 745–757. 1939.
(8) WHITTIER, E. O. Bureau Dairy Ind. Private communication, Apr. 3, 1946.

THE AMMONIA CONTENT AND FORMOL TITRATION OF ROLLER-DRIED BUTTERMILKS AS INDICES OF THE QUALITY OF THE SOURCE CREAMS[1]

V. C. VAN DEVENDER, JR.,[2,3] H. G. LYON[4] AND E. W. BIRD

Iowa Agricultural Experiment Station, Ames

Dried buttermilk is considered satisfactory for human consumption if clean, sweet cream is the source and if no deterioration occurs after the churning process. In the United States 0.2 per cent acidity is the arbitrary point of demarkation between sweet and no. 1 sour cream. Therefore, cream can be classified as sweet, although some slight acidity development has occurred. This project was undertaken to determine whether or not proteolysis occurred in sufficient amount in the cream, prior to churning, or in the buttermilk to be reflected in the ammonia content and formol titration values of roller-dried buttermilks and, if it did, at what acidity development in the cream the proteolysis was detectable.

METHODS AND APPARATUS

The formol titration method employed was that of Walker (4), except that a 10-g. sample of liquid or reconstituted buttermilk was employed and the results were calculated as the quantity equivalent to 100 g. serum solids.

A modified Choi *et al.* (1) method was employed for the ammonia determination. The modifications were: (a) Use of a 10-g. sample of liquid or reconstituted buttermilk; (b) removal of precipitated proteins by centrifuging in 50-ml. tubes, in an International clinical centrifuge for 10 min. at 2,000 r.p.m.; (c) catching about 21 ml. of distillate (7 min.) in a 25-ml. volumetric flask containing 3 ml. 0.01 *N* H_2SO_4, warming to room temperature and making to 25 ml. volume; (d) transfer of the 25 ml. distillate (after mixing) into a 125-ml. Erlenmeyer flask and agitating thoroughly before the sample was pipetted for color development; (e) use of a 30-min. color development period in a metal water bath (with lid) at room temperature; (f) calculation of results on the basis of 100 g. serum solids; and (g) use of the apparatus shown in fig. 1 for distillation of ammonia from the protein-free aliquot.

Distilled water, containing 15 ml. of a solution of $K_2Cr_2O_7$ and H_2SO_4 (5 g. and 5 ml. c.p. conc., respectively, per 100 ml. H_2O) per 18 l. of water was re-distilled in a Pyrex glass still, with a quartz condenser inner tube. This re-distilled water, to 18 l. of which 15 ml. of the $K_2CR_2O_7$–H_2SO_4 mixture were added, was employed in the steam boiler (fig. 1, 2). Powdered pumic was added to

Received for publication Aug. 16, 1951.

[1] Journal Paper no. J-2000 of the Iowa Agricultural Experiment Station, Ames. Project no. 1023.

[2] Presented by the senior author as a thesis in part fulfillment for the degree Master of Science in Dairy Industry.

[3] Present address: Mississippi State Board of Health, Jackson, Miss.

[4] Present address: Bridgeman-Russell Co., Duluth, Minn.

prevent bumping. The boiler water was changed when the dichromate color began to fade.

The trap (fig. 1, 4) was joined to the boiler by a standard-taper fitting. The inlet to the trap projected through the trap wall and was bent downward, extending to within 0.75 in. of the bottom of the trap. The stopcock at the bottom of the trap was opened slightly during the determination to bleed condensate from the trap. The lower end of the condenser tube (fig. 1, 7) was sealed to a 5-mm., i.d., glass tube to facilitate introduction of the condenser tube into the acid contained in the receiving, volumetric flask (fig. 1, 8).

A Coleman model 11 spectrophotometer was employed in evaluating the developed color. Readings were made at 610 mμ in square cuvettes. The blank

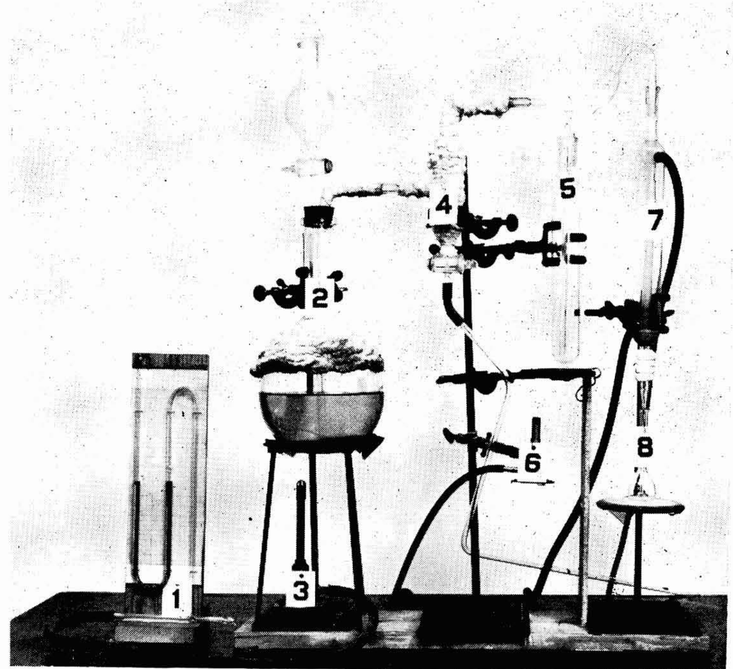


Fig. 1. Apparatus employed for the ammonia determination.

was set at 100 per cent transmittance. The cuvettes were matched at the stated wave length with 0.35 per cent chromic chloride solution in 95 per cent ethanol.

Ten g. dry buttermilk were reconstituted with 93 g. freshly re-distilled water at 43° C., by agitating for 5 min. in a Waring blendor.

Fat and total solids determinations were made on all liquid and reconstituted buttermilks by the Mojonnier method (2).

The creams were neutralized by adding the calculated amount of sodium sesquicarbonate in a 15 per cent aqueous solution. All creams were pasteurized in a water-jacketed laboratory pasteurizer at 160–165° F. for 20 min., unless otherwise noted in fig. 2. The creams were neutralized in large beakers and then

were divided among the small pasteurizing "vats." Each vat plus cream was weighed before pasteurization and again after pasteurization and cooling. Boiled distilled water was added to the pasteurized cream so that the weight was equivalent to that before pasteurization.

The creams were held at 35 to 40° F. overnight after pasteurization. They were churned at this temperature in glass-lidded, quart fruit jars in a shaker with reciprocating action.

The buttermilks, which were to be dried, were centrifuged for 20 min. in 250-ml. bottles at 1,800 r.p.m. in a size 2 International centrifuge. The bottles were chilled to solidify the fat layer. The buttermilks as drawn off had sufficiently low fat contents to dry satisfactorily.

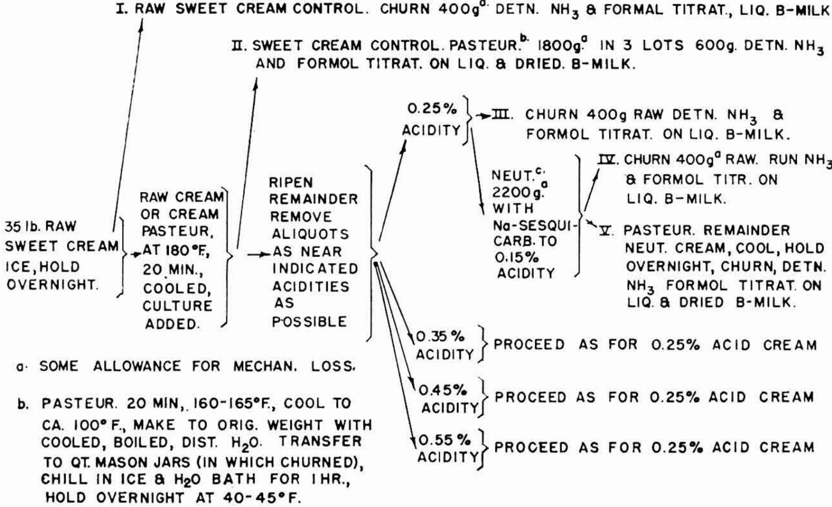


FIG. 2. Flow-sheet of methods of handling samples.

The buttermilks were dried with a double roll, atmospheric, model Buflovac drier (the steel rolls were 6 in. in diameter and 7.5 in. long). The drier did not operate satisfactorily until baffles of aluminum sheet were made to: (a) prevent the milk from fouling the knife blade on the underside by splashing over the rolls and (b) prevent splashing milk over the end of the rolls into the dry milk receivers. The blades were sufficiently flexible that it was necessary to place additional set-screws in the blade-guards, 0.25 in. from the ends of the blade-guards and mid-way between each pair set in the blade-guards by the manufacturer.

The final runs, designed to determine the increase in the ammonia content and formol titration of the liquid or reconstituted buttermilks as the quality of the cream decreased, were conducted as indicated in fig. 2.

RESULTS

Evaluation of the method.

The method for the determination of ammonia was studied to determine the accuracy of the method in our hands. Considerable difficulty was experienced in obtaining agreement among blank determinations. It was necessary to temper the blanks in a covered, room-temperature metal water bath; even under these conditions considerable variation occurred.

Russell (3) has shown that the pH of the medium in which the color is de-

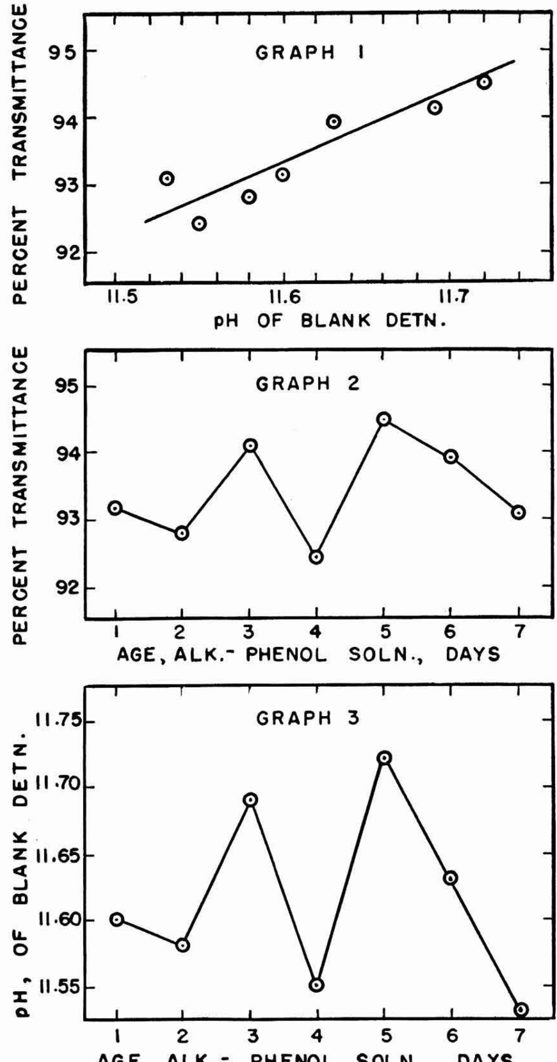


FIG. 3. Relationships among the age of the alkaline-phenol reagent, the pH of the final colored solution read and the percent transmittance of the colored solution.

veloped is important; maximum color development was indicated at about pH 12.0. Graph 1 of fig. 3 shows that the transmittance of the blank is a function of the pH value of the colored solution in the pH range specified by Russell; a variation of 0.15 pH unit will cause a change in transmittance of 1.7 per cent. Neither the transmittance change (graph 2 of fig. 3) nor the pH of the colored solution (graph 3 of fig. 3) is a function of the age of the alkaline-phenol reagent.

There was some question as to the effect of the anion of the ammonium salt upon color development, since volatile acids and lactic acid might distill and be

TABLE 1

The difference between the ammonia added to the solution and that calculated for the solution from the regression equations[a], as the percentage of weights added

NH_3 in 10 ml. soln. read (1)	$(NH_4)_2SO_4$ solutions		$(NH_4)_2C_2O_4$ solutions	
	NH_3 calc. from regression equation (2)	(1)−(2) as % error of (1) (3)	NH_3 calc. from regression equation (4)	(1)−(4) as % error of (1) (5)
(γ/10 ml.)	*(γ/10 ml.)*		*(γ/10 ml.)*	
1.0	0.883	11.70	0.99	1.00
1.0	0.730	27.00	0.81	19.00
1.0	0.796	20.04	....	....
2.0	1.511	24.45	2.26	13.00
2.0	1.906	4.70	1.88	6.00
3.0	2.915	2.83	2.94	2.00
3.0	2.948	1.73	....	....
3.0	3.051	1.70	....	....
4.0	3.630	9.25	4.50	12.50
4.0	4.242	6.05	4.04	1.00
5.0	5.069	1.38	5.14	2.80
6.0	6.546	9.10	5.95	0.83
6.0	6.152	2.53	....	....
7.0	7.436	6.22	7.19	2.71
8.0	7.477	6.53	....	....
9.0	9.476	5.29	9.32	3.55
10.0	9.661	3.39	10.03	0.30
10.0	10.180	1.80	....	....
11.0	11.568	5.16	11.17	1.54
12.0	11.578	3.51	11.56	3.66
13.0	13.553	4.25	13.22	1.69
14.0	13.918	0.58	13.33	4.78
15.0	15.536	3.57	15.28	1.86
18.0	17.872	0.71	17.41	3.27
19.0	19.762	1.38	19.25	1.31
22.0	21.103	4.07	22.26	1.18
23.0	23.648	2.81	24.11	4.82
26.0	25.361	2.45	25.24	2.92

[a] Equations presented in table 2.

present in the final mixture. For this reason standard reference curves were established using ammonium sulfate and ammonium oxalate. The data of table 1 show that when the concentration of ammonia in the colored solution read is 3γ or more per 10 ml., the deviation of the values from the regression are within the limits anticipated for biological work and, that one equation is about as satisfactory as the other. Table 2 indicates that at the same transmittance reading the two curves can be used interchangeably in the region 20 to 90 per cent. These data indicate that the anion involved should not affect the results.

Difficulty was encountered in removing precipitated proteins by filtration, as was recommended by Choi *et al.* (1), because some filter papers add considerable ammonia to the filtrate and the filtration is slow. Fritted-bottomed glass crucibles were likewise slow. The proteins could be satisfactorily and easily removed by centrifuging (table 3). This method was employed in obtaining the data presented below.

TABLE 2

NH_3 calculated by the regression equations[a] obtained from $(NH_4)_2SO_4$ ano $(NH_4)_2C_2O_4$ standard solutions. The difference between the calculated values as percentage of the $(NH_4)_2SO_4$ value

G reading selected (1)	NH_3 $(NH_4)_2SO_4$ regression (2)	NH_3 $(NH_4)_2C_2O_4$ regression (3)	Diff. (2)−(3) (4)	Diff. as % $(NH_4)_2SO_4$ value (5)
	(γ/10 ml.)	(γ/10 ml.)	(γ/10 ml.)	
90.0	1.12	1.09	0.03	2.68
68.0	4.92	4.79	0.12	2.44
46.5	10.08	9.72	0.36	3.57
32.5	14.94	14.65	0.28	1.88
22.5	19.93	19.96	−0.03	0.15
14.5	25.89	26.70	−0.81	3.13

[a] $(NH_4)_2C_2O_4$ regression equation:
$\text{Log } G = 1.9929 - 0.03493\ (\gamma\ NH_3) - 0.000141\ (\gamma\ NH_3)^2$
$(NH_4)_2SO_4$ regression equation:
$\text{Log } G = 1.99023 - 0.03201\ (\gamma\ NH_3)$

The recoveries of ammonia by the modified method employed were satisfactory if quantities of ammonia added were 30 γ or more per sample (table 4). Thirty γ per sample are equivalent to 3.75 γ in the colored solution read. The limitations of the method are virtually those encountered in preparing the reference curves (table 1).

TABLE 3

The effect of the method of removal of proteins on the ammonia values obtained for reconstituted dry buttermilk

Sample treatment	Av. NH_3 content of dry buttermilk			
	Trial 1	Trial 2	Trial 3	Trial 4
		(mg./100 g.)		
Centrifuged[a]	5.28	5.27	1.69	1.92
Centrifuged[a] filtered through filter paper[b]	6.93	5.36	3.20	2.38
Centrifuged[a] filtered through sintered glass crucible[c]	5.17	5.43	2.53	1.02

[a] Samples centrifuged 10 min. at 2,000 rpm. after treatment with H_2SO_4 and sodium tungstate.
[b] No. 42 Whatman, 12.5 cm. papers.
[c] Medium-porosity, Corning crucibles.

The replicability of the method was determined by analyzing three lots of dried buttermilk. Three samples were taken from each lot and each sample was analyzed in triplicate. No significant differences were encountered. The determinations were accurate to 0.242 mg. ammonia and 0.126 ml. 0.1 *N* NaOH

(formol titration) per 100 g. of serum solids. For this reason one analysis was made per sample of buttermilk.

Ammonia and formol titration values of fluid and dried buttermilks.

The outline of the experiment is presented in fig. 2. The experiments were run on a laboratory scale because of shortage of sweet cream and fat costs. With the exception of two instances (in which two runs were made with cream obtained at different times from the same producer), each cream was obtained from a different producer in order to introduce as much variation as possible.

The small scale operation can be criticized in that the bacterial flora of these creams will be much less uniform than would those of creams that were mixtures from a number of herds. Therefore, these data may show much greater variation than would have been obtained in a commercial operation. Despite these shortcomings, these data should yield valuable preliminary information.

TABLE 4

Recoveries of ammonia from aqueous solutions and reconstituted dry buttermilks

NH_3 added to sample	Per cent recovery of ammonia from					
	Aqueous solutions			Reconstituted dry buttermilk		
	Run 1	Run 2	Av.	Run 1	Run 2	Av.
(γ/10 ml.)						
	80.00	89.60		60.00	117.60	
10	72.00	86.40	82.00	109.60	128.80	104.00
	64.00	84.00		106.40	81.20	
20	72.00	86.40	76.60	89.20	90.00	91.70
	107.00	93.33		111.73	82.13	
30	95.46	95.53	98.08	111.73	82.13	98.29
	97.60	96.00		100.80	106.88	
50	100.00	97.28	97.72	102.72	103.52	103.48
	98.98	93.87		99.41	91.52	
75	97.92	93.76	96.13	102.82	92.69	96.61
	91.36	95.20		98.64	95.52	
100	95.20	95.52	94.32	100.80	92.16	96.78
	97.28	98.89		97.40	91.52	
125	99.20	96.64	98.00	94.81	91.58	93.82
	99.73	93.97		94.13	96.48	
150	98.13	96.00	96.95	93.33	97.06	95.25

The ammonia values for the liquid buttermilks from the non-neutralized creams (tables 5 and 6) were plotted against the raw cream acidities (graph not presented). A virtually linear graph was obtained which indicated that ammonia values as high as 40 to 45 mg. per 100 g. of serum solids might be expected for a buttermilk from a cream with 0.2 per cent acidity, obtained from a single producer.

Detailed data for buttermilks from naturally soured creams are presented in table 5 and the average trends in fig. 4. The ammonia values increase in nonlinear manner with the acidity of the raw cream and are not appreciably affected by neutralization and pasteurization. Drying liberates the ammonia from the

TABLE 5

Variation in ammonia content and formol titration[a] of buttermilk from naturally soured creams with: 1. increase in acidity, 2. neutralization, 3. pasteurization, and 4. drying of buttermilk from same source cream

Buttermilk source	Sample[b] designation	Cream 1				Cream 2				Cream 3				Cream 4			
		Cream acidity		Values per 100 g. serum solids		Cream acidity		Values per 100 g. serum solids		Cream acidity		Values per 100 g. serum solids		Cream acidity		Values per 100 g. serum solids	
		Raw	At churn	NH_3	Formol titration	Raw	At churn	NH_3	Formol titration	Raw	At churn	NH_3	Formol titration	Raw	At churn	NH_3	Formol titration
		(%)	(%)	(*mg.*)		(%)	(%)	(*mg.*)		(%)	(%)	(*mg.*)		(%)	(%)	(*mg.*)	
1. Raw cream	Sweet	0.14	0.14	6.08	203.0	0.14	0.14	5.40	215.5	0.16	0.16	22.40	230.3	0.20	0.20	22.44	236.1
	1	0.30	0.30	39.52	242.2	0.30	0.30	41.92	226.1	0.24	0.24	31.44	234.7	0.32	0.32	68.36	244.0
	2	0.43	0.43	47.09	280.5	0.42	0.42	207.82	293.4	0.35	0.35	192.30	297.8	0.50	0.50	172.08	293.8
	3	0.52	0.52	43.48	249.1	0.51	0.51	367.24	307.9	0.45	0.45	375.40	342.2	0.62	0.62	162.84	286.5
	4	0.58	0.58	44.76	234.4	0.55	0.55	297.80	274.5	0.57	0.57	402.96	346.5	0.71	0.71	151.56	308.5
2. Neutralized raw cream	1	0.30	....	43.94	242.9	0.30	0.16	50.76	233.9	0.24	0.14	40.82	234.3	0.32	0.17	60.50	231.1
	2	0.43	....	46.60	272.9	0.42	0.16	282.32	320.9	0.35	0.15	158.56	257.5	0.50	0.18	131.35	256.6
	3	0.52	....	50.26	265.5	0.51	0.17	363.32	366.6	0.45	0.15	414.46	361.6	0.62	0.16	144.06	289.2
	4	0.58	....	55.56	246.4	0.55	0.16	353.08	326.5	0.57	0.15	366.22	337.8	0.71	0.17	144.20	291.3
3. Neutralized & past. cream	Sweet (not neut.)	0.14	0.14	7.50	200.4	0.14	0.14	9.20	219.3	0.15	0.15	27.40	223.0	0.20	0.20	32.29	245.8
	1	0.30	0.14	44.37	190.9	0.30	0.15	35.54	220.7	0.24	0.14	43.46	228.8	0.32	0.16	75.60	262.5
	2	0.43	0.15	42.07	193.4	0.42	0.14	133.50	304.7	0.35	0.14	129.30	254.9	0.50	0.18	128.79	266.2
	3	0.52	samp. lost	samp. lost	samp. lost	0.51	0.16	324.74	330.9	0.45	0.12	313.24	295.2	0.62	0.15	146.60	275.3
	4	0.58	samp. lost	samp. lost	samp. lost	0.55	0.15	384.78	311.2	0.57	0.13	360.40	328.5	0.71	0.16	150.68	296.8
4. Dried buttermilk (buttermilk from 3.)	Sweet	0.14	0.14	3.15	164.6	0.14	0.14	4.16	200.7	0.15	0.15	6.81	198.4	0.20	0.20	28.15	194.1
	1	0.30	0.14	3.84	140.6	0.30	0.15	3.80	195.5	0.24	0.14	9.17	205.0	0.32	0.16	33.68	178.1
	2	0.43	0.15	4.17	132.4	0.42	0.14	7.38	159.3	0.35	0.14	12.23	226.2	0.50	0.18	44.23	192.3
	3	0.52	samp. lost	samp. lost	samp. lost	0.51	0.16	13.56	188.5	0.45	0.12	16.18	213.6	0.62	0.15	24.74	197.7
	4	0.58	samp. lost	samp. lost	samp. lost	0.55	0.15	23.11	191.9	0.57	0.13	23.24	203.0	0.71	0.16	31.51	203.6

[a] Expressed as ml. 0.1*N* NaOH.

[b] In each buttermilk source, the term sweet and the numbers 1, 2, 3, 4, refer to buttermilk from the source cream in 1.

TABLE 6

Variation in ammonia content and formol titration[a] of buttermilk from cream ripened with Streptococcus lactis, Escherichia coli, and Aerobacter aerogenes with: 1. increase in acidity, 2. neutralization, 3. pasteurization, and 4. drying of buttermilk from the same source cream

Buttermilk source	Sample[b] designation	*Streptococcus lactis*				*Escherichia coli*				*Aerobacter aerogenes*			
		Cream acidity		Values/100 g. serum solids		Cream acidity		Values/100 g. serum solids		Cream acidity		Values/100 g. serum solids	
		Raw or cultured	At churn	NH_3	Formol titration[a]	Raw or cultured	At churn	NH_3	Formol titration[a]	Raw or cultured	At churn	NH_3	Formol titration[a]
		(%)	(%)	(*mg.*)		(%)	(%)	(*mg.*)		(%)	(%)	(*mg.*)	
Raw cream	Sweet or no. 1	0.23	0.23	53.26	298.2	0.14	0.14	11.45	211.2	0.16	0.16	23.40	268.0
1. Heated cream	Heated	0.21	0.21	36.55	115.4	0.14	0.14	10.96	212.8	0.14	0.14	20.00	221.0
2. Heated ripened cream	1	0.46	0.46	57.28	293.6	0.25	0.25	3.46	203.9	0.22	0.22	70.40	232.0
	2	0.49	0.49	55.26	269.7	0.33	0.33	9.59	194.6	0.31	0.31	140.00	277.0
	3	0.50	0.50	48.31	239.3	0.41	0.41	20.08	207.5	0.36	0.36	182.00	332.0
	4	0.55	0.55	51.09	255.7	0.65	0.65	351.64	300.1	0.41	0.41	168.00	273.0
3. Neutralized heated ripened cream	1	0.46	0.14	48.68	253.8	0.25	0.13	3.06	210.5	0.22	0.13	94.64	263.0
	2	0.49	0.16	52.34	252.0	0.33	0.12	8.43	203.3	0.31	0.13	144.80	267.0
	3	0.50	0.17	57.07	289.3	0.41	0.12	18.89	209.7	0.36	0.14	131.00	284.0
	4	0.55	0.20	49.42	256.1	0.65	0.14	293.81	273.8	0.41	0.11	152.00	327.0
4. Neutralized and pasteurized heated ripened cream	Heated	0.21	0.21	36.55	115.4	0.14	0.14	10.96	212.8	0.14	0.14	20.00	221.0
	1	0.46	0.12	55.76	211.9	0.25	0.12	3.62	183.8	0.22	0.13	77.68	223.0
	2	0.49	0.15	59.29	214.4	0.33	0.11	9.08	196.2	0.31	0.12	136.60	258.0
	3	0.50	0.17	55.28	216.4	0.41	0.12	18.89	196.6	0.36	0.13	143.20	274.0
	4	0.55	0.20	54.47	217.0	0.65	0.14	345.25	305.4	0.41	0.11	164.80	300.0
5. Dried buttermilk (buttermilk from 4.)	Heated	....	0.21	........[c]	........[c]	0.14	0.14	1.70	177.2	0.14	0.14	5.86	207.0
	1	....	0.12	........[c]	........[c]	0.25	0.12	1.70	175.6	0.22	0.13	7.02	203.0
	2	0.49	0.15	8.20	150.0	0.33	0.11	1.89	177.4	0.31	0.12	7.84	208.0
	3	0.50	0.17	7.26	133.1	0.41	0.12	1.45	183.3	0.36	0.13	2.02	175.0
	4	0.55	0.20	6.87	137.6	0.65	0.14	10.38	182.0	0.41	0.11	1.55	242.0

[a] Expressed as ml. 0.1*N* NaOH.
[b] In each buttermilk source, the term sweet or no. 1 and the nos. 1, 2, 3, 4, refer to buttermilk from the source raw cream in 1.
[c] Unable to dry samples on experimental drier.

buttermilks obtained from neutralized, pasteurized creams. The ammonia values would be of no value as an index of the quality of the raw cream. However, the ammonia values of the liquid buttermilk may serve as an index of its quality for central drying plants, if samples were taken and iced by the tank truck operator. The ammonia value of butter serum might likewise serve as an index of the quality of the cream from which it is made.

The formol titration values (table 5, fig. 5) in the buttermilks increase as the acidity of the raw cream rises and then stabilizes. Neutralization and pas-

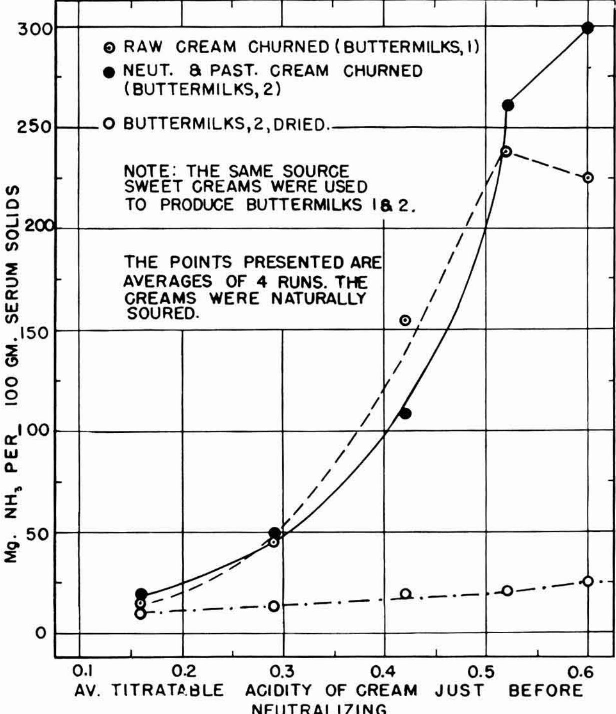


FIG. 4. The relationship between the acidity of cream and the ammonia content of the buttermilk from that cream.

teurization reduced the average values somewhat in the raw cream acidity range 0.2 to 0.5 per cent. Drying affects the value in much the same way as it does the ammonia values.

With both the ammonia determination and the formol titration, the variations encountered among different lots of buttermilk were sufficiently great (tables 5 and 6) that these determinations would have been of no value for predicting the quality of the raw creams. The data of tables 5 and 6 suggest that the variation among samples might have resulted from differences in bacterial flora of the raw creams. Large batches of commercial creams should have flora that

are more nearly alike from batch to batch and might yield buttermilks that would have less variation among samples than those found in this study.

Table 6 indicates that there are considerable differences among the quantities of ammonia and the formol titration values produced when the creams were inoculated with *Streptococcus lactis*, *Escherichia coli* or *Aerobacter aerogenes*. The inoculated creams were handled as indicated in fig. 2; they were ripened at room temperature. The time elapse to produce the maximum acidity was longer with the cultured than with the naturally soured creams: *S. lactis*, 23 hr., *E. coli*, 29 hr. and *A. aerogenes*, 32 hr.

With *S. lactis* (1 per cent inoculum) the ammonia values and formol titrations showed no tendency to increase at acidities up to 0.55 per cent. *E. coli*

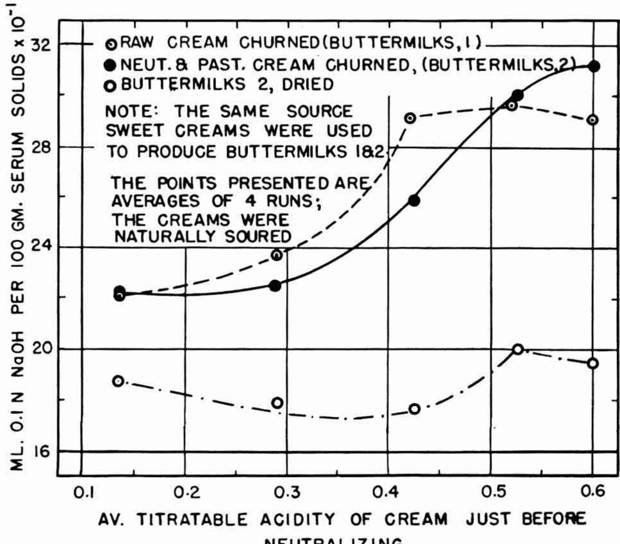


FIG. 5. The relationship between the acidity of cream and the formol titration of the buttermilk from that cream.

(1.7 per cent inoculum) reduced the ammonia and formol titration values as the cream acidity increased to 0.35 per cent, but increased these values between 0.35 to 0.65 per cent acid in the cream. *A. aerogenes* (1.7 per cent inoculum) increased the ammonia values of the liquid buttermilk as the cream acidity rose; the formol titration values rose slowly until the cream acidity was 0.31 per cent and more rapidly thereafter.

CONCLUSIONS

The method for the determination of ammonia adapted to dry milk by Choi *et al.* (1) was modified slightly and used in this study. This method and the formol titration method of Walker (4) were shown to have an accurracy of 0.242 mg. ammonia and 0.126 ml. 0.1 *N* NaOH, respectively, per 100 g. serum solids.

For naturally ripened creams, ammonia values of liquid buttermilks increased

as the acidity at the time of neutralization and pasteurization increased. Neutralization and pasteurization had little effect on the magnitude of the ammonia value of the liquid buttermilk. Drying expelled ammonia from the sample and virtually eliminated differences among the values of buttermilks from creams of widely different acidities. Formol titration values increased to a lesser degree with cream acidity and were lowered to a greater degree by neutralization and pasteurization than were the ammonia values; such differences as existed were largely eliminated by drying.

Results with pure cultures of organisms depended upon the type of organism in the cream and the results with any single organism are not necessarily in accord with the results obtained with naturally ripened creams. These data suggest that the variations among the values obtained with different naturally ripened creams result from the particular mixed flora of organisms present in the cream from a single producer.

Ammonia and formol titration values can not be employed to estimate the quality of cream from which a lot of dry buttermilk was produced. There is a possibility that the ammonia value may be used to determine the quality of liquid buttermilk received at drying stations.

ACKNOWLEDGMENT

The authors express appreciation to the Fairmont Foods Co., Omaha, Neb., which supported this study in part and to F. E. Nelson and E. B. Collins, Iowa Agricultural Experiment Station, for the pure cultures employed.

REFERENCES

(1) CHOI, R. P., O'MALLEY, C. M., AND FAIRBANKS, B. W. Colorimetric Determination of Ammonia in Milk. J. Dairy Sci., **24**: 645–649. 1946.

(2) MOJONNIER BROS. CO. Instruction Manual for Setting Up and Operating the Mojonnier Milk Tester. Bull. 101. Mojonnier Bros. Co., Chicago, Ill., 1925.

(3) RUSSELL, J. A. The Colorimetric Estimation of Small Amounts of Ammonia by Phenol Hypochlorite Reaction. J. Biol. Chem., **156**: 457–467. 1944.

(4) WALKER, W. O. A Rapid Method for Determining the Percentage of Casein in Milk. J. Ind. Eng. Chem., **6**: 131–133. 1914.

NUTRITIVE VALUES OF CROPS AND COWS' MILK AS AFFECTED BY SOIL FERTILITY. II. THE ESSENTIAL AMINO ACIDS IN COLOSTRUM AND MILK PROTEINS[1,2]

C. W. DUNCAN, GERTRUDE I. WATSON, K. M. DUNN[3] AND R. E. ELY[4]
Departments of Agricultural Chemistry and Dairy, Michigan Agricultural Experiment Station, East Lansing

As a result of conflicting reports in the literature, a 10-yr. project was started at this Station in 1945 to study the characteristics of certain plant species grown on natural soils seriously depleted of mineral nutrients and on the same kind of soil supplied with adequate amounts of mineral fertilizers and minor elements, with the specific objective of investigating the nutritive quality of these plant products from the standpoint of animal nutrition as distinguished from quantitative yield.

Smooth bromegrass and timothy were chosen as the hays most likely to grow satisfactorily on both depleted and highly fertilized soils. Since these hays are naturally low in protein, soybeans are being grown and fed as a protein supplement. Hybrid corn, winter wheat and spring oats are being raised on both the fertilized and depleted soils to supply the components of the grain rations.

The crops grown under these conditions are being fed to two groups of cows as their sole rations. Ten pairs of heifers, each pair consisting of half-sisters, were started on the experiment. The pairs are divided into two groups, one half-sister is in the group which receives the feeds grown on the fertilized soil and the other half-sister is in the group which receives the feeds grown on the depleted soil. Each pair of cows has been bred to the same bull in order to maintain similarity in the genetic make-up of their calves. Details in regard to the initiation and purpose of this experiment have already been published (2).

Under the rigid conditions imposed by this experiment it was thought desirable, among other things, to determine whether the same crops grown on highly fertilized and depleted soils of the same type and fed to the two groups of cows would change the amino acid pattern of the colostrum and milk proteins and to obtain more comprehensive knowledge of the amino acid composition of colostrum and milk proteins at various stages of lactation, since there is an acute paucity of this kind of information in the literature.

EXPERIMENTAL

Samples. The samples analyzed represent "first" colostrum puerperium and colostrum collected 24 hr. postpartum. Fifteen samples of "first" colostrum

Received for publication Aug. 18, 1951.

[1] Published with the approval of the Director of the Michigan Agricultural Experiment Station as Journal article no. 1269.

[2] This work is supported in part by a grant from the National Dairy Council on behalf of the American Dairy Association and was presented in part at the forty-fifth annual meeting of the American Dairy Science Association, Ithaca, N. Y., June, 1950.

[3] Deceased.

[4] Present address: Bureau of Dairy Industry, U. S. D. A., Washington 25, D. C.

were obtained before the calves had an opportunity to suckle and 16 samples were collected 24 hr. postpartum. Two of the cows had freshened for the first time, five for the second, seven for the third and one for the fourth time. Colostrum was obtained from three cows on successive parturitions (K4, K9, K18). Fifteen 60-day composite milk samples representing that produced on the 59th, 60th and 61st days of lactation were collected for analysis. The samples were obtained from four cows during their first lactation, nine cows during their second and two cows during their third lactation. Milk samples were obtained from two cows (K12, K16) on successive lactations. Thirteen terminal milk samples were obtained on the last day of lactation before the cow was started on her dry period. Four cows had completed their first lactation, seven their second and two their third lactation. Terminal samples were obtained from three cows (K9, K12, K16) on successive lactations. All of the colostrum and milk samples were collected over a 2-yr. period.

Animals. The cows numbered K1 through K12 represent the Jersey breed, whereas those with numbers above 12 represent the Holstein breed. The odd numbered cows receive the feeds grown on the unfertilized soil and the even numbered cows receive the feeds grown on the highly fertilized soil. Both groups of cows are maintained on the same plane of nutrition, are being fed for full milk production and are living under identical conditions of care and management. None of the cows has received green feed or silage since the inception of the investigation.

TABLE 1

Organisms, media and range of standards used for determining specific amino acids

Amino acid	Organism	Standard curve increment	Medium[b]
		(γ/*tube*)	
Arginine	*S. faecalis* (9790)[a]	10	II
Histidine	"	5	II
Isoleucine	*L. plantarum* 17-5 (8014)[a]	5	I[c]
Leucine	"	5	I
Lysine	*L. mesenteroides* P-60 (8042)[a]	25	III
Methionine	"	5	V
Phenylalanine	*L. plantarum*	5	I
Threonine	*S. faecalis*	5	II
Tryptophan	*L. plantarum*	1	IV
Valine	"	5	I[c]

[a] American Type Culture Collection, Georgetown University, Medical School, Washington, D. C.

[b] See table 2 for composition of media.

[c] 20 ml. of tomato eluate added per 500 ml. of medium (7).

Methods. Total nitrogen was determined by the Kjeldahl method. The total crude protein content calculated for each colostrum and milk sample was obtained by multiplying the nitrogen value by 6.38.

The hydrolyzates for the determination of arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine and valine were prepared according to the method of Stokes *et al.* (14). Ten ml. of colostrum or 20 ml. of

milk were diluted with an equal volume of concentrated HCl and autoclaved for 8 hr. at 15 lb. pressure. The hydrolysates were cooled, neutralized to pH 6.6 to 6.8 with 18N NaOH, made up to 100 ml., filtered, covered with a few drops of toluene and stored in the refrigerator for subsequent analysis. For the tryptophan assay, the enzymatic digestion procedure was essentially that proposed by Wooley and Sebrell (16). Five ml. of colostrum or 10 ml. of milk were pipetted into 100-ml. volumetric flasks, 20 mg. of pepsin and 40 ml. of 0.1N H_2SO_4 were added to each flask and the flasks incubated at 37° C. for 24 hr. with constant shaking. The contents of the flasks then were transferred to 100-ml. beak-

TABLE 2

Composition of media used in amino acid assays[a] *(Per 500 ml. of double-strength medium)*

Composition of medium:	I (13)	II (3)	III (10)	IV (6)	V (9)
Casein hydrolyzate (g.)	....	....	....	5.0	....
H_2O_2 treated peptone (g.)	....	....	....	....	7.5
DL(−)-Alanine (mg.)	200	100	200	....	....
L(+)-Arginine · HCl (mg.)	50	50	100	....	....
L-Asparagine (mg.)	200	200	200	....	....
L(−)-Cystine (mg.)	100	200	200	200	100
L(+)-Glutamic acid (mg.)	400	400	400	....	....
Glycine (mg.)	20	20	100	....	....
L(+)-Histidine · HCl · H_2O (mg.)	50	50	100	....	....
DL-Isoleucine (mg.)	200	200	200	....	....
DL-Leucine (mg.)	200	200	200	....	....
L(+)-Lysine · HCl · H_2O (mg.)	200	200	200	....	....
DL-Methionine (mg.)	100	100	200	....	100
DL-Phenylalanine (mg.)	100	100	100	....	....
L(−)-Proline (mg.)	50	50	50	....	....
DL-Serine (mg.)	50	50	200	....	....
DL-Threonine (mg.)	200	200	200	....	....
DL-Tryptophan (mg.)	50	100	100	....	100
L(−)-Tyrosine (mg.)	50	100	100	....	100
DL-Valine (mg.)	200	200	200	....	....
Glucose (g.)	20	20	20	20	20
Na acetate (anhyd.) (g.)	20	....	20	20	12
Na citrate · H_2O (g.)	....	25	....	....	....
NH_4Cl (g.)	....	....	....	....	6
KH_2PO_4 (mg.)	500	....	500	500	500
K_2HPO_4 (mg.)	500	5000	500	500	500
$MgSO_4 \cdot 7H_2O$ (mg.)	200	200	200	200	200
$FeSO_4 \cdot 7H_2O$ (mg.)	10	10	10	10	10
$MnSO_4 \cdot 4H_2O$ (mg.)	10	10	10	10	10
NaCl (mg.)	10	10	10	10	10
Adenine $SO_4 \cdot 2H_2O$ (mg.)	10	10	10	10	10
Guanine · HCl · $2H_2O$ (mg.)	10	10	10	10	10
Uracil (mg.)	10	10	10	10	10
Xanthine (mg.)	10	10	10	....	......
Thiamin · HCl (mg.)	0.5	0.5	0.5	0.1	1.0
Pyridoxine · HCl (mg.)	1.0	1.0	1.0	0.1	2.0
DL-Ca pantothenate (mg.)	0.5	0.5	0.5	0.1	2.0
Riboflavin (mg.)	0.5	0.5	0.5	0.2	2.0
Nicotinic acid (mg.)	1.0	1.0	1.0	0.4	2.0
p-Aminobenzoic acid (mg.)	0.1	0.1	0.1	0.1	0.01
Biotin (mg.)	0.001	0.001	0.001	0.2	0.005
Folic acid (mg.)	0.01	0.01	0.01	....	0.0015
pH before autoclaving	6.6–6.8	6.6–6.8	6.6–6.8	6.6–6.8	6.9–7.0

[a] The amino acid to be assayed is omitted from the medium.

ers, 3.0 g. of K_2HPO_4 were added to each beaker and the pH adjusted to 8.4 with 3*N* NaOH. The solutions were transferred again to 100-ml. volumetric flasks, 20 mg. of trypsin were added and allowed to incubate at 40° C. for 24 hr. with constant shaking. The contents of the flasks were cooled, adjusted to pH 7.0, diluted to 100 ml., centrifuged, filtered, preserved with toluene and stored in the refrigerator. In all cases, the assays were run after the proper dilutions had been made.

Assay standards. DL-Configurations of isoleucine, leucine, methionine, phenylalanine, threonine and valine were used in the preparation of standards

TABLE 3

Essential amino acids in colostrum from cows receiving fertilized feeds
(Amino acid values expressed as per cent of the crude protein)

Cow no.	K4	K6		K4	K10	K12	K18	K18	K16		S.D.
Freshening no.	2	2		3	3	3	1	2	2	Av.	±
				"First" colostrum							
Arginine	5.06	4.85		4.62	4.56	4.76	4.30	4.44	4.27	4.61	0.097
Histidine	2.74	2.29		2.63	2.92	2.37	2.64	2.67	2.35	2.58	0.078
Isoleucine	5.98	5.27		5.14	5.09	5.72	5.47	5.66	5.17	5.44	0.115
Leucine	9.07	8.59		8.37	8.50	8.27	8.48	8.41	8.59	8.54	0.086
Lysine	7.84	7.70		7.13	7.50	7.68	7.13	6.90	7.16	7.38	0.121
Methionine	1.96	1.76		1.68	1.91	1.59	1.77	1.73	1.71	1.76	0.042
Phenylalanine	4.54	4.70		4.59	4.69	4.52	4.73	4.37	4.59	4.59	0.042
Threonine	7.62	7.46		7.15	7.58	7.67	7.44	7.61	7.39	7.49	0.061
Tryptophan	1.74	1.77		1.63	1.85	1.92	1.55	1.92	1.74	1.77	0.047
Valine	8.55	8.13		8.26	8.62	8.66	8.25	8.25	8.08	8.35	0.080
Total protein %	13.89	15.08		17.05	7.80	19.22	6.45	13.97	15.72	13.65	1.553
Cow no.	K8	K10	K12	K4	K10	K12	K18	K18	K16		
Freshening no.	2	2	2	3	3	3	1	2	2		
				24-hr. colostrum							
Arginine	4.32	4.19	4.34	4.32	4.70	4.27	4.36	4.18	4.02	4.30	0.061
Histidine	2.48	2.75	2.50	2.96	2.91	2.49	2.81	2.94	2.61	2.72	0.068
Isoleucine	6.15	5.84	5.46	6.19	5.99	5.73	5.45	5.73	5.57	5.79	0.093
Leucine	8.85	8.61	8.45	8.30	8.49	8.61	8.48	8.51	8.55	8.54	0.050
Lysine	7.80	7.65	7.92	7.92	7.80	7.68	7.56	7.26	7.37	7.66	0.077
Methionine	1.85	1.93	1.64	2.00	1.90	1.66	2.03	1.89	1.64	1.84	0.051
Phenylalanine	4.62	4.98	4.23	4.67	4.91	4.26	4.97	4.24	4.56	4.60	0.103
Threonine	7.25	7.42	7.46	7.30	7.59	7.08	7.56	7.30	7.01	7.33	0.066
Tryptophan	1.87	1.93	1.99	1.42	1.55	1.66	1.59	1.49	1.73	1.69	0.067
Valine	8.16	8.59	7.95	8.52	7.93	8.57	8.24	8.07	8.52	8.28	0.090
Total protein %	9.61	6.47	11.29	8.22	4.64	12.99	4.59	5.03	9.68	8.06	1.022

for these amino acids, whereas the L-configurations were used for the preparation of the arginine, histidine, lysine and tryptophan standards.

The organisms, concentration ranges and the media used for determining the specific amino acids are given in table 1. The composition of the various media are given in table 2.

Assay procedure. For the assay of the various amino acids, 5-ml. quantities of the appropriate basal medium (table 2), free of the amino acid to be assayed, were placed in lipless culture test-tubes. A standard curve of eleven levels was prepared by adding, in triplicate, amounts ranging from 0 to 5 ml. of the

standard amino acid solution to separate tubes of medium. To another set of tubes containing 5 ml. of the particular amino acid-free medium were added in duplicate 1.0-, 2.0- and 3.0-ml. quantities of the appropriate dilution of the protein hydrolyzate to be assayed. The total volume in all tubes was brought to 10 ml. with water. The tubes were capped and sterilized by autoclaving at 15 lb. pressure for 10 min. Each tube was inoculated aseptically with one drop of the appropriate organism suspension and then incubated for 72 hr. at 37° C. to permit the development of lactic acid to the maximum degree permitted by the available amino acid. After incubation, the lactic acid in each tube of the

TABLE 4

Essential amino acids in colostrum from cows receiving unfertilized feeds
(Amino acid values expressed as per cent of the crude protein)

Cow no.	K3	K5	K9	K11	K9	K17	K15		S.D.
Freshening no.	3	3	3	3	4	1	2	Av.	±
			''First'' colostrum						
Arginine	5.65	5.33	5.10	4.88	4.89	4.98	5.59	5.20	0.124
Histidine	2.50	2.93	2.52	2.82	2.60	2.66	2.74	2.68	0.077
Isoleucine	4.95	5.24	5.29	5.54	5.29	4.81	5.19	5.19	0.091
Leucine	8.38	8.49	7.99	8.02	8.55	8.10	8.16	8.24	0.087
Lysine	7.79	8.13	7.94	7.50	7.41	7.93	7.69	7.77	0.097
Methionine	1.62	2.00	1.68	1.94	1.81	1.96	1.90	1.84	0.055
Phenylalanine	4.00	4.46	4.42	4.77	4.65	4.63	4.73	4.52	0.100
Threonine	7.40	7.62	7.01	7.08	7.41	7.68	7.30	7.36	0.095
Tryptophan	1.87	1.55	1.70	1.67	1.80	1.76	1.91	1.75	0.047
Valine	8.37	8.15	8.06	8.28	8.45	8.13	8.38	8.26	0.056
Total protein %	22.97	11.92	20.28	11.13	8.02	11.13	12.76	14.03	2.059
Cow no.	K3	K5	K3	K11	K9	K17	K15		
Freshening no.	3	3	2	3	4	1	2		
			24-hr. colostrum						
Arginine	5.70	5.55	5.66	5.14	5.18	5.30	5.87	5.49	0.107
Histidine	2.82	2.73	2.51	3.01	2.89	2.79	2.86	2.80	0.059
Isoleucine	5.71	6.24	5.82	5.89	5.60	6.38	5.78	5.92	0.108
Leucine	8.89	8.57	8.61	8.77	8.48	8.81	8.13	8.61	0.097
Lysine	7.58	8.33	7.78	7.27	7.83	7.12	7.56	7.64	0.151
Methionine	1.60	1.94	1.72	2.06	2.04	2.11	1.96	1.92	0.072
Phenylalanine	4.43	4.37	4.47	5.03	4.58	4.70	4.75	4.62	0.089
Threonine	7.91	7.77	7.58	7.69	7.16	7.73	7.43	7.61	0.094
Tryptophan	1.77	1.64	1.89	1.52	1.54	1.68	1.39	1.63	0.063
Valine	7.60	8.09	7.97	7.86	8.20	8.29	8.18	8.03	0.090
Total protein %	7.90	6.23	10.20	6.11	4.61	6.21	4.55	6.54	0.745

medium was titrated electrometrically with 0.1N NaOH to pH 7.0. The reliability and reproducibility of the various amino acid determinations used in this work have been reported previously (12).

RESULTS AND DISCUSSION

The results of the microbiological determinations of the essential amino acids in the ''first'' and 24-hr. colostrum samples from each cow receiving the feeds grown on the highly fertilized soil are compiled in table 3 and the data obtained from each cow receiving the unfertilized feeds are presented in table 4. These data are expressed as the per cent of the individual amino acid based on the

amount of total crude protein in the sample. It is recognized that the amino acid data expressed as per cent of total protein may have questionable value (5) since the per cent of total protein was obtained by calculation. The calculated protein and amino acid values are extremely useful for comparative purposes, because the amounts of the various amino acids in the colostrum and milk proteins are reduced to an approximately equivalent basis. All of the values can be easily recalculated to milligrams of the appropriate amino acid per 100 ml. of colostrum or milk and the per cent of total protein can be recalculated to per cent of nitrogen. The data obtained from the 60-day composite milk sample

TABLE 5

Essential amino acids in 60-d. composite milk
(Amino acid values expressed as per cent of the crude protein)

Cow no.	K4	K6	K8	K10	K12	K12	K16	K16	K18		S.D.
Lactation no.	2	2	2	2	2	3	1	2	1	Av.	±
	Cows received fertilized feeds										
Arginine	4.08	4.18	3.84	3.88	3.77	3.95	4.27	3.86	3.87	3.97	0.057
Histidine	2.61	2.80	2.91	2.99	2.95	2.93	2.40	2.71	3.07	2.82	0.071
Isoleucine	6.76	6.57	6.91	7.07	6.69	6.82	6.51	6.93	6.57	6.76	0.064
Leucine	9.73	9.57	9.58	10.17	9.68	9.85	9.80	10.04	9.61	9.78	0.070
Lysine	7.74	7.97	7.87	7.69	8.04	7.71	7.84	7.76	7.85	7.83	0.040
Methionine	1.97	2.36	1.88	1.86	1.82	2.38	2.28	2.01	2.19	2.08	0.074
Phenylalanine	4.78	5.00	4.55	4.37	4.38	4.66	4.35	4.33	4.77	4.58	0.080
Threonine	4.89	4.86	4.40	4.76	4.53	4.93	4.71	4.55	4.59	4.69	0.062
Tryptophan	1.83	1.55	1.66	1.70	1.63	1.39	1.40	1.42	1.33	1.55	0.055
Valine	7.23	7.17	7.16	7.49	7.49	7.63	7.41	7.47	7.42	7.39	0.054
Total protein %	3.48	4.14	3.61	3.38	3.68	3.41	2.55	2.77	2.83	3.32	0.169
Cow no.	K3	K5	K11	K9	K15	K17					
Lactation no.	2	2	2	3	1	1					
	Cows received unfertilized feeds										
Arginine	4.37	4.40	4.31	4.40	3.63	4.25				4.23	0.122
Histidine	3.17	2.81	2.69	3.14	2.45	2.44				2.78	0.131
Isoleucine	6.56	6.45	6.45	6.42	6.93	6.71				6.59	0.082
Leucine	9.94	10.06	9.38	9.01	8.64	8.56				9.27	0.262
Lysine	7.69	7.64	8.47	7.92	7.75	8.32				7.97	0.142
Methionine	2.04	2.54	2.32	2.24	2.39	2.47				2.33	0.166
Phenylalanine	5.05	4.93	5.02	4.74	4.85	4.83				4.90	0.049
Threonine	4.69	4.48	5.04	4.72	4.11	4.69				4.62	0.126
Tryptophan	1.52	1.39	1.60	1.33	1.40	1.30				1.42	0.047
Valine	7.29	7.16	7.37	7.12	7.36	7.57				7.31	0.067
Total protein %	3.28	3.35	2.87	3.77	2.80	2.92				3.17	0.152

from each individual cow in both groups are compiled in table 5 and the data concerning the composition of the terminal milk from each cow are presented in table 6.

The data in table 3 show the relative percentages of the ten essential amino acids found in the "first" and the 24-hr. samples of colostrum obtained from each of the Jersey and Holstein cows which had been maintained solely on the feeds produced on highly fertilized soil. From an examination of both the individual and averaged data, there is relatively little difference in the amino acid pattern of the "first" colostrum and that obtained 24 hr. later when these values

are expressed as per cent of the total protein. The greatest concentration of each amino acid occurred, however, immediately after parturition and then decreased rapidly during the next 24 hr. as mammary secretion progressed. The average amount of total protein in the 24-hr. sample was approximately 60 per cent of that present in the "first" colostrum, but individual cows varied markedly.

Table 4 presents the data secured from the Jersey and Holstein cows maintained solely on the same species of plants grown on soil that was severely depleted of mineral elements. Again, there is little difference in the amino acid

TABLE 6

Essential amino acids in terminal milk
(Amino acid values expressed as per cent of the crude protein)

Cow no.	K4	K8	K12	K12	K16	K16	K18		S.D.
Lactation no.	2	2	2	3	1	2	1	Av.	±
				Cows received fertilized feeds					
Arginine	3.84	3.92	3.86	3.88	4.28	4.25	4.04	4.01	0.070
Histidine	2.58	2.74	2.91	2.74	2.86	2.96	3.14	2.85	0.069
Isoleucine	6.62	6.57	6.65	7.02	7.07	6.85	6.65	6.78	0.077
Leucine	8.04	8.64	8.22	8.31	8.42	8.56	8.31	8.36	0.077
Lysine	7.28	7.92	7.95	7.46	7.57	7.57	7.32	7.58	0.101
Methionine	2.24	2.46	2.52	2.28	2.07	2.37	2.32	2.32	0.056
Phenylalanine	4.55	4.87	4.44	4.61	4.55	4.69	4.89	4.66	0.065
Threonine	5.39	5.60	5.46	5.18	5.20	5.75	5.04	5.37	0.095
Tryptophan	1.12	1.41	1.45	1.25	1.29	1.29	1.33	1.31	0.041
Valine	7.56	7.98	7.55	7.70	7.65	7.46	7.67	7.65	0.063
Total protein %	4.75	5.05	4.73	4.56	5.83	4.73	4.21	4.84	0.191
Cow no.	K1	K3	K9	K11	K9	K15			
Lactation no.	1	2	2	2	3	1			
				Cows received unfertilized feeds					
Arginine	3.86	3.77	3.75	4.07	4.27	4.08		3.97	0.084
Histidine	2.58	2.84	2.76	2.83	2.46	2.47		2.66	0.072
Isoleucine	6.86	7.29	7.15	6.65	6.77	6.67		6.90	0.108
Leucine	8.91	9.11	9.22	9.02	9.58	8.99		9.14	0.098
Lysine	7.98	7.88	7.91	7.88	7.77	7.68		7.85	0.043
Methionine	2.29	2.43	2.47	2.20	2.44	2.23		2.34	0.048
Phenylalanine	4.84	4.98	4.76	5.25	5.17	4.83		4.97	0.082
Threonine	5.11	5.54	5.43	5.47	5.57	5.47		5.43	0.068
Tryptophan	1.45	1.32	1.66	1.42	1.41	1.20		1.41	0.062
Valine	7.47	7.12	7.18	7.47	7.27	7.33		7.31	0.059
Total protein %	5.06	4.25	4.73	4.99	4.76	3.75		4.59	0.204

patterns of the "first" and 24-hr. colostrum samples. The first noticeable difference observed in these two sets of data is the consistently higher arginine values present in the "first" and 24-hr. samples obtained from the cows receiving the unfertilized feeds. The concentration of all of the other amino acids are within ± 10 per cent of those found for the group receiving the fertilized feeds. The significance of the higher arginine values is not evident. The average amount of total protein in the 24-hr. sample was less than 50 per cent of that present in the "first" colostrum. This observation is of interest because both groups of cows received comparable protein intakes in their rations. The decrease in total protein during the first 24 hr. postpartum is in contrast to the findings of Miller

et al. (11) who reported that the highest total nitrogen and amino acid concentrations occurred in human colostrum on the fifth to eighth day postpartum. Attention should be called again to individual cow differences in regard to the amount of total protein secreted in the "first" and 24-hr. samples.

Table 5 presents the individual cow data obtained from the analysis of fifteen 60-day composite milk samples for both groups of cows. These samples were taken at a definite stage of lactation, not only to compare the amino acid patterns but also to study the characteristics of the mature milk proteins in regard to rat nutrition. It was thought that a comparison of these milks might help to elucidate the nutritive characteristics of the milk proteins produced by cows receiving feeds grown on highly fertilized soil and on depleted soil. The results of this research will be the basis of a subsequent publication (1). An examination of these data shows very little difference in the amino acid patterns of the milk proteins that can be attributed to the method of producing the feeds. The average methionine value of the milk produced by the cows receiving the unfertilized feed was 12.0 per cent higher than that produced by the cows receiving the feed grown on the fertilized soil, whereas the tryptophan content of the milk produced by the cows receiving the fertilized feed was 9.2 per cent higher than that produced by the cows receiving the unfertilized feeds. The differences between all of the other amino acids, however, are less than $\pm$ 7.0 per cent. The average amount of crude protein secreted in the milk was almost identical for both groups of cows. The differences observed in the protein and amino acid contents of these milks are attributed to the inherited characteristics of each cow and not to the feeds.

When the concentrations of the various essential amino acids in the 60-day composite samples are compared with the concentrations in "first" colostrum, certain differences are apparent. The amounts of arginine, threonine, tryptophan and valine are noticeably higher in the "first" colostrum than in the milk, whereas the amounts of isoleucine, leucine and methionine are markedly higher in milk. The amounts of histidine, lysine and phenylalanine are approximately the same in milk and "first" colostrum. These differences and similarities are found in both groups of cows and can not be attributed to any differences in agronomic practices of producing the feeds.

It was thought that if any differences between the two groups were manifest, they should appear in the terminal milk at the completion of 10 mo. of lactation. No pronounced difference was evident in any of the essential amino acids in the milk proteins of these two groups (table 6), except that the average leucine content of the milk produced by the cows receiving the unfertilized feed was 9.3 per cent higher.

The average amounts of arginine, threonine, tryptophan and valine in terminal milk proteins are considerably less than that found in "first" colostrum, whereas the amount of isoleucine and methionine are increased. The amounts of histidine, lysine and phenylalanine in the terminal milk proteins are the same as those found in "first" colostrum. With the exception of threonine, the terminal milk proteins of both groups are the same as those found in the 60-day milk samples. Threonine, however, was consistently (16 per cent) higher in the ter-

minal milk proteins than in the 60-day samples in both groups. The significance of the bovine secreting larger amounts of this amino acid at the end of lactation is obscure.

A comparison of the "first" colostrum data obtained from the three cows (K4, K18, K9) shows good agreement in their individual amino acid patterns on successive parturitions (table 3, 4). With the exception of the isoleucine, lysine and methionine contents in the colostrum of cow K4 and in tryptophan (K18), there was less than ± 10 per cent difference in concentration among all of the other amino acids of individual cows on successive parturitions. The amount of total protein in the "first" colostrum increased on each succeeding parturition of cows K4 and K18, but decreased in cow K9.

The 60-day milk samples obtained from two cows (K12, K16) show regular uniformity in their individual amino acid patterns; however, the methionine content of the milk proteins of both cows, the arginine and histidine contents in the milk of cow K16 and the tryptophan content in the milk of cow K12 varied more than ± 10 per cent on successive lactations (table 5). All of the other amino acids varied less than ± 10 per cent. The total protein content increased in the milk of cow K16 on succeeding lactations, but decreased in the milk of cow K12.

The milk samples obtained from three cows (K9, K12, K16) also show good uniformity in their individual amino acid patterns at the end of succeeding lactations (table 6). The methionine content in the milk proteins of cows K12 and K16, the tryptophan content in the milk of cows K12 and K9 and the arginine and histidine contents in the milk of cow K9 varied by more than ± 10 per cent at the time the cows ceased to milk. The variation was less than ± 10 per cent in the case of all of the other amino acids. The amount of total protein in the terminal milk of two cows (K9, K12) was the same at the end of each succeeding lactation, but the amount was markedly less in the case of cow K16. When the above comparisons are examined critically, there is nothing to indicate that feeds grown on the fertilized soil have either superior or inferior nutritive properties to the same kind of feeds grown on depleted soil.

Since no major differences were noted in the amino acid patterns of the colostrum or milk protein secreted by these two groups of cows that could be attributed to feeds grown on highly fertilized or depleted soils, the data for each group were weighted and combined to illustrate the changes in protein distribution which occur as lactation progresses. The results of these calculations (table 7) indicate that: (a) the amounts of arginine and valine decreased during the first 60 days postpartum and then remained constant throughout the rest of the lactation period; (b) isoleucine and methionine increased throughout the entire lactation period; (c) histidine, lysine and phenylalanine remained unchanged in both colostrum and milk; (d) leucine was higher in milk secreted 60 days postpartum than in colostrum or terminal milk; (e) threonine was higher in colostrum, markedly lower in milk secreted 60 days postpartum and intermediate in terminal milk; whereas, (f) tryptophan decreased progressively from colostrum to terminal milk.

The ratios of the individual amino acids in colostrum and milk also are shown in table 7. The distribution of histidine, lysine and phenylalanine is relatively

unchanged in the transition from colostrum to milk and milk contains more isoleucine, leucine and methionine and less arginine, threonine, tryptophan and valine than colostrum.

Kuiken and Pearson (8), Miller *et al.* (11) and Ray Sarkar *et al.* (12) have recently reviewed the literature on the amino acid composition of colostrum and whole milk proteins. In most instances, the values recorded in tables 3 to 7 are in good agreement with published values, but comparative data of this nature are extremely meager and unavailable for terminal milk proteins. Consistently higher arginine values were found (*ca* 16 per cent) in colostrum and milk proteins, both in this work and that published previously (12), than has been reported by Kuiken and Pearson (8) and Hodson and Krueger (4), but the other average values were comparable. Sutton and Esh (15) reported an average value for tryptophan of 3.85 mg. per gram (range 1.42 to 5.94) from the chemical

TABLE 7

Comparison of the amounts of the essential amino acid in "first" colostrum, colostrum secreted 24 hr. postpartum, milk secreted 60 d. postpartum and terminal milk

(All values expressed as per cent of the total protein)

Amino acid	Colostrum		Milk		Ratio
	0-hr.	24-hr.	60-d.	Term.	M/C[a]
Arginine	4.89	4.82	4.07	3.99	0.83
Histidine	2.63	2.76	2.80	2.76	1.06
Isoleucine	5.32	5.85	6.69	6.84	1.26
Leucine	8.40	8.57	9.58	8.72	1.14
Lysine	7.56	7.65	7.89	7.51	1.04
Methionine	1.80	1.88	2.18	2.33	1.21
Phenylalanine	4.56	4.61	4.71	4.80	1.03
Threonine	7.43	7.45	4.66	5.40	0.63
Tryptophan	1.76	1.66	1.50	1.36	0.85
Valine	8.31	8.17	7.36	7.49	0.89
Total protein %	13.83	7.40	3.26	4.72	0.24

[a] 60-d. milk and 0-hr. colostrum.

analysis of ten first-milking colostrum samples. The average value found in this investigation by a microbiological method was 2.45 mg. per milliliter (range 1.00 to 4.29) and 2.53 in a previous report (12). Miller *et al.* (11) found 2.05 mg. per milliliter as the average amount present in human colostrum.

From the results obtained in this investigation, the amount and kind of amino acids in colostrum and milk proteins apparently are very similar irrespective of whether the feeds consumed by the cows are grown on highly fertilized or badly depleted soil of the same type. In case of a deficiency of protein or any essential nutrient in the feed, the first defense mechanism would operate to limit milk production rather than to alter the amount or kind of protein in the milk. The importance of this and related fields of research is obvious, both from the standpoint of plant, animal and human nutrition.

SUMMARY AND CONCLUSIONS

The essential amino acids in colostrum and milk proteins of cows maintained on feeds grown on highly fertilized and on highly depleted soil of the same type have been determined.

There was no essential difference between the amino acid pattern of the "first" colostrum and that obtained 24 hr. postpartum when the values are expressed as per cent of the crude protein, except that the arginine content was higher in the colostrum secreted by the cows receiving the unfertilized feed. The amount of crude protein in colostrum declined approximately 50 per cent within 24 hr. postpartum. The genetic differences between cows appear to be greater than differences between groups.

The amino acid concentrations of the 60-day composite milk samples obtained from each group of cows varied less than ± 10 per cent, except that the methionine content of the milk produced by the cows receiving the unfertilized feeds was 12 per cent higher than that produced by the cows receiving the fertilized feeds. The average amount of total crude protein in the milk was almost identical in both groups. Individual cow differences were larger than group differences.

The average amino acid values in the terminal milk proteins from each group of cows deviated less than ± 10 per cent, except that the leucine content of the milk produced by the cows receiving the unfertilized feeds was consistently higher than that produced by the cows receiving the fertilized feeds. The average total protein content in the milk produced by both groups was approximately the same.

The amounts of isoleucine and methionine increased as lactation progressed, whereas arginine and tryptophan decreased progressively from colostrum to terminal milk. Threonine decreased markedly during the first 60 days postpartum and then increased slightly as lactation progressed. The other amino acids were relatively unchanged.

The concepts that badly depleted soils produce crops of lower nutritive value than highly fertilized soils or that commercial fertilizers decrease the nutritive properties of crops are not confirmed by this investigation. Insofar as the amino acid composition of milk proteins is concerned, evidence is lacking which indicates that the nutritive value of feeds is dependent chiefly on soil fertility.

ACKNOWLEDGMENT

The authors are most grateful to Margaret A. Ohlsen, head of the Department of Foods and Nutrition, for her interest and financial support in this research and to Carolyn Tull and Joana R. Boniece for technical assistance.

REFERENCES

(1) CEDERQUIST, DENA C. *et al.* Unpublished Data. Mich. Agr. Expt. Sta. 1951.

(2) DEXTER, S. T., DUNCAN, C. W., CEDERQUIST, DENA C., DUNN, K. M., MILLAR, C. E., TURK, L. M., WEAVER, E., AND PRIOR, R. Nutritive Values of Crops and Cows' Milk as Affected by Soil Fertility. 1. The Research Problem and Procedures. Mich. Agr. Expt. Sta. Quart. Bull., **32**: 352–359. 1950.

(3) GREENHUT, I. T., SCHWEIGERT, B. S., AND ELVEHJEM, C. A. The Amino Acid Requirements of *Streptococcus faecalis* and the Use of this Organism for the Determination of Threonine in Natural Products. J. Biol. Chem., **162**: 69–76. 1946.

(4) HODSON, A. Z., AND KRUEGER, G. M. Essential Amino Acid Content of Casein and Fresh and Processed Cows' Milk as Determined Microbiologically on Hydrolyzates. Arch. Biochem., **10**: 55–64. 1946.

(5) JONES, D. B. Factors for Converting Percentages of Nitrogen in Foods and Feeds into Percentages of Proteins. U. S. D. A. Circ. 183. Rev. 1941.

(6) KREHL, W. A., STRONG, F. M., AND ELVEHJEM, C. A. Determination of Nicotinic Acid. Modifications in the Microbiological Method. Ind. Eng. Chem., Anal. Ed., **15**: 471–475. 1943.

(7) KUIKEN, K. A., NORMAN, W. H., LYMAN, C. M., HALE, F., AND BLOTTER, L. The Microbiological Determination of Amino Acids. I. Valine, Leucine, and Isoleucine. J. Biol. Chem., **151**: 615–626. 1943.

(8) KUIKEN, K. A., AND PEARSON, P. B. The Essential Amino Acid (except Tryptophan) Content of Colostrum and Milk in the Cow and Ewe. J. Nutrition, **39**: 167–176. 1949.

(9) LYMAN, C. M., MOSELEY, O., BUTLER, B., WOOD, S., AND HALE, F. The Microbiological Determination of Amino Acids. III. Methionine. J. Biol. Chem., **166**: 161–171. 1946.

(10) MCMAHAN, J. R., AND SNELL, E. E. The Microbiological Determination of Amino Acids. I. Valine and Arginine. J. Biol. Chem., **152**: 83–95. 1944.

(11) MILLER, S., RUTTINGER, V., RUTLEDGE, M. M., FRAHM, R., MAURER, S., MOYER, E. Z., KAUCHER, M., MACY, I. G., PRATT, J. P., AND HAMIL, B. M. Human Milk Studies. XXVII. Essential Amino Acids in Human Colostrum and Transitional Milk. J. Nutrition, **40**: 499–514. 1950.

(12) RAY SARKAR, B. C., LUECKE, R. W., DUNCAN, C. W., ELY, R. E., WATSON, G. I., AND RYKALA, A. J. The Amino Acid Composition of Bovine Colostrum and Milk. J. Dairy Sci., **32**: 671–678. 1949.

(13) SCHWEIGERT, B. S., MCINTIRE, J. M., ELVEHJEM, C. A., AND STRONG, F. M. The Direct Determination of Valine and Leucine in Fresh Animal Tissues. J. Biol. Chem., **155**: 183–191. 1944.

(14) STOKES, J. L., GUNNESS, M., DWYER, I. M., AND CASWELL, M. C. Microbiological Methods for the Determination of Amino Acids. II. A Uniform Assay for the Ten Essential Amino Acids. J. Biol. Chem., **160**: 35–49. 1945.

(15) SUTTON, T. S., AND ESH, G. C. The Nutrition of the Newborn Calf. 1. Changes in the Tryptophan Content of the Blood Plasma Following Birth and the Ingestion of Colostrum. J. Dairy Sci., **31**: 183–187. 1948.

(16) WOOLEY, J. G., AND SEBRELL, W. H. Two Microbiological Methods for the Determination of *l*(–)-Tryptophane in Proteins and Other Complex Substances. J. Biol. Chem., **157**: 141–151. 1945.

CYTOCHEMICAL REACTIONS OF BOVINE SPERMATOZOA AND SEMINAL PLASMA

R. M. MELAMPY, L. F. CAVAZOS AND J. C. PORTER

Departments of Zoology and Entomology and Animal Husbandry, Iowa State College, Ames[1]

The use of cytochemical procedures permits the characterization and localization of various components such as nucleoproteins, lipids, carbohydrates and enzymes which constitute cells. Results of such investigations not only are of importance in an evaluation of cellular morphology but also are useful in the functional analysis of cells and tissues. Wislocki (20) made a comprehensive study of seasonal changes in the male reproductive tract of deer, using histochemical methods, and, in a later study (21), presented results of cytochemical investigations on human spermatozoa and seminal plasma. An investigation of the chemical composition of bovine spermatozoa has been reported by Porter *et al.* (19).

This paper presents cytochemical reactions of bovine spermatozoa and seminal plasma.

MATERIAL AND METHODS

Samples of bovine semen were collected by means of an artificial vagina from bulls maintained for breeding purposes at Iowa State College. Motile spermatozoa were present in all samples used. In experiments where it was desirable to remove adsorbed materials such as seminal plasma from the spermatozoa, the cells were washed by repeated centrifugation, using a Ringer-phosphate solution at pH 6.8. Smears of semen or washed spermatozoa were prepared soon after the collections were made and allowed to dry for 1 to 24 hr. before fixation.

The following fixatives, reactions and staining procedures were used in this study:

Acid phosphatase. Smears were fixed for 4 hr. in acetone at approximately 4° C. After fixation, the acid phosphatase reaction was carried out at pH 5.0 for 48 and 72 hr. at 37° C., as outlined by Gomori (8). Sodium glycerophosphate and adenosine triphosphate were used as substrates.

Alkaline phosphatase. Smears were fixed for 12 hr. in 80 per cent ethyl alcohol at approximately 4° C. This reaction was carried out at pH 9.0 for 48, 72 and 96 hr. at 37° C., according to Gomori (7). Sodium glycerophosphate and hexose diphosphate (fructose-1,6-diphosphate) were used as substrates.

Gram's stain. Hucker's modification of Gram's method as described by Guyer (9) was used.

Feulgen reaction. Smears were fixed 1 to 12 hr. with picric-sulfosalicylic acid mixture and stained 18 to 24 hr. with Feulgen's staining solution (Schiff's

Received for publication Aug. 20, 1951

[1] This project is supported in part by funds furnished by Dairy Genetics, Des Moines, Ia.; Eastern Iowa Artificial Breeding Association, Cedar Rapids, Ia.; and Northwest Iowa Federated Breeders Coop., Sheldon, Ia. Project *936*. Journal paper no. J-1967.

reagent) diluted with distilled water to one-half strength, according to Lhotka and Davenport (15).

Toluidin blue. Smears were fixed for 24 hr. in 4 per cent basic lead acetate solution followed with 24 hr. in 10 per cent formalin. They then were stained in a 0.5 per cent toluidin blue in 5 per cent alcohol solution for 30 min., as suggested by Wislocki (21). After staining, the slides were washed rapidly in 95 per cent alcohol, dehydrated and mounted in clarite.

Baker's acid hematein test for phospholipids. This method and its control procedure (pyridine extraction) were carried out as outlined in detail by Bloom and Wislocki (2) and Wislocki (21).

Sudan black B. Smears were fixed in 10 per cent neutral formalin for 6 hr. Following fixation, they were stained in a saturated solution of Sudan black B in 70 per cent alcohol for times varying from 1 min. to 12 hr. After staining, the slides were dipped in 70 per cent ethyl alcohol, washed in distilled water and mounted in glycerine jelly.

Bodian's protargol method. Smears were fixed in Bodian's fixative no. 2 (90 ml. of 80 per cent ethyl alcohol, 5 ml. of 37 per cent formaldehyde and 5 ml. of glacial acetic acid). The staining procedure was carried out in accordance with the directions of Dawson and Barnett (3), as recommended by Wislocki (21). The preparations were dehydrated, cleared and mounted in clarite.

Periodic acid-Schiff procedure. Smears were fixed for 12 hr. in Orth's fluid (2.5 g. potassium bichromate and 1 g. sodium sulfate were dissolved in 100 ml. of distilled water. To 100 ml. of this solution were added 10 ml. of 37 per cent formaldehyde before using.) Following fixation, the smears were washed in distilled water, stained by the periodic acid-Schiff procedure according to McManus (17) and then dehydrated, cleared and mounted in clarite. The three types of controls for this method were as follows: (a) Smears stained with the Schiff reagent without previous treatment with periodic acid were prepared. Staining was considered significant only after periodic acid action. (b) In order to remove polysaccharides such as glycogen, hydrolyzable by salivary amylase, smears were treated with saliva for 1 hr. at 37° C. before treatment with periodic acid-Schiff reagent. (c) In order to remove glycolipids, smears were extracted for 16 hr. with a mixture of equal parts of methanol and chloroform at 54° C. prior to treatment with periodic acid-Schiff reagent.

Glycogen. Smears were fixed for 12 hr. in Rossman's fluid (90 ml. of absolute alcohol saturated with picric acid and 10 ml. of 37 per cent formaldehyde) in a refrigerator. After fixation, the smears were washed in absolute alcohol followed by distilled water before staining with the Bauer-Feulgen and periodic acid-Schiff methods. Control smears were treated with saliva for 1 hr. at 37° C. before staining.

Schiff reaction (Plasmal reaction of Feulgen and Voit). Smears were fixed in 10 per cent formaldehyde for 12 hr. and the staining procedure of Herman (10) was followed.

RESULTS

Acid phosphatase. Using sodium glycerophosphate as a substrate at pH 5.0, positive reactions were obtained in spermatozoa and seminal plasma. The post-

PLATE 1

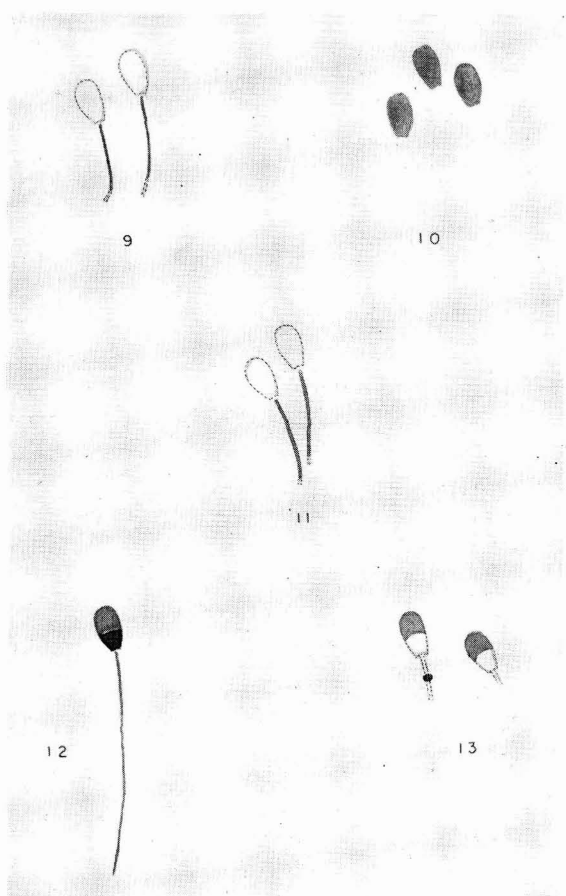
Explanation of figures

All figures are of bovine spermatozoa as observed in smears of semen. Drawn with an X95 objective and X10 ocular.

1 Spermatozoön stained for acid phosphatase. Smear incubated 48 hr.
2 Spermatozoön stained for acid phosphatase. Smear incubated 72 hr.
3 Spermatozoön stained for alkaline phosphatase. Smear incubated 48 hr.
4 Spermatozoön stained for alkaline phosphatase. Smear incubated 72 hr.
5 Spermatozoön stained for alkaline phosphatase. Smear incubated 96 hr.
6 Spermatozoa stained by Gram's method.
7 Spermatozoa stained for the Feulgen reaction.
8 Spermatozoa stained with toluidin blue.

nuclear cap of the head stained more intensely than the acrosome, with a faintly staining elliptical zone separating the two (fig. 1 and 2). The neck, middle-piece, main-piece and tip of the tail showed a positive reaction. The staining intensity of the spermatozoa was reduced with washing but the pattern of the response was not altered. A positive reaction was observed in the neck, middle-

PLATE 2



Explanation of figures

All figures are of bovine spermatozoa as observed in smears of semen. Drawn with an X95 objective and X10 ocular.

9 Spermatozoa stained by Baker's acid hematein method.
10 Spermatozoa stained by Baker's control method (pyridine extraction).
11 Spermatozoa stained with Sudan black B.
12 Spermatozoön stained by Bodian's protargol method.
13 Spermatozoa stained by the periodic acid-Schiff procedure.

piece and tail when adenosine triphosphate was used as a substrate at pH 5.0.

Alkaline phosphatase. Using sodium glycerophosphate as a substrate at pH 9.0, the acrosome stained more intensely than the post-nuclear cap. The middle- and main-pieces showed a positive reaction, but the neck remained unstained (fig. 3, 4, and 5). Seminal plasma was negative for this enzyme. There was no indication in the head of an unstained elliptical zone, as observed in the acid phosphatase reaction. As in the case of acid phosphatase, the staining intensity

of spermatozoa was reduced by washing, but the pattern remained the same. Hexose diphosphate (fructose-1,6-diphosphate) also was used as a substrate at pH 9.0 and a positive reaction was noted in the head, neck, middle-piece and tail. Furthermore, there was a slight staining in the seminal plasma with this substrate.

Gram's stain. Bovine spermatozoa were uniformly Gram positive throughout the head (fig. 6). There was no reaction in the seminal plasma.

Feulgen reaction. A uniformly positive reaction was obtained throughout the head and there was no line of demarcation between the acrosome and the post-nuclear cap (fig. 7).

Toluidin blue. The heads were stained throughout and there was no indication of a metachromatic reaction (fig. 8). In addition, there was no staining in the seminal plasma.

Baker's acid hematein test for phospholipids. The presence of phospholipid in the middle-piece was demonstrated by a positive reaction with this method (fig. 9). Preparations treated according to Baker's control procedure, consisting of pyridine extraction prior to staining, showed a uniform bluish-grey coloration throughout the head, whereas the middle-piece remained unstained (fig. 10), indicating the removal of the phospholipid fraction from this region. Seminal plasma was negative after Baker's acid hematein test, but the control preparations were characterized by a bluish-grey stippling.

Sudan black B. The presence of lipid in the middle-piece was demonstrated with this stain (fig. 11). The chief constituents of this region are generally believed to be of mitochondrial origin. It should be indicated, however, that the test was rather faint in all the preparations studied, as might be expected, since the total lipid content of moisture-free spermatozoa was 1.2 per cent according to Porter *et al.* (19). Furthermore, the seminal plasma showed a grey staining, indicating the presence of lipid material.

Bodian's protargol method. The heads of the spermatozoa were distinctly stained by this procedure. The acrosome was stained a uniform greenish-grey. The post-nuclear cap was heavily impregnated with silver and was separated from the acrosome by a narrow unstained elliptical zone. Furthermore, heads of spermatozoa observed in profile showed the lightly staining anterior portion, the unstained zone and the relatively dark posterior region. The neck did not stain, whereas the entire tail showed a faint positive reaction which became only slightly perceptible toward the distal end of the main-piece (fig. 12). Seminal plasma did not show a reaction with this method.

Periodic acid-Schiff reaction. The acrosome gave a weak reaction with the Schiff reagent after oxidation with periodic acid (fig. 13). There was no difference in the reaction of preparations treated with saliva or extracted with heated methanol and chloroform mixture and that of untreated cells, indicating absence of both glycogen and glycolipid. In addition, a mucopolysaccharide does not appear to be concerned in the reaction because of the absence of metachromatic reaction with toluidin blue. A slight positive reaction was obtained with seminal plasma. The cytoplasmic droplet occasionally found in varying positions on the tail also gave a consistently strong periodic acid-Schiff reaction (fig. 13).

Glycogen. The presence of glycogen in bovine spermatozoa could not be demonstrated by either the Bauer-Feulgen or periodic acid-Schiff reaction.

Schiff plasmal reaction. Bovine spermatozoa did not stain with this procedure.

DISCUSSION

Phosphatase reactions. In this study it was observed that when sodium glycerophosphate was used as a substrate at pH 5.0 the post-nuclear cap gave a more intense reaction than the acrosome, whereas at pH 9.0 the reverse was true. Since there was a reduction in phosphatase activity with washing, it appears that these enzymes are concentrated on the surface of the cell. However, this does not explain the apparent localizations of acid and alkaline phosphatase reactions, as shown in figures 1, 2, 3, 4 and 5. Since the acrosome and post-nuclear cap are of differing origins, as shown by Gatenby and Beams (5), it is likely that these coverings possess dissimilar molecular constitutions and, therefore, may exhibit different adsorptive capacities for various seminal constituents such as enzymes and substrates. It is of interest that when sodium glycerophosphate was used as a substrate at pH 5.0, the neck gave a positive phosphatase reaction, whereas when the same substrate was employed at pH 9.0 there was no localization of enzymatic activity in this region.

MacLeod and Summerson (16) demonstrated that human spermatozoa washed free of seminal plasma could not hydrolyze *beta*-glycerophosphate, glucose-1-phosphate, hexose-6-phosphate and fructose diphosphate but could act upon adenosine triphosphate. It was possible to confirm their finding in this investigation with a cytochemical procedure, as spermatozoa showed a positive reaction in the neck, middle-piece and tail when adenosine triphosphate was used as a substrate at pH 5.0. The importance of this phosphate in sperm metabolism is suggested by the work of Lardy *et al.* (12), who reported that epididymal spermatozoa incubated aerobically esterified inorganic phosphate to produce an ester which appeared to be adenosine triphosphate.

It is suggested that the enzymatic activity of bovine spermatozoa on hexose diphosphate (fructose-1,6-diphosphate) may in some way be related to the utilization of fructose by these cells. In a study of the fructose content of the semen of 36 bulls by Melampy *et al.* (18) it was found that the concentration of this sugar varied from 60 to 1,070 mg. per 100 ml. of semen, with an average of 510 mg. per 100 ml.

Wislocki (21) observed in the case of human spermatozoa a similarity in the staining pattern of acid phosphatase and the deposition of silver by Bodian's protargol method. This also was observed to be the case in the bovine, as shown in figure 12.

Gram's stain and Feulgen reaction. Bovine spermatozoa were Gram positive (fig. 6). In some cases washed sperm suspensions were treated with 0.1 *M* potassium iodide for 24 to 48 hr. before making smears, to study the effect of this salt on the staining capacity of the cells. It was observed after such treatment that the spermatozoa became Gram negative. Such Gram negative spermatozoa, however, showed a positive Feulgen reaction. This experiment suggests that the

ribonucleic acid present was extracted by the potassium iodide solution, whereas the desoxyribonucleic acid remained in the cell. The Gram positive reaction of bacteria according to Dubos (4) is due to the ribonucleic acid present in the cells.

Toluidin blue. Metachromatic components were absent from the head of bovine spermatozoa and seminal plasma. However, Wislocki (21) observed metachromasia as well as blue staining in human seminal plasma.

Lipids. Using Sudan black B for localization of lipid, a positive reaction was obtained in the middle-piece. It should be pointed out that Wislocki (21) observed a spiral arrangement of lipid material in the middle-piece of human spermatozoa, whereas in the same region bovine spermatozoa had a more uniform appearance (fig. 11.) Baker's acid hematein procedure was used for the localization of phospholipids as this method is considered specific for this type of lipid. Baker (1) observed the presence of phospholipid in the middle-piece of the mouse spermatozoön and Wislocki (21) confirmed this finding and reported a similar localization in human spermatozoa. Bovine spermatozoa also showed the same reaction for phospholipid, as shown in figure 9.

Periodic acid-Schiff reaction. This method was developed by McManus (17) and later critically evaluated from a chemical standpoint by Hotchkiss (11). According to Leblond (13), under the conditions that the method is used, it is likely that the substances detected are (a) glycogen, which may be removed from preparations by hydrolysis with salivary amylase, (b) mucoproteins and (c) such mucopolysaccharides as mucoitin sulphuric acid, heparin and hyaluronic acid. Gersh (6) has observed the presence of a glycoprotein in the Golgi apparatus of intestinal epithelium which gave a positive reaction.

In the present investigation a weak positive reaction was observed in the acrosome of ejaculated sperm with the Schiff reagent after periodic acid oxidation (fig. 13). This reaction probably is related to similar staining observed in preceding developmental stages of this cell. In a study of this reaction in bull testes, it was observed that the cytoplasm of the interstitial cells, walls of blood vessels, lamellated connective tissue and basement membrane of the seminiferous tubules gave a positive periodic acid-Schiff reaction. Within the seminiferous tubules the cytoplasm of Sertoli's cells gave a positive reaction. The development of the acrosome was traced back as far as the spermatocytes where periodic acid-Schiff positive material was found to occur in the region of the Golgi apparatus.

The cytoplasm of the spermatids gave a positive reaction and the acroblast was distinctly visible as a bright red bead. Subsequently, this bead spread over the nucleus to form a thin cap (acrosome), as described in man by Gatenby and Beams (5). This reaction showed that this cap covered the anterior two-thirds of the sperm head in accordance with the findings of Leuchtenberger and Schrader (14) for the bull. The presence of the periodic acid-Schiff positive bead and its spreading over the nucleus was comparable to that reported for the rat by Leblond (13). During the formation of the acrosome, there was an elongation of the nucleus and the cytoplasm passed toward the tail. This cytoplasmic mass carried with it the excess periodic acid-Schiff positive granules and

formed the so-called protoplasmic droplet, as shown in figure 13. It appeared that the amount of positive material produced by the cell was in excess to that needed for the production of the acrosome and this excess was discharged in the extruded cytoplasm. Experimental results of Leuchtenberger and Schrader (14) indicate a possible relationship between the hyaluronidase and the periodic acid-Schiff positive material of the acrosome.

SUMMARY

By using accepted cytochemical techniques the following reactions were observed in bovine spermatozoa: Acid phosphatase was present throughout with a slight reaction in the equatorial segment when sodium glycerophosphate was used as a substrate at pH 5.0. Alkaline phosphatase was present, with the exception of the neck, when the same substrate was employed at pH 9.0. Enzymatic activity was found in the neck, middle-piece and tail when adenosine triphosphate was used at pH 5.0. The occurrence of an enzyme acting upon hexose diphosphate (fructose-1,6-diphosphate) at pH 9.0 was noted in the head, neck, middle-piece and tail. Gram's stain and the Feulgen reaction were positive throughout the head, indicating the presence of both ribonucleic and desoxyribonucleic acids. Metachromatic staining was not obtained with toluidin blue, indicating the absence of mucopolysaccarides. Fat, including phospholipid, was localized in the middle-piece. The acrosome was characterized by a slight positive reaction with the periodic acid-Schiff reaction which was not due to glycogen, glycolipid or mucopolysaccharide. Furthermore, the protoplasmic droplet which occasionally was present on the tail gave an intense reaction with the periodic acid-Schiff procedure.

Seminal plasma gave the following reactions: Acid phosphatase was positive with sodium glycerophosphate as a substrate at pH 5.0 and alkaline phosphatase was characterized by a weak response with hexose diphosphate (fructose-1,6-diphosphate) at pH 9.0. Gram's stain and toluidin blue gave no reactions. Baker's acid hematein test for phospholipid was negative, but the control preparations were characterized by a bluish-grey stippling and a positive test was obtained with Sudan black B. No reaction was observed with Bodian's protargol method. A slight positive reaction was obtained with the periodic acid-Schiff reaction.

REFERENCES

(1) BAKER, J. R. The Histochemical Recognition of Lipine. Q. J. M. Sci., **87**: 441–470. 1946.

(2) BLOOM, M. L., AND WISLOCKI, G. B. The Localization of Lipids in Human Blood and Bone Marrow Cells. Blood, **5**: 79–88. 1950.

(3) DAWSON, A. B., AND BARNETT, JULIA. Bodian's Protargol Method Applied to other than Neurological Preparations. Stain Technol., **19**: 115–118. 1944.

(4) DUBOS, R. J. *The Bacterial Cell.* Harvard University Press, Cambridge. 1945.

(5) GATENBY, J. B., AND BEAMS, H. W. The Cytoplasmic Inclusions in the Spermatogenesis of Man. Q. J. M. Sci., **78**: 1–30. 1935.

(6) GERSH, I. A Protein Component of the Golgi Apparatus. Arch. Path., **47**: 99–109. 1949.

(7) GOMORI, G. Microtechnical Demonstration of Phosphatase in Tissue Sections. Proc. Soc. Exptl. Biol. Med., **42**: 23–26. 1939.

(8) GOMORI, G. An Improved Histochemical Technic for Acid Phosphatase. Stain Technol. **25**: 81–85. 1950.

(9) GUYER, M. F. *Animal Micrology*, 4th ed., University of Chicago Press, Chicago. 1936.

(10) HERMAN, E. Histochemical and Cytological Techniques. Department of Anatomy, Harvard Medical School, Boston. (Mimeographed) 1950.

(11) HOTCHKISS, R. D. A Microchemical Reaction Resulting in the Staining of Polysaccharide Structures in Fixed Tissue Preparations. Arch. Biochem., **16**: 131–141. 1948.

(12) LARDY, H. A., HANSEN, R. G., AND PHILLIPS, P. H. The Metabolism of Bovine Epididymal Spermatozoa. Arch. Biochem., **6**: 41–51. 1945.

(13) LEBLOND, C. P. Distribution of Periodic Acid-reactive Carbohydrates in the Adult Rat. Am. J. Anat., **86**: 1–50. 1950.

(14) LEUCHTENBERGER, C., AND SCHRADER, F. The Chemical Nature of the Acrosome in the Male Germ Cells. Proc. Nat. Acad. Sci., **36**: 677–683. 1950.

(15) LHOTKA, J. F., AND DAVENPORT, H. A. Differential Staining of Tissue in the Block with Picric Acid and the Feulgen Reaction. Stain Tech., **22**: 139–144. 1947.

(16) MACLEOD, J., AND SUMMERSON, W. H. The Phosphatase Activity of Human Spermatozoa. J. Biol. Chem., **165**: 533–539. 1946.

(17) MCMANUS, J. F. A. Histological Demonstration of Mucin after Periodic Acid. Nature, **158**: 202. 1946.

(18) MELAMPY, R. M., HOMEYER, P. G., AND PORTER, J. C. Fructose and Volume Relationships of Bovine Semen. Endocrinol., **49**: 596–600. 1951.

(19) PORTER, J. C., SHANKMAN, S., AND MELAMPY, R. M. The Chemical Composition of Bovine Spermatozoa. Proc. Soc. Exptl. Biol. Med., **77**: 53–56. 1951.

(20) WISLOCKI, G. B. Seasonal Changes in the Testes, Epididymides and Seminal Vesicles of Deer Investigated by Histochemical Methods. Endocrinol., **44**: 167–189. 1949.

(21) WISLOCKI, G. B. Cytochemical Reactions of Human Spermatozoa and Seminal Plasma. Anat. Rec., **108**: 645–662. 1950.

THE INTRACELLULAR PROTEINASES OF CERTAIN ORGANISMS FROM CHEESE AND THEIR RELATIONSHIP TO THE PROTEINASES IN CHEESE[1,2]

L. E. BARIBO[3] AND E. M. FOSTER

Department of Bacteriology, University of Wisconsin, Madison

In cheese ripening the breakdown of casein represents one of the most important changes. This breakdown has been attributed at one time or another to the natural proteinase in milk, to the proteinases in rennet, or to proteinases of microbial origin. In recent years new evidence has been presented to direct attention to the microbial proteinases. In studying the proteinases of cheddar cheese, Peterson *et al.* (10) found that the initial enzyme content was relatively low but it increased steadily as the cheese ripened. This increase could be produced only by microorganisms growing during the ripening period.

The predominance of *Streptococcus lactis* and *Lactobacillus casei* in cheese during manufacture and ripening has led to the belief that these species are important in casein breakdown and would therefore be the most probable sources of microbial proteinase. If this is true, the proteolytic enzymes extracted from cheese should possess, in some degree, the characteristics of the proteolytic enzymes from these organisms.

Peterson *et al.* (9) determined some of the characteristics of enzyme extracts from cheddar cheese. In the present investigation a study similar to theirs was done with extracts from 1-yr.-old cheddar cheese made from pasteurized milk and also with intracellular proteinases extracted from single strains of *S. lactis*, *L. casei* and *Micrococcus freudenreichii*. The latter organism was included because Alford and Frazier (1) reported that several strains of this species improved the flavor of cheese when they were added with the starter. Pasteurized milk was used for making the cheese to avoid complications caused by the natural milk proteinases and proteinases from a mixture of organisms such as would be found in cheese made from raw milk.

The characteristics of each of the microbial proteinases were compared with those of cheese extract. By this comparison it was hoped that information could be obtained on the role of each organism in casein breakdown during ripening.

EXPERIMENTAL METHODS

Organisms. *S. lactis* 171 and *L. casei* 142 were isolated from raw-milk cheddar cheese. They were selected from a group of 250 isolates of lactic rods and

Received for publication Aug. 20, 1951.

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

[2] Supported in part by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation and in part by funds supplied by the National Cheese Institute, Inc.

[3] Current address: The Western Condensing Co., Appleton, Wis.

cocci because of their ability to produce the greatest increase in nonprotein nitrogen (NPN) for the particular species when grown in milk containing $CaCO_3$. The streptococci were identified as *S. lactis* by the descriptions of Sherman (11) and Niven *et al.* (8). The lactobacilli were identified by reference to Breed *et al.* (4). *M. freudenreichii* 325 was obtained from Alford and Frazier (1).

Preparation of enzyme extracts. Large quantities of cells of *S. lactis* 171 and *L. casei* 142 were obtained by growing the organisms in carrot-liver extract broth of the following composition: 100 ml. liver extract, 100 ml. carrot extract, 5 g. glucose, 5 g. K_2HPO_4, 2.5 g. Difco neopeptone, 2.5 g. Difco peptonized milk, 1 g. lactose, 0.5 g. Tween 80 and 800 ml. distilled water. The cells of *M. freudenreichii* 325 were produced in a medium of the same composition with the exception that 1 g. of yeast extract replaced the Tween 80 and lactose. The media were adjusted to pH 6.8, dispensed in 16-l. quantities in pyrex carboys and autoclaved at 121° C. for 45 min. The carrot and liver extracts were prepared by the methods described by Barber and Frazier (2). Either pork or beef liver was extracted by heating in an autoclave to 121° C. as rapidly as possible.

Young broth cultures were used for inoculation at the rate of 1.25 per cent. All of the cultures were incubated at 30° C., *S. lactis* for 5 to 7 days, *L. casei* for 7 to 9 days and *M. freudenreichii* for 3 days. The cultures were neutralized with sterile 2 *N* NaOH as often as necessary to maintain a pH above 6.0.

The cells were collected in a Sharples Super Centrifuge operated at 35,000 to 40,000 r.p.m. They were washed with distilled water by mixing in a Waring blendor and again collected in the Sharples Super Centrifuge. The washing was repeated four or five times. The cells were stored at 5° C. and used within 5 days.

Enzyme extracts of *S. lactis* 171 and *L. casei* 142 were prepared as follows: Cell paste was mixed with glass glow beads at the rate of 1 g. of paste to 20 g. of beads. Approximately 210 g. of this mixture were placed in a 250-ml. centrifuge bottle; the air was replaced by flushing with CO_2 and the bottle was closed with a rubber stopper. The mixture was shaken for 15 min. at 475 strokes per minute in a mechanical shaker. This disrupted about 98 per cent of the cells. The mixture was kept cold, but not frozen, with dry ice. After shaking, 2 ml. of distilled water per gram of cell paste were added and the mixture was placed in the refrigerator for 24 hr. The extract then was removed from the beads by centrifugation. It was kept under toluene at 5° C. and used within 2 days.

The cells of *M. freudenreichii* were disintegrated with a Booth-Green mill (3) in a cold room. One ml. of distilled water was added per gram of cell paste and the mixture was ground for 60 min. The resulting mixture was diluted to approximately 4 ml. per gram of paste with distilled water. Intact cells were removed by centrifugation at 3,500 to 4,000 r.p.m.

Cheese enzyme extracts were prepared by the method of Peterson *et al.* (9) with the following exceptions: A 1 : 5 dilution of the cheese was prepared instead of 1 : 10 and the extracts were further purified by filtering through coarse filter paper, then through a Seitz filter to remove the bacteria. This filtration was necessary to avoid contamination in the reaction mixtures. Contamination was

not a problem for Peterson *et al.* because they used a short incubation time at a fairly high temperature.

Preparation and analysis of reaction mixtures. Difco sodium caseinate prepared according to the method of Peterson *et al.* (9) was used as the substrate. Foaming was reduced by adding 1 ml. of octyl alcohol to the mixture in the Waring blendor. The casein suspension was adjusted to pH 5.1. This value also was used for the complete reaction mixtures because it is near the pH of the cheese and also is close to the reaction used by Peterson *et al.*

The reaction mixture consisted of 2.5 ml. of substrate, 0.5 to 2.0 ml. of enzyme extract, 0.5 ml. of buffer, 0.5 ml. of reducing agent, 0.5 ml. of metal activator and 0.1 ml. of toluene. The volume was adjusted to 6.1 ml. with distilled water. Unless otherwise specified, 0.1 *M* acetate solution adjusted to pH 5.1 was used as the standard buffer, 0.1 *M* cysteine hydrochloride as the standard reducing agent, and 0.1 *M* calcium lactate as the standard metal ion activator in the reaction mixture. The enzyme extract steamed for 5 min. was used as a control.

The reaction mixture was shaken approximately 20 times, then incubated in stoppered tubes at 30° C. for 36 hr. or at 11° C. for 7 days. One-ml. aliquots were taken for NPN determinations immediately before and after incubation. An increase in NPN represented casein hydrolysis and therefore the amount of proteinase activity. Duplicate determinations were made for each reaction mixture and the values were averaged.

The NPN was determined by precipitating the protein with trichloroacetic acid and analyzing the protein-free filtrate for total Kjeldahl nitrogen. For this purpose, 1 ml. of the reaction mixture was mixed thoroughly with 19 ml. of 0.3 *M* trichloroacetic acid. The mixture was filtered through paper and analyzed for nitrogen by the colorimetric micro-Kjeldahl method described by Johnson (7) with the following modifications: After addition of the acid digestion mixture, the samples were placed in an oven at 110° C. for 12 to 18 hr. to remove the excess water. The samples were kept in a hot sand bath for the necessary time, as determined by inspection. Also, the digestion was aided by the addition of one drop of superoxal per tube.

The micro-Kjeldahl method was reported by Johnson (7) to have a 3 per cent error between duplicate samples. On this basis analyses showing 45 γ of nitrogen per sample were considered to have an error of 1.35 γ. When calculated on the basis of the results reported here, differences of more than 0.09 mg. of NPN per milliliter of enzyme extract were significant.

The formula for calculating the data is as follows:

$$\frac{\text{Increased NPN in } \gamma \times \text{dilution with trichloroacetic acid} \times \text{ml. of reaction mixture}}{\text{Ml. of enzyme extract in reaction mixture} \times \text{ml. of trichlororoacetic acid dilution analyzed} \times 1{,}000} = \text{NPN in mg. per ml. of enzyme extract}$$

RESULTS

Effect of temperature of incubation on enzyme activity. The first characteristic of the enzyme extracts to be determined was the effect of temperature on activity. Figure 1 shows that the enzyme extract from year-old cheddar cheese

was most active above 45° C. However, there was a secondary optimum between 11 and 18° C., showing that there were at least two enzymes in the cheese extract. By extrapolating the line between 30 and 23° C. it can be shown that the enzyme with the higher temperature optimum would have relatively less activity at cheese ripening temperatures than would the enzyme with the lower optimum. Therefore, on the basis of temperature, the enzyme with the lower optimum might be considered the more important in casein breakdown.

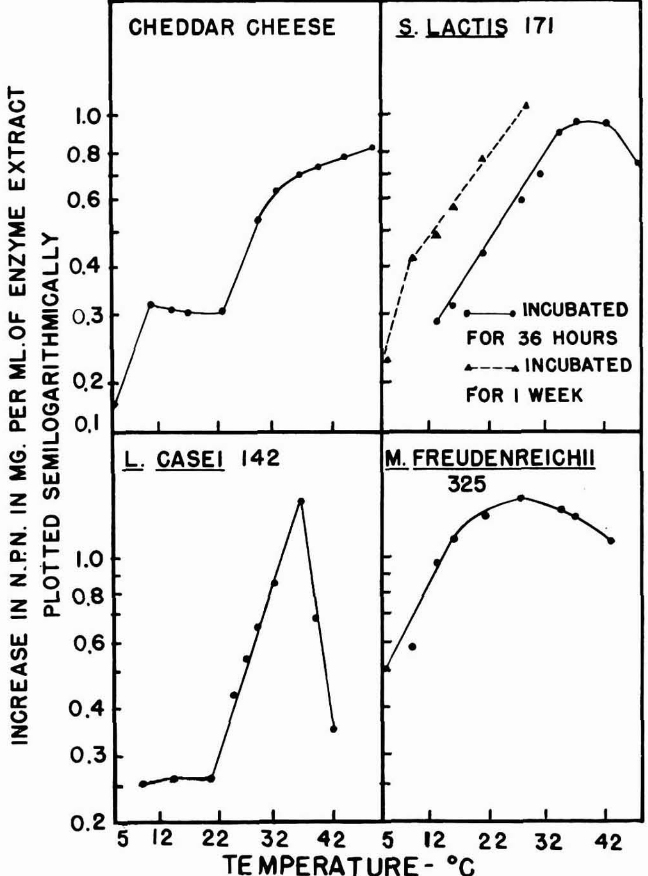


FIG. 1. Effect of temperature of incubation on activity of the proteinases.

It should be pointed out that Peterson *et al.* (9, 10) determined proteolytic activity of cheese extracts at 40° C. With a mixture of enzymes such as those present in cheese, each having a different temperature optimum, the activity occurring at high temperatures does not necessarily reflect that which occurs under cheese ripening conditions. Therefore, in this study the activity was determined at both 11 and 30° C. in most of the experiments with enzyme preparations that showed evidence of having two temperature optima.

The activity curve for the enzyme extract of *L. casei* 142 resembled that of the cheese extract in that both showed a low-temperature optimum as well as greater activity at a higher temperature. The rapid decrease in activity of the *L. casei* extract above 37° C., however, shows that enzymes from other sources were active in the cheese extract. Even so, the similarity in the two curves below 37° C. is somewhat surprising in view of the fact that the cheese was made from pasteurized milk and therefore would not be expected to contain appreciable numbers of *L. casei.*

The enzyme extract of *S. lactis* 171 was most active at 40 to 42° C. Thus, it might account in part for the proteolytic activity of the cheese extract at the higher temperatures. The *S. lactis* extract did not clearly show two temperature optima, although after long incubation of the low temperature samples there was a change in the slope of the line, indicating a secondary optimum similar to that of the cheese extract. Although the curve for the *S. lactis* enzyme extract does

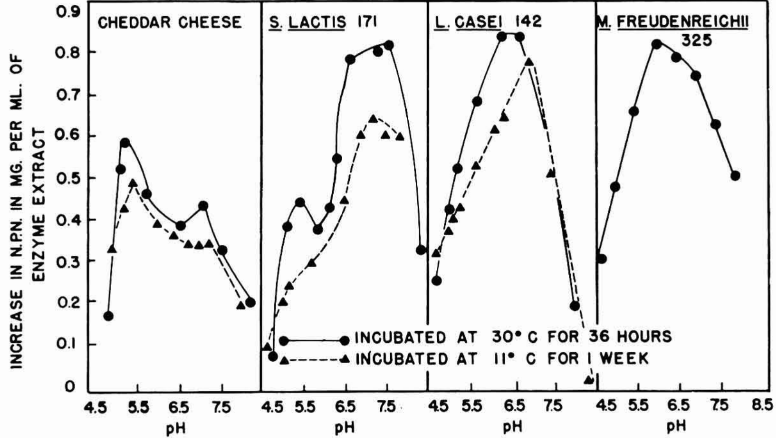


FIG. 2. Effect of pH of incubation on activity of the proteinases.

not resemble that of cheese, it can be seen that considerable activity would occur at cheese ripening temperatures. *M. freudenreichii* 325 showed only one optimum, at 30° C. If large numbers of this organism were present in cheese they also might contribute significantly to casein breakdown. Because there was no indication of a secondary temperature optimum, further tests with this organism were run only at 30° C.

Effect of pH on enzyme activity. The pH of the reaction mixture was regulated by use of a composite 0.5 *M* acetate, 0.5 *M* phosphate and 0.5 *M* borate buffer adjusted to the desired pH with 2 *N* NaOH or 2 *N* H_2SO_4. The data in figure 2 show the effect of pH on activity of the enzyme extracts at 11 and 30° C. Generally, there was little difference between the samples incubated at these two temperatures. It was expected that the two enzymes indicated by the temperature optima might have different pH optima. This then would be expressed by different pH activity curves at 11 and 30° C. It has been found throughout this

study, however, that with few exceptions the characteristics of the enzyme extracts were similar when incubated at either 11 or 30° C. The data show that enzyme extracts from the year-old cheddar cheese were most active at pH 5.0 to 5.5. A secondary optimum for activity occurred near neutrality, indicating at least two enzymes based on pH optima. These data are similar to those reported by Peterson *et al.* (9).

By contrast, the microbial enzyme extracts were most active at pH values near neutrality and only one, *S. lactis* 171, showed evidence of a secondary optimum near pH 5.0 to 5.5. This dissimilarity in the appearance of the curves for the cheese and microbial extracts is difficult to understand in terms of information now available, but it suggests that cheese must contain enzymes from sources other than microorganisms like those used in this study to account for the activity in the cheese extracts at pH 5.0 to 5.5. Davis and Davies (5) reported that rennet contains, in addition to pepsin, proteolytic enzymes with pH optima at 4.7 and 6.2, which could account for the enzymes in the cheese extract with the optimum pH in the acid range. In view of the results reported here, however, this explanation could be acceptable only if it is assumed that microbial enzymes with pH optima near neutrality are first formed in the cheese but later are inactivated during ripening. There is no evidence for this assumption.

Peterson *et al.* (10) reported a relatively low proteinase content in cheese in the early stages of ripening, as compared with that after several months. From their data it appeared that the contribution of rennet to casein breakdown was negligible compared to that of the enzymes of microbial origin. From the evidence, then, it must be assumed that the organisms which produced the proteinases measured by Peterson *et al.* were different than the ones used in this study, otherwise the activity would have been greater near neutrality than at pH 5.0 to 5.5.

Stability of the proteinases. For determination of the pH stability, the enzyme extracts were adjusted to pH values from 3.4 to 8.6 and incubated at 30° C. for 50 hr. After incubation, the enzyme was mixed with the remainder of the reaction mixture containing 0.5 ml. of 1 *N* acetate buffer adjusted to pH 5.1. The final pH of the reaction mixture was 5.1 ± 0.05. Activity was determined at 30° C. The proteinase in each of the enzyme extracts was relatively stable over a considerable pH range, including the pH values normally found in cheese.

The heat stability of the enzyme extracts was determined by heating them at pH 5.1 and 60° C. for different lengths of time. A heated control, which was autoclaved for 10 min., and an unheated control were used for comparison. Activity was measured at 11 and 30° C. The enzyme extract obtained from the year-old cheddar cheese consisted entirely of heat-stable proteinase, as determined at both 11 and 30° C. (fig. 3). The extract of *S. lactis* 171 incubated at 11° C. also was heat-stable, but the same extract incubated at 30° C., appeared to consist of both heat-stable and heat-labile enzymes. The extract of *L. casei* 142 showed heat-stable and heat-labile fractions at both 11 and 30° C. incubation. The proteinase of *M. freudenreichii* 325 was heat-labile.

These data suggest that the heat-labile fraction of the microbial extracts, if

ever present in cheese, is destroyed during ripening. Further investigations of month-old cheddar cheese made with a commercial starter showed that it also did not contain heat-labile proteinases. However, with lots of cheese in which 0.25 per cent each of *S. lactis* 171 and *L. casei* 142 were added with the regular com-

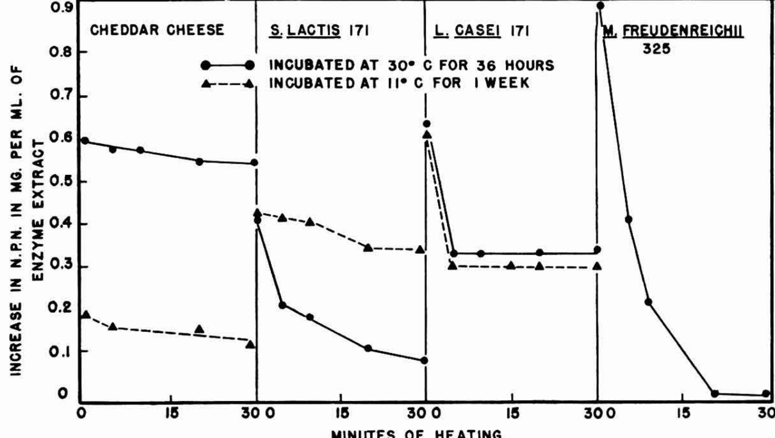


FIG. 3. Effect of time of heating at 60° C. on activity of the proteinases.

mercial starter, the product at 1 mo. contained both heat-stable and heat-labile enzymes. In this cheese it is evident that the enzymes of *S. lactis* 171 and *L. casei* 142 were detected. However, it raises a question as to whether the heat-labile fraction is ever present normally in cheese.

Effect of reducing agents on enzyme activity. One-half-ml. amounts of 0.1 *M* solutions of various reducing agents were added to the reaction mixtures. Ac-

TABLE 1

Effect of reducing agents on the activity of proteinases from cheddar cheese and from microorganisms

Reducing agent	Increase in NPN in mg. per ml. of enzyme extract at the temperatures indicated						
	Cheese		*S. lactis*		*L. casei*		*M. freudenreichii*
	11° C.	30° C.	11° C.	30° C.	11° C.	30° C.	30° C.
None	0.26	0.18	0.22	0.64	0.29	0.34	0.12
Cysteine HCl	0.61	0.31	0.54	0.95	0.45	1.08	0.70
Thioglycollate	0.42	0.32	0.75	1.10	0.35	0.99	0.20
Na_2SO_3	0.37	0.36	0.49	1.09	0.42	1.14	0.07
Ascorbic acid	0.49	0.23	0.77	1.19	0.35	0.63	0.92
KCN	0.44	0.23	1.02	1.50	0.74	0.94	....

tivity at pH 5.1 was measured at 11 and 30° C. The enzyme extract to be tested for activity was prepared without the usual precautions of maintaining anaerobic conditions, *i. e.*, without preparing and storing under CO_2. Table 1 shows that all of the enzyme extracts were activated by reducing agents, although there was

much variation between the reducing agents with different enzyme extracts. Peterson *et al.* (10) noted a greater amount of cysteine-activated proteinase in raw-milk cheddar cheese than in pasteurized-milk cheese and suggested that cysteine-activated proteinase was of microbial origin. Judging from the results in table 1, the organisms used in this study could have contributed to the cysteine-activated proteinase found in cheese.

TABLE 2

Effect of buffers on the activity of proteinases from cheddar cheese and from microorganisms

Buffer	Increase in NPN in mg. per ml. of enzyme extract at the temperatures indicated						
	Cheese		*S. lactis*		*L. casei*		*M. freudenreichii*
	11° C.	30° C.	11° C.	30° C.	11° C.	30° C.	30° C.
Acetate	0.39	0.74	0.39	0.70	0.51	1.44	0.44
Phosphate	0.29	0.69	0.31	0.65	0.31	1.02	0.68
Phthalate	0.15	0.48	0.34	0.61	0.47	0.93	0.70
Citrate	0.24	0.51	0.32	0.51	0.59	0.97	0.53

Effect of buffers on enzyme activity. Several compounds with buffering capacity near pH 5.1 were tested for their effect on the enzyme extracts. One-half ml. of a 0.1 *M* solution of the buffer adjusted to pH 5.1 with 2 *N* H_2SO_4 was added to the reaction mixture. Activity was determined at both 11 and 30° C. Table 2 shows that acetate generally was the best buffer, except for the enzyme

TABLE 3

Effect of metal ions on the activity of proteinases from cheddar cheese and from microorganisms

Metal ion	Increase in NPN in mg. per ml. of enzyme extract at the temperature indicated						
	Cheese		*S. lactis*		*L. casei*		*M. freudenreichii*
	11° C.	30° C.	11° C.	30° C.	11° C.	30° C.	30° C.
None	0.31	0.49	0.24	1.13	1.04	0.85	0.57
Ca^{++}	0.28	0.54	0.28	0.92	1.06	1.01	0.53
Mn^{++}	0.23	0.43	0.20	0.92	0.70	0.78	0.45
Mg^{++}	0.31	0.51	0.10	0.63	0.61	0.81	0.54
Cu^{++}	0.35	0.31	0.21	0.96	0.37	0.45	0.12
Zn^{++}	0.29	0.46	0.04	0.83	0.76	0.71	0.08
Co^{+++}	0.31	0.51	0.11	0.99	0.67	0.92	0.55
Fe^{++}	0.32	0.52	0.19	1.02	0.96	0.82	0.54
Fe^{+++}	0.30	0.52	0.30	0.80	0.99	0.54	0.54

extract of *M. freudenreichii* 325. For this organism, phosphate and phthalate appeared to be superior. Peterson *et al.* (9) also found acetate to be the most effective buffer for cheese extracts. There was no consistent difference between the samples of the enzyme extracts incubated at 11 and 30° C. in so far as their activity in the different buffers is concerned.

Effect of metal ions on enzyme activity. Preparations of the enzyme extracts were tested for activation by different metal ions. One-half-ml. amounts

of the following solutions were used: 0.1 *M* $Ca(C_3H_5O_7)_2 \cdot 5H_2O$, $FeCl_3 \cdot 6H_2O$, $FeSO_4 \cdot 7H_2O$, $MnSO_4 \cdot 4H_2O$, $MgSO_4 \cdot 7H_2O$, and $ZnSO_4 \cdot 7H_2O$, and 0.01 *M* $CuSO_4 \cdot 5H_2O$ and $CoCl_3$. Activity at pH 5.1 was determined at 11 and 30° C. For the most part, the metal ions either had no effect or were inhibitory (table 3). In the initial trials, which were made with *L. casei* extract, calcium was slightly stimulatory; hence it was used in the standard reaction mixture. There was no consistent difference between samples incubated at 11 and 30° C. in so far as their activity in the presence of different metal ions is concerned.

The sodium salts of the anions chloride, sulfate and lactate had no effect on activity of the enzyme preparations.

Effect of rennet plus microbial enzymes on casein breakdown. Davis *et al.* (6) showed a much greater increase in nonprotein nitrogen on prolonged incubation of cultures of lactic bacteria in skimmilk containing sterile rennet than in similar cultures without rennet or in the milk containing rennet but no bacteria. Their results might be interpreted in either of two ways: (a) that the products of rennet action stimulated growth of the lactic acid bacteria, thus causing more bacterial enzymes to be produced; or (b) that the bacterial enzymes released on autolysis acted more readily on the products of rennet action than on the whole proteins of the milk. Both of these explanations have been advanced from time to time to account for the proteolytic activities occurring in cheese. There is no doubt that the first can be true, but, as far as is known, there are no published results of critical experiments done to test the second. If it is true that the products of rennet action are more suitable substrates for proteinases of the lactic acid bacteria, then greater casein breakdown should result with a mixture of rennet (A) and microbial enzyme (B) than the sum of their separate actions.

To test this possibility, the amount of casein degradation products formed by a mixture of A and B was compared with the sum of the products formed by the same concentrations of A and B tested separately. The products were measured as trichloroacetic acid-soluble and as acetic acid-soluble nitrogen. Activity at pH 5.1 was measured at 11 and 30° C. Using each of the microbial enzyme extracts as B, it was observed that a mixture of A and B, without exception, caused no more casein breakdown than the total for A and B determined separately. In fact, the value for the mixture was always slightly less, although the difference usually was not significant. This was true whether the products were measured as trichloroacetic acid- or as acetic acid-soluble nitrogen. Thus, as far as the organisms used in this study are concerned, the products of rennet action are not a more suitable substrate for the bacterial proteinases than is casein.

DISCUSSION

It is too much to hope that a study of this type with only three strains of bacteria could explain the complex proteolytic changes that occur in cheese and the role of bacteria therein. The proteolytic activity in cheese is the result of the action of several enzymes. Not only are these from different sources, such as rennet and a variety of microbial species, but also from each type of organism may come an entire system of proteolytic enzymes. With such a mixture, the

activity resulting under a given set of conditions may be such that certain enzymes, if not denatured, are relatively inactive. Therefore any combination of temperature, pH, buffers, reducing agents, metal ions, etc. selects those enzymes whose activity is measured. At the present state of knowledge it is impossible to duplicate in the test tube under controlled conditions the environment that exists in cheese. Complicating any attempt to do this is the fact that the environment in cheese changes from day to day.

Even though it is simple to control the temperature, pH, buffers, reducing agents and other ingredients of an enzyme reaction mixture in a test tube, there are other factors not so easily controlled which may be important in regulating the activity of specific enzymes in cheese. For example, the products of one enzyme may become the substrate for another, resulting eventually in the complete breakdown of casein to amino acids and ammonia.

In this study there was considerable similarity between the characteristics of the cheese enzyme extracts and the microbial extracts. All of the microbial extracts were fairly active at the normal temperature of cheese ripening. They also resembled the cheese extracts in that they were affected by the same buffers, metal ions and reducing agents.

There were, however, two major differences between the cheese extracts and those of the organisms. These differences were in the pH for optimum activity and in the heat stability. From the pH-activity curves (fig. 2), a large part of the proteinase extracted from the cheese apparently did not come from organisms like those used in this study. If it had, the cheese extract would have been more active near pH 7.0. There are two possible sources of the enzymes most active at pH 5.0 to 5.5. One is rennet, the other is organisms similar to those used here but whose proteolytic enzymes have lower pH optima. Tarnanen (12) showed that extracts of *Bacterium casei* were most active at about pH 6.0 when incubated at 42° C. This is slightly lower than the optimum shown by *L. casei* extracts used in this study (fig. 2) but still is not low enough to account for the optimum of pH 5.0 to 5.5 possessed by the cheese extracts, even if it were assumed that *L. casei* grew in the cheese made from pasteurized milk. Whether there are other lactic acid organisms whose enzyme extracts show lower pH optima is undetermined. It should be emphasized that the results of this study represent only two strains of the lactic acid bacteria that generally are considered to function in cheese ripening and a strain of a *Micrococcus* species that has been suggested as a possible agent in flavor production during ripening. The latter organism is not commonly present in large numbers in cheese and would not be expected to have much function in protein breakdown.

It also is possible that organisms such as those used in this study grow in cheese and release their enzymes, which are active for a period of time and then are inactivated. This would leave the enzymes from rennet as the main proteolytic agents in an old cheese—providing they were not also inactivated—and could explain the low pH optimum observed for the extract of the year-old cheese used in this study. Experiments with cheeses of different ages might confirm or deny this suggestion. It should be re-emphasized that the extracts of

the organisms used in this study were relatively stable at the pH of cheese for a period of 50 hr. at 30° C. No information is available, however, on their stability over a period of weeks or months.

Although the temperature curve for cheese extract indicated the presence of at least two enzymes with different temperature optima, the differences in pH for optimum activity, pH and heat stability, and other characteristics on incubation at 11 and 30° C. were slight. This likewise was true of the extract of *L. casei.* It is difficult to conceive that the properties of two enzymes with widely separated temperature optima would be so much alike in other characteristics. Unfortunately it is impossible at this stage of investigation to state with certainty what part, if any, *S. lactis* and *L. casei* play in casein breakdown during ripening of cheddar cheese. Perhaps further work with other strains of these organisms and with cheeses of different ages will help to answer part of the questions that remain.

Some of the results of this study are difficult to reconcile with those of Peterson *et al.* (10). The large increase in the proteinase content of cheese that these investigators reported could be caused only by growth of microorganisms. Since they made their activity tests at 40° C., however, the increase that they measured may be of less significance than would be inferred from their results. Proteinase analyses of 15 samples of cheese varying in age from 1 to 24 mo. were made in this laboratory by following their method closely. The values obtained were only about one-tenth as high as the ones they reported. Also the increase in proteinase content of cheese up to 8 mo. of age was considerably less than they reported. It appears that further studies with other cheeses are necessary to explain these differences.

SUMMARY AND CONCLUSIONS

Some of the characteristics of the intracellular proteinases of one strain each of *L. casei, S. lactis* and *M. freudenreichii* were determined. The characteristics of the microbial enzymes were compared with those of the proteolytic enzymes extracted from a year-old cheddar cheese. From this comparison it was concluded that the organisms used in this study possessed enzymes which could account for only a part of the proteinases found in the cheese. Enzymes from other sources are necessary to account for the remainder. It is suggested that rennet may provide part or all of the other proteolytic enzymes.

REFERENCES

(1) ALFORD, J. A., AND FRAZIER, W. C. Effect of Micrococci on the Development of Flavor when Added to Cheddar Cheese Made from Pasteurized Milk. J. Dairy Sci., **33**: 115–120. 1950.

(2) BARBER, F. W., AND FRAZIER, W. C. Dissociants of Lactobacilli. J. Bact., **50**: 637–649. 1945.

(3) BOOTH, V. H., AND GREEN, D. E. A Wet-crushing Mill for Micro-organisms. Biochem. J., **32**: 855–861. 1938.

(4) BREED, R. S., MURRAY, E. G. D., AND HITCHENS, A. P. *Bergey's Manual of Determinative Bacteriology.* 6th ed. Williams and Wilkins Co., Baltimore, Md. 1948.

(5) DAVIS, J. G., AND DAVIES, W. L. The Nature of the Proteolytic Enzymes in Rennet. J. Soc. Chem. Ind., **53**: 1083. 1934.

(6) DAVIS, J. G., DAVIES, W. L., AND MATTICK, A. T. R. Studies in Cheddar Cheese. VI. The Degradation of Milk Proteins by Lactic Acid Bacteria Isolated from Cheese, Alone, with Sterile Rennet and with Whole Rennet. J. Dairy Research, **8**: 238–244. 1937.

(7) JOHNSON, M. J. Isolation and Properties of a Pure Yeast Polypeptidase. J. Biol. Chem., **137**: 575–586. 1941.

(8) NIVEN, C. R., SMILEY, K. L., AND SHERMAN, J. M. The Hydrolysis of Arginine by Streptococci. J. Bact., **43**: 651–660. 1942.

(9) PETERSON, M. H., JOHNSON, M. J., AND PRICE, W. V. Determination of Cheese Proteinase. J. Dairy Sci., **31**: 47–54. 1948.

(10) PETERSON, M. H., JOHNSON, M. J., AND PRICE, W. V. Proteinase Content of Cheddar Cheese during Making and Ripening. J. Dairy Sci., **31**: 55–61. 1948.

(11) SHERMAN, J. M. The Streptococci. Bact. Rev., **1**: 3–97. 1937.

(12) TARNANEN, J. Über die proteolytischen Enzyme der Milchsäurebakterien. Akademische Abhandlung. Univ. of Helsinki, Finland. 1930.

OBSERVATIONS ON THE TACTUAL FLAVOR QUALITIES OF HEATED MILK[1, 2]

STUART PATTON AND DONALD V. JOSEPHSON

Department of Dairy Husbandry, The Pennsylvania Agricultural Experiment Station, State College, Pa.

Many of the off-flavors of milk have been given comprehensive study. However, the literature pertaining to tactual flavor defects of beverage milk is limited. Interest in this subject has increased materially as a result of the armed forces' unfavorable experience with reconstituted dry milk during World War II. The tactual flavor qualities of this product have been characterized by the terms "chalky," "rough," "powdery," "astringent," etc. A similar defect may be noted consistently in evaporated milk. According to Trout (4), homogenized milk occasionally is criticized as being chalky. Such a defect seems to be rarely encountered in fluid raw or pasteurized milks.

Preliminary observations have suggested that heat treatment of milk is a principal factor in the development of tactual flavor defects (2). The purpose of this study was to investigate the significance of heat treatment and to determine if possible what milk constituents are involved in production of such defects.

EXPERIMENTAL

Sample preparation: Fresh raw milk, adjudged to be of good quality, was secured each morning of the day tasting sessions were held. In order more or less completely to de-fat the milk, it was separated in a DeLaval E 19 separator at a temperature of 30° C. The various samples for tasting were made from the raw skimmilk as follows: (a) *Raw skimmilk:* No further treatment. (b) *Raw whey:* Raw skimmilk was set with rennet at the rate of 4 oz. per 100 gal. of skimmilk at a temperature of 30° C. The curd was cut after 30 min. and heated for an additional 30 min. at 46 to 48° C. to expel the whey. Following this, the whey was drained off and filtered through a Buchner filter with vacuum, employing no. 1 filter paper. (c) *Heated skimmilk:* Raw skimmilk was heated momentarily to 95° C. in a Pyrex beaker over a direct flame. To prevent "burning-on," a mechanical glass agitator with adjustable speed was used. Time to come up to temperature was standardized at approximately 12 min. The sample was cooled immediately to 20° C. by means of a water bath. Further cooling was accomplished in a refrigerator. (d) *Heated whey:* Raw whey was processed using the same procedure as for heated skimmilk. (e) *Centrifuged heated skimmilk:* Raw skimmilk was heated in the usual manner (95° C.). While still hot, the skimmilk was clarified by passing it through a Sharples super-centrifuge at 35,000

Received for publication Aug. 23, 1951.

[1] Portions of this research were performed by the authors while in residence at the Dairy Technology Department, Ohio State University, Columbus.

[2] Authorized for publication as paper no. 1683 on August 8, 1951 in the Journal Series of The Pennsylvania Agricultural Experiment Station.

rpm. Following clarification, the skimmilk was cooled immediately in a water bath to 20° C. and further cooled in a refrigerator. (f) *Centrifuged heated whey:* Raw whey was heated and centrifuged according to the procedure described for centrifuged heated skimmilk. (g) *Thirty-min. centrifuged heated skimmilk:* Treatment was the same as for centrifuged skimmilk, except that a batch centrifuge bowl was used and centrifuging at 35,000 rpm. was continued for a period of 30 min. (h) *Thirty-min. centrifuged heated whey:* This procedure was the same as for 30-min. centrifuged, heated skimmilk.

Tasting procedure: The tasting was performed by five trained observers. Tasting sessions were held from 2 to 5 p.m. During the period of the taste tests only one observer[3] at a time, a person to present samples and a data recorder were permitted in the room. The length of the taste session for any one O was limited to 30 min. and the number of presentations to 24. While tasting, the O would sit relaxed in a chair. The samples were maintained at 37° C. in a constant temperature bath out of sight of the O. All samples were tasted by the O without knowledge as to sample makeup or treatment. Each O was instructed to give "a complete qualitative description" of his taste responses to each sample. The samples were presented using the aspersion technique. Essentially, this consisted of drawing up 3 ml. of sample into a 3-ml. pipette by means of an attached rubber bulb, then spraying the sample onto the anterior two-thirds of the O's tongue. Cool tap water was used for the purpose of rinsing the mouth. The samples were presented in random order.

TABLE 1

Tactual flavor responses to skimmilk and its rennet whey[a]

Flavor response	Raw skimmilk	Raw whey	Heated skimmilk	Heated whey
	(%)	(%)	(%)	(%)
Rough	15.5	5.9	42.2	57.0
Slightly rough	19.3	28.2	20.0	17.8
Smooth	28.1	22.2	8.1	5.2

[a] 135 presentations of each sample treatment.

RESULTS

Although gustatory and olfactory responses to each sample presentation were recorded, only the tactual responses are presented here, since these are most pertinent to the present study. In addition, the term "rough" has been used throughout to denote the many terms used to characterize the tactual defect(s). Although this term may oversimplify the phenomena involved, the purpose has been to make a clear contradistinction to "smoothness."

The tactual flavor qualities of raw and heated skimmilk were compared with those of raw and heated whey. A compilation of results pertaining to these experiments is presented in table 1. It will be noted that the total of tactual flavor responses for any sample reported in the tables does not equal 100 per cent.

[3] Hereinafter referred to as O.

This resulted from failure of the O's to report tactual observations after every sample presentation. These data demonstrate that heating skimmilk or rennet whey samples momentarily to 95° C. materially increases the number of rough taste responses to such samples and lowers the number of smooth responses. The data also infer that casein does not contribute to this heat-generated flavor defect.

The heating of rennet whey was observed to produce a considerable amount of precipitated material. The possibility that this material contributes to the

TABLE 2

The effect of centrifugation on the tactual flavor qualities of heated whey[a]

Flavor response	Raw whey	Heated whey	Centrifuged heated whey	30-min. centrifuged heated whey
	(%)	(%)	(%)	(%)
Rough	0.0	66.7	22.2	8.9
Slightly rough	14.5	8.9	30.0	30.0
Smooth	41.1	3.3	11.1	20.0

[a] 90 presentations of each sample treatment.

tactual flavor defect was investigated through the effect of its removal by super-centrifugation from heated whey. The data in table 2 clearly reveals that the flocculated material in heated whey contributes to roughness of the product. Clarification alone significantly reduced the number of rough responses and increased the number of smooth responses to the heated whey. This trend was amplified by batch centrifugation of the whey for 30 min.

In order to confirm further that the flocculated material from heated whey is responsible for the tactual flavor defect, residue deposited on the centrifuge bowl

TABLE 3

The effect of incorporating centrifuge residue from heated whey into raw whey upon its tactual flavor qualities[a]

Flavor response	Raw whey	Raw whey plus residue	Heated whey	Centrifuged heated whey
	(%)	(%)	(%)	(%)
Rough	0.0	76.6	80.0	23.3
Slightly rough	23.3	6.7	0.0	26.7
Smooth	36.7	0.0	0.0	3.3

[a] 30 presentations of each sample treatment

from heated whey was redispersed in raw whey. Tactual responses of the taste group to this and related samples are given in table 3. These data also show the heat-coagulable whey constituents are of primary importance in the tactual flavor defect. Raw whey to which no centrifuge residue had been added elicited few rough and a considerable number of smooth responses from the taste group. Raw whey containing the centrifuge residue was distinctly rough tasting and produced no smooth responses from the group. An additional trial in which the

centrifuge residue from heated whey was incorporated in raw skimmilk gave results essentially the same as those obtained with raw whey containing the residue.

The question arose as to whether substances responsible for roughness could be removed from heated skimmilk in the same manner as was found possible with heated whey. It may be seen from table 4 that the substances responsible for roughness are not nearly so readily removed from heated skimmilk as from heated whey. Centrifuging the heated skimmilk for 30 min. did not materially reduce the number of rough responses, although the data indicate the degree of roughness was diminished. As suggested previously (3), casein appears to behave in the manner of a protective colloid toward the heat-coagulable whey proteins. This may account for the observed difficulty in removing the substances responsible for roughness from heated skimmilk.

TABLE 4

The effect of centrifugation on the tactual flavor qualities of heated skimmilk[a]

Flavor response	Raw skimmilk	Heated skimmilk	Centrifuged heated skimmilk	30-min. centrifuged heated skimmilk
	(%)	(%)	(%)	(%)
Rough	3.3	50.0	40.0	26.7
Slightly rough	30.0	10.0	20.0	26.7
Smooth	20.0	0.0	0.0	3.3

[a] 30 presentations of each sample treatment

This study has not established the precise identity of the heat-coagulable substances from whey which are responsible for the tactual flavor defect of heated milk. However, recent research (1) has indicated that both the serum proteins and milk salts may be involved in the phenomenon.

SUMMARY

Five experienced taste observers were used to investigate tactual flavor changes induced in milk by momentary heating to 95° C. Such heat treatment of milk increased materially the number of "rough"–"chalky" type of taste responses elicited by the group and lowered the number of "smooth" responses. Heat coagulable substances from the whey portion of milk were found responsible for the flavor defect. These substances may be removed rather readily from heated whey by supercentrifuging. However, they are not readily recovered from heated skimmilk, apparently because of protective colloid action by casein.

ACKNOWLEDGMENTS

This paper reports research undertaken by The Ohio State University Research Foundation in cooperation with The Quartermaster Food and Container Institute for the Armed Forces and has been assigned number 352 in the series of papers approved for publication. The views and conclusions contained in

this report are those of the authors. They are not to be construed as necessarily reflecting the views or endorsements of the Department of the Army.

REFERENCES

(1) HUTTON, J. T., AND JOSEPHSON, D. V. A Tactual Flavor Defect of Dried Milk (abs.). J. Dairy Sci., **34**: 488. 1951.

(2) PATTON, S., AND JOSEPHSON, D. V. Some Observations on the Chemistry of Heat-induced Flavors in Milk (abs.). J. Dairy Sci., **30**: 538. 1947.

(3) PATTON, S., AND JOSEPHSON, D. V. Observations on the Application of the Nitroprusside Test to Heated Milk. J. Dairy Sci., **32**: 398–405. 1949.

(4) TROUT, G. M. *Homogenized Milk*. Michigan State College Press, East Lansing. p. 103. 1950.

FLUORESCENCE AND ASSOCIATED CHANGES PRODUCED UPON STORAGE OF EVAPORATED MILK[1]

H. D. SIMONSON AND N. P. TARASSUK

Division of Dairy Industry, University of California, Davis

The browning and fluorescence characteristics that develop upon sterilization of evaporated milk have been discussed by the authors in a recent publication (5). Here it was observed that many of the associated changes, such as production of CO_2, consumption of O_2 and increase in color, continue during storage. On the basis of analyses of aged samples of evaporated milk, it appeared that fluorescence also continues to increase during storage. Pearce (3) and Olcott and Dutton (1) have indicated that this is true in the case of stored dried eggs. Pearce (4) stated that milk powder samples showed an increase in fluorescence upon storage and that fluorescence measurements have been used to give an indication of the storage history of wheat germ (2). The behavior and separation of the fluorescent material appear to vary somewhat with different products. Studies on evaporated milk have shown that enzymatic hydrolysis of the separated protein material is the most satisfactory method for releasing fluorescence (5). Data pertaining to the development of fluorescence in stored food products are not extensive. Consequently, it appeared advisable to study the nature of the fluorescence development in stored evaporated milk, observing the effect of temperature of storage upon the rate of development, and how the fluorescence is associated with other phenomena, such as milkfat separation, color development and pH changes. In addition, it was desired to determine whether evaporated milk prepared by companies in different localities behaved similarly with regard to these various aspects.

Further studies were carried out on another series of samples with the purpose of studying changes brought about by storing samples of evaporated milk prepared by high-temperature, short-time sterilization procedures. The data are discussed in the second part of this report.

PART I. MATERIALS AND METHODS

The evaporated milk samples used in the first part of this study were obtained from three commercial companies in California. Some cans were removed from the line prior to sterilization, while the remainder were sterilized according to the conventional process for evaporated milk. The sterilization times and temperatures varied somewhat with the company. Sterilized samples from each company were stored at room temperature (70 to 80° F.) and in a refrigerator at 40° F. The cans were inverted every 2 wk. Samples of the sterilized and unsterilized milks were analyzed immediately for fluorescence, color and pH ac-

Received for publication Aug. 26, 1951.

[1] This study was supported in part by funds from the California Dairy Industry Advisory Board.

cording to methods previously described (5), and subsequently, the sterilized samples were analyzed each month over a period of 11 mo. In addition, the milk was checked each month for any visible indication of separation, gelation or other defects, and the relative degree of variation between the samples stored at the two temperatures was noted.

EXPERIMENTAL RESULTS AND DISCUSSION

Figure 1 illustrates the course of fluorescence development over a period of 11 mo. for milk from companies A, B and C. A large initial increase in fluorescence occurred upon sterilization, as indicated previously (5). From the time of sterilization, differences were apparent in fluorescence values of the samples from different companies. This is to be expected, since it is known that relatively small variations in the time and temperature of sterilization have a con-

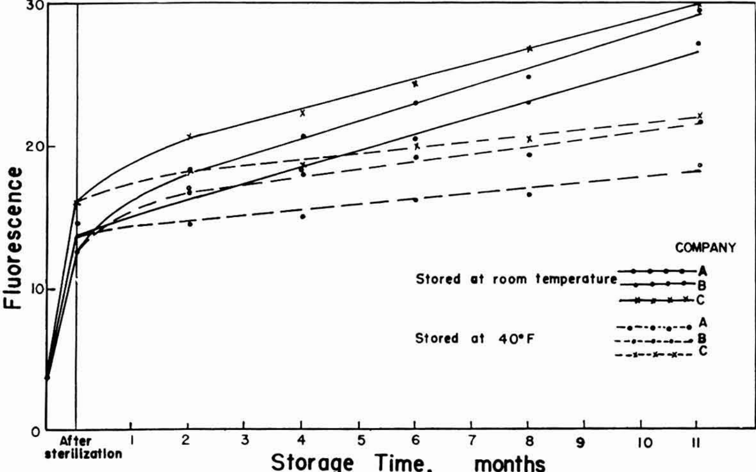


FIG. 1. Effect of time and temperature of storage on the fluorescence of evaporated milk.

siderable effect upon the fluorescence. Fluorescence developed more slowly following sterilization, but at a constant rate in each of the milks over the 11-mo. period. At all times during the storage period the samples stored at 40° F. were lower in value than those stored at room temperature, indicating that the changes taking place are retarded at the lower temperature. Data pertaining to samples stored at a relatively high temperature would be advantageous for comparative purposes. Such data, but on a different series of samples, will be given in the next part of this study. Observation of changes in the stored milks indicated that, after approximately 5 mo., separation was visible in the samples stored at room temperature. It appeared that this corresponded to a fluorescence value of 18 to 20 units. Separation was not evident in the samples stored at 40° F., even after 11 mo. At this time the fluorescence value of these samples was approaching 18 to 20 units. Whether this value might be critical as far as separation of

fat is concerned in samples prepared and treated according to the conventional process, was not ascertained.

Figure 2 illustrates the changes in the color of evaporated milk upon storage. Milk exhibiting the greatest fluorescence is the darkest in color. Samples from each of these companies behaved similarly, and color increments from 1 mo. to the next are not significantly different. The samples stored at 40° F. were consistently lighter in color than those stored at room temperature. This was evidenced visibly, as well, indicating that the color-measuring technique was not sufficiently sensitive. No doubt the values obtained were affected considerably by physical and chemical changes taking place in the milk, since they were based upon reflection from the surface of the milk. Webb & Holm (7), using Munsell equipment, state that the color developed upon sterilization of evaporated milk is different in character from that developed during the course of storage. Using

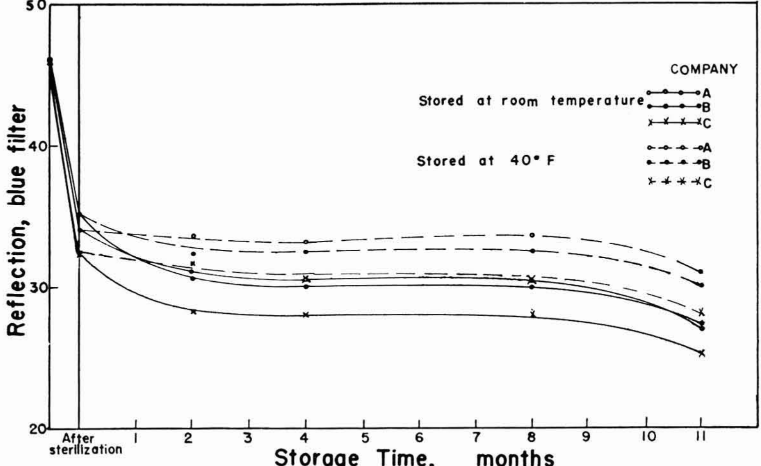


FIG. 2. Effect of time and temperature of storage on the color of evaporated milk.

their terminology, the hue and brilliance during storage remain relatively unchanged, whereas the variation in chroma is responsible for the color change. The Munsell equipment appeared to be far more sensitive to relatively small changes in color than the reflection method used in this study.

The changes in pH upon storage of evaporated milk are shown in figure 3. The pH data are illustrated together for each of the series of milks from companies A, B and C. It appears that the pH changes in the milks from each of the companies are similar. There is a decided decrease in the pH of the samples stored at room temperature. However, in each case the final pH reading on the samples stored at 40° F. is higher than the initial pH. While this variation is possibly within the error of the pH meter reading, it does not explain satisfactorily the reason for the consistency of the observation in the previous months as well. Separation of milkfat began occurring at a pH of approximately 6.1. Webb and Deysher (8) indicate that evaporated milk remains in good condition during storage until its reaction is below pH 6.0. Observations in this study do

not confirm a pH as low as this. If pH were a critical measure of acceptability, this factor as well as the fluorescence values would indicate that the samples stored at 40° F., even for 11 mo., constitute a very acceptable product.

PART II. MATERIALS AND METHODS

In the second part of this work, a study was undertaken of the effects of temperature and time of storage upon evaporated milk sterilized at higher temperatures and shorter times than those used in the conventional process. The samples of evaporated milk were obtained from a local commercial plant just prior to

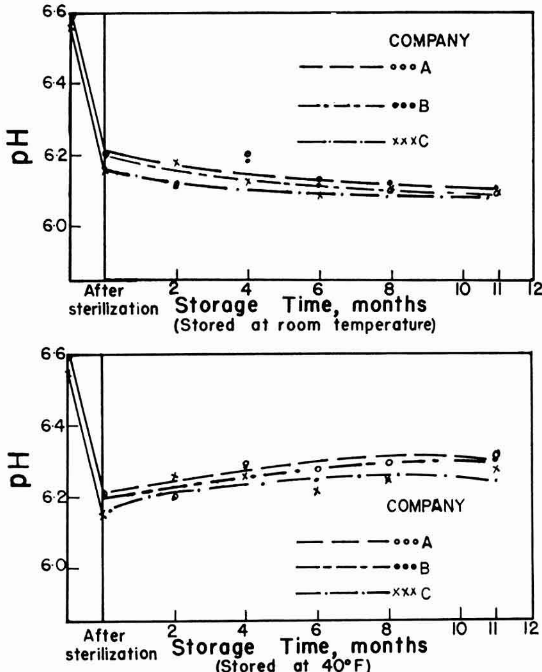


FIG. 3. Effect of time and temperature of storage on the pH of evaporated milk.

sterilization and heated in a Fort Wayne pilot batch sterilizer[2] at the following temperatures and times:

Series	Come-up time	Sterilization temperature	Sterilization time	Cooling time
	(min.)	*(° F.)*	*(min.)*	*(min.)*
I	9.0	243	15.0	10
II	9.5	249	7.0	10
III	10.0	255	3.5	10
IV	9.0	261	1.5	10

[2] The sterilizer was adapted for sterilization at high temperatures by adjustment of the safety valve to release at 30 lb. pressure. Temperatures were obtained manually by the steam inlet valve. In this manner, it is possible to obtain any one of the indicated temperatures in 8.5 min. or more. The come-up time effect was minimized by keeping this time within 9 to 10 min.

A number of cans from each series was stored at 92° F., room temperature (70 to 80° F.) and 40° F. These cans never were inverted during storage and consequently, fat separation was allowed to proceed undisturbed. The samples were tested initially and every 2 mo. over a period of 8 mo. for fluorescence and pH. They also were observed for any physical changes that might occur.

EXPERIMENTAL RESULTS AND DISCUSSION

The fluorescence data obtained are illustrated in figure 4. In general, development of fluorescence has a similar trend regardless of the manner of preparation of the samples. The samples of series I exhibited the greatest amount of

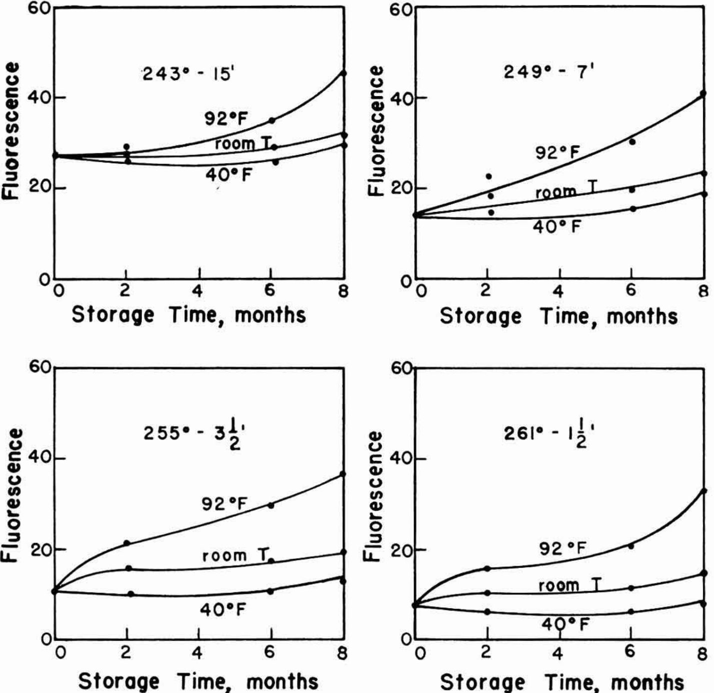


FIG. 4. Effect of time and temperature of storage on fluorescence of evaporated milk sterilized at various temperatures.

fluorescence, followed by series II, III and IV, respectively. For each of the series the temperature of storage apparently had a considerable effect on the fluorescence development. This is particularly true for the samples stored at 92° F. The degree of variation among storage temperatures is not so characteristic in the samples sterilized at 243° F. for 15 min.; these initially had a higher fluorescence. It is of particular interest to note that initially and throughout the storage period, at all storage temperatures, samples sterilized by high-temperature, short-time sterilization processes never fluoresced to the same extent as those sterilized at 243° for 15 min. Likewise, there was less development of

fluorescence as the temperatures of sterilization were increased and the times correspondingly decreased. Physical examination indicated that milkfat separation occurred in all the series stored at room temperature and at 92° F. Analysis of the data indicates that the increments in fluorescence varied from 19 to 26 in the samples stored at 92° F., from 5 to 9 in the samples stored at room temperature, and from 1 to 4 in the samples stored at 40° F. It appears that, if there is not to be separation, the fluorescence increase during an 8-mo. storage period must not exceed that incurred in the samples stored at 40° F. All the samples stored at this temperature were of good quality at the termination of the study.

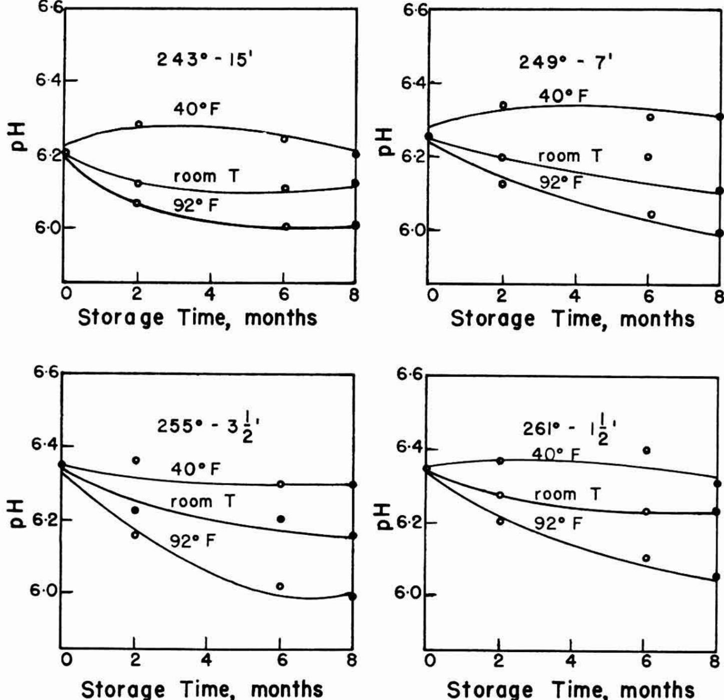


FIG. 5. Effect of time and temperature of storage on pH of evaporated milk sterilized at various temperatures.

The pH values obtained on the samples over the 8-mo. period are shown in figure 5. There was very little change in pH in samples stored at 40° F. The decrease was considerably greater in samples stored at room temperature and most pronounced in the samples stored at 92° F.

Associated with the decrease in pH and increase in fluorescence were changes in the physical composition of the milk. Upon sterilization, the color of the product became progressively lighter and less viscous as the temperature of sterilization was increased for the recorded time. Consequently, the samples of series IV, sterilized at 261° F. for 1.5 min., were very light in color and low in viscosity.

The 2-mo. examination revealed fat separation in all the samples stored at 92° F. for each of the series, the separation becoming more severe as the temperature of sterilization increased. There was no evident separation in any of the samples stored at room temperature or 40° F. The 4-mo. examination revealed a similar separation in the samples stored at room temperature as well, and furthermore the series IV samples stored at 92° F. now exhibited a gel formation. At this time there was no separation in the samples stored at 40° F. The term "gelation" as used here refers to the defect known to be associated with high-temperature, short-time sterilization commonly termed "sweet curd," or simply "partial coagulation." The milk is smooth and thin after sterilization but gradually thickens to a gel in storage. In many instances, this gel is reversible. On shaking, it reverts to a sol which on standing again converts to a gel. Gelation serves to differentiate this defect from another form of coagulation due to excessive heat during sterilization. Coagulation by heat is not a reversible phenomenon, and, furthermore, the coagulum differs in composition from the liquid phase. In the case of gelation, it was found that the composition of gel and liquid phases is essentially the same except for the greater percentage of fat in the gel phase. The 6-mo. examination showed an increase in the severity of separation, with the samples of series III stored at 92° F. having formed a gel. The samples stored at 40° F. showed no fat separation or gel formation. The 8-mo. examination indicated gel formation in all the samples stored at 92° F., and the samples of series III and IV showed wheying off. In addition, the samples of series IV stored at room temperature now showed gel formation. The samples stored at 40° F. were still free from fat separation and gelation. These observations serve again to emphasize the important part that storage temperature plays in the keeping quality of evaporated milk. This is especially true in samples prepared by the high-temperature, short-time sterilization process where the viscosity is low. The physical effects observed here are only briefly considered, since a more complete discussion is given in another publication (6).

SUMMARY

Fluorescence and associated changes that take place in evaporated milk upon sterilization proceed, but at a much slower rate, during subsequent storage. The extent of these changes is dependent upon the sterilization treatment and the storage temperature. The high-temperature, short-time sterilization process results in the production of a milk lower in fluorescence and lighter in color than the regular sterilization process of 243° for 15 min. Temperature of storage is an important factor in the rate of deterioration of the stored milk. The observed changes, particularly fluorescence, are greatly inhibited in milk stored at 40° F. and accelerated at 92° F.

Fat separation and gelation likewise are directly associated with the sterilization temperature and the temperature of storage. Both defects are accented in evaporated milk prepared by the high-temperature, short-time sterilization process. However, even after 11 mo. of storage at 40° F., milk treated according to this process showed no fat separation or gelation and represented a superior

product with respect to color and flavor. This was particularly true of the samples sterilized at 261° F. for 1.5 min.

It appears that until such a time as fat separation and gelation are controlled, a superior quality of evaporated milk with respect to brown discoloration and cooked flavor may best be produced by high-temperature, short-time sterilization and subsequent storage at a low temperature (40° F.).

REFERENCES

(1) OLCOTT, H. S., AND DUTTON, H. J. Changes In Stored Dried Eggs. Ind. Eng. Chem., **37**: 1119–1121. 1945.

(2) PEARCE, J. A. Effect of Storage on Thiamin Content and on Development of Rancidity in Wheat Germ. Can. J. Research, **21**: 57–65. 1943.

(3) PEARCE, J. A. Behavior and Nature of the Fluorescing Substances in Dried Egg Powders. Can. J. Research, **21**: 98–107. 1943.

(4) PEARCE, J. A. Fluorescence Development in Various Food Products. Can. J. Research, **22**: 87–95. 1944.

(5) TARASSUK, N. P., AND SIMONSON, H. D. Browning and Fluorescence of Evaporated Milk. Food Technol., **4**: 88–92. 1950.

(6) TARASSUK, N. P., AND SIMONSON, H. D. Gel Formation and Fat Separation in Evaporated Milk as Affected by the State of Milk Proteins. Abs. of paper presented at meeting of American Dairy Science Association, Knoxville, Tenn., June, 1951.

(7) WEBB, B. H., AND HOLM, G. E. Color of Evaporated Milk. J. Dairy Sci., **13**: 25–39. 1930.

(8) WEBB, B. H., AND DEYSHER, E. F. Effect of Storage Temperature on Properties of Evaporated Milk. Abs. of paper presented at meeting of American Dairy Science Association, Knoxville, Tenn., June, 1951.

THE RELATIONSHIP OF MILK ENERGY AND TOTAL PROTEIN TO PER CENT FAT IN BROWN SWISS HERD MILK

S. D. MUSGRAVE AND G. W. SALISBURY
Department of Dairy Science, University of Illinois, Urbana

INTRODUCTION

Investigations to date have resulted in two interpretations as to the actual relation of Calories per kilogram of milk and per cent fat, and between per cent total protein and per cent fat. Bonnier *et al.* (1) presented data based on 2,152 samples of milk from monozygotic and dizygotic twins showing that the relationship between Calories per kilogram of milk and the per cent fat of that milk was not linear. The fitted curvilinear regression line representing their data was concave in an upward direction. These data were not in accord with the earlier work of Gaines and Overman (3), who showed that milk energy was related linearly to the per cent fat of that milk. Overman and Gaines (4), using three sets of available data, have shown two instances in which milk energy was related to the per cent fat in other than a linear way. The smoothed curves representing this relationship were found to be concave downward in both instances. The third set of data, based on 305-day partial lactations, definitely showed a linear regression of milk energy on the per cent fat.

Bonnier *et al.* (1) have shown that the regression of per cent total protein on per cent milk fat was not linear. They found that protein followed the quadratic function of fat more closely than it did the linear function of fat.

It is the purpose of this paper to present additional evidence on the relationship of milk energy and milk total protein to the per cent of milk fat.

DATA

The data presented here are based on the results of laboratory analyses[1] of 494 samples of Brown Swiss herd milk[2] taken from 39 Brown Swiss herds located in 16 states. Samples were taken at approximately monthly intervals during the time of sampling for each herd. The mean number of samples per herd was 12.7 and ranged from 10 to 17. Each sample was a representative composite of two consecutive milkings starting with the night milking. The period of sampling for all herds ranged from December, 1946, to May, 1950. Methods of obtaining and preserving samples and analytical procedures for the determination of Calories per kilogram, per cent total protein, and per cent fat have been described previously (5).

RESULTS AND DISCUSSION

The energy value of the 494 samples ranged from 669.02 to 914.28 Cal. per kilogram of milk with a mean value of 755.60. Likewise, per cent fat of these

Received for publication Sept. 1, 1951.

[1] Analyses were made by the late O. R. Overman, R. J. Keirs and E. M. Craine.

[2] Supported in part by the Brown Swiss Cattle Breeders' Association, Beloit, Wis.

samples ranged from 3.22 to 5.32 with a mean value of 3.97. A correlation of +0.92 was found between Calories per kilogram and per cent fat for the 494 samples. This value is not as high as that previously reported by Gaines and Overman (3), where individual cow samples were dealt with. The distribution of Calories per kilogram of milk when plotted on per cent of milk fat is shown in figure 1. The linear regression line *A*, of figure 1 is fitted from the equation

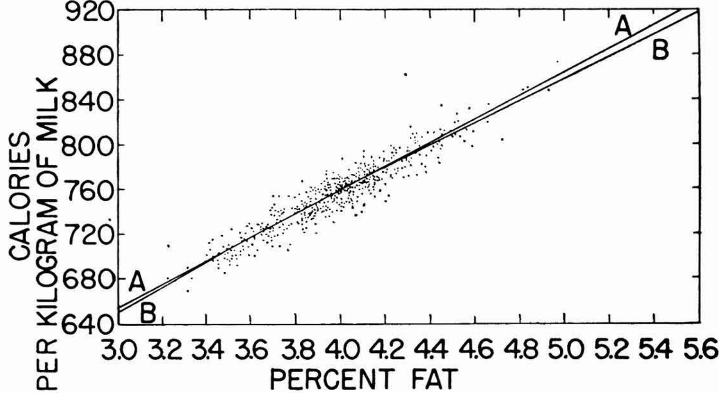


FIG. 1. Calories of milk energy plotted against per cent fat (494 samples of Brown Swiss herd milk). Line *A* represents the linear regression equation, $E = 343.483 + 103.833 \pm 1.965)\ f$. Line *B* represents the curvilinear regression equation, $E = 287.062 + 132.127\ f - 3.529\ f^2$.

$E = 343.483 + 103.833\ (\pm\ 1.965)f$. The model used here was of the type $y = a + bx$. Fitting the same data to a curvilinear equation of the type $y = a + bx + cx^2$ gave the equation $E = 287.062 + 132.127f - 3.529f^2$. This equation is fitted to the data as line *B*, of figure 1. In these equations *E* is Calories per kilogram, *a* is a constant and *f* is per cent fat. As may be seen in figure 1, line *B* is slightly concave downward. This trend is not in agreement with the data of Bonnier *et al.* (1), but agrees with the findings of Overman and Gaines (4). The mean square (table 1) due to curvilinearity of regression, is not statistically significant, while that due to linear regression is significant at the one per cent level (6). Here then is evidence, based on data of a slightly different nature (herd milk samples instead of individual cow milk samples), that the best estimate of the relationship

TABLE 1

Regression of Calories per kilogram of milk on per cent fat

Source	Degrees of freedom	Mean square	F
Total	493		
Due to linear regression	1	441,290.9069	279.0216**
Due to curvilinear regression	1	122.8286	0.7766
Deviation from curvilinearity of regression	491	158.1565	

** = confidence limits at the 99% level.

between Calories per kilogram and per cent fat of cows' milk is that described by a linear regression equation.

Using the linear equation, $E = 343.483 + 103.833f$, it is possible to predict the energy in Calories for herd milk of differing fat content, as was done previously by Gaines and Davidson (2) for the milk of individual cows in deriving their fat corrected milk (FCM) formula. In the present equation, when f is equal to 4.0, $E(4.0)$ is equal to 758.815 Calories. Division of the right side of this linear equation by 758.815 gives $0.45 + 0.137f$. It is necessary to multiply the factor $0.137f$ by 100 to convert this part of the formula from per cent fat to units of fat. Therefore, the formula reads $FCM = 0.45M + 13.7F$ and these values correspond to the formula of Gaines and Davidson (2) ($FCM = 0.4M + 15F$), where M is milk and F is milk fat, all in the same unit of weight. While the values obtained from the equation presented here differ quantitatively from those of Gaines and Davidson (2) for individual cows, the end result in terms of FCM as determined by the two formulas differs very little for milk ranging from 2.00 to 6.00 per cent fat as shown in table 2. Obviously the differences

TABLE 2

A comparison of the amount of FCM per pound of milk of differing fat percentages when computed by the formulas $FCM = 0.45M + 13.7F$, and $FCM = 0.4M + 15F$

Fat	Amount FCM/lb. of milk	
	FCM = 0.45M + 13.7F	FCM = 0.4M + 15F
(%)	(*lb.*)	(*lb.*)
2.0	0.72	0.70
2.5	0.79	0.78
3.0	0.86	0.85
3.5	0.93	0.93
4.0	1.00	1.00
4.5	1.07	1.08
5.0	1.14	1.15
5.5	1.20	1.23
6.0	1.27	1.30

shown in table 2 are greatest at the extremes of per cent fat. For Brown Swiss herd milk the extreme range is less than that shown in table 2, suggesting that no real difference exists in the formula for calculation of FCM from the present data on Brown Swiss herd milk and that calculated earlier by Gaines and Davidson (2) from data on the milk of individual cows.

Per cent total milk protein in the same 494 samples ranged from 3.10 to 4.11 with a mean of 3.52. The relationship of per cent total protein to per cent fat of these samples is described by a correlation coefficient of +0.30, a linear regression equation of $P = 2.785 + 0.186f \pm 0.0265$ (line C, of figure 2), and a curvilinear regression equation of $P = 2.480 + 0.3387f - 0.01899f^2$ (line D of figure 2). Here P represents per cent total protein and f represents per cent fat. The distribution of the 494 samples is shown in figure 2 when per cent total protein is plotted on per cent fat. The mean square due to curvilinearity of regression is not statistically significant (table 3), while that due to linear regression is signficant at the 1 per cent level. This is not in agreement with the data

of Bonnier *et al.* (1), but does support the data of Gaines and Overman (3). As a result of the present data and earlier work, it is felt that the best estimate of the relationship of per cent total protein and per cent fat of cows' milk is one expressed by a linear equation.

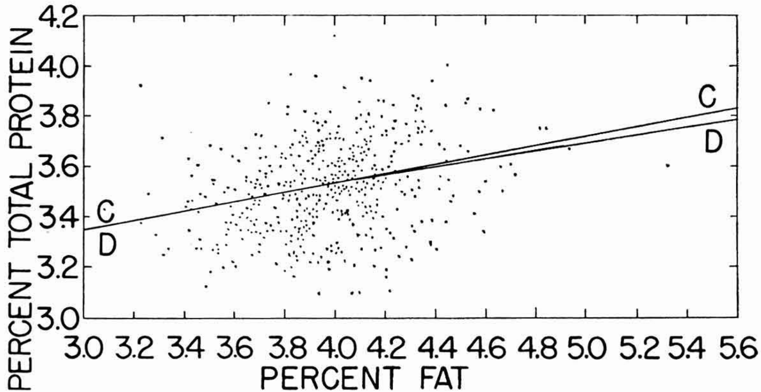


FIG. 2. Per cent total protein plotted against per cent fat (494 samples of Brown Swiss herd milk). Line *C* represents the linear regression equation, $P = 2.785 + 0.186\ (\pm\ 0.0265)\ f$. Line *D* represents the curvilinear regression equation, $P = 2.480 + 0.3387\ f - 0.01899\ f^2$.

SUMMARY

An analysis of the relationships between Calories per kilogram and per cent fat and between per cent total protein and per cent fat has been made from data based on detailed analytical determinations of the chemical composition and energy content of 494 samples of Brown Swiss herd milk.

TABLE 3

Regression of per cent total protein on per cent fat

Source	Degrees of freedom	Mean square	F
Total	493		
Due to linear regression	1	1.4229	49.578**
Due to curvilinear regression	1	0.0036	0.125
Deviation from curvilinearity of regression	491	0.0287	

** = confidence limits at the 99% level.

A highly significant (1 per cent level) linear relationship existed between Calories per kilogram and per cent fat and between per cent total protein and per cent fat of composite samples of cows' milk. Tests of the significance of the departure from linearity indicated that the minor effect of curvilinearity was not statistically significant in either case.

From the data available an FCM formula was calculated for Brown Swiss herd milk having the values $FCM = 0.45M + 13.7F$. However, while quantitatively different from the original $FCM = 0.4M + 15F$ of Gaines and Davidson, the

values obtained from the use of this formula indicate that no important increase in accuracy of estimating the comparative energy equivalents of Brown Swiss market milk of differing fat content would be achieved by its use.

REFERENCES

(1) BONNIER, G., HANSSON, A., AND JARL, F. Studies in the Variations of the Calory Content of Milk. Acta Agr. Suecana, **2**: 159–169. 1946.

(2) GAINES, W. L., AND DAVIDSON, F. A. Relation between Percentage Fat Content and Yield of Milk. Ill. Agr. Expt. Sta. Bull. 245. 1923.

(3) GAINES, W. L., AND OVERMAN, O. R. Interrelations of Milk-fat, Milk-protein, and Milk-energy Yield. J. Dairy Sci., **21**: 261–271. 1938.

(4) OVERMAN, O. R., AND GAINES, W. L. Linearity of Regression of Milk Energy on Fat Percentage. J. Animal Sci., **7**: 55–59. 1948.

(5) OVERMAN, O. R., KEIRS, R. J., AND CRAINE, E. M. Composition of Brown Swiss Herd Milk. Ill. Agr. Expt. Sta. Bull. (In Press).

(6) SNEDECOR, G. W. *Statistical Methods*, 4th ed. Iowa State College Press, Ames. 1946.

VOLUME XXXV FEBRUARY, 1952 NUMBER 2

JOURNAL OF DAIRY SCIENCE

ABSTRACTS OF LITERATURE

Prepared in cooperation with the
International Association of Ice Cream Manufacturers
and the Milk Industry Foundation

CHEESE

A. C. DAHLBERG, SECTION EDITOR

53. Sur le chancre superficiel des fromages à pâte ferme (Rotten spots at the surface of hard cheeses). J. KEILLING, J. CASLAIS and N. MAURO. Lait, **31**, 307: 353–360. July–Aug., 1951.

A defect of hard cheeses, in which the surface becomes pitted, cavitied and discolored is described. Economic significance as well as earlier research on the problem are discussed.

Two groups of microorganisms are concerned. Yeasts which readily tolerate salt and utilize lactic acid create favorable conditions for growth of caseolytic fungi which produce the cavities. Prevention of the defect is facilitated by repeatedly scrubbing the cheeses with clean water during the 1st 3–4 wk. In addition, wooden utensils should be kept clean, free of rot and dry when not in use. The microbiological condition of brines should be closely followed and all possible measures to prevent surface contamination of the cheeses should be taken. S. Patton

54. Le fromage blanc comme aliment exclusif au presque exclusif du rat blanc (Cottage cheese as an exclusive or nearly exclusive diet of the white rat). C. RICHET and R. MARET. Lait, **31**, 307: 361–367. July–Aug., 1951.

White rats fed cottage cheese exclusively survived 6 mo. or more. However, multiple deficiencies were manifest on this diet. Reproduction was delayed and the young, continued on cheese alone, rapidly succumbed. Better results were obtained when cottage cheese was supplemented with green vegetables. The 2nd generation on this diet, although hardier than that receiving cheese alone, developed nephritic lesion and also died prematurely. S. Patton

DAIRY BACTERIOLOGY

P. R. ELLIKER, SECTION EDITOR

55. Thermal resistance of a faculative aerobic spore-forming bacterium in evaporated milk. E. H. RUYLE and P. SOGNEFEST, Am. Can Co., Maywood, Ill. J. Milk & Food Technol., **14:** 173–175. Nov.–Dec., 1951.

The custard-like consistency of commercially canned evaporated milk is due to the growth of *Bacillus subtilis* in the product. Detection of this spoilage is difficult because there is no apparent change in pH or gas formation. The heat resistance of the organism depends on the number of spores/unit volume. A minimum of 16.4 min. at 243° F. is necessary to control this defect. Regular bacteriological examination of the raw milk supply is recommended, along with adequate cleaning and sterilization of all milk equipment. H. H. Weiser

56. Psychrophylic bacteria in Edmonton milk and cream. I. Numbers. I. E. ERDMAN and H. R. THORNTON, Univ. of Alberta, Edmonton. Can. J. Technol., **29,** 5: 232–237. May, 1951.

Ten samples of winter raw milk, 23 of summer raw milk and 28 of summer churning cream were collected and plated at 35.5° C., as well as at 10.5 and 4.5° C. The 10.5° C. counts expressed as a percentage of the 35.5° C. counts varied from 36–350%. The 4.5° C. counts were considerably lower. The major source of the psychrophiles is non-sterile utensils. They rarely survive pasteurization and their presence in freshly pasteurized products indicates inefficient plant sanitation. O. R. Irvine

57. Psychrophilic bacteria in Edmonton milk and cream. II. Kinds. I. E. ERDMAN and H. R. THORNTON, Univ. of Alberta, Edmonton. Can. J. Technol., **29,** 5: 238–242. May, 1951.

From the bacterial plates discussed in the preceding paper, 722 psychrophiles were isolated. These were studied as to morphology, Gram reaction and certain other cultured characteristics and found to be quite heterogeneous, especially at higher incubation temperatures. 190 cultures were classified into the following genera: *Pseudomonas, Lactobacillus, Streptococcus* (lactic acid streptococci), *Aerobacter, Flavobacterium* and *Escherichia.* No spore formers were encountered and no culture grew at 35.5° C. All isolates produced flavor defects in skimmilk, bitter flavor predominating. O. R. Irvine

58. Bacteriological aspects of the evaluation of adequacy of pasteurization. F. W. BARBER, Natl. Dairy Research Lab., Oakdale, N. Y. J. Milk & Food Technol., **14:** 170–172 and IX. Nov.–Dec., 1951.

Use of Micrococcus MS-102 as a typical heat-resistant test organism in determining the effi-

ciency of pasteurization is suggested. The thermal death time of various pathogens should be determined in different dairy products. Studies of this kind would be useful in showing any combination of time and temperature in determining satisfactory pasteurization. H. H. Weiser

59. Conservation du lait par des méthodes autres que les méthodes classiques (Conservation of milk by methods other than the classic methods). G. Ray. Lait, **31,** 307: 375–383. July–Aug., 1951.

The methods discussed include inhibition or destruction of bacteria by heavy metals, mechanical abrasives (such as fine carborundum or glass beads), ultra-violet rays, a combination of ultra-violet and infra-red rays, ultra-sonic vibrations and high intensity electronic impulses.

S. Patton

60. Average plate count ratios of dairy products calculated for periods of six months. J. L. Courtney, Dept. Public Health, Oak Ridge, Tenn. J. Milk & Food Technol., **14:** 176–178. Nov.–Dec., 1951.

Average standard plate count ratios between dilutions 1:100 and 1:1,000 were determined on 12,109 samples of dairy products during the 3 yr. of grading periods. The average ratio was 1.8. This may be useful in securing comparable results in different control laboratories, as well as encouraging laboratory technicians to attain greater skill in their work. This study is based on U.S.P.H.S. recommendation that average ratios should not be over 2.0 for those samples for which 2 dilutions show between 30 and 300 colonies.

H. H. Weiser

61. Etude bactériophagique du colostrum. I. Lipska. Lait, **31,** 307: 383–385. July–Aug., 1951.

In order to demonstrate that the beneficial effects of administering polyvalent bacteriophage to the new born infant were not arising from phagic activity of the mothers' colostrum, 60 colostrums and 60 milks were analyzed for phage activity. The results indicated that the microflora of colostrum and milk are very similar and that neither, in these instances, contained bacteriophage. S. Patton

62. A study, by means of paper chromatography of the growth-stimulating properties of liver extract, yeast solubles and trypsin digest of casein for Lactobacillus casei. I. W. Coleman, Univ. of Manitoba, Winnipeg. Can. J. Med. Sci., **29,** 4: 151–158. Aug., 1951.

Transverse sections of linear partition chromatograms on filter paper when eluted with basal medium for *L. casei* showed Wilson liver fraction L to contain at least 2 growth-stimulating factors for this organism. Three such factors were demonstrable by this technique in trypsin digest of casein, in contrast to dried yeast solubles which contained 1 such factor. The factor from yeast was separated and partially purified. The evidence suggests it is peptide in nature.

O. R. Irvine

DAIRY CHEMISTRY

H. H. SOMMER, SECTION EDITOR

63. Fat emulsion in milk from a chemical standpoint. H. H. Sommer, Univ. of Wisconsin, Madison. Milk Dealer, **41,** 1: 58–74. Oct., 1951.

The chemistry of fat emulsion in milk is summarized. Milk fat and butter fat are not strictly synonymous. Besides tri-glycerides, milk fat contains various lipids in traces, including phospholipids at about 0.7–0.8 g./100 g. of fat. Triglycerides of milk fat contain from 13–20 different fatty acids; the exact number, chosen for purposes of discussion, will depend on the decision as to amounts that are significant. With 18 different fatty acids, 5,832 different tri-glycerides are theoretically possible. Rules have been stated for computing the numbers of several different types of glycerides. Analytical evidence indicates that glyceride composition of milk fat is in harmony with expectations based on random distribution of fatty acids. A fat globule 1 μ in diameter will contain about 380 million tri-glyceride molecules; a 10 μ globule will contain 380 billion. There is no good evidence to support the belief that composition of globules differs according to their size, except that in the case of small globules the lipids that coat the surface constitute a larger fraction of the total globule. A theory has been advanced to account for the assortment of globule sizes in terms of concentration and rate at which glycerides form and collect, as opposed to the rate at which their surface tends to become sealed off against further growth.

Calculations have been offered in support of the theory that globules are covered with a monolayer of phospholipids. The reactive groups in the phospholipids have been emphasized. Attention has been called to the formation of complexes by the phospholipids with fats as well as with proteins. The evidence as to the nature of proteins or proteins associated with the surface of fat globules is confusing and needs re-study in light of expanding knowledge of milk proteins and techniques for their study. In such study the several factors need to be controlled, including particularly the effect of previous temperature history. Temperature activation of lipolysis is reproducible with synthetic emulsions and with pancreatic lipase, but phospholipids must be provided. The phenomenon is observed when the enzyme is applied after the cooling-warming-cooling treatment. Effects of temperature manipulations are visualized in terms of fractional melting of fat and resulting changes in composition and orientation of the molecules represented in the globule surface. C. J. Babcock

64. Le test de la phosphatase appliqué à la recherche de la pasteurization des laits de fromageria (Use of the phosphatase test to study pasteurization of milks at the cheese plant). A. Camus and R. Alifax. Lait, **31,** 307: 367–374. July–Aug., 1951.

Appearance of phosphatase activity of microbial origin in cheese prepared from adequately pasteurized milk prompted an investigation of

the phenomenon as a function of storage time and point of sampling in the cheese. Phosphatase activity in soft cheese was greatest at the surface of the cheese and essentially absent at the center; activity tended to increase with storage time. In the case of Gruyère, phosphatase activity was evident throughout the cheese and tended to reach a maximum in approximately 1 mo., after which time it decreased. The authors suggest that proteolysis of the enzyme is responsible for the decrease. Practical implications of the results, with respect to checking pasteurization of milk by testing the cheeses, are discussed.

S. Patton

65. The precipitation of whey proteins using waste sulphite liquor. J. H. Hare and B. E. Baker, Macdonald College, Quebec. Can. J. Technol., **29,** 7: 332–336. July, 1951.

Whey obtained from a muriatic-acid casein plant was treated with sulphite waste liquor at rates of 1–15 g./100 ml. whey. Solid sodium lignosulphonate also was used at rates of 0.1–2.5 g./100 ml. whey. The maximum proportions of protein precipitated were 73.5 and 81.9% respectively. Acidification to pH 2.0–3.5 aided precipitation. Attempts to isolate the whey proteins from the lignosulphate complex have been unsuccessful. Preliminary rat-feeding tests indicated that the lignin-protein complex could be well tolerated when it supplied 10% of the crude protein of the diet.

O. R. Irvine

DAIRY ENGINEERING

A. W. FARRALL, SECTION EDITOR

66. Agitation of milk by air. R. E. Storck, Dairymen's League Co-op., New York. Milk Dealer, **41,** 1: 52, 127, 130. Oct., 1951.

Air agitation of milk in holding tanks offers advantages, both from an economic and sanitary standpoint. It takes only 2.5 min. to agitate a tank of milk with air, whereas a minimum of 20 min. is required to do the work with the average mechanical agitator. By the latter method a 4,000 gal. tank calls for the use of from 2–3 h.p., depending upon the design of the agitator. With air, only 0.5 h.p. is needed and it will do a more thorough job. With air agitation, milk in the tank can be mixed down to the last 4 in. There will be air in milk if it is raised to the point where it will fall and form a number of spheres or drops. In a properly designed air agitation system, the volume of air supplied is regulated so that milk will rise to the surface with the air and roll to the sides; the milk will never be agitated so violently that it will splash above the surface and fall back to that level in drops. A technical system of agitating by air and the proper equipment and installation are discussed.

C. J. Babcock

67. Cold milk separators. Anonymous. Milk Dealer, **41,** 1: 50–51. Oct., 1951.

Cold milk separators differ from the conventional type in that milk at 40° F. can be run through them and efficiently separated. They also can be used to separate hot milk. Cold milk separation is economical as the heating and cooling of the product in the hot milk process costs from 30–50¢/hr./5,000 lb. of milk separated. Also the equipment made for this heating and cooling can be eliminated or released for other plant operations. This reduces the labor required for wash-up and equipment assembly. Other advantages claimed for cold milk separation are that the viscosity of the cream is increased at least 10% and it has a lower bacterial count. To obtain maximum efficiency with the cold milk separator, it is necessary to reduce the capacity to about half that of the hot milk separator. Precautions should be taken to prevent the butterfat in the milk from becoming churned because it will have a tendency to plug. Back pressure on the cream and skimmilk discharges of the separators should be kept to a minimum.

C. J. Babcock

DAIRY PLANT MANAGEMENT AND ECONOMICS

L. C. THOMSEN, SECTION EDITOR

68. Distributing milk through vending machines. Anonymous. Milk Dealer, **41,** 1: 46, 114–117. Oct., 1951.

Ritting Bros. operate approximately 500 milk-vending machines in Buffalo, Rochester and New York City. Experience has been acquired with both glass and paper containers in pints, half-pints and third-quarts. Sales are approximately 51% white milk, 36% chocolate drink, 12.5% orange drink and 0.5% buttermilk. Variations occur during certain seasons or in certain types of outlets but over a period of time the average generally follows this pattern. An increase in price sharply reduces sales. When changing economic conditions made it necessary to increase the price from 5 to 10¢, volume dropped 20–25%. A machine which does not produce sales of 30 units a day minimum should be moved to a new location. Selecting locations, machine maintenance and operations are discussed.

C. J. Babcock

69. Frozen concentrated orange juice on milk routes. Anonymous. Milk Dealer, **41,** 1: 42–43, 85–97. Oct., 1951.

Extensive study of sale of frozen foods on retail milk trucks, initiated 4 yr. ago, showed that frozen orange juice concentrate had the greatest possibilities. This study was conducted in Washington, D. C. and Detroit, Mich. Extensive study and development also has been carried on for the last 18 mo. on retail routes in Detroit, Boston, Bridgeport and Washington, D. C. The accomplishment in each of the above cities is given. Well-developed merchandising patterns are being successfully followed by a number of milk dealers. These should be studied before the distribution of frozen orange juice concentrate is started.

C. J. Babcock

70. Statistics vital to ice cream management. A. S. Aronson, Natl. Dairy Prod. Corp., New York City. Ice Cream Trade J., **47,** 9: 50, 76. Sept., 1951.

Internal statistics relating to facts concerning the operation of a particular business and external statistics relating to things which happen outside a particular business may be used advantageously by management in arriving at sound business decisions.

Profit and loss statements, balance sheets and inventories, make it possible for the manager to make comparisons with previous years business. Statistics on the operation of each department make it possible to operate more efficiently. These should include sales, manufacturing and administration.

External statistics are available from many sources such as the Internatl. Assoc. of Ice Cream Manufacturers, various government agencies, Bureau of the Census, Bureau of Agriculture Economics, Bureau of Labor Statistics, United Nations, Dept. of Commerce and others.

W. H. Martin

71. Trends in ice cream costs. Anonymous. Ice Cream Trade J., **47,** 6: 24–26, 89. June, 1951.

Annual expense comparisons for the years 1945–49 for the ice cream industry, show that total cost of ice cream has increased each year since 1945. In 1949 products and manufacturing expenses accounted for a little over 70% of the total and distributing costs were 25–29%. Product costs were 48.95% of total costs. There has been a marked increase in the sale of novelties and packaged ice cream and a large decrease in sale of bulk ice cream during the 4-yr. period 1946–49. Manufacturing costs increased 68%, delivery costs 88 and total costs 26%. W. H. Martin

72. What are you doing about labor turnover? E. R. QUACKENBUSH, Borea, O. Milk Dealer, **41,** 1: 98–102. Oct., 1951.

Too large a labor turnover is costing the dairy industry much in actual money and, indirectly, has its effect on morale and standards of service. Observation of methods employed by concerns which have not had such a high turnover shows that they have several points in common: (a) They have a continuous list of prospective employees which is kept alive and up to date. (b) Using such an organized list of prospects, these firms secure all the information possible and carefully select those men who give indication of the best possible chance for permanent success. (c) They realize that a new man needs to be given confidence in his ability to satisfy his employer and is most alert to any positive suggestions during the first few months. They spend considerable time and attention getting him started off "on the right foot," making him feel "at home" in his organization and proud of it.

C. J. Babcock

ICE CREAM

C. D. DAHLE, SECTION EDITOR

73. First history of the ice cream industry. V. M. RABUFFO. Ice Cream Trade J., New York, N. Y., **47,** 5: 38. May, 1951.

Items covered include early history, evolution of delivery equipment, ice cream history down through the ages, first trade paper, development of the ice cream freezer, early advertisements, the first trade association, development of ice cream items, the ice cream sandwich, the story of the ice cream cone, package development, stick confections and other novelties and many other topics relating to the development of the ice cream industry. W. H. Martin

74. Problems in the manufacture of soft ice cream mixes. J. J. SHEURING and P. F. ROSSI, Univ. of Ga. Ice Cream Trade J., **47,** 10: 74–76, 106–107. Oct., 1951.

In this study 95 mixes of variable composition were frozen into soft ice cream. Observations were made on rate of freezing, amount of overrun, appearance of ice cream and quality factors. An excellent product can be made from a mix containing 6% fat or above, 9% milk solids not fat, 0.15% emulsifier and 0.40% stabilizer. Powdered eggs tend to improve the whipping properties, smoothness, firmness and luster of soft ice cream. A drawing temperature of 20° F. gave the best results for mixes studied.

W. H. Martin

75. H.T.S.T. pasteurization of the ice cream mix. F. W. BARBER, Natl. Dairy Research Lab., Inc., Oakdale, Long Island, N. Y. Ice Cream Trade J., **47,** 6: 52, 95–96. June, 1951.

Ice cream mixes were inoculated with test organism and pasteurized at 165, 175 and 185° F. for 25 sec. and 190, 210, 240 and 260° F. for 1.4 sec. Portions of the same mixes were vat-pasteurized and pasteurized in test tubes in the laboratory at 155° F. for 30 min. The results showed that at 175° F. and above using 25 sec. holding time and at 190° F. and above with a minimum holding time of 1.4 sec., the bacterial destruction was comparable to that obtained at 155° F. for 30 min. in the laboratory and by batch pasteurization. The U.S.P.H.S. has granted tentative approval for the pasteurization of ice cream mix at 175° F. for 25 sec. W. H. Martin

76. Production tips. W. J. CAULFIELD, Iowa State College, Ames. Ice Cream Trade J., **47,** 9: 42. Sept., 1951.

Prolonged storage of ice cream mix at high temperature may result in high bacterial count and increase the amount of refrigeration required for freezing.

The maximum safe storage temperature at 40° F. is 4 days if the standard plate count is to be maintained at less than 50,000/gram and the coliform count at 10 or less organisms/gram. By lowering the mix temperature 10° F. the refrigeration load may be reduced 8%. Prolonged holding of the mix may result in development of undesirable flavors and result in ice cream which may show a greater tendency toward shrinkage. Dating of mix cans and the use of recording thermometer in storage rooms are suggested.

W. H. Martin

77. Wil Wright markets a 25 per cent butter fat ice cream. Anonymous. Ice Cream Trade J., **47,** 8: 34, 69. Aug., 1951.

Wright's ice cream which is reported to contain 25% butter fat and weigh 2 lb./qt. is made by hand in French pots, with mechanical mixing and automatic controls. The pots rotate in a brine solution in spherical tanks. The brine is brought to a precise temperature within a given length of time and the freezing through rotating action is controlled to a given number of minutes by circuit-breaking regulators. The paddling by hand begins in about 12 min. when the brine reaches the right temperature and continues for 26 min. The ice cream is sold in Wright's own stores in Los Angeles and also is shipped to Dallas, Kansas City, El Paso and out-of-town points. The bulk prices range from $1.50/qt. for some special flavors. Wright's stores have standardized special designs and colors. W. H. Martin

78. Candies for ice cream production. A. MANN, MANN'S Candies, Los Angeles, Cal. Ice Cream Trade J., **47,** 8: 36, 81. Aug., 1951.

Mann has developed a special process for making a candy which may be used for flavoring ice cream without the danger of jamming fruit feeders. 3–6 lb. of candy is recommended for each 5 gal. of mix. Candy is available in a variety of flavors, including peppermint, lemon stick, butter pecan crunch, almond, hazelnut, English toffee, butter brittle and midget marshmallows.
W. H. Martin

79. Processed nuts for use in ice cream. C. A. PETERSON, Cleveland, O. Ice Cream Trade J., **47,** 6: 40, 92. June, 1951.

A brief history of the most common nuts used in ice cream is presented. Only 1st grade nuts, uniform in quality, size, texture and flavor must be available to insure a quality ice cream. All pieces of shells, and inferior nuts should be removed, as well as any foreign matter, and the nuts carefully tested. To insure best results nuts should be stored in the cooler, kept dry and used as cold as possible. W. H. Martin

80. Ice cream sandwiches by the millions. Anonymous. Ice Cream Trade J., **47,** 8: 38, 81–82. Aug., 1951.

An automatic process for making ice cream sandwiches has been developed for use by the Pioneer Ice Cream Div. of Borden Co. in New York City. The sandwiches are made in Anderson Bros. Model no. 193, which is hooked up directly with 3 150-gal. Vogt continuous freezers. The ice cream is fed to the machine and is extruded between wafers which fall into place from chutes on either side of the extruder. A pusher cuts the ice cream into proper size and moves sandwiches to the automatic bagger, and from there to a conveyor where they are placed in trays to be transferred to the hardening tunnel. As they emerge from the tunnel they are boxed before being placed in the hardening room.
W. H. Martin

81. Ice cream bon-bons. Anonymous. Ice Cream Trade J., **47,** 10: 50, 51. Oct., 1951.

The Borden Co. in New York, Canada and California is making ice cream bon-bons, bite-size portions of ice cream coated with chocolate. Five bon-bons weighing approximately 2.5 fluid oz. retail for 10–15¢ in theaters.

The production of this item is completely automatic with 3 persons employed in a double set-up capable of producing 2,400 doz. cartons each containing 5 bon-bons in an 8-hr. day.
W. H. Martin

82. Hood's introduce a $2\frac{1}{2}$ oz. nickel ice cream bar. Anonymous. Ice Cream Trade J., **47,** 10: 48–49, 156. Oct., 1951.

Hoodsie bars is a new 2-flavored wrapped piece produced by automatic means at the rate of 750 doz./hr. with newly developed equipment. The bar wholesales to dealers for 45¢/doz.

Ice cream is frozen dry to 20° F. in 2 150-gal. Vogt freezers, chocolate in one and vanilla in the other. The 2 flavors are piped directly to an extruder head by means of swivel-jointed sanitary pipe. The 2 flavors meet at the center of the extruder head and are cut off in regular pieces weighing 2.5 fluid oz. and dropped to an 8-ft. canvas-treated conveyor. The conveyor moves the bars into a freezing tunnel for hardening at –30 to –35° F. The bars are wrapped and packed 3 doz. to the box and returned to the hardening room. W. H. Martin

83. Insulated overwraps the latest phase in packages. Anonymous. Ice Cream Trade J., **47,** 8: 28, 29, 32, 85. Aug., 1951.

The insulated overwrap package for ice cream, first introduced by Plantation Food in Florida, now is being used by H. P. Hood & Sons in New England, Bowman Dairy Co. in Chicago and the General Ice Cream Co. in New York and New England. In some plants the insulated wrapper is formed by machinery from a large roll of light corrugated paper, after which the package is overwrapped with a colorful printed wrapper. In other plants, the insulated sleeve is purchased completely made up and scored lightly so that it may be tucked in at the sides, following which the over wrapping is applied. The estimated cost of the wrapper is 8–12¢/gal. W. H. Martin

84. New walk-in delivery truck. Anonymous. Ice Cream Trade J., **47,** 7: 40–41, 98. July, 1951.

A new-type ice cream delivery truck designed to provide for greater pay loads, faster loading and delivery, more selling time for the driver, readier accessibility to merchandise on the truck and a larger variety of items available, has been built by the Pioneer Div. of the Borden Co., New York.

The new truck body was built on a Ford 1.5 ton chassis, the interior is lined with shelves on both sides so the driver can walk into the truck and select his items quickly. The interior has 5′ $8\frac{1}{2}$″ of head room. There are 2 14-gauge steel shelves on either side and the floor makes the third shelf. The body built to aproximate 1,200-gal. capacity will hold a mixed load equivalent to what a body of that size will hold, plus several hundred gallons which may be stacked in the aisle without interfering with driver activities. Faster loading is accomplished by running a skate-type conveyor through the tall rear door. The aisle space meas-

ures 26 in. The truck has a 3-ft. shorter wheel base than the conventional type and is equipped with a Copeland 2 H. P. compressor. The body consists of a wooden frame lined with steel inside. It has 5 in. of hard rubber board insulation on the floor, ultra-lite 8 in. side walls, ultra-lite in the room and aluminum sheets on sides of the body and the steel roof. W. H. Martin

85. Building half-gallon sales with a price promotion. V. F. Hovey, Jr., Gen. Ice Cream Corp., Schenectady, N. Y. Ice Cream Trade J., **47,** 11: 36. Nov., 1951.

Increases as high as 500% on the sale of 0.5-gal. packages of ice cream resulted from a sales promotion program in which wholesale prices were reduced from $1.85/gal. net to $1.60. The resale price was reduced from $1.15 to 98¢/package. During the month of promotion, the percent of 0.5-gal. sales to the total sales of the plants increased from 7.0–16.9%. The 0.5-gal. carton, by making ice cream available in the house, will substantially increase consumption.

W. H. Martin

86. Franklin ice-cream-a-teria. Anonymous. Ice Cream Trade J., **47,** 10: 60–61. Oct., 1951.

The Franklin Ice Cream Co. in Cleveland, O., has applied the principles of food cafeterias to the dispensing of ice cream. The customer choses a glass dish and the serving girl places the dips of ice cream in the dish. The customer then moves to the syrup department where he chooses the topping for his ice cream. At the end of the line he picks up spoons, straws, napkins and a paper cup of water, while the clerk adds up the sale on a register. Malts and sodas also are available. W. H. Martin

87. State standards for 1951. Anonymous. Ice Cream Trade J., **47,** 10: 136. Oct., 1951.

The minimum percent of fat and milk solids for plain, fruit and nut ice cream and milk sherbets and the weight standards of ice cream/gal. are presented in tabular form. Twenty-three states have minimum standard of 10% fat for plain ice cream, 5 have an 8% standard, 16 a 12% standard and 4 have a standard of above 12%. All states except 7 allow for 2% less fat in fruit and nut ice cream, 3 in plain ice cream.

Sixteen states have a minimum weight of food solids/gal. and 22 states have a minimum weight/gal. One state (Ill.) limits the overrun to 100/can and 1 state (Iowa) has a minimum weight of 18 oz./qt. in factory-filled packages.

W. H. Martin

88. Texas defines vegetable fat frozen dessert. Anonymous. Ice Cream Trade J., **47,** 10: 46. Oct., 1951.

The Texas State Health Department has promulgated a legal definition for vegetable fat frozen dessert, and has ruled that the product must be called *Mellorine.*

"*Mellorine* is a frozen or unfrozen product made from edible fat, solids and sugar, with or without a natural flavoring, and contains not less than 6% edible fat and not less than 30% of all solids including fats and may contain not more than 1% of a stabilizer approved by the State Health Officer, and may contain 1 or more of the following optional ingredients: eggs, fruit, salt, nuts, extracts, harmless solvent, chocolate or cocoa and sucrose, dextrose, fructose and any other mixture approved by the State Health Officer.

"The use of the word *cream* or its phonetic equivalent, however spelled, in connection with the labeling, advertising, branding or sale of this product is prohibited by article 708, penal code of Texas.

"The manufacturers of *Mellorine* shall meet the same rules and regulations that govern the production and manufacture of ice cream and other manufactured products as promulgated by the State Health Dept., Jan. 1, 1946."

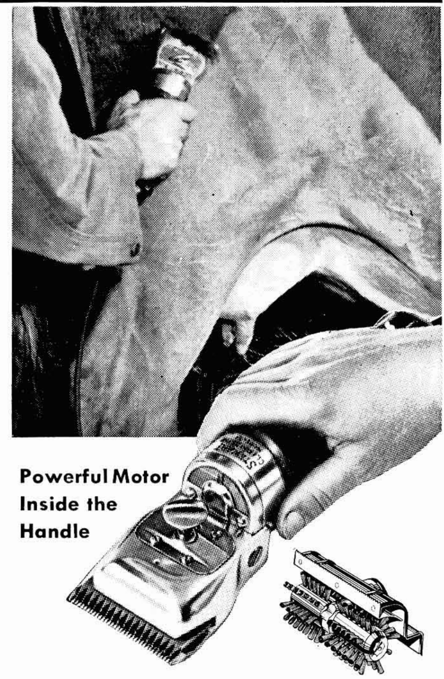
W. H. Martin

EMPHASIZE REGULAR CLIPPING
for the production of quality dairy products

Sunbeam
STEWART
ELECTRIC
CLIPMASTER

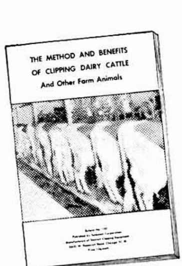
Good sanitary practice calls for a regular clipping program. Clipped cows are easier to keep clean. Clean cows mean less sediment and a lower bacteria count. Milk with a lower bacteria content is more desirable.

Leading health authorities say: "A regular clipping program means more wholesome milk. It is an essential step in the production of quality dairy products." Emphasize the advantages of regular clipping. It reduces sediment, lowers bacteria, avoids contamination and increases profits from production of cleaner, higher quality milk.



Handy, interchangeable electric Grooming Brush head fits Clipmaster.

Free!



Bulletin 100—"The Method and Benefits of Clipping Dairy Cattle and Other Farm Animals." This handy manual points out the advantages of a regular clipping program and illustrates the 5 simple steps in clipping dairy cattle that can be easily learned by everyone. Contains no advertising. Send for your free copy.

An electric grooming brush saves time and does a more thorough job of cleaning than hand brushing.

Sunbeam CORPORATION (formerly Chicago Flexible Shaft Co.) **Dept. 141, 5600 West Roosevelt Road, Chicago 50, Illinois**

Your advertisement is being read in every State and in 45 Foreign Countries

BACK COPIES
of
Journal May Be Available

The Association has available back copies of the Journal of Dairy Science. If you need back copies, please write and inquire as to whether the particular one that you need is available. In some cases we have only a few volumes and we do not sell them unless the complete set of volumes is purchased. In many cases we have six or eight volumes complete with 50 or 100 copies available of certain numbers such as the November or December issue.

Volumes	ADSA Members	Non-members and institutions
1–16 (if available)	5.00	6.50
17–32 (if available)	6.00	8.00
33–	8.00	10.00

If you are interested in procuring back copies please write to the Sec'y-Treas., American Dairy Science Assn., c/o Ohio State University, Columbus 10, Ohio. Make all checks payable to the

AMERICAN DAIRY SCIENCE ASSOCIATION

SUBSCRIPTION ORDER

To THE AMERICAN DAIRY SCIENCE ASSOCIATION
Publishers of the Journal of Dairy Science
Ohio State University, Columbus, Ohio

Please find enclosed Ten Dollars in payment of subscription to the *Journal of Dairy Science* for one year beginning with January, 19....

Name ....

Address ....

....

Foreign postage 50 cents additional.

Checks, etc., should be drawn to the order of the *American Dairy Science Association* and forwarded to P. R. Ellsworth, Ohio State University, Columbus 10, Ohio.

GBI
Reg. U. S. Pat. Off.

Complete Stabilizers and Emulsifiers

BODY-GUARD®—for the finest grade of ice cream, high active ingredient content—83¢/lb.

PRIDEX®—for ice creams with pleasing mellow consistency—economically priced—57¢/lb.

Straight Stabilizers

HY-GUARD®—for ice creams, milk sherbets, ices and stick confections—excellent stabilization—57¢/lb.

PRIDE®—for deliciously smooth textured ice cream, with maximum stabilization at low cost—53¢/lb.

Straight Emulsifier

EMEX® — for increasing whipping ability of ice cream and controlling overrun when used with good stabilizers—44¢/lb.

• • •

Ask your Dairy Supply Jobber or us for testing samples and bulletins.
®—Registered U.S. Patent Office

WRITE FOR

Write for literature describing G.B.I. Ice Cream Stabilizers and Emulsifiers

GENERAL BIOCHEMICALS, INC.
20 LABORATORY PARK • CHAGRIN FALLS, OHIO

An enzyme of growing interest in Dairy and Cheese circles

Catalase

Wherever the economical decomposition of hydrogen peroxide is indicated we will be glad to discuss the application of this new stable Catalase preparation.

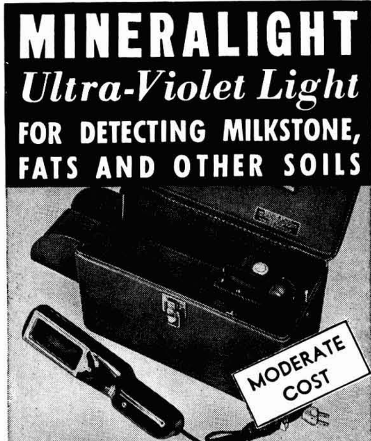
Other Paul-Lewis products for the DAIRY INDUSTRY

LACTIVASE (for prevention of oxidized flavor)
RENNET, Extract & Powder (high activity-always uniform)
PHOSPHATASE REAGENTS
BUTTER COLOR
BROM THYMOL BLUE
BROM CRESOL PURPLE

For Complete Information on the Paul-Lewis Products

Paul Lewis Laboratories, Inc.
MANUFACTURING CHEMISTS
4253 N. Port Washington Rd., Milwaukee, Wis.

MINERALIGHT
Ultra-Violet Light
FOR DETECTING MILKSTONE, FATS AND OTHER SOILS



For Sanitarians, Field Men and Inspectors

Mineralight is a compact portable long wave ultra-violet light which causes fluorescence in milkstone, fats, and other soils not readily seen by the eye. Used like a flashlight. Operates 110 V-AC or batteries. Adapter available for 110 V-DC. Carrying case optional but necessary for battery operation. Moderate cost. Valuable aid to any size plant. Indispensable in improving sanitary standards. Write for literature.

KLENZADE PRODUCTS, INC.
BELOIT • WISCONSIN

"HANSEN'S"
DAIRY PREPARATIONS
For High Quality Dairy Products

CHEESE RENNET AND COLOR
COnAGE CHEESE COAGULATOR
ANNAno BunER COLOR
DANDELION BunER COLOR
CERTIFIED BunER COLOR
STARTER DISTILLATE
ICE CREAM COLOR
LACTIC FERMENT CULYURE

ODORLESS TYPE DAIRY FLY SPRAY
CULTURE CABINETS
TESTING SOLUTIONS

Write for Literature

CHR. HANSEN'S LABORATORY, INC.
MILWAUKEE 14, WISCONSIN

Mention
JOURNAL OF
D A R y 5 (E N (E

This helps both the Advertiser and you

When Answering Advertisements

Copies of the 20-Year Index covering Volumes I to XX, inclusive are available at the following prices:

MEMBERS

Cloth Bound	$2.35
Paper Bound	2.00

NON-MEMBERS

Cloth Bound	$5.50
Paper Bound	5.00

NOTICE TO CONTRIBUTORS

Authorship of Original Articles and Reviews.—Space in the JOURNAL is reserved for the publication of original research voluntarily submitted by members of the Association to the JOURNAL and review articles by invitation. In the case of joint authorship, the membership ruling applies to one author only.

Papers that already have appeared in print or that are intended for simultaneous publication elsewhere will not be accepted.

Manuscripts.—Manuscripts should be submitted in double spacing on one side of suitable 8½″ × 11″ paper. The original copy should be furnished. All illustrative and tabular material should accompany the manuscript.

Except in cases of invited reviews, papers must be limited to 12 printed pages unless previous permission from the editor is obtained. When non-review articles exceed 12 pages, a charge of $5 per over page is made.

Manuscripts will be published in the order of their receipt. They should be sent to the Editor, F. E. Nelson, Dept. of Dairy Industry, Iowa State College, Ames, Iowa. In order to speed publication, one author should be designated to assume the responsibility of checking the galley on all papers of multiple authorship. All galleys should be returned in the minimum possible time to avoid delay in publication.

Figures.—Original drawings, diagrams and charts should be done in India ink on tracing cloth (or white board) not larger than standard letter size (8½″ × 11″). All lettering should be inked in block style and be of such size that the lettering will be not less than ⅛ in. in height when the figure is reduced to 4 in. in maximum dimension. Typewritten labeling of axes and axis units is not acceptable. Original drawings should be submitted, rather than photographs of such drawings. When suitable drawings are not furnished, the author will be charged for the preparation of drawings of satisfactory quality by an independent agency.

Photographs.—Photographs for halftone reproduction should be glossy prints free of all imperfections.

Legends.—Legends for figures and photographs should be typed on a sheet separate from the illustrative material and should be made as concise as possible while retaining their descriptive character.

Tabular Material.—Tabular material should be clear, concise and accurate. Often data can be condensed and presented in summarized tabular form. Tables of only one or two lines should be avoided except in most unusual cases. Excessively large or complicated tables are almost impossible to print satisfactorily. Headings should be as concise as possible, yet descriptive in character. Data may be presented in either tabular form or in figures, but the same data must not be presented in both forms. Each table should be placed on a separate sheet and not in the body of the manuscript. The letters *a, b, c,* etc., should be used for footnote designations. If possible, tables should be so organized that they may be set across the page, rather than the length of the page.

References.—Literature reviews should be limited to only the most pertinent references. Reference lists should be double spaced and arranged alphabetically as to author and by chronological appearance of the journals cited under a given author. Papers by a single author always precede papers by that author and associates. References to multiple authors are arranged in the alphabetical order of the several authors. Give only initials rather than full first names of male authors. Citations in the text should be made by the number in parentheses, corresponding to the number in the reference list.

Each reference should contain the following: Reference number, author(s), title of article, name of journal, volume number, first and last page numbers, and year of publication. Titles of all articles should appear in complete untranslated form. Consult recent published articles in the JOURNAL for proper citation. Publications are abbreviated according to the form given in CHEMICAL ABSTRACTS, vol. 40, no. 24, part 2. 1946.

Sample of journal citation: (1) JONES, L. W., AND SMITH, J. D. Effect of Feed on Body of Butter. J. DAIRY SCI., 24: 550–560. 1941.

Sample of book citation: (1) LANDSTEINER, K. The Specificity of Serological Reactions. Rev. Ed. Harvard University Press, Cambridge, Mass. 1945.

For Experiment Station publications, the citation should be as follows: (1) COULTER, S. T., AND JENNESS, R. Packing Dry Whole Milk in Inert Gas. Minn. Agr. Expt. Sta. Tech. Bull. 167. 1945.

The more common abbreviations used in the text are: cm., centimeter(s); cc., cubic centimeter(s); g., gram(s); mg., milligram(s); γ, microgram(s); ml., milliliter(s); mμ, millimicron(s); C., Centrigrade; F., Fahrenheit; lb., pound(s); oz., ounce(s).

Where configurational structures of chemical compounds are used, drawings suitable for reproduction by photoengraving are to be furnished by the author.

In preparing manuscripts, use of first person should be avoided.

Your advertisement is being read in every State and in 45 Foreign Countries

DIFCO

for Microbiological Assay

of

VITAMINS and AMINO ACIDS

Bacto dehydrated media containing all the necessary nutriments and growth factors for the microbiological assay of vitamins and amino acids are now available from Difco. These basal media require only the addition of graduated amounts of the substance under assay to obtain linear growth of the test organism for construction of the standard curve. The vitamin or amino acid content of the material under assay is determined by adding appropriate concentrations of the test substance to the basal medium and comparing the growth response obtained with the standard.

BACTO-FOLIC ACID ASSAY MEDIUM
BACTO-NIACIN ASSAY MEDIUM
BACTO-PANTOTHENATE ASSAY MEDIUM
BACTO-PYRIDOXINE ASSAY MEDIUM
BACTO-RIBOFLAVIN ASSAY MEDIUM
BACTO-THIAMIN ASSAY MEDIUM
BACTO-TRYPTOPHANE ASSAY MEDIUM
BACTO-VITAMIN B_{12} ASSAY MEDIUM
BACTO-B_{12} ASSAY MEDIUM U S P

The method employed in carrying stock cultures of the test organisms and preparing the inoculum for microbiological assay is important. The following media have been developed especially for carrying stock cultures and for preparation of the inoculum.

BACTO-MICRO ASSAY CULTURE AGAR
BACTO-MICRO INOCULUM BROTH
BACTO-NEUROSPORA CULTURE AGAR

BACTO-VITAMIN FREE CASAMINO ACIDS, dehydrated, is an acid hydrolysate of vitamin free casein prepared especially for laboratories investigating microbiological assay of vitamins.

Descriptive literature is available upon request.

Specify "DIFCO"

THE TRADE NAME OF THE PIONEERS
In the Research and Development of Bacto-Peptone and Dehydrated Culture Media

DIFCO LABORATORIES
DETROIT 1, MICHIGAN